Université de Lille

Ecole Doctorale Biologie Santé

THESE

Pour l'obtention du

DOCTORAT DE L'UNIVERSITE DE LILLE

Discipline : Immunologie

Présentée et soutenue publiquement par

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Le 12 Octobre 2023

Contribution de la sénescence cellulaire à la vaccination anti-tumorale chez l'individu âgé

Devant le jury composé de:

Mme. le docteur **Corinne ABBADIE**	Président du Jury
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Equipe « I2M »

Université de Lille

Ecole Doctorale Biologie Santé

Dissertation submitted to the University of Lille for the degree of

Doctor in Immunology

Presented and defended by

Özmen ÇOBANOĞLU, M.Sc

12 October 2023

Contribution of senescent cells to anti-tumoral vaccine responses in the elderly

Composition of the Jury:

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I, Özmen ÇOBANOĞLU confirm that the work submitted by me in this dissertation is an outcome of my independent and original work, except where information has been derived from other sources indicated by references in the text.

Acknowledgements

I would like to thank Dr. Delphine SAUCE, Dr. Christophe PAGET, Dr. Corinne ABADIE, and Dr. Dmitry BULAVIN for accepting the invitation to be part of the jury and contributing to my scientific journey.

I would like to extend my gratitude to Dr. François TROTTEIN for his supervision and the opportunity that gave me the independence to carry out a scientific investigation and create a unique project with a novel hypothesis. I am thankful to him for giving me a space to do mistakes and allowing me to learn from them. I appreciate the time, presence, and guidance he provided throughout the project, especially when I faced challenges. Many times, I learned and came to a point of realization with his feedbacks and comments that have helped me with my research and professional understanding. I highly value all the meetings we had which kept me on track and provided me encouragments for further steps.

My gratitude also extends to my team members, Corinne, Cyril, Fabiola, Gemma, Isabelle, Loic, Lou, Lucie, Patricia, Severine and all former teammates whose support has been invaluable throughout this long and challenging journey. Every single one of you played a contributing role in this process. I appreciate the practical help you provided, but also simply being present with your smile, bearing with me at the days I was grumpy, sharing a moment over a lunch break, cake, or coffee, and enriching my learning French when I have been utterly confused. Thank you for your kind help and co-operation. Many thanks to Severine Heumel and Lucie Deruyter for constant support and technical assistance they have provided since the early times of my project.

I would like to acknowledge the financial support that made this research possible. This work was made possible through the funding provided by Europen Commission Marie Curie Skladowska Action. I would also like to express my deep appreciation to the members of the PAVE consortium who have been integral to this research journey. Their collaboration, expertise, and valuable insights have enriched the scope and quality of my work.

I would like to thank to my CSI committee members Olivier Pluquet and Victor Appay. Over the years, your constructive feedback, expert insights, and thoughtful suggestions have helped me to navigate my work.

Further I would like to thank our collaborators, particularly Professor Philip Beckhove for the opportunity to work in his lab and all the members of AG Beckhove for their support during my time at Leibniz Institute for Immunotherapy in Germany. Special thanks to Abir Hussein, for being such a good friend and co-worker.

Special thanks to my friends Bea, Cemre, Dimi, Ilay, Lobna, Mûge, Mustafa who have handled the stressfull moments with me and supported me with their presence and friendship along this time.

Above all, I am deeply grateful to my parents, my dad Tahsin and my mom Meliha for their love and support. I always felt that you believed in me and wanted the best for me. My sisters, Yenigul, Selma and Selda thank you for always being there for me and giving me strength when I need. And my dear niblings, Gôksin, Îmran, Zehra, Gôksu, Hamza Ali, Zeynep, Duru, Derin and Berrak thank you for being a great source of inspiration and encouragement for me.

“Two there are who are never satisfied — the lover of the world and the lover of knowledge.”

Rumi

Dedicated to all minorities

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Introduction

Background and Significance

Chronic diseases and cancer are major health concerns associated with aging. Aging is the primary risk factor for these diseases. The ability of the immune system to detect and respond to transformed cells, as well as the efficacy of vaccines, decreases significantly in older individuals because of reduced antibody production and altered cellular responses that can contribute to the development of cancer. The immune system experiences significant changes with age, which affects its response to immune challenges. As a result, the response patterns are highly age-dependent. There is increased dysfunction of both innate and adaptive immune responses, which contributes to impaired immune responses to pathogens and greater mortality and morbidity. One of the fundamental aging mechanisms that contribute to immune dysfunction in the elderly population is cellular senescence, which occurs naturally with aging. Cellular senescence is characterized by stable cell growth arrest and the acquisition of a specific senescence-associated secretory phenotype (SASP), which is marked by the release of inflammatory cytokines, growth factors, and various immune modulators. The need for enhanced immune responses in older adults has spurred research efforts in the development of more effective vaccines and the implementation of innovative strategies, despite our limited understanding of immunosenescence and its impact on immunity in the elderly. Therefore, understanding senescence becomes more important with aging, as it leads to diverse remodeling processes in the immune system. In this regard, the assessment of vaccine efficacy in the aged population is critical. Considering the rapid aging of the population, a better understanding of aging and its contributors is not only crucial for individual welfare but also vital for maintaining the prosperity and political stability of the global community.

1 - Aging and Immune Function

Aging is an inescapable and natural process in human life. Improved hygiene conditions and the availability of vaccines have increased the global human lifespan. United Nations' population projections indicate a steady increase in the foreseeable future. The global population aged ≥ 65 years is projected to increase from 10 percent by 2022 to 16 percent by 2050. According to these projections, by 2050, approximately two-thirds of the global population aged 60 years and above will reside in low- and middle-income countries, posing significant healthcare challenges. Although current technologies and interventions have contributed to the mitigation of certain age-related conditions, obstacles remain to be addressed to prevent multiple age-related diseases and enhance overall responses. (Anon n.d.) These challenges primarily stem from unresolved questions regarding the processes underlying aging.

Aging is a multi-faceted biological process. Moreover, it entails numerous physiological and cellular changes that result in a gradual decline in multiple bodily functions. The immune system displays significant changes during aging, leaving individuals more susceptible to diseases, such as cancer, disorders, and other age-related ailments. (Weyand & Goronzy 2016) These changes in immune function can be attributed to various factors, including modifications in the production and functionality of immune cells. Owing to their increased susceptibility, older individuals experience increased morbidity and mortality rates. The gradual deterioration in the function and number of immune cells is collectively called immunosenescence, and is characterized by differences in various aspects of adaptive and innate immunity. Senescent cells are types of cells permanently stop dividing, and their presence tends to increase naturally in different tissues and organs during aging. This increase in senescent cells is often caused by damage or stress such as genetic, oxidative, or inflammatory stress, leading to persistent low-grade inflammation. Although many studies have shown that aging affects the immune response to vaccines, there is a lack of research on how senescent cells contribute to the immune dysregulation associated with aging. (Di Micco et al. 2021) Senescence cells are yet to be fully defined, since each cell displays a different phenotype and function during differentiation.

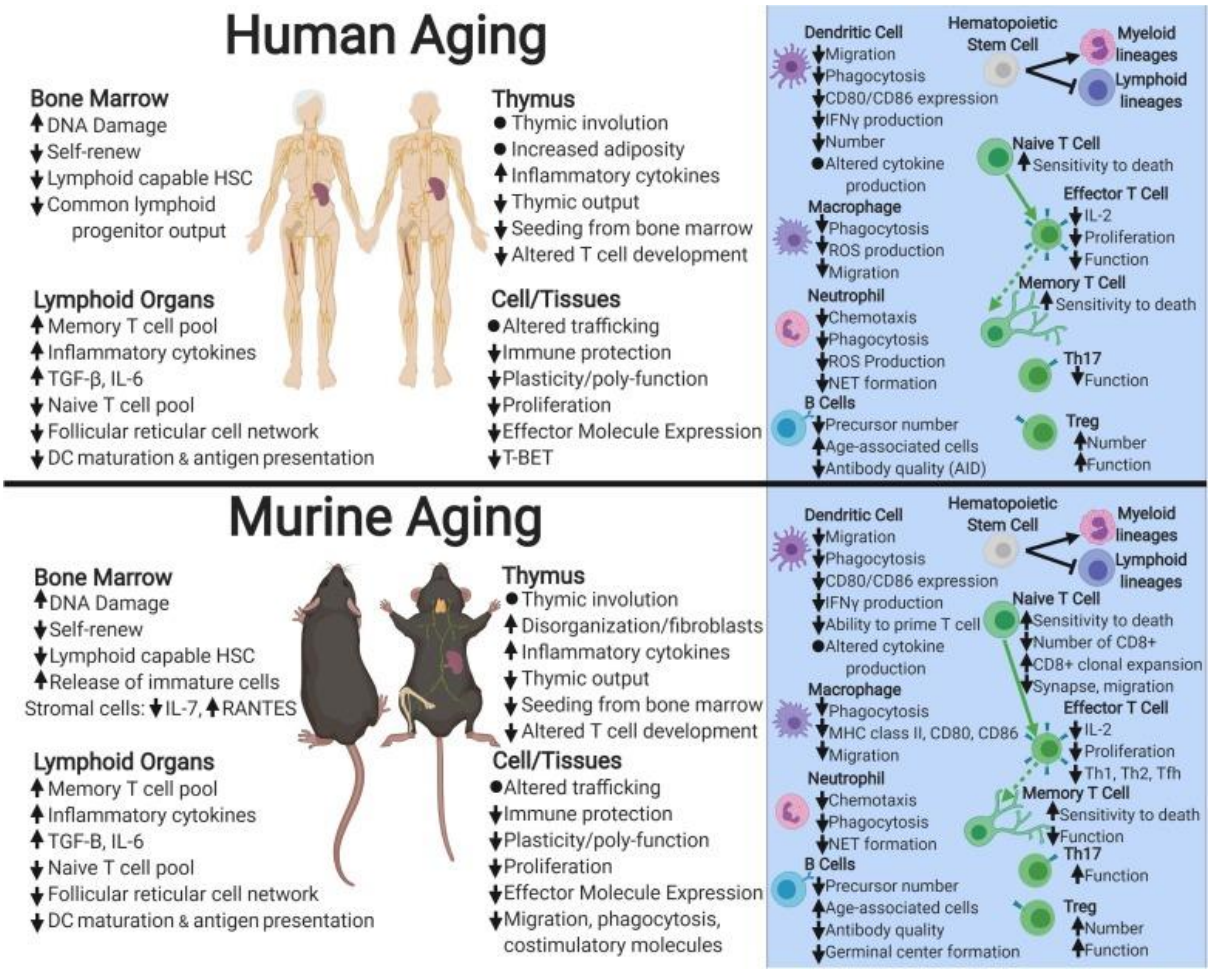

Fig. 1. Summary of age-related changes to the immune systems of humans and mice. The direction of arrows pointing upward or downward indicates whether there is an increase or decrease in the number of cells or specific functions. (Figure from Keilich et al. 2019)

1-1- Impact of Aging on Innate Immune System

The innate immune system functions as the body's initial point of defense against foreign invaders, and is essential for maintaining an appropriate immune response. To identify pathogens, the innate immune system uses specific molecular pathways known as pathogen-associated molecular patterns (PAMPs). Different cell types continuously express pattern recognition receptors (PRRs), which enable the recognition of pathogens and their microbial ligands and induce a proper host response to stop the spread and invasion of pathogens. Granulocytes, macrophages, dendritic cells, and innate-like lymphocytes, such as NK cells, are

cell types that play a role in innate immunity. These cells respond to bacterial, viral, and fungal pathogens in a nonspecific manner. (Mogensen 2009)

The innate immune response is rapidly activated in a short period of time, within hours or even minutes, through the receptors on the cells, leading to the activation of a transmembrane signal that activates NF-kB and other related pathways. When innate immune cells identify pathogens, they coordinate their killing activity and alert the adaptive immune system. The cytokines and chemokines produced by granulocytes and antigen-presenting cells draw more innate cells to the site of pathogen encounter. Activated tissue macrophages produce chemokines that recruit neutrophils and help to clear foreign substances. Secreted cytokines form a complex signaling network that can affect the environment and facilitate cell-to-cell communication. Some cytokines, including IL-6, IL-1B, and TNF-α, may counteract or enhance these effects. These cytokines contribute locally to the activation of inflammatory cells. Once the local activation of inflammatory cytokines reaches a high intensity, the increased production of cytokines is released into the circulation, where the production of inhibitory cytokines such as IL-10 may constrain the acute response. (Takeuchi & Akira 2010)

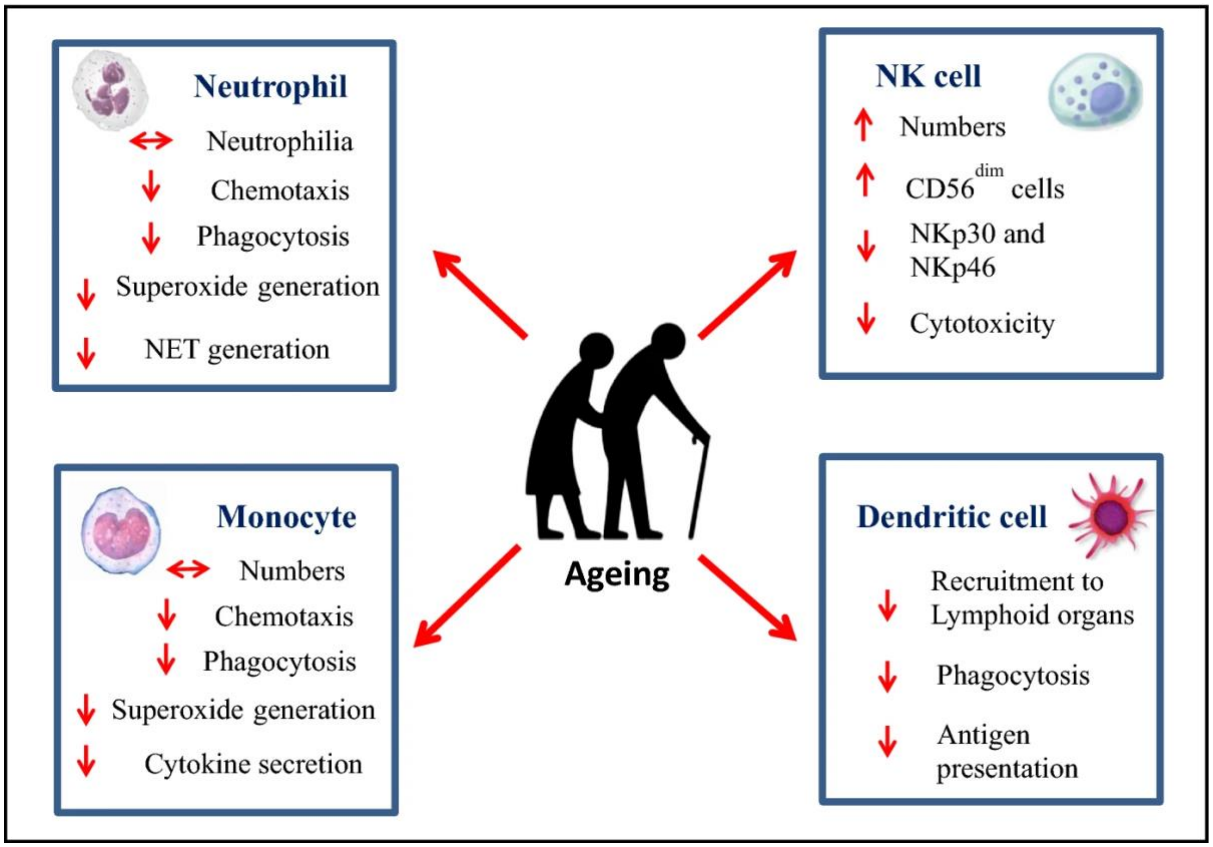

Fig. 2. Age related alterations in innate immune cells. (Figure from Duggal 2018)

1-1-1- Neutrophils

As is well known, aging is accompanied by cellular and molecular impairments affecting the innate immune system. Dysregulated signaling pathways and altered transcriptional programming reduce the ability of the host to respond effectively to foreign encounters and pathogens. The functions of different innate immune cells such as NK cells, macrophages, and neutrophils decrease with age. (Franceschi, Bonafè, Valensin, et al. 2000) In neutrophils it is indicated as reduced chemotaxis. This impairment is accompanied by compromised recruitment to the infection site and delayed egress, which contributes to the persistence of chronic inflammation in older individuals. Neutrophils also exhibit altered functionality by forming extracellular traps (NETs) that phagocytose microbes and generate reactive oxygen species (ROS). For instance, decreased chemotaxis, impaired NET formation, and reduced reactive oxygen species production have been observed in aging neutrophils. Neutrophils form NETs via a process known as NETosis. (Nauseef & Borregaard 2014; Van Avondt et al. 2023; Hazeldine et al. 2014)

Netosis can occur via different mechanisms such as suicidal NETosis, vital NETosis, and a third mechanism involving the release of mitochondrial DNA by neutrophils, as described by Yousefi et al. These mechanisms depend mainly on reactive oxygen species (ROS). (Vorobjeva & Chernyak 2020) Several studies have suggested that aged mice exhibit lower levels of autophagic activity and reduced ROS generation, resulting in decreased NET formation. (Xu et al. 2017) However, stimulation with TLR ligands successfully induced ROS-dependent NET formation in young mice. NETs have also been implicated in tumor-associated immune responses and have the potential to exhibit both anti-tumorigenic and pro-tumorigenic activities. (Ireland & Oliver 2020) Evidence suggests that NETs formed by tumor-associated neutrophils are in close contact with sarcoma cells, linking NET formation to cancer development. (Masucci et al. 2020) It is important to fthe ully understand mechanism of these changes and their impact on the overall health and disease outcomes in the elderly population.

1-1-2- Macrophages

Macrophages play an essential role in innate immunity. Pathogen engulfment (phagocytosis) and release of various cytokines, chemokines, and growth factors are some of the primary immune regulatory functions mediated by macrophages. (Kumar 2019) The coordination and proper management of immune responses depend on the flawless operation of these functions.

However, recent research has shown that macrophage functional capacity induces phenotypic changes, which eventually result in diminished function during aging. (Jackaman et al. 2017) Along with the accumulation of various immune complexes, hormones, free fatty acids, and immunoglobulins in the body, macrophages generate low-level tissue-specific inflammation. Owing to these changes and the generation of a new inflammatory environment, the phenotypic characteristics of macrophages are altered. (Oishi & Manabe 2016) Generally, according to their polarization status, macrophages can be classified into two main groups: classically activated M1 macrophages (lipopolysaccharide-induced) or activated M2 macrophages (induced by Th2 cytokines). Once macrophages enter an active state, they release cytokines and chemokines, leading to inflammation and the further recruitment of other immune cells. These cytokines include TNF-α, IL-6, and IL-12, as well as chemokines, such as CXCL9 and CXCL10, which attract effector CD8+ and helper CD4+ T cells. In addition to pro-inflammatory responses, anti-inflammatory or repair responses can be induced by the subsequent activation of macrophages. For instance, regulatory CD4+ T cells play an essential role in wound healing and are attracted to macrophages that release CCL17, CCL22, CCL24, and CXCL13. In mice and humans, these factors promote wound healing and injury repair but can also impact angiogenesis and tumor growth. Studies have suggested that macrophages in aging individuals exhibit a more pro-inflammatory M1 phenotype, characterized by increased production of inflammatory mediators. (Pence & Yarbro 2018) As a result of this shift towards a pro-inflammatory phenotype, an imbalance occurs in the immune response, potentially contributing to the development of age-related diseases. Some studies have highlighted that age-related functional changes may be tissue-specific and may stem from the origin of macrophages. (Becker et al. 2018) Recently Duong et. al have shown the role of macrophages in cancer during aging. (Duong et al. 2018) In their study they found that mesothelioma tumors grew faster in aged mice and the tumor-associated macrophages found to be positively correlated with tumor size. This increase in tumor-associated macrophages was suggested to be due to the large supply from the bone marrow, as bone marrow macrophages have shown enhanced proliferation in response to mesothelioma in the elderly. In contrast to bone marrow macrophages, splenic macrophages found to be decreased suggesting that it may be further exacerbated with cancer which leads to replicative senescence in macrophages.(Duong et al. 2018; Duong et al. 2021) More information can be found on macrophage polarization and its role with age related diseases elsewhere (Cui et al. 2019; Becker et al. 2018; Oishi & Manabe 2016)

Additionally, the transition of macrophages from a pro-inflammatory to reparative phenotype is dysregulated with age. Studies have shown that the ability of macrophages in elderly hosts to phagocytose apoptotic cells decreases. This is associated with a poor ability to resolve inflammation. It has also been shown that macrophages and other specialized scavenger cells from elderly humans and rodents exhibit a decreased capacity to engulf particles in vitro and reduced clearance of apoptotic cells in aged mice, displaying impaired phagocytic activity. (Lloberas & Celada 2002; Plowden et al. 2004; Stout & Suttles 2005)

1-1-3- Natural Killer Cells

Furthermore, NK cells exhibit age-related changes in addition to neutrophils and macrophages. In addition to their ability to recognize and destroy virally infected or tumor cells, NK cells are large granular cells with direct cytotoxicity through the secretion of cytokines and chemokines that have an immunoregulatory function. (Brauning et al. 2022) By their receptor is divided into two main categories: CD56dim and CD56bright, which have different properties. In older adults, immunosenescence of NK cells contributes to increased infection rates.(Hazeldine & Lord 2013) Over the past decade, their roles have expanded from only the killing activity of virally infected and malignant cells to recognizing and eliminating senescent cells, participating in antimicrobial defense, contributing to the resolution of inflammation, and modulating adaptive immunity. (Nikzad et al. 2019; Wagner et al. 2017; Iannello & Raulet 2013; Kim et al. 2022; Przemska-Kosicka et al. 2018) Consequently, senescence features or age-related changes in NK cells have far greater effects on the health of older individuals than young adults. Studies have shown that a lower number of NK cells is associated with a higher mortality rate in elderly individuals. (Remarque & Pawelec 1998) Additionally, the cytotoxic activity of NK cells was observed to change in elderly donors. (Franceschi et al. 2018; Hazeldine & Lord 2013). Like other lymphoid cells, they originate from common progenitors and mature lymphoid tissues such as the spleen and bone marrow. NK cells react rapidly upon stimulation and play an essential role in antiviral anti-tumor responses. The killing response occurs via a signal received by the inhibitory and activating receptors expressed on the surface of these cells. Signals are regulated by two types of receptors, Ig-like and C-type lectins. As with many other cell types, the number and function of NK cells are modified during aging. Several studies have shown that in older adults, decreased overall cytotoxicity may stem from impaired binding to targets or perforin content. (Ovadya et al. 2018; Hazeldine et al. 2012) In older adults, IL-2 induction of IFN-y and IFN-alpha decreases, whereas IL-1, IL-6, IL-8, IL-

10, and TNF-α levels increase. Additionally, NK cells produce less IFN-γ upon IL-2 stimulation in older adults. It has also been shown that expansion of the CD56dim population and shrinkage of the CD56bright population in older people diminishes the amount of cytokines produced by these cells, such as IFN-γ and GM-CSF. (Chidrawar et al. 2006) NK cells play different roles in various age-related pathologies during aging. For instance, regarding age-related cancers and NK cell function, some cancers have evolved NK escape mechanisms where tumor cells can secrete soluble NKG2D, which induces a false NK response by competing with the true NKG2D for binding on its receptor side, while evading CD8 recognition. (Groh et al. 2002; Terme et al. 2008). The percentage of CD57+ mature NK cells was the dominant circulating cell type with age. Another crucial step in NK cell-mediated defense is patrolling function and migration to the infection site. Murine studies have shown impairment in the migration of NK cells with age (Fang et al. 2010; Beli et al. 2011; Beli et al. 2014). It has also been shown that reduced responses to challenges in older people may be responsible for the increase in senescent cells found in aged tissues. (Sagiv et al. 2013)

1-1-4- Dendritic Cells

Several age-related immune dysfunctions are associated with antigen-presenting dendritic cells (DCs) in the innate immune system. (Agrawal et al. 2017; Agrawal et al. 2012) DCs are well known as effective antigen-presenting cells with high levels of MHC II, CD80, and CD86, which are involved in antigen presentation upon activation. They are well distributed throughout the body under the epithelial cell barrier and are continuously surveyed for both external and internal danger-associated signals using a variety of pattern recognition receptors, such as TLRs, C-type lectin receptors, and NOD-like receptors. Dendritic cells can be divided into two significant classes: plasmacytoid dendritic cells (pDCs), which are known for their ability to produce high amounts of type I interferons (IFNs), and myeloid dendritic cells (mDCs), which play a role in antigen presentation derived from myeloid progenitors. pDCs play a critical role in antiviral defense by collaborating with other immune cells such as T and B cells. They can be distinguished from other immune cells based on the expression of specific surface markers such as CD123 and CD303. In contrast, mDCs are primarily responsible for capturing and processing antigens to T cells, and can be identified by the expression of CD11c, CD1c, and CD141. Aging significantly affects hematopoiesis, with a decline in the functional capacity of HSCs skewing towards myeloid lineages and accumulation of myeloid cells in the peripheral blood of older individuals. (Zhang et al. 2020) pDCs numbers are reduced with age

in circulation and, therefore, are the source of type I IFNs. (Molony et al. 2017; Feng et al. 2021) Knowing that dendritic cells are involved in the recruitment of other immune cells, such as macrophages and NK cells, through direct cell-to-cell contact or by secreting soluble mediators, a reduction in type I IFNs affects the recruitment and activation of macrophages and NK cells. Consequently, the bridge between the innate and adaptive immune systems is altered to effectively eliminate pathogens and tumors. However, mDCs are reported to remain unchanged in the aged population, with a decrease only in CD141+ mDCs in circulation. (Agrawal et al. 2016) Monocyte-derived dendritic cells (moDCs) then be added as a third subset. Although they function similar to myeloid DCs, they are derived from circulating monocytes, which are capable of activating naïve T cells to differentiate into effector T cells. They have been widely studied in the context of autoimmune diseases and cancer immunotherapy because their ability to prime T cell responses makes them attractive candidates for therapeutic applications. DCs play a pivotal role in bridging innate and adaptive immunity. However, according to Zhao et al., defects displayed by aged mouse DCs migrating to draining lymph nodes eventually result in lower T-cell responses. (Zhao et al. 2011) Moreover, Dendritic cells from aged mice were shown to have impaired T cell stimulating capacity *in vitro* (Grolleau-Julius et al. 2008) and *in vivo* (Li et al. 2012) This functional impairment and lack of ability to induce T cell responses may alter the effectiveness of dendritic cells in anti-tumor T cell responses, therefore contribute to cancer progression in elderly people. (Gardner et al. 2017) In contrary, the study of Gardner et al. demonstrated that elderly moDCs maintain their ability to activate antigen specific T cells in response to LPS/IFN-y mediated induction. However, these cells may have less efficient function in recruiting neutrophils and monocytes to the site of infection due to their reduced ability to stimulate Th2 and Th17 responses, affecting the quality of their anti-pathogen and anti-tumor responses. (Gardner et al. 2018) Knowing the lowered chemotaxis abilities of neutrophils in aging, this also raises the question whether or not if those impaired functioning between neutrophils and dendritic cells might contribute to the low-grade chronic inflammation in the elderly individuals.

Additionally, reduction in pDCs function with advancing age leads to decreased secretion of type I IFN in response to activation through TLR7 and TLR9 ligands, compared to younger individuals. (Panda et al. 2010) Evidence also suggests that thymic DCs could poorly participate in central tolerance formationbecauserofg aging due to shrinkage in the thymus, which alters the abundance and function of these cells, contributing to an increased risk of

autoimmunity. Regarding the role of TLRs in aged subjects, Panda et al. (2010) reported defective functioning in both mDCs and pDCs, with reduced production of cytokines upon activation. Furthermore, a decrease in IL-12 production by DCs from peripheral blood mononuclear cells (PBMCs) of aged subjects has been reported in comparison with their younger counterparts. (Della Bella et al. 2007)

In contrast to pDCs and mDCs, moDCs display increased secretion of proinflammatory cytokines, such as TNF-α, CXCL-10, and IL-6, in response to LPS. (Agrawal et al. 2007) It is suggested that the decrease in PI3kinase/AKT pathway signaling, as a negative regulator of TLR signaling, contributes to the increased production of pro-inflammatory cytokines. This impaired function of AKT stems from the increased PTEN expression in the DCs of aged subjects. Moreover, there is a significant age-associated decrease in the production of IL-10, which is crucial for the modulation of inflammation and stems from an inherent defect in the DCs of aged subjects.

1-2- Impact of Aging on Adaptive Immune System

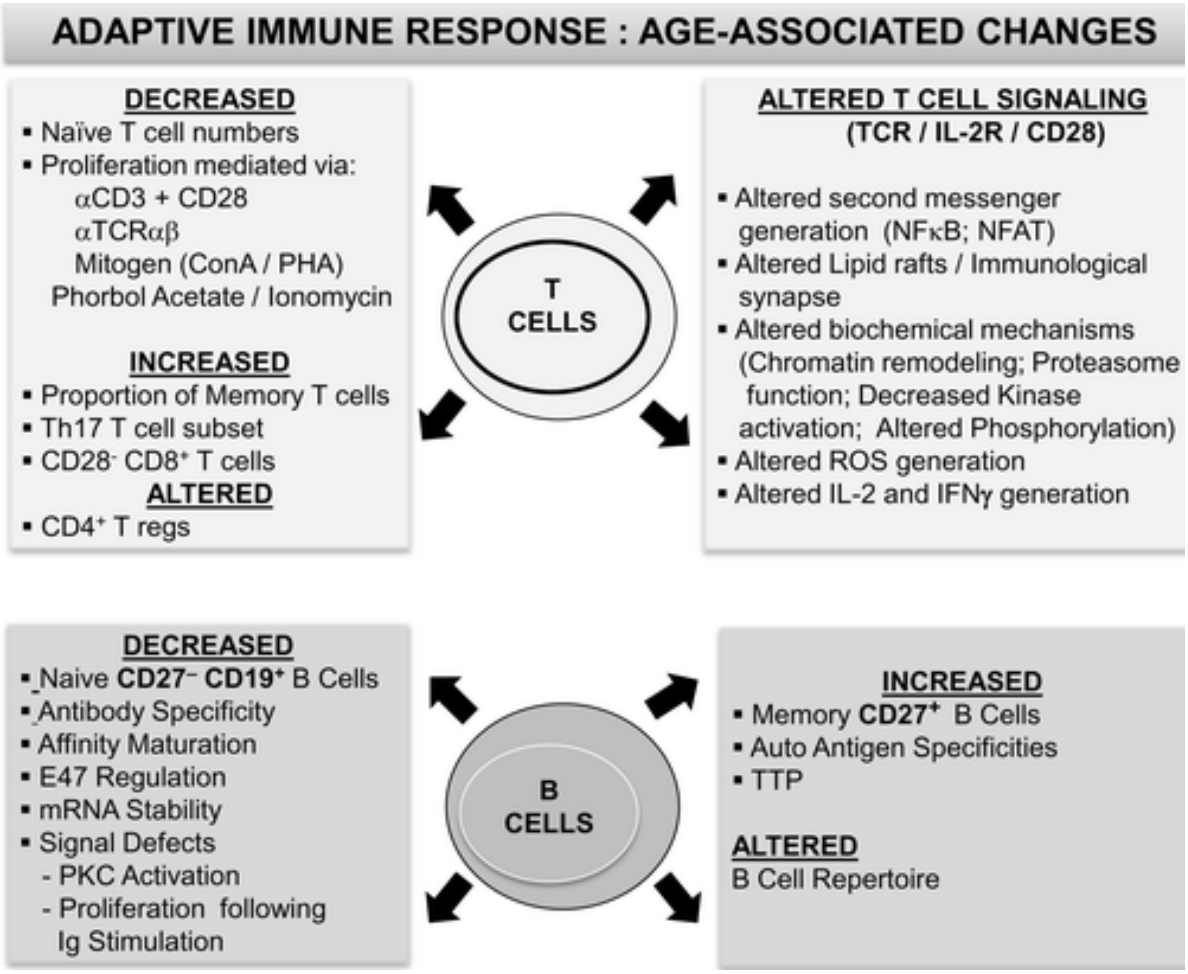

Fig. 3. Summary of age-associated alterations in adaptive immunity: T lymphocytes and B lymphocytes. (Figure from Ponnappan & Ponnappan 2011)

1-2-1- T cells

Although age-related changes occur in the innate and adaptive arms of the immune system, knowledge regarding age-related changes is accumulated and better understood in the adaptive part of immune defense, particularly T cells. One notable change in the immune system during aging is the thymus involution. (Liang et al. 2022) Thymic involution affects the production of newly generated naive T-cells, leaving the organism lacking adaptive immunity to new pathogens. Functional tissues of the thymus, such as the cortex and medulla, are replaced with fat during aging. Owing to thymus involution, there is almost no output of newly generated naïve T cells by the age of 50 years. A reduced frequency of naïve T cells occurs in peripheral and lymphoid organs, particularly within the CD8+ T cell compartment. This is the most

consistent and prominent marker of immune ageing in healthy older adults. (Goronzy et al. 2015)

In comparison, the composition of the CD4 + T-cell compartment remained stable. (Goronzy et al. 2015) Challenges with lifelong antigenic stimulation. This reduction places pressure on existing naïve T cells, which may turn them into virtual memory cells by acquiring phenotypic memory markers. (Lanzer et al. 2018) While this hypotdemonstrated has been shown in murine models, TCR sequencing studies do not support this hypothesis as a frequent event occurring during aging in older humans. This indicates a difference in age between mice and humans. (Taylor et al. 2017; Leignadier et al. 2011) Aging T cells also reduce the T-cell receptor (TCR) repertoire. In healthy adults, circulating T cells exhibit a repertoire of several million TCR β sequences; however, this number is significantly reduced in older age. (Qi et al., 2014; Warren et al., 2011; Yoshida et al., 2017).

Additionally, although IL-7 regulates homeostasis, survival, and proliferation of peripheral T cells, available data regarding IL-7 levels and their use with age are contradictory. Aiello et al. proposed that IL-7 levels decrease in relation to thymic involution characterized by atrophy, and that reduction of IL-7 could contribute to the decline in the number of thymocytes and naïve T cells with age. (Aiello et al. 2019; Andrew & Aspinall 2002) In contrast, previously published data by Gruver et al. and Sempowski et al. reported no decrease in IL-7 levels with age. (Sempowski et al. 2000) Moreover, with involution of the thymus and decreasedT-cellc activity, T cell diversity cannot be increased. This leads to contraction of the T cell repertoire, which impairs the foreign antigen response.

In contrast, studies of thymopoiesis in adult humans have shown that T-cell receptor rearrangement and the generation of diverse TCRV beta repertoires still exist, demonstrating that the adult thymus remains active late in life and contributes to the functional T cell pool. T cell receptor excision circles (TRECS), as evidence of recent TCR rearrangement, were found in peripheral CD4 + T cells even at 78 years of age. The average TREC content was measured by den Braber et al. in naïve T cells from mice and humans to understand the thymic output compared to mice and humans. (den Braber et al. 2012) Although naïve T cells of mice dit differ much, the TREC content with a naïve phenotype in humans was found to decrease with age. This suggests that naïve T cell maintenance differs between mice and humans and between their young and old counterparts.(Nikolich-Žugich 2014) Therefore, insights from mice to human,s or vice vers,a are needed for the production of naïve T cells. Despite the homeostatic

pro-liberation of previously generated naïve T cells, which represents a compensatory mechanism to counteract the loss of output, the frequency of naïve T cells is reduced in the periphery and lymphoid organs in old age, particularly within the CD8+ T cell compartment (Lazuardi et al. 2005; Fagnoni et al. 2000; Flavell et al. 2013)

CD8 + T cells play a crucial role in the recognition and elimination of intracellular pathogens. CD8 T cells use CD28 as a co-stimulatory activator signal and its ligands, CD80 and CD86, on the surface of antigen-presenting cells. With continuous stimulation, activation, and proliferation, CD28 expression is downregulated on the surface of CD8+ T cells. This eventually results in the accumulation of CD28 − CD8+ T cells, and CD28 downregulation is more evident in the CD8 compartment. The loss of CD28 expression is linked to rapid telomerase inactivation, resulting in shorter telomeres in CD8+ cells. However, CD28- cells can still proliferate under certain conditions, such as when cytokines such as IL-15 provide the necessary co-stimulatory molecules. Although this does not permanently immerse these cells in a senescent state, they create a senescence-like phenotype. As CD28 downregulation occurs, there is an increase in CD57expression. This was linked to the reduced expression of genes related to DNA repair and apoptosis. In contrast and not very surprisingly, these cells showed enhanced production of proinflammatory cytokines. These cells accumulate in many disorders, including tumor microenvironments and chronic viral infections such as CMV. (Tsukishiro et al. 2003; Derhovanessian et al. 2011) Due to their pro-inflammatory phenotype, CD28- CD8+ T cells may contribute to low levels of chronic inflammation in old age. (Franceschi et al. 2018; Naismith et al. 2019). Although these cells may not be fully senescent, evidence indicates that their presence is detrimental and may contribute to disease.

The loss of CD28 and gain of CD57 expression generates a differentiated profile of T cells akin to a pro-inflammatory phenotype. It has been found that the SASP phenotype may be present in peripheral CD28- CD57+ CD27- T cells. (Pangrazzi et al. 2020; Pangrazzi & Weinberger 2020) Despite this, no T cell population with features of all senescence-associated markers has been identified to date. Therefore, a combination of several different markers is critical for the identification of senescent T-cells.

Moreover, senescence phenotypes in the CD8+ and CD4+ compartments may also differ. The senescence profile within the CD4 compartment exacerbates itself with mitochondrial dysfunction and has the potential to be reversed by blocking p38 mitogen-activated protein kinase (MAPK), which has been observed in CD45RA+ CD27- CD4+ T cells with a SASP-

relatedpro-inflammatory phenotype. (Callender et al. 2020; Di Mitri et al. 2011) An additional senescence-associated cell profile in the CD4 compartment occurs in aged cells after homeostatic proliferation of naïve T cells. Homeostatic proliferation of naïve T cells results in conversion to a memory phenotype (MP) with higher CD44 expression on CD4+ T cells. Due to thymic involution and a decrease in the T cell output memory phenotype, T cells become predominant with decreased TCR-mediated proliferation and IL-2 production. This is followed by an increased expression of PD1, and to a lesser extent, CD153 molecules defined assenescence-associated T cells. (Minato et al. 2020) Although senescence-assos are found to have reduced production of regular T cell cytokines, they produce pro-inflammatory cytokines, and chemokines are abundant in these cells. In aged mice, senescence-associated T cells are found in high numbers in lymphoid organs such as the spleen, lymph nodes, and bone marrow. They are primarily located in the follicular region of the white pulp in the spleen, where B cells reside, and are often associated with germinal centers that develop with age. Additionally, these cells exhibited an increase in the expression of cell cycle inhibitors, such as Cdkn1a and Cdkn2b, as well as in the levels of SA beta-galactosidase, which is the most well-known marker of senescence. For instance, in lupus-prone female NZB/W F1 (BWF1) mice, PD1+ CD153+ SA T cells were elevated in association with the development of spontaneous GC reactions as the disease progressed. They promote autoantibody production by secreting abundant OPN in GCs.

Given the comprehensive examples from humans and mice, the current literature may not provide sufficient conclusions regarding the role of T-cells in aging. However, the incidences of cancer and infectious diseases increase with age. Accordingly, Palmer et al. suggested that a decline in T cell output of the immune system is the primary factor for increasing cancer incidence.

1-2-2- B cells

Additionally, there is progressive modification in humoral responses and impairment caused by aging, in addition to cellular responses. Several studies have been conducted in mice and humans to investigate age-related changes in the B cell compartment. (Scholz et al. 2013; de Mol et al. 2021; Hagen & Derudder 2019; Johnson & Cambier 2004) B cells arise from the progenitors in the bone marrow. There are additional stages of development that occur within

the B-cell lineage because of the variable regions of immunoglobulins that are present. As a result, aging appears to disrupt B cell development in the mouse bone marrow, and the pre-B cell compartment appears to be the most affected compartment in the process. (Frasca & Blomberg 2009; Miller & Allman 2003; Alter-Wolf et al. 2009) Impaired B cell development appears to be due to affected RAG and SLC expression, which alter pre-BCR and BCR formation, and decreased sensitivity to IL-7 signals. (Ratliff et al. 2015) Ratliff et al. have demonstrated that levels of the SLC protein λ5 determine the relative susceptibility of pro-B cells to apoptosis induced by TNFalpha and TGFB. They showed that pro-B cells in the bone marrow of aged mice are low in λ5 SLC expression, resulting in resistance to apoptosis, which was also linked to IL-7-dependent B cell precursor growth. The latter was reduced in aged mice and coincided with the increased reactivity of B cells to the self-antigen. Therefore, apoptosis resistance of pro-B cells in aged bone marrow is likely the direct consequence of reduced SLC expression. Furthermore, IL-7 from young adult bone marrow was inhibited upon challenge with either TNFα or TGFb. In old mice, pro-inflammatory TNFalpha-producing B cells and other inflammatory cells increase apoptosis, leading to the preferential death of SLChigh pro-B cells, consequently affecting B cell development and the antibody repertoire. Interestingly, inhibition of mesenchymal progenitor cell senescence correlates with increased expression of IL-7 and CXCL12. (Gao et al. 2018) Therefore, aging and a pro-inflammatory environment could potentially interfere with support in the bone marrow by limiting the availability of IL-7 and CXCL12 during B cell generation. Systemic levels of CXCL12 in the bone marrow of adult mice are negatively affected by the presence of inflammatory mediators such as TNFα, in combination with or without IL1B. A remarkable alteration of the mature B cell compartment in mice during aging is the accumulation of age-associated B cells (ABCs), which are detrimental to naïve follicular B cells (Fo B cells). (Ratliff et al. 2013) The generation of ABCs depends on pro-inflammatory signals, and the stimulation of TLRs, especially TLR7, contributes to the formation of ABC cells in mice. (Hao et al. 2011) Moreover, in vitro ABC cells from aged mice secrete sufficient TNFalpha to impair the generation of young pro B cells, suggesting a possible contribution of bone marrow resident ABCs to altered B cell development. (Ratliff et al. 2015; Hagen & Derudder 2019) The age-associated B cells (ABC) were first defined in companion publications by Hao et al. and Rubtsov et al. and defined as B220+ CD19+ splenic cells that lack CD21, CD23, CD95 and CD43 by Hao et al. whereas CD11c on B220+ CD19+ splenocytes were the identification criteria by Runtsov et al. (Rubtsov et al. 2011; Hao et al. 2011) In response to BCR cross-linking, ABCs do not divide, but remain viable under these conditions, making them distinguishable from MZ and TR B

cells, which die rapidly following BCR cross-linking. In contrast, ABCs proliferate robustly in response to a stimulus and engage the endosomal nucleic acid-sensing Toll-like receptors TLR7 and TLR9. Hao et al. suggested that most naturally arising ABCs do not represent de novo production of a unique pre-immune B cell type by progenitors prevalent in the aged bone marrow, but are instead members of a slowly accumulating population, probably derived from pre-immune peripheral B cell compartments. ABCs are likely to originate from antigen-experienced B cells that arise in T cell-dependent immune responses and persist as a distinct Bmem population. (Russell Knode et al. 2017) It was revealed that naturally accumulating ABCs are products of antigen-driven B cell responses, with a probability of involving cognate T cell help. Furthermore, many ABC immunoglobulin heavy and light variable chain region sequences are somatically mutated, implying a GC origin and, by extension, a requirement for cognate T cell help and co-stimulation. Based on this finding, ABCs can arise following antigen-driven activation. In contrast, an alternative view of ABC generation has also suggested that in some instances, they may arise without the engagement of exogenous antigens, and with advancing age, an increasing proportion of primary responses to influenza infection involve ABCs. (Swain et al. 2017) Current data suggest that the ABC pool reflects a combination of formative routes, whose relative contributions may vary with age, antigenic load, and other variables. The gradual increase in ABCs with age raises the question of the functional role that may play in immunological changes with aging. These concerns include reduced B-cell genesis, systemic and local increases in inflammatory mediators, contribution to inflammation, and altered responses to primary and recall challenges. Using a combination of in vitro and in vivo approaches, ABCs have been found that ABCs impede early B cell development through the production of TNF-α. These effects were direct via the induction of apoptosis in pre-B cells and indirect through systemic inflammatory effects on the bone marrow microenvironment. Initial descriptions regarding the contribution of these cells to inflammation were observed through the propensity for IL-6 and IFN-γ production, coupled with their ability to skew T cell polarization towards inflammatory effector subsets.

2 - Aging and Vaccines

Vaccines have been one of the most important medical interventions in human history. Vaccines generate antibodies that confer protection against infections by binding to pathogens

to prevent infection and spread. Vaccines control pathogen invasion through the formation of memory cells by inducing the formation of memory cells. Memory cells can help in defense as they can rapidly proliferate and turn into effector cells. In particular, memory B cells turn into antibody-secreting plasma cells and can re-enter the germinal center for further expansion. On the other hand, memory CD8 T cells turn into effector CD8 T cells that can directly attack and kill virus-infected cells, whereas memory CD4 T cells support multiple different immune cell types and help promote effective CD8+ T cell and antibody responses. Cell-mediated immunity functions as a complement to antibody-mediated immunity to ensure protection and to improve vaccine efficacy. While both cellular and humoral responses are important for ensuring the efficacy of vaccines, the production of protective antibody responses is considered to be the main correlate of protection in vaccine-induced immunity. Vaccine-induced antibodies can be generated through two different mechanisms: the extrafollicular response and germinal center reaction. The extrafollicular response creates an early post-immunization stage, where antigen-primed B cells differentiate into plasmablasts in the extrafollicular foci of the spleen or medullary cords of lymph nodes. Plasmablasts are important in the early phase of active infections as they can undergo expansion and differentiation to form short-lived plasma cells that secrete protective antibodies. Germinal centers are the main source of long-lived antibody-secreting plasma cells. GCs consist of two distinct zones: dark and light zones. In the dark zone, centroblast germinal center B cells undergo somatic hypermutation of their immunoglobulin genes, with activation induced by cytidine deaminase AID. Later, B cells undergo a process of selection in the light zone for the selection of high-affinity functional B Cell receptors (BCRs). The functionality of acquired BCRs is further tested by binding to antigens that are present on the surface of follicular dendritic cells, which later present processed antigens to T follicular helper cells (Tfh cells) via MHC II. GC B cells that are able to interact with Tfh cells survive with the help of cytokines such as IL-21 and IL-4. GC B cells with low-affinity or autoreactive BCRs cannot receive survival signals, which eventually cause apoptosis. In GCs, plasma cells migrate to the bone marrow with CXCR4/CXCL12 receptor expression, where they produce high antibody titers. Memory B cells circulate in the blood, secondary lymphoid tissues, and bone marrow to differentiate into plasma cells upon antigen exposure. Since germinal centers are very important and central for the generation of vaccine-specific antibodies, the mechanism behind age-related decline in vaccine responses can be better understood by deciphering how aging influences the GC reaction.

Aging has been associated with a decline in the number and size of GCs, and the magnitude and volume of GC responses were found to be significantly lower in aged mice than in young mice after immunization.(Lee, Fra-Bido, et al. 2022; Stebegg et al. 2020; Siegrist & Aspinall 2009) In result, this has been shown to associated with a decline in the number of long-lived bone marrow plasma cells and vaccine induced antibody titers in aged mice. However, defects in the GC response of older humans are less characterized owing to the limitations in accessing the draining secondary lymphoid tissues after vaccination. Circulating Tfh cells in the blood and lymph strongly correlate with vaccine-induced antibody production in humans. For instance, in people aged 65 years and older, the frequencies of hemagglutinin-specific cTfh cells in the peripheral blood are reduced after influenza vaccination, which correlates with reduced hemagglutinin-specific antibody responses. (Herati et al. 2014; Hill et al. 2021) Lower titers of vaccine-specific antibodies, an increase in self-reactive antibodies, and a shorter duration of the antibody response are among the major effects of aging on vaccine responses. Importantly, age-associated reduced responses to vaccination and protection in the elderly are not correlated with a single cell type, but rather affect multiple cells from both the innate and adaptive compartments. It has been shown that the upregulation of IFN- signaling pathways is impaired with age. (Connors et al. 2022; Li et al. 2015) Due to such impairment, pathogen-specific antibody responses are affected, as is the case for influenza vaccinations. In some cases, such as influenza, the annual administration of vaccines may elicit poor innate immune responses due to pre-existingting and cross-reactive antibodies. (Hagan et al. 2022) Decreased MHC peptide expression on the surface of dendritic cells from age mice eventually contributes to reduction in sufficient level of signaling thus impacting the strength of T cell activaton. Recently, lymph node macrophages have been shown to initiate responses to influenza vaccination by releasing interferon-β (IFNB) and interleukin-1 alpha (IL-1α), which encourage cellular reactions in the lymph nodes.. (Picard et al. 2022)

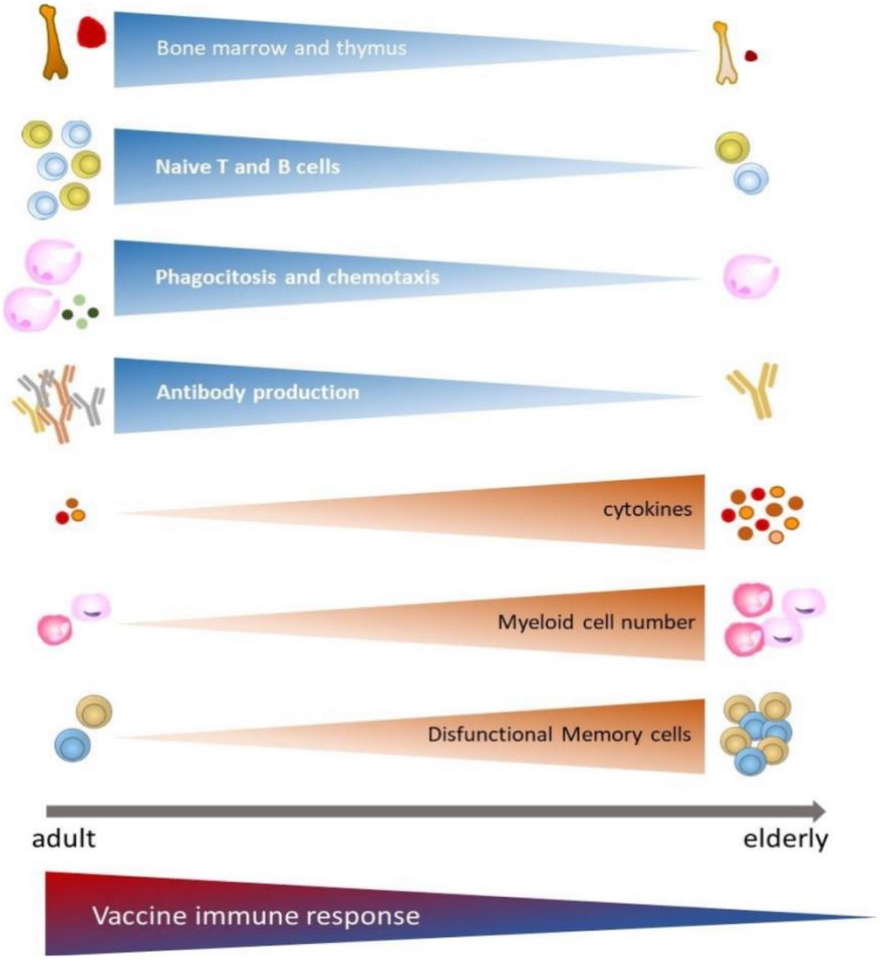

Fig. 4. Vaccine immune response in function of changes of the innate and adaptive immune system associated with aging. As individuals age, both their innate and adaptive immune responses diminish, resulting in reduced effectiveness of vaccinations. This phenomenon is known as immunosenescence, which involves the shrinking of primary lymphoid organs such as the bone marrow and thymus, leading to a decrease in B and T cell progenitors. Additionally, there is a buildup of dysfunctional memory cells due to chronic antigenic stimulation, including but not limited to CMV, as well as a reduction in phagocyte functions such as chemotaxis and phagocytosis. This is accompanied by an increase in pro-inflammatory cytokine production. All of these changes contribute to a decline in the immune system's ability to respond to vaccinations. (Figure from Ciabattini et al. 2018)

2-1 Aging and Cancer Vaccines

The studies on cancer vaccines discussed below suggests that vaccination is less effective in older individuals than in younger ones, but it is still viable to customize vaccination for older ages. For example, studies conducted by Yung and colleagues found that vaccination with a DC-OVA vaccine derived from young mice was less effective against B16-OVA melanoma tumors in older mice compared to younger ones. This demonstrates the impact of the altered

tumor microenvironment at older ages on vaccination. (Grolleau-Julius et al. 2009; Grolleau-Julius et al. 2008) Zhang's group discovered that the tumor microenvironment (TME) is different in old mice compared to young mice. They showed that there were more myeloid-derived suppressor cells (MDSC) in the TME of old mice, and that these cells played a role in making the T cells less responsive to stimuli in old mice. (Grizzle et al. 2007) The age factor is often ignored in human clinical trials with cancer immunotherapy. Moreover, various groups have shown that also cancer vaccination is less effective at older age.(Castro et al. 2009) The research group of Provinciali reported that CD4 and CD8 T cells were present in tumors of young but hardly detectable in tumors of old mice, while macrophages and neutrophils were detected at both ages. However, protective memory responses that could reject tumor cells upon re-challenge of tumor-free mice was only obtained in young mice. (Provinciali et al. 2003) Lustgarten and colleagues also found that cancer vaccination was less effective at old than at young age. They showed that young but not old mice developed long-lasting memory responses to a pre-B-cell lymphoma (BM-185). (Lustgarten et al. 2004) Additionally Posnett and colleagues used DNA based tumor antigen vaccines against melanoma and showed that old mice were not protected. Moreover in their study they have shown that Vaccines incorporating fusions of the tumor antigen with microbial adjuvant proteins OmpA (E. Coli) or Vp22 (Herpes simplex virus-1) dramatically improved protection of old mice. (Posnett et al. 2009)While the effect of cancer vaccination on growth of tumors and metastases could be strongly improved by tailoring the vaccine to older age, as shown in the preclinical studies analyzed here, in most cases improvement was not the result of T cell activation but rather the result of other immune cells stimulated by the vaccine. Although various functions of NK and NKT cells are decreased at old age, it is significantly less dramatic than the age-related decline in T cell function, and both cells play an important role in anti-tumor responses. For instance, NK cells and TCRγδ NKT cells could be activated by attenuated Listeria and/or αGalCer and exhibit anti-tumor activity. Since the number of TCRγδ NKT and NK cells also increases with age, Listeria-based vaccination in combination with αGalCer could enhance vaccine-induced T cell responses against cancer at older age. For instance, the problem of lack of naïve T cells, one of the most important changes at older age, could be avoided by immunizing at young age when sufficient naïve T cells are present and boosted to generate memory T cells, followed by recall at old age to reactivate memory T cells.

3- Causes and Mechanisms of Cellular Senescence

In 1961, cellular senescence was first described as a state of permanent proliferation arrest triggered by various stressors, such as telomere dysfunction, oncogene activation, and persistent DNA damage in vitro. This process has been linked to important biological phenomena such as tumor suppression, tissue repair, embryonic development, and aging in organisms. In 1961, Hayflick and Moorhead made a groundbreaking discovery that normal cultured human cells have a limited capacity for cell division before entering an irreversible growth arrest called replicative senescence. This finding led to the hypothesis that tissue aging may be caused by the progressive loss of cell division, which is necessary for replacing old cells that accumulate naturally over time. Cellular senescence is a stable and irreversible state of growth arrest, rendering cells incapable of proliferating even under favorable growth conditions and mitogenic stimuli. Additionally, senescent cells exhibit increased resistance to apoptotic cell death due to the upregulation of cell survival pathways, such as the BCL-2 family of anti-apoptotic proteins.

The process of senescence is dynamic and involves overlapping yet distinct molecular pathways that are activated at various stages, ranging from cell cycle exit to the later stages of senescence.

Cells can become senescent in response to a variety of intrinsic and extrinsic stimuli, including progressive telomere shortening, changes in telomeric structure, mitogenic signals, oncogenic activation, radiation, oxidative and genotoxic stress, epigenetic changes, chromatin disorganization, perturbed proteostasis, mitochondrial dysfunction, inflammation, and/or tissue damage signals, irradiation, or chemotherapeutic agents, nutrient deprivation. (Kumari & Jat 2021)

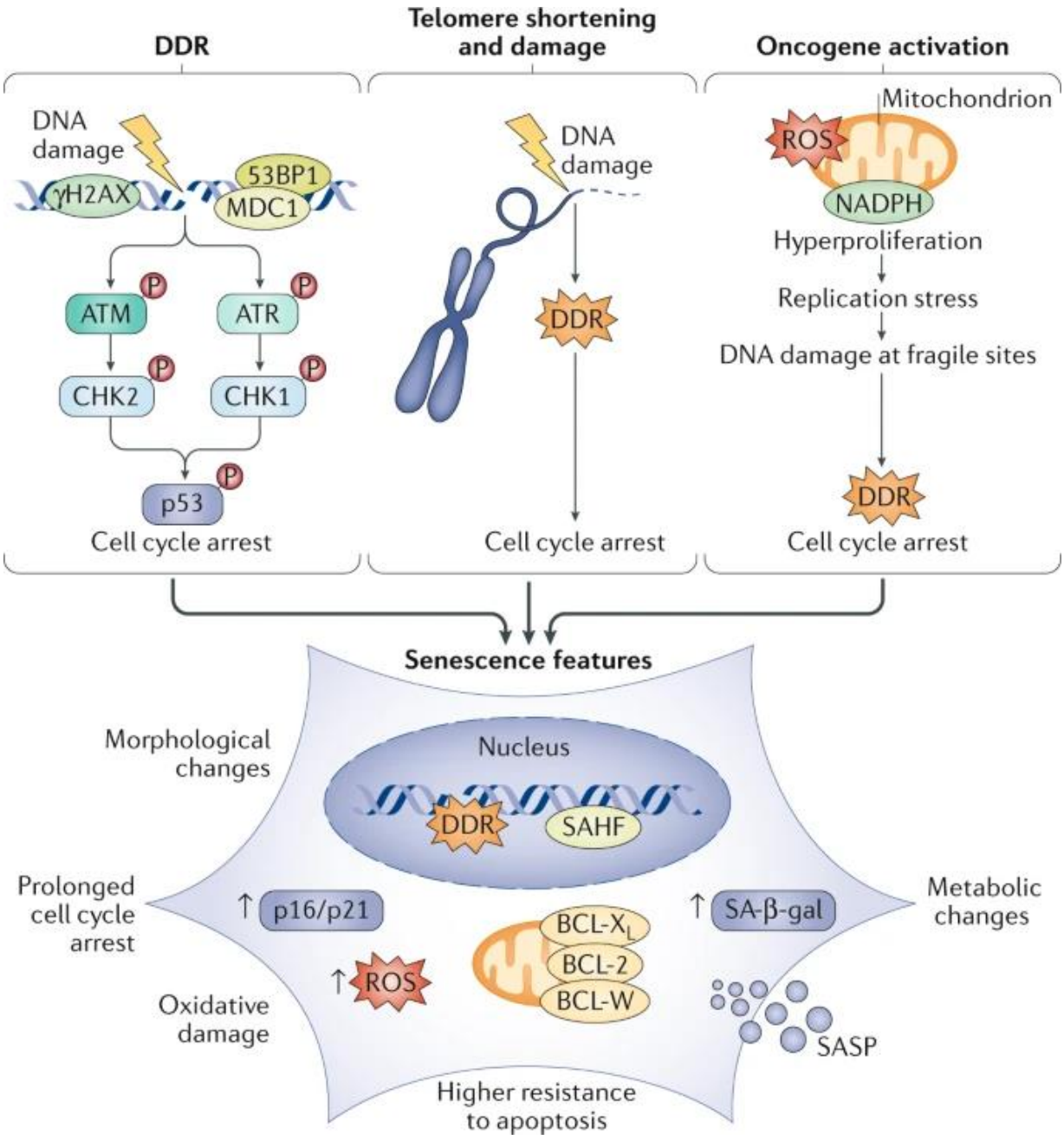

Fig. 5 : Senescence drivers and phenotypes. (Figure from Di Micco et al. 2021)

3-1 DNA Damage Response (DDR) and Cellular Senescence

Nuclear DNA damage is often caused by DNA double breaks. When DNA damage is detected, it triggers the DNA damage response pathway (DDR), resulting in the accumulation of DDR factors at the damaged sites, forming visible nuclear foci in the cytology. These foci consist of phosphorylated histones H2AX, MDC1, 53BP1, and activated ATM. If DNA damage persists

without repair, it leads to extended signaling in the DDR pathway and prolonged cellular senescence. Severe or accumulated DNA damage can cause cellular senescence or apoptosis and contribute to the aging process. Different types of stress signals, such as telomere shortening, mitogenic signals, radiation, oxidative stress, and genotoxic stress trigger distinct types of senescence. DDR involves multiple DNA damage sensors and protein kinases, including ATM and ATR, and is characterized by DNA damage foci and telomere dysfunction-induced foci. Although DDR markers may not exclusively identify senescent cells in vivo, they can still serve as indicators of cellular senescence, as other mechanisms independent of DDR can also induce senescence. DDR can also be triggered by DNA-damaging stimuli that do not necessarily result in senescence but are involved in physiological responses or transient, reversible DNA damage. These different types of stress signals lead to various forms of senescence, such as telomere-dependent replicative senescence, programmed senescence, and non-telomeric stress-induced premature senescence, including oncogene-induced senescence (OIS), unresolved DNA damage-induced senescence, epigenetically induced senescence, and mitochondrial dysfunction-associated senescence.

3-2 Telomere Shortening and Cellular Senescence

One of the initial mechanisms characterized for cellular senescence induction is telomere shortening. Due to the inadequacy of the standard DNA replication machinery in replicating chromosomal DNA ends, the absence of telomere maintenance mechanisms such as telomerase expression or recombination among telomeres results in telomeres becoming shorter with each round of DNA replication. As telomere length decreases, the loss of telomere-capping factors or protective structures becomes critical. Short telomeres resemble one-ended double-strand breaks, and thus trigger a DNA damage response that is very similar to that triggered by double-strand breaks. (d'Adda di Fagagna et al. 2003; Herbig et al. 2004) The presence of DDR proteins at telomeres has been linked to telomere dysfunction, which in turn leads to senescence and increased aging in various mammalian tissues (Hewitt et al. 2012; Kaul et al. 2011; Fumagalli et al. 2012) and having only one or a few critically short telomeres is sufficient to trigger a DDR and lead to cellular senescence in vivo, despite having a majority of otherwise long telomeres. (Hemann et al. 2001) As mammalian tissues age, the levels of DDR markers and TAFs tend to rise. It has been observed that mice exhibit an age-dependent increase in dysfunctional telomeres in both tissues that undergo proliferation and those that do not. (Birch et al. 2015; Anderson et al. 2019; Bloom et al. 2023) The analysis of telomere length in tissue

samples from 952 individuals revealed that 21 out of the 24 tissues examined exhibited age-related telomere attrition. (Demanelis et al. 2020) Senescence in a human alveolar epithelial-like cell line is triggered by telomere dysfunction, resulting in an upregulation of both transcriptional and proteomic hallmarks of the Senescence-Associated Secretory Phenotype (SASP) (Sullivan et al. 2021) Therefore, the persistent activation of tDDR is the common causative factor responsible for both replicative cellular senescence triggered by critically short telomeres and the senescence-like state in non-replicating cells resulting from damaged telomeres.

3-3 Oncogene Induced Senescence

Oncogene activation is a strong inducer of cellular senescence. The expression of oncogenes triggers an initial phase of hyperproliferation that is inherently altered DNA replication is linked with the activation of DDR pathways, which ultimately leads to senescence. (Di Micco et al. 2006; Bartkova et al. 2006) Telomeres are highly sensitive to DNA replication stress, which can be caused by oncogenes and the accumulation of telomeric dysfunction induced by oncogenes, and this sensitivity has been observed in the development of hyperplastic cancer lesions in humans. (Suram et al. 2012) In addition to their well-known function as DNA-damaging agents, ROS can also act as signaling molecules that promote oncogene functions in tumors. (Ogrunc et al. 2014) The process of oncogene-induced senescence (OIS) occurs without the involvement of telomere erosion and can activate both the TP53/CDKN1A and p16/RB signaling pathways. OIS cells typically have elevated p16 expression, which can allow them to bypass senescence and re-enter the cell cycle when p16 levels are low. (Rayess et al. 2012) The function of primary OIS is well established: it acts as an early, intrinsic tumor-suppressive mechanism. Several studies have demonstrated that the activation of oncogenic KRASV12 in lungs and pancreas leads to neoplastic transformation in pre-malignant cells, which are characterized by senescence markers. (Collado et al. 2005) The tumor-suppressive function of OIS is also apparent in the mouse prostate following the depletion of the tumor suppressor PTEN, but not when both PTEN and TP53 are lost in advanced prostate cancer. (Chen et al. 2005) Importantly, in vitro and in vivo studies on human melanocytic naevi (moles) have revealed OIS as a significant obstacle to tumorigenesis. (Michaloglou et al. 2005) Up to 80% of these naevi have the BRAFV600E mutation, which is the most common mutation found in melanomas. These melanocytes with the BRAFV600E mutation remain in a state of growth

arrest and exhibit several characteristics of OIS, such as increased expression of p16 and positive SA-β-gal staining.

3-4-Senescence Associated Secretory Phenotype (SASP)

The senescence-associated secretory phenotype is a characteristic that appears in senescent cells following their entry into a state of irreversible growth arrest. This phenotype is linked to aging and various age-related diseases and features a variety of bioactive molecules, including cytokines, chemokines, growth factors, and proteases. Although senescence helps prevent the proliferation of damaged or potentially cancerous cells, it can have both positive and negative impacts on the surrounding tissue and the entire organism. (Campisi 2005) Several stress factors can also induce cellular senescence. These stresses include telomeric dysfunction, mitochondrial deterioration, oxidative stress, DNA damage, chromatin disruption, and expression of certain oncogenes. (Di Micco et al. 2021) When cells undergo senescence, they experience significant changes in protein expression and release a variety of substances, including pro-inflammatory cytokines, chemokines, growth factors, and proteases. This complex secretion pattern, known as the senescence-associated secretory phenotype (SASP), has significant implications for the surrounding tissue environment. Initially, it was believed that the SASP emerged due to constant activation of the DNA damage response (Rodier et al. 2009), but subsequent investigations revealed that the primary regulating factor was p38MAPK. The suppression of p38MAPK activity, either through chemical inhibitors or RNA interference, resulted in a significant decrease in the levels of the majority of SASP factors that are secreted. A study by Freund et al. showed that p38MAPK regulates the SASP through activation of the transcription factor NF-κB, which is responsible for increased expression of many SASP genes. (Freund et al. 2011; Freund et al. 2010)

The senescence-associated secretory phenotype (SASP) can include a variety of pro-inflammatory and destructive molecules, such as TNFα, IL-6, IL-8, and others, that can cause damage to tissues and contribute to the development of diseases. (Coppé et al. 2010) It can also include chemokines that attract and activate immune cells, matrix metalloproteinases (MMPs) that break down tissue, TGFβ family members that can cause fibrosis and dysfunction of stem cells and progenitors (Xu et al. 2015), activins and inhibins that can induce dysfunction and dysdifferentiation of these cells, factors that can cause blood clotting and fibrosis, growth

factors that can promote tumor growth (Yang et al. 2017), bioactive lipids that contribute to inflammation and tissue dysfunction (Trayssac et al. 2018), micro-RNAs that can contribute to stem and progenitor cell dysfunction and inflammation, (Xu & Tahara 2013; Victoria et al. 2017) and exosomes that can carry cytotoxic and senescence-inducing molecules locally and systemically. (Davalos et al. 2010; Young & Narita 2009)

IL-6 and IL-8 are signaling molecules that have been found to have both cell-autonomous effects and paracrine effects on cellular processes. These molecules can cause cellular senescence within individual cells, thereby decreasing the likelihood of cancer. Additionally, they can influence nearby cells through the promotion of EMT, leading to the progression of tumors in the surrounding cellular environment. (Ortiz-Montero et al. 2017) Other molecules such as MCP-1 and IL-8 also appear to play a role in the cellular response aimed at recruiting immune cells, which helps to eliminate senescent cells and facilitate tissue repair. (Krizhanovsky et al. 2008) However, it's important to note that the activity of SASP factors may not always result in positive outcomes. While they aid in immune response activation, they can also cause unintended consequences such as promoting chronic inflammation and damaging the surrounding tissue.(Tripathi et al. 2021)

The complex interplay of these factors has been strongly implicated in the development and progression of various age-related diseases, emphasizing the importance of understanding the balance between senescence induction, immune response modulation, and inflammation regulation. A deeper comprehension of these mechanisms could lead to innovative therapeutic interventions that target these processes to alleviate the burden of age-related diseases and improve overall health and longevity.

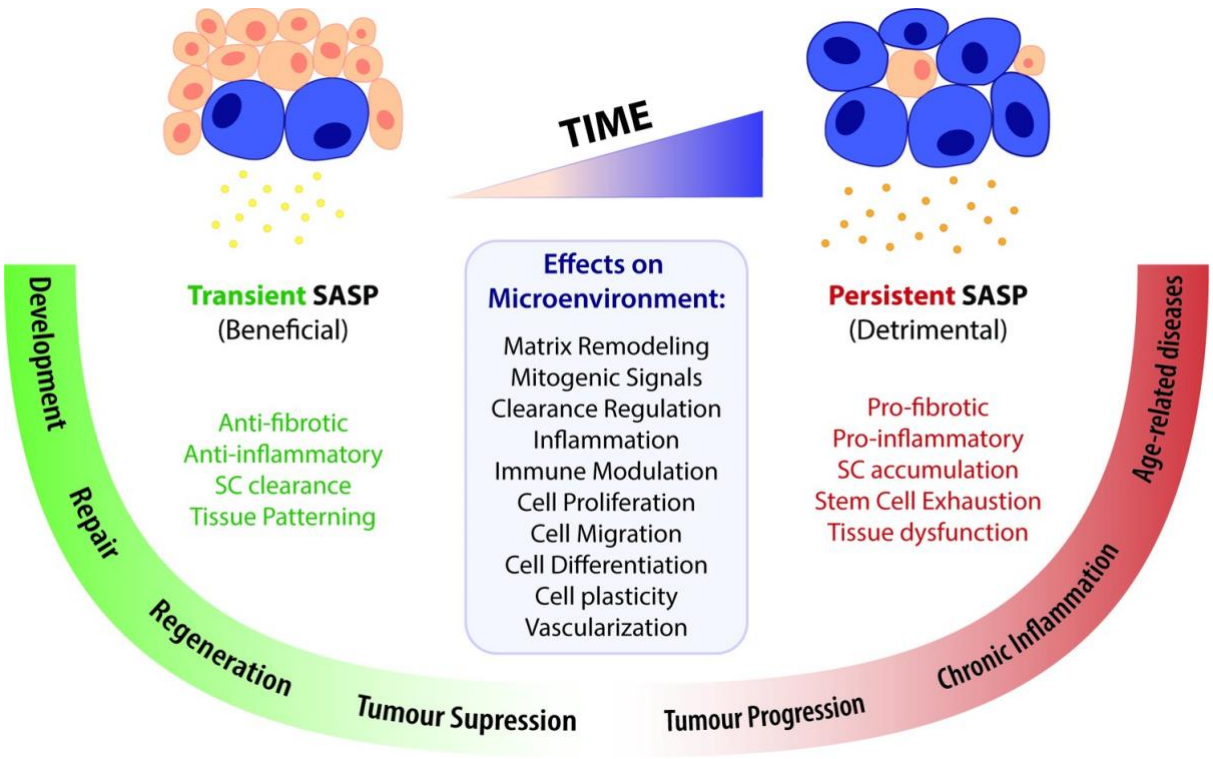

Fig. 6. : A time-gated model for the senescence-associated secretory phenotype (SASP)-mediated biological activities of cellular senescence.

The SASP has a wide range of effects on the surrounding microenvironment, including matrix remodeling, mitogenic signaling, clearance regulation, inflammation, immune modulation, cell proliferation, migration, differentiation, and plasticity, as well as vascularization. The effects of the SASP can be beneficial or detrimental, depending on the duration of the senescence program and the associated SASP response. A transient SASP is normally correlated with anti-fibrotic and anti-inflammatory effects, which favor tissue repair and regeneration, as well as immune-mediated clearance of SCs to avoid their accumulation and persistence. A short-term SASP is also fundamental for tissue patterning during development. In contrast, long-lasting SASP responses have detrimental pro-fibrotic and pro-inflammatory effects on the microenvironment, leading to tissue dysfunction and a broad spectrum of aging-related diseases. Persistent senescence responses can also deplete stem cell progenitor pools, impairing the repair/regenerative capability of affected tissues. The role of the SASP in cancer is more ambiguous, as SCs can both promote tumor suppression and tumor progression/invasiveness. However, current knowledge suggests that the SASP suppresses tumor growth in early stages,

while supplying pro-tumorigenic chronic inflammatory environments in later stages. (Figure from Paramos-de-Carvalho et al. 2021)

4 - Discovery of Senolytics and Senescence Cell Removal

A wealth of evidence in the past decade has established a link between the accumulation of senescent cells, age-related diseases, and their impact on lifespan and healthspan, leading to the pursuit of therapeutic agents that can specifically target these cells. An article by the Sharpless group in 2004 (Krishnamurthy et al. 2004) sparked J.L. Kirkland's interest in seeking interventions that eliminate senescent cells. From 2005 onwards, they attempted to create fusion proteins consisting of a senescent cell recognition site and a toxin to kill senescent cells. They conducted high-throughput screenings of human senescent non-senescentcent cells in search of chemicals that could selectively kill senescent cells, but to no avail. The screens proved unsuccessful due to the challenge of identifying appropriate controls. To prevent replication, parallel non-dividing and non-senescent cells had to be either serum-starved or confluent, but both methods can cause artificial effects. Alternativenon-senescentcent cells that divide could be used as controls, but doing so introduces a confounding factor since these cells must be compared to senescent cells, which cannot divide and therefore interferes with the interpretation of the results. They then shifted their focus to a hypothesis-driven, mechanism-based approach to drug discovery, which led to the development of the first senolytic drugs. Their strategy was based on the observation made by E. Wang in 1995 that senescent cells are resistant to apoptosis, and in some respects, senescent cells are similar to cancer cells that do not divide, including their resistance to apoptosis and metabolic shifts. The researchers discovered that small interfering RNA (siRNA) that reduced expression of BCL-xL or other BCL-2 family members was effective in triggering programmed cell death (apoptosis) in senescent versus non-senescent human umbilical vein endothelial primary cells (HUVECs). (Zhu et al. 2015) They identified 46 potentially senolytic compounds using bioinformatics approaches, including the tyrosine kinase inhibitor Dasatinib (D) which has been approved for clinical use in the United States since 2006, and Quercetin (Q), the naturally occurring flavonoid. (Kirkland & Tchkonia 2020)

Navitoclax is a B-cell lymphoma 2 (BCL-2) family protein inhibitor that was generated during the clinical development of ABT-737. Preclinical studies of ABT-737 revealed its poor bioavailability and physicochemical properties, which hindered the progress of clinical studies. (Tse et al. 2008) Preclinical studies have demonstrated that ABT-737 exhibits maximum efficacy when administered continuously on a daily basis. In pursuit of enhancing the drug's potency, pharmacokinetics, and pharmacodynamics, the modification of ABT-737's structure into ABT-263 is being pursued, which could potentially increase its anti-cancer potential. (Bruncko et al. 2007) One study has demonstrated a high affinity profile of navitoctowardsward BCL-2 family proteins (Chen et al. 2011) The regulation of the intrinsic apoptotic pathway by BCL-2 family proteins is crucial for the survival of cancer cells. It has been observed that multiple myeloma cell lines express high levels of anti-apoptotic proteins, such as BCL-2, BCL-XL, and BCL-W. To develop cancer treatments, one strategy is to encourage the natural process of programmed cell death. A transcriptomic analysis between senescent cells and proliferating cells revealed elevated expressionprosurvivalival/anti-apoptotic genes, such as Bcl-xL (a Bcl-2 family protein that regulates programmed cell death by inhibiting caspase activation). (Zhu et al. 2016) Therefore, BCL-2 family proteins that promote survival are potential targets for this therapeutic approach. A study has revealed the mechanism by which navitoclax induces apoptosis in cancer cells by disrupting the interaction between anti-apoptotic proteins and BH3 domain binding proteins family inhibitors, which have been explored as anti-cancer drugs. Navitoclax is one such inhibitor. As a result, BH3-only activators cause the translocation of BAX to the mitochondria, leading to the onset of mitochondrial outer membrane permeabilization (MOMP). (Han et al. 2019) The secretion of cytochrome c from the mitochondrial intermembrane space into the cytoplasm, triggered by MOMP, cascades into downstream signaling of intrinsic apoptosis through interactions with caspase proteins. (Bender & Martinou 2013) Despite this, the mechanism by which navitoclax mediates anti-tumor activity in various cancer types by recruiting BCL-2 family proteins is still not well understPreclinical

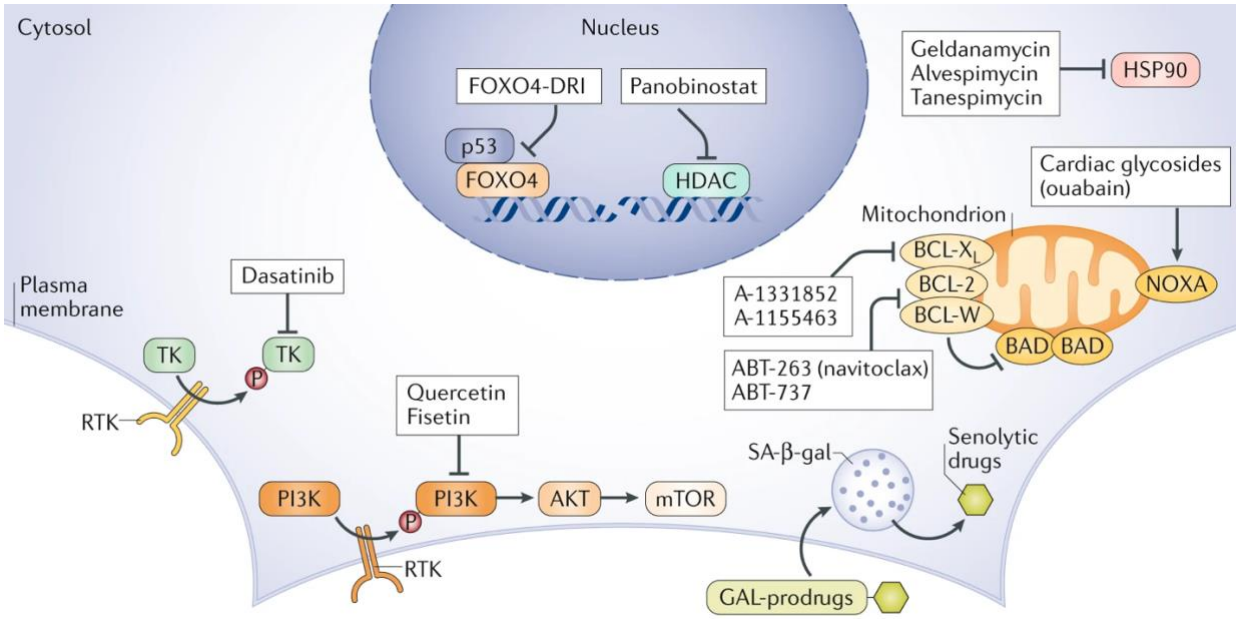

Fig. 7 : Senolytic therapeutic interventions.

The range of sensitivities exhibited by senescent cells in response to pharmacological treatments that can trigger their death is extensive. A variety of known mechanisms of senolytic action have been identified; specific compounds that target these pathways are also known. For example, dasatinib, when used alone or in combination with quercetin, can activate tyrosine kinase to induce the death of certain senescent cell types. Quercetin and fisetin are natural flavonoids that affect mTOR signaling. Inhibitors of the BCL-2 family of antiapoptotic proteins can promote mitochondrial-mediated death in senescent cells, a process that can also be initiated by cardiac glycosides like ouabain. HSP90 or HDAC inhibitors have also been suggested to selectively induce apoptosis in senescent cells. Additionally, the use of a small peptide can disrupt the binding of FOXO4 to p53, a process that occurs in senescent cells, and activate apoptosis. Galactose-conjugated senolytic prodrugs (GAL-prodrugs) are processed by SA-β-gal to selectively target senescent cells. (Figure from Di Micco et al. 2021)

4-1-Preclinical applications of senolytic drugs

An article published on senolytics highlighted that the combination of D + Q effectively decreases the burden of senescent cells in multiple tissues and enhances the function of naturally aged animals. (Zhu et al. 2015) Drugs such as D + Q, fisetin, navitoclax, and others have been demonstrated to alleviate multiple conditions through senolytic therapy. The intervention resulted in various positive outcomes in different models, including improved

cardiac function, enhanced vascular reactivity, decreased vascular calcification, restored vascular reactivity, decreased loss of intervertebral disk glycosaminoglycans and spondylosis, decreased gait disturbance, improved pulmonary function, reduced pulmonary fibrosis, decreased liver fibrosis, decreased insulin resistance, improved cognitive function, reduced neuroinflammation, improved neurogenesis, decreased uterine fibrosis, increased healthspan and lifespan in old mice. (Kirkland & Tchkonia 2017; Xu et al. 2018; Palmer et al. 2019; Chang et al. 2016; Lewis-McDougall et al. 2019; Roos et al. 2016; Schafer et al. 2017; Moncsek et al. 2018; Ogrodnik et al. 2021; Ogrodnik et al. 2017; Kirkland et al. 2017) D + Q and F have been demonstrated to decrease the abundance of senescent cells in vivo without damaging non-senescent cells. (Xu et al. 2018; Yousefzadeh et al. 2018) D + Q reduces the burden of senescent cardiac cells, as demonstrated by decreased levels of p16Ink4a, and partially reverses age-related enlargement of cardiomyocytes and fibrosis in the left ventricle. (Lewis-McDougall et al. 2019)

(Tse et al. 2008) were the first to show the inhibitory effect of navitoclax on anti-apoptotic proteins of the BCL-2 family for various tumor therapies including SCLC. Navitoclax was found to have a high affintowardsward BCL-2 and BCL-XL proteins, but not to MCL-1 protein. n vivo study on SCLC xenograft models found that daily oral dosing of navitoclax effectively attenuates tumor progression. Further study conducted aimed to elucidate the efficacy of navitoclax in several SCLC cell lines (in vitro) and xenograft models (in vivo). Navitoclax efficacy on different SCLC xenograft models was varied, yet the treatment showed the induction of BAX translocation, cytochrome c release and caspase-3 stimulation were dose-dependent through the cellular level. Nonetheless, the results displayed a good correlation between in vitro cellular potency and in vivo tumor efficacy. (Shoemaker et al. 2008)

4-2-Clinical Applications of Senolytic Drugs

With encouraging outcomes in preclinical studies, more than 20 clinical trials of senolytic therapies have been completed, are ongoing, or are planned. (Kirkland & Tchkonia 2020). As the full side effects of senolytics in humans are not yet understood, the initial clinical trials are currently being conducted on patients suffering from severe health conditions, such as diabetic kidney disease, Alzheimer's disease, frailty, and IPF, in order to optimize the benefit-risk ratio. An open-label pilot study was conducted, involving 14 patients with IPF who were treated with

intermittent D + Q on 3 d per week for 3 weeks. (Jn et al. 2019). The results indicated that senolytics enhanced physical function in frail patients, and a post hoc analysis of a study involving 20 patients with IPF revealed that urine levels of the 'geroprotective' factor α-Klotho were elevated following oral D + Q treatment compared to before treatment. (Zhu et al. 2022). A 3-day course of oral D + Q administered to 9 patients with diabetic kidney disease was sufficient to decrease the burden of senescent cells, inflammation, fibrosis, and circulating SASP factors for at least 11 days after the last dose of senolytics, indicating target engagement and suggesting that an intermittent dosing regimen may be effective in humans (Hickson et al. 2019). A clinical trial of Navitoclax was conducted for a year involving 47 patients with small-cell lung cancer or pulmonary carcinoids. (Gandhi et al. 2011). In this study, intermittent and continuous treatments with navitoclax were administered to evaluate the safety profile and pharmacokinetics of the drug in humans. Navitoclax has been found to affect the expression of the BCL-2 protein, which can be reflected by the increase in pro-gastrin releasing peptide (pro-GRP) levels. The pro-GRP was found to function as a proxy for BCL-2 amplification and its fluctuations were linked to changes in tumor size. (Tahir et al. 2010; Gandhi et al. 2011). A phase 1 dose-escalation study was conducted on adult patients with various relapsed or refractory lymphoid tumors, including chronic lymphoid leukemia (CLL), diffuse large B-cell lymphoma (DLBCL), mantle-cell lymphoma (MCL), follicular lymphoma (FL), small lymphocytic lymphoma (SLL), classic Hodgkin's lymphoma, NK/T-cell lymphoma, and marginal-zone lymphoma. In this study, intermittent and continuous dose-escalation regimens were administered for 21 days, and patients were enrolled in different regimen groups. Researchers discovered a new pharmacological effect of navitoclax on the treatment of lymphoid tumors, which was characterized by its ability to significantly decrease platelet levels and T-cell levels in the peripheral blood, by targeting the BCL-2 and BCL-XL proteins with high affinity. (Wilson et al. 2010). Another phase I clinical trial carried by (Roberts et al. 2012) on 29 patients with relapsed or refractory CLL. A schedule of dose escalation for navitoclax was initiated, in which 15 patients were administered navitoclax for 14 days at dosages of 10, 110, 200, or 250 mg/day, while another 14 patients received navitoclax for 21 days at dosages of 125, 200, 250, or 200 mg/day in each 21-day cycle. Their research showed that Navitoclax exhibits potent solitary-agent activity against circulating, nodal, and splenic disease in patients with CLL. A significant decrease in the number of leukemic cells was observed within a few days, and maximum therapeutic responses were typically seen within the first 4 months, particularly in terms of circulating lymphocyte levels. Preclinical studies of navitoclax in combination with lymphocytic tumors demonstrated thrombocytopenia due to BCL-XL

inhibition, which is consistent with the previously observed results. (Roberts et al. 2012). A phase IIa clinical trial of navitoclax has been conducted in patients with recurrent and progressive small-cell lung cancer, as the therapeutic options for this condition are limited. To avoid the risk of severe thrombocytopenia, a phase I study was conducted before phase IIa to determine the optimal and safe dose of navitoclax for patients with various solid tumors. The resulting acceptable dose of 325 mg per day was determined and demonstrated that the severity of thrombocytopenia could be managed in phase II. (Rudin et al. 2012). The study found that the development of major adverse events corresponded to the findings reported earlier by (Gandhi et al. 2011). It was observed that approximately 40% of patients in grades III-IV experienced low blood platelet count as a side effect of navitoclax. (Rudin et al. 2012).

5-Senescence and Cancer

Cancer is the second leading cause of death worldwide and the leading cause for middle aged and elderly people in industrialized countries. In accordance with increasing number of individuals living to older ages, the number of new cancer cases increase, especially among adults aged ≥60 years. (White et al. 2019). Although the reason for this increase cannot be defined by a single path, contribution of somatic mutations is non-negligible as they in turn can affect several biological events including cell division, replication or repair of DNA and genomic instability, especially during aging. (Vijg 2014; Vijg & Montagna 2017; Risques & Kennedy 2018). Throughout an organism's lifespan, cells are subjected to a diverse array of stresses, including both intrinsic and extrinsic factors. In cases where cell damage is irreparable, cells may undergo programmed cell death, known as apoptosis. These cells may either undergo senescence or stably exit the cell cycle. Senescent cells can either have positive or negative effects in various physiological and pathological situations. Furthermore, oncogenic signaling that is excessive or the loss of specific tumor suppressor genes can trigger senescence. (Serrano et al. 1997; Krtolica et al. 2001; Liu & Hornsby 2007). Biologically speaking, senescence has traditionally been viewed as a potent tumor-suppressive mechanism because of its persistent cell cycle arrest. Senescent cells may have a beneficial role by suppressing tumors through p53 (Rufini et al. 2013) or p16 (Rayess et al. 2012) pathways (Campisi 2005). Senescent cells can also participate in local anti-tumor immunity. The process of inducing senescence in liver tumor cells by restoring p53 expression promotes the clearance

of these cells in in vivo hepatocellular carcinoma models (Xue et al. 2007) These findings show that senescence arrest, both prior to and following the formation of cancer, can be advantageous in preventing malignancies. As a result, several drugs have been developed to induce senescence in cancer cells. CDK4/6 inhibitors are currently being employed in preclinical and clinical trials for the treatment of solid and liquid tumors (Geoerger et al. 2017). It has been also shown that cytokine induced senescence can stably arrest cancer cells through activation of IFN-JAK1-STAT1 signaling pathway by inducing p16 and p21 to act as a tumor suppressor mechanism (Brenner et al. 2020).

Cellular senescence is an example of evolutionary antagonistic pleiotropy, which is a special cellular program that can have both beneficial and detrimental effects, depending on the pathophysiological context (Gorgoulis et al. 2019). Moreover, senescent cells actively produce and release a large quantity of pro-inflammatory molecules, including cytokines, chemokines, growth factors, and proteases, which collectively impact neighboring cells, a phenomenon known as the senescence-associated secretory phenotype (SASP) (Coppé et al. 2008). The anti-cancer treatments such as chemotherapy are intended to decrease the size of tumors by triggering the programmed cell death of cancer cells. However, these approaches often lead to significant side effects, especially the induction of cellular senescence names as therapy-induced senescent (TIS), which can be predominant in the tumor micro environment. The buildup of senescent stromal cells caused by therapy can have negative consequences and reduce the effectiveness of cancer treatment, and the byproduct is harmful to the organism and contributes to the development of the SASP. On the other hand, some cancer cells can also gain stem-like properties through exposure anti-cancer agents and enter a senescence state. The cell-autonomous mechanism known as senescence-associated stemness allows for highly aggressive growth upon escape from cancer cell growth arrest, while promoting the adaptability of cancer cells and facilitating disease recurrence in the post-treatment phase (Liu et al. 2022). Two of the most prominent pro-inflammatory cytokines in the SASP, IL-6 and IL-8, are responsible for the emergence and sustenance of cancer stem cells (CSCs). IL-6 plays a crucial role in converting non-CSCs into CSCs by activating the IL-6-JAK1/STAT3 signaling pathway (Kim et al. 2013). On the other hand, IL-8 promotes the formation of CSCs through activation of the PI3K/Akt signaling pathway (Ma et al. 2021). Therefore, SASP may also act as a driver to escape from senescence by secretion of factors that can contribute to tumor growth in a non-cell autonomous manner (Saleh et al. 2018). The study carried out by Bianchi-Frias and colleagues revealed that the aged microenvironment in prostatic cancer regulates the growth of

tumor cells and metastatis contributing to the progression of prostate cancer (Bianchi-Frias et al. 2019). Another study demonstrated the age dependent splenic immune influence on tumor development from adolescent, young adult, middle-aged and old mice comparisons (Beheshti et al. 2015).

Recent studies have focused on developing therapeutic agents that can inhibit the harmful effects of senescent cells, which can disrupt the organism's homeostasis due to their long-term persistence (Gorgoulis et al. 2019; Myrianthopoulos et al. 2019). Senotherapy, a promising approach that selectively targets senescent cells, has emerged to date (Myrianthopoulos et al. 2019; Kim & Kim 2019; Raffaele & Vinciguerra 2022). The application of ABT-263 following etoposide or doxorubicin treatment led to a notable and prolonged suppression of tumors, which was achieved by obstructing the interaction between BCL-xL and BAX in lung and breast cancer cell-bearing animals. Senolytic therapy, when used in conjunction with conventional anti-cancerr treatment, has been shown to enhance the therapeutic outcomes (Saleh et al. 2020). However, it appears that the effects of ABT-263 may not be universal, as it only causes rapid and selective cell death in a subset of chemotherapy-treated cancer cells (Shahbandi et al. 2020). Stopping the growth and development of SASP is also another viable solution to curtail the deleterious effects of senescent cells. Metformin, an antidiabetic drug, has been shown to effectively regulate the secretion of pro-inflammatory factors in the SASP pathway. This is accomplished by obstructing the nuclear translocation of NF-κB, impeding the phosphorylation of IκB and IKKα/β, and prohibiting the activation of NF-κB, all of which are critical for full-spectrum SASP expression (Moiseeva et al. 2013).

6- Research Questions and Objectives

Regarding this context, several questions emerged.

What are the significant changes in the immune system with age, and how do these changes influence its response to immune challenges?

How does cellular senescence, a natural aging mechanism, contribute to immune dysfunction in the elderly population, and what are its key characteristics?

What could be the potential outcomes of senescent cell depletion prior to immunization on immune responses triggered by vaccines?

During my thesis, I focused on this later question. Our main objectives were to decipher:

1. The potential consequences of senescent cell removal (ABT-263) on vaccine efficacy (humoral and cellular immune responses) in older individuals (the mouse system was used),

2. The consequences of senolysis on anti-tumor responses of vaccinated individuals.

Results

The age-related immune decline is a critical issue for vaccine-based prophylactic and therapeutic approaches in older adults. The main objective of my PhD thesis was to investigate the effect of senolysis on vaccine efficacy in the elderlies. To address this issue, we compared the ability of two different vaccines to induce immune responses in young mice and old mice, pretreated or not with ABT-263 (Navitoclax). We used ABT-263 as it specifically targets Bcl-2 expressing (senescent) cells and because it is used in the clinics. Our initial objective was to validate the efficacy of ABT-263 (i) to deplete senescent cells in vitro and in vivo and (ii) to lower SASP. Very few studies have analyzed in details the nature of immune cells impacted by ABT-263. This question is of crucial importance. In this study, we attempted to better understand the effects of ABT-263 treatment on immune cell number and functions. The second objective was to investigate the consequences of senescent cell depletion prior to vaccination on immune response. To address this question, we depleted senescent cells long before vaccination in order to eliminate any potential confounding effects due to senolytic interference with the ongoing immune response. Two different adjuvants were used and ovalbumin (OVA) was used as the antigen. Lastly, the efficacy of the immune response of vaccinated mice against cancer development was studied. To this end, we used a preclinical melanoma model (OVA-expressing B16F10 cells). We initiated this study without any starting hypotheses since the depletion of pre-existing senescent cells might have both positive and negative outcomes on vaccine effectiveness. The data are presented below. The format utilized is the one used for the manuscript submitted in Aging Cell and entitled "*Depletion of pre-existing B cell lymphoma (Bcl)-2 -expressing senescent cells before vaccination impacts antigen-specianti-tumorumor immune responses in old mice*". This manuscript is presently accepted for publication (Aging Cell). The main message of this study is that depletion of senescent cells modifies immune responses upon vaccination and caution should be taken for the use of senolytics in vaccine-based cancer therapy.

Depletion of pre-existing B cell lymphoma-2-expressing senescent cells before vaccination impacts antigen-specific antitumor immune responses in old mice

Running title: Age-related senescent cells on vaccine effectiveness

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SUMMARY

The age-related decline in immunity reduces the effectiveness of vaccines in older adults. Immunosenescence is associated with chronic, low-grade inflammation and the accumulation of senescent cells. The latter express Bcl-2 family members (providing resistance to cell death) and exhibit a pro-inflammatory, senescence-associated secretory phenotype (SASP). Pre-existing senescent cells cause many aging-related disorders, and so therapeutic means of eliminating these cells have recently gained attention. The potential consequences of senescent cell removal on vaccine efficacy in older individuals have not yet been reported. We used the Bcl-2 family inhibitor ABT-263 to investigate the effects of pre-vaccination senolysis on humoral and cellular immune responses in old mice. Two different ovalbumin (OVA)-containing vaccines (containing a saponin-based or a CpG oligodeoxynucleotide adjuvant) were tested. ABT-263 depletes senescent cells (apoptosis) and ablates the basal and lipopolysaccharide-induced production of SASP-related factors in old mice. Depletion of senescent cells prior to vaccination (prime/boost) had little effect on OVA-specific antibody and T cell responses (slightly reduced and augmented, respectively). We then used a preclinical melanoma model to test anti-tumorumor potential of senolysis before vaccination (prime with the vaccine and OVA boost by tumor cells). Surprisingly, ABT-263 treatment abrogated the vaccine's ability to protect against B16 melanoma growth in old animals, an effect associated with reduced antigen-specific T cell responses. Some, but not all, of the effects were age-specific, which suggests that pre-existing senescent cells were partly involved. We conclude that the depletion of senescent cells modifies immune responses to vaccines in some settings and that caution should be taken when incorporating senolytics into vaccine-based cancer therapies.

KEYWORDS: aging, cellular senescence, senolytics, Bcl-2, vaccination, immune responses, tumor growth

1 – INTRODUCTION

Vaccines are less effective in older people, due to a functional decline in innate and adaptive immune responses (Nikolich-Žugich 2018; Crooke et al. 2019). Changes in the numbers and functional activity of various immune cells (ranging from antigen-presenting cells to effector cells) contribute to age-related defects in immunity (Nikolich-Žugich 2014). The cellular and molecular impairments affecting the innate immune system include dysregulated signaling cascades and altered transcriptional programming, which notably reduce the host's ability to respond to adjuvants (e.g. pathogen-associated molecular patterns). With regard to the adaptive immune system, the involution of primary lymphoid organs (thymus) and reduced cellularity in the bone marrow impair B and T lymphopoiesis and thus contribute to an age-associated fall in the relative abundance of naive T cells (mostly $CD8^+$) and B cells (Nikolich-Žugich 2014; Nikolich-Žugich 2018; Crooke et al. 2019). Aged B and T cells also display intrinsic changes in their activation threshold, effector capacity, homeostasis, and trafficking (Nikolich-Žugich 2014; Frasca et al. 2017; Lee, Fra-Bido, et al. 2022) Along with age-related changes in the microenvironment (lymphoid organs and non-lymphoid tissues) and levels of circulating factors, these changes weaken cellular (effector) immune responses and humoral immune responses in older individuals. A decline in the immune response with chronological aging is associated with a state of chronic, low-grade inflammation status known as inflammaging, which is characterized by elevated concentrations of pro-inflammatory cytokines like interleukin (IL)-6 and IL-1□. The accumulation of senescent cells is an important contributor to inflammaging in older individuals (Baker et al. 2011; Xu et al. 2018; Cai et al. 2020).

Cellular senescence is a permanent state of cell cycle arrest that occurs in proliferating cells exposed to various types of damage or stress, including inflammatory and metabolic insults, DNA damage, and telomere shortening. Senescent cells overexpress anti-apoptotic molecules (such as the B cell lymphoma-2 (Bcl-2) family of proteins) and are resistant to cell death (Zhu et al. 2016). Although senescent cells do not proliferate, they are transcriptionally and metabolically active and secrete a range of pro-inflammatory and immunomodulatory cytokines, growth factors and proteolytic components as part of the senescence-associated secretory phenotype (SASP), with potent effects on the surrounding cells and tissues (Childs et al. 2017; Gorgoulis et al. 2019; Birch & Gil 2020). Cellular senescence has various physiological roles throughout life. Firstly, it has an essential role in tissue and organ formation

during embryonic development. Throughout adulthood, cellular senescence exerts functions in wound healing and protects against oncogenic insults.

With chronological aging, senescent cells accumulate as a result of several factors including elevated levels of stress factors and defective immune surveillance (Ovadya et al. 2018). The progressive accrual of senescent cells is responsible, partially via the induction of a chronic SASP, for many aging-related disorders: chronic inflammation, a decline in the regenerative potential and function of tissues, degenerative disorders, and cancer (Van Deursen & M 2014). Experiments on mouse models have shown that the removal of senescent cells (using pharmacological and/or genetic approaches) prevents the onset of (or at least attenuates) age-related disease pathologies and increases life span and fitness (Chang et al. 2016; Baker et al. 2016; Farr et al. 2017; Ogrodnik et al. 2021). Therefore, therapeutic strategies designed to target and deplete senescent cells (i.e. senolytics) have gained attention in the field of aging research and hold great promise for the treatment of age-related dysfunction (Kirkland & Tchkonia 2020; Chaib et al. 2022). Senolytic agents include natural, diet-derived factors (such as certain flavonoids, albeit with nonspecific activity) and synthetic inhibitors of the anti-apoptotic molecules that are upregulated in senescent cells. Senolytic drugs can be divided broadly into two main classes, depending on the mechanism of action; kinase inhibitors and Bcl-2 family inhibitors (Kirkland & Tchkonia 2020). As a result of the promising research in mouse models mentioned above, senolytics have been used in the clinic to treat senescence-related diseases (Di Micco et al. 2021; Chaib et al. 2022). For safety reasons, the first senolytic treatment to be studied in humans was a combination of dasatinib (a multi-tyrosine kinase inhibitor) and quercetin (a flavonol that targets the PI3K/AKT pathway) (Kirkland & Tchkonia 2020). This cocktail was well tolerated and was shown to remove senescent cells, decrease local and systemic inflammation, and alleviate physical dysfunction in idiopathic pulmonary fibrosis (Jn et al. 2019; Hickson et al. 2019). These results prompted the initiation of large clinical trials of treatments targeting fundamental aging processes and senescence-related diseases.

With regard to the clinical value of senolytic drugs, it is essential to investigate the latter's potential effects on vaccine efficacy. To our surprise, we were unable to find any publications on the consequences of senolytic treatment on vaccine efficacy in old mice. In view of the putative harmful effects of senescent cells on the immune system (Palacio et al. 2019; Lorenzo et al. 2022), one can speculate that senescent cell depletion might lead to an increase in vaccine efficacy. In contrast, one must bear in mind that certain senescent immune and/or stromal cells might have key roles in immune responses to vaccines. In the present study, we investigated

the effects of senolysis (using the drug ABT-263) on the humoral and cellular immune responses in vaccinated, old mice and (control) young mice. ABT-263 targets the Bcl-2 family of prosurvival proteins (Zhu et al. 2016) and is already used to treat cancer in the clinic. We sought to determine whether or not ABT-263 would interfere with the immune response to the administration of two difference ovalbumin (OVA)-containing vaccines. One vaccine contained Quil-A® (a saponin-based adjuvant known to induce antibodies in aged mice) and the other contained a CpG oligodeoxynucleotide (ODN) adjuvant. To study the effect of senolysis on anti-tumorumor response, OVA-vaccinated mice were grafted with OVA-expressing B16 melanoma cells (antigenic boost by tumor cells). We found that ABT-263 treatment depleted senescent cells and abrogated the production of systemic, SASP-related factors in old mice. In the classical vaccine prime/boost system, and compared to vehicle-treated old animals, ABT-263 treatment had little effect on antigen-specific antibody (slightly reduced) and T cell (slightly augmented) responses. In the vaccine prime and tumor boost model, ABT-263 treatment was associated with low antibody production and T cell response, although these effects were not statistically significant. Strikingly, the depletion of senescent cells prior to vaccination was associated with a low vaccine's ability to restrict tumor growth. Some (but not all) of these effects were age-specific. We conclude that the senolytic drug ABT-263 influences the immune response and strongly affects anti-tumorumor response in vaccinated, old mice.

2 - RESULTS

ABT-263 inhibits lipopolysaccharide (LPS)-induced, SASP-related cytokine production by old splenocytes in vitro.

We first assessed the effects of senescent cell depletion on the production of SASP-related pro-inflammatory cytokines by old murine immune cells *ex vivo*. To this end, cells were isolated from the spleen – a major secondary lymphoid organ involved in successful immune responses. Even though old (22-month-old) mice had a larger spleen, the number of splenocytes collected was lower than in young (2-month-old) mice (Supplementary Figure. 1A). Although young and old animals did not differ significantly in terms of the frequencies of splenic T cells and dendritic cells, old mice had lower proportions of B cells and natural killer (NK) cells and higher proportions of macrophages and neutrophils (Supplementary Figure. 1B). Lysosomal senescence-associated β-galactosidase activity (SA-β-Gal) is a good marker of cellular senescence (Cai et al. 2020). Using flow cytometry with the fluorogenic β -galactosidase substrate C12FDG, we detected β -galactosidase activity in splenocytes from old mice and in none of the splenocytes from young mice (Figure 1A, Figure 1B and Supplementary Figure 1C for the gating strategy). Most of the β-galactosidase activity was detected in CD45-positive (i.e. hematopoietic) cells.

Senescent splenocytes were depleted by exposure to ABT-263, which is toxic at high doses in non-senescent cells. To avoid cytotoxicity as a confounding factor, we determined the ABT-263 dose level at which >90% of the splenocytes collected from young adults were viable after 24 h of treatment. As a result, cells were treated with 1 μM ABT-263 in subsequent experiments (Supplementary Figure. 1D). The proportion of old splenocytes expressing SA-β-Gal was strongly reduced after ABT-263 treatment (Figure 1B, *lower* panel). After having established ABT-263's ability to eliminate senescent cells *ex vivo,* we then stimulated ABT-263-treated splenocytes with LPS - a well-known Toll-like receptor 4 agonist. As expected, LPS induced the production of IL-6, IL-1β, monocyte chemoattractant protein 1 (MCP-1), IL12p40, and IL-10 (Figure 1C). Upon LPS stimulation, the production of the prototypical SASP-related factors IL-6, IL-1β and MCP-1 was higher in splenocytes from old animals than in splenocytes from young animals (Figure 1C, *upper* panel). Regarding immune regulatory cytokines, the LPS-

induced production of IL-12p40 was identical in young and old splenocytes, whilst that of IL-10 was lower in old splenocytes (Figure 1C, *lower* panel). ABT-263 treatment prior to LPS stimulation dramatically restricted the secretion of IL-6, IL-1β and MCP-1 by old splenocytes but not by young splenocytes. Intriguingly, ABT-263 treatment was associated with greater IL-12 production by young and old splenocytes but lower production of IL-10 (a cytokine with pleiotropic immunosuppressive effects) by the young splenocytes. These results show that the *ex vivo* depletion of ABT-263-sensitive cells among old splenocytes is associated with lower production of LPS-induced, SASP-related cytokines. The results also suggest that ABT-263 has indirect effects, independently of the direct depletion of senescent cells.

ABT-263 inhibits senescence-associated signatures in old mice *in vivo*

We next analyzed the effect of ABT-263 on senescence signatures *in vivo* and *ex vivo*. To this end, old animals were gavaged for four days with 50 mg/kg ABT-263 (a standard protocol in the mouse system (Chang et al. 2016)) and euthanized on day 5 and 9 (Figure 2A). Beta-galactosidase staining of spleen sections from old mice revealed a higher number of SA-β-Gal-positive cells, relative to young counterparts (Figure 2B, *left* panel). It is noteworthy that most SA-β-Gal-positive cells were located in the marginal zone around the lymphoid follicles, a region with high frequencies of B cells and macrophages. ABT-263 treatment led to a reduced proportion of SA-β-Gal-positive cells in old spleen (Figure 2B, *right* panel). This associated with enhanced expression of cleaved caspase-3 in spleen as assessed by immunohistochemistry (Figure 2C, *upper* panel). The presence or absence of the prototypical senescence marker $p16^{INK4a}$ (referred to hereafter as p16) was then assessed using immunohistochemistry. It was clear that p16-positive cells accumulated in old spleens and were located at the same sites as SA-β-Gal-positive cells (Figure 2C, *middle left* panel). Few p16-postive cells were detected in young spleens (Supplementary Figure. 2A). Old mice treated with ABT-263 had fewer p16-positive cells than controls did (Figure 2C, *medium right* panel). The immunohistochemistry experiments indicated that levels of Bcl-2 and Bcl-xL expression were far higher intense in the spleen of old mice than in the spleen of young counterparts (Supplementary Figure. 2B). ABT-263 treatment resulted in lower intensities of Bcl-2 and Bcl-xL labelling in aged spleen (Figure 2C, *lower* panels). To substantiate ABT-263's impact *in vivo*, we analyzed the drug's effect on senescent $CD4^+$ T cells (defined as $CD44^{high}$ $CD62L^{low}$ $PD1^+$ $CD153^+$ in the mouse system

(Yoshida et al. 2020)). As depicted in Figure 2D and Supplementary Figure. 2C (for the gating strategy), senescent $CD153^+$ $CD4^+$ T cells were detected in old spleen but not in young spleen. ABT-263 treatment reduced the frequency of $CD153^+$ $CD4^+$ T cells, albeit not significantly (P = 0.20). Analysis of annexin V expression revealed a higher (almost 2 fold, P = 0.082) level of apoptosis in CD45-positive cells collected from ABT-263-treated old mice relative to controls (Figure. 2E). B cells and T cells were particularly affected (Figure. 2E, Supplementary Figure. 2D and not shown). It is noteworthy that 4 days post-treatment, ABT-263 did not overtly influence the proportion of splenocytes in old mice, although a slight reduction of macrophages was noted (Supplementary Figure. 2E).

Aging is associated with elevated systemic levels of inflammatory cytokines, and senescent cells are a major contributor to the inflammaging process (Cai et al. 2020). As expected, serum IL-6 and MCP-1 levels (IL-1 β was not detected) were much higher in old mice than in young mice (Figure 2F). ABT-263 treatment strongly reduced the blood IL-6 and MCP-1 levels. In contrast, ABT-263 treatment did not have a significant effect on IL-12p40 expression (IL-10 was not detected). Spleens from ABT-263-treated mice were collected, and the splenocytes were stimulated with LPS. The levels of IL-1 β and MCP-1 production were higher in splenocytes from old animals than in splenocytes from young animals (Figure 2G). The same was true (albeit to a lesser extent) for IL-6 and IL-12p40. The anti-inflammatory cytokine IL-10 was less induced in old splenocytes. Remarkably, *in vivo* ABT-263 treatment significantly reduced the production of IL-1β, IL-6 and (albeit to a lower extent) MCP-1 in old mice. In contrast, *in vivo* ABT-263 treatment had no effect on IL-12p40 production by old splenocytes and was associated with greater IL-10 production. Taken as a whole, these data showed that the *in vivo* removal of ABT-263-sensitive cells in old mice was associated with the impaired production of SASP-associated cytokines

ABT-263 treatment prior to vaccination has a small impact on humoral and cellular immune responses in old mice

To investigate the effect of senolysis on the immune response elicited by vaccination, old mice were treated twice with ABT-263 during four days with a four-days interval between the two treatments (Figure 3A, *upper* panel). Mice were inoculated with the OVA Quil-A® vaccine three days after the second ABT-263 treatment and, a week later, received a booster vaccination. Samples were collected 13 days after the booster. To study the primary humoral response, we also collected serum after priming. ABT-263 treatment did not significantly influence body weight gain in young animals and body weight loss in old animals (Figure 3A, *lower* panel). At sacrifice, the serum levels of IL-6 and MCP-1 in old mice were lower after ABT-263 treatment - indicating that senolysis had a long-term effect on chronic low-grade inflammation (Figure 3B and Supplementary Figure 3A). Accordingly, mRNA expression of the genes coding for p16 and Bcl2 was lower in ABT-263-treated, old mice than in controls (Supplementary Figure 3B).

The antibody production induced by vaccination is a key parameter in vaccine effectiveness. Primary humoral immune responses and responses evoked by memory B cells are both altered in older individuals. We first analyzed the effect of senolysis on the primary humoral response. The anti-OVA IgM response was much higher in old mice than in young animals, whilst the opposite was seen for anti-OVA IgG (Figure 3C). This finding confirmed the presence of a class-switching defect in old animals. Depletion of senescent cells prior to vaccination did not affect anti-OVA IgM and IgG production in old and young mice. The secondary antibody response was then evaluated after the boost (Figure 3D). The anti-OVA IgM response was similar in old mice and young animals, whereas the anti-OVA IgG response was still less intense in old mice. In both age groups, ABT-263 treatment did not significantly (slight reduction) modify the levels of OVA-specific IgM or IgG. IgG1 (a T helper 2-related isotype) was the dominant isotype in this context (Supplementary Figure 3C). To examine the OVA-specific T cell response, draining lymph node (LN) cells and splenocytes were restimulated with whole OVA or OVA peptides. LN cells (old but not young mice) and splenocytes (young but not old mice) collected from vaccinated animals produced interferon gamma (IFN-y in response to whole OVA and to the predominantly MHC class I-restricted OVA peptide SIINFEKL (Supplementary Figure 3D). Of note, IFN-y was not (LN) or hardly (spleen) detected in response to the MHC class II-restricted OVA peptide ($OVA_{223-239}$, not shown).

ABT-263 treatment had no significant effect on IFN-y production by restimulated LN cells and spleen (slight augmentation) cells (Figure 3E). Hence, ABT-263 treatment prior to vaccination has no significant effect on humoral and cellular immune responses in old mice.

ABT-263 treatment prior to vaccination accelerated tumor growth in aged mice

To analyze the potential effects of senolysis on anti-tumorumor response resulting from vaccination, CpG ODN was used as an adjuvant. Briefly, senescent cells were depleted (as shown in Figure 3) and mice were primed with OVA + CpG ODN three days after the discontinuation of ABT-263 treatment (Figure 4A). Ten days after this priming, OVA-expressing B16 tumor cells were engrafted. This vaccination regimen (comprising priming only and then a challenge with B16-OVA) was effective in abrogating tumor growth in young animals (Supplementary Figure. 4A). Of note, ABT-263 treatment had no effect on tumor growth in unvaccinated animals. The effect of ABT-263 was then studied in young and old vaccinated mice. At the time of sacrifice (day 23 after tumor inoculation), mRNA expression of the genes coding for p16 and Bcl2 was lower in ABT-263-treated, old mice than in controls (Supplementary Figure 4B). The OVA-specific IgM response was higher in old animals than in young animals (Figure 4B). In contrast, there was no difference in the IgG response (mostly IgG1) between the two age groups. Although ABT-263 treatment prior to vaccination had no effect on the IgM response, it significantly improved the IgG response (both IgG1 and IgG2a) response in young animals. Strikingly, ABT-263 treatment in old animals resulted in a lower (though not significant) IgM and IgG responses (Figure 4B and Supplementary Figure. 4C). The T cell response was then examined after restimulation with whole OVA and OVA peptide. Compared with LN cells from young mice, LN cells from vaccinated old mice produced more IFN-□ in response to both OVA and OVA peptide (Figure 4C, *upper* panel and Supplementary Figure. 4D). When the spleen cell response was examined, the contrary was observed (Figure 4C, *lower* panel). In response to OVA and OVA peptide, ABT-263 treatment decreased (albeit not significantly) IFN-□ production by old, but not young, LN cells (Figure 4C). On the contrary, ABT-263 treatment reduced (albeit not significantly) IFN-y production by young, but not old, splenocytes. Lastly, we investigated the potential effect of ABT-263 treatment on subcutaneous B16-OVA tumor growth. Relative to young animals, B16 melanoma tumor growth was delayed in old mice (Figure 4D). In this setting (a single immunization 10 days

before the B16 graft), vaccinated old mice were partially protected against subcutaneous tumor outgrowth. Surprisingly, ABT-263 treatment of old mice completely abrogated the vaccine's protective effect: tumor growth was faster in treated, vaccinated animals than in non-vaccinated animals. The observation was slightly different in young mice; after ABT-263 treatment, vaccinated young mice were still protected but to a lesser extent. We conclude that ABT-263 treatment before vaccination influenced the immune response in old mice (with an impaired humoral and cellular response) and dramatically increased tumor outgrowth. Differences (in the humoral response) and similarities (in the T cell response and tumor development) were also observed in young animals – suggesting that the observed effects were underpinned by age-dependent mechanisms and age-independent mechanisms.

3 – DISCUSSION

The age-related decline in immune responsiveness is a critical issue for vaccine-based prophylaxis and therapy in older adults. This decline is caused by combinations of impairments in various pathways, including cellular senescence (Van Deursen & M 2014; Palacio et al. 2019; Budamagunta et al. 2021; Yousefzadeh et al. 2021; Agrawal & Weinberger 2022; Lee, Flores, et al. 2022). Cellular senescence might be of particular interest in the setting of vaccination and is potentially amenable to prophylactic and/or therapeutic developments. To the best of our knowledge, the impact of senescent cell depletion on the vaccine response in older people or in preclinical models of aging has not been studied previously. Here, with the hope of alleviating immune dysfunction in older individuals, we used a senolytic approach. We focused on the broad Bcl-2 family inhibitor ABT-263, a drug currently used in the clinic to target apoptosis-resistant tumor cells. Our objective was to explore this potential new senotherapeutic strategy for enhancing vaccine responses in older adults.

We initiated this study without any starting hypotheses, since the depletion of pre-existing senescent cells might have both positive and negative effects on vaccine efficacy. As mentioned above, the aging process involves changes in (among others) immune cell numbers and functions, cellular organization in lymphoid organs and non-lymphoid tissues, and levels of circulating factors that interact with both immune cells and their microenvironment to ensure appropriate immune responses (Nikolich-Žugich 2014; Frasca et al. 2017; Mittelbrunn & Kroemer 2021). Age-associated inflammation, which is due in part to a chronic SASP, blunts immunity in aged individuals (Freund et al. 2010; Goldberg et al. 2020; Pereira, Xu, et al. 2020) Hence, the SASP might be a key element in the weak immune responses to vaccines. By

eliminating pre-existing senescent cells and thus by reducing levels of the SASP-related inflammatory factors involved in inflammaging, one could expect to ameliorate vaccine responses. By way of an example, SASP factors reduce T cell proliferation and the B-cell class-switching and hypermutation required for the generation of high-affinity antibodies (Frasca et al. 2014; Palacio et al. 2019). Decreasing the expression of immunosuppressive cytokines by senescent cells (IL-10, for instance (Acosta et al. 2013)) might also improve immune responses. Moreover, the depletion of pre-existing senescent cells in older individuals might enable danger-associated signals in the microenvironment to act as adjuvants and promote effective immune responses. Hence, the use of ABT-263 to eliminate age-related senescent cells might conceivably have a beneficial effect on immune responses to vaccines. However, one must also bear in mind that in older individuals, almost all immune cells express senescent markers (including the *bona fide* p16) – albeit at different levels (Hall et al. 2016; Grosse et al. 2020; Martínez-Zamudio et al. 2021; Yousefzadeh et al. 2021). These cells include macrophages, NK cells, T cells, and (memory) B cells. One should also consider that non-senescent immune cells including T and B cells express Bcl-2 members and that Bcl-2 expression changes in aging (Delbridge et al. 2016; Rohner et al. 2020). Hence, ABT-263 could eliminate critical (non-senescent and/or senescent) immune cells and have a catastrophic effect on immune responses to vaccines. Our experimental data, however, do not support this hypothesis because ABT-263 treatment did not abrogate immune responses, at least in the classical prime/boost protocol. One could also hypothesize that the removal of pre-existing senescent cells could reduce the production of components required for immune responses after vaccination. During an immune response, senescence and energy-sensing signaling pathways converge to regulate functional responses in immune cells – including B cells (Akbar 2017). Some SASP factors signal to different immune cells. For instance, IL-1α, IL-6, IL-7 and TGF are central players in CD4 T cell homeostasis, stimulation and differentiation and thus have an impact on adaptive immune responses (Ben-Sasson et al. 2009). The production of extracellular matrix proteins, proteases and growth factors (typically SASP factors) might also be important in immune responses triggered by the vaccine. The production of these factors by pre-existing immune and non-immune senescent cells might be important for vaccine-associated immune responses.

To study the effect of senolysis on immune responses following vaccination, we designed an experimental protocol in which the effect of ABT-263 was restricted to pre-existing senescent cells and not new, stress-induced Bcl-2-expressing cells. Indeed, studies of transgenic mice and gene-targeted mice have revealed that Bcl-2 family members have roles in immune responses

(Smith et al. 2000). Since ABT-263 has a short *in vivo* half-life (8-12h) and the treatment regimen ended three days before vaccine administration in our experiments, it is unlikely that the treatment impacted the future immune response triggered by stressed Bcl-2-expressing cells (*e.g*. cells stressed by the adjuvant in the vaccine formulation). ABT-263 treatment effectively removed senescent cells in old mice, as assessed by enhanced apoptosis, lower p16 and Bcl-2 expression, a lower level of β-Gal activity, and lower blood levels of SASP-related factors. Despite the age environment, senescent cells (or at least those involved in the production of circulating SASP factors (Figure. 3B) failed to repopulate mice 23 days after the discontinuation of ABT-263 treatment, which indicates that the drug had a long-term effect. Immune responses to vaccines comprise several steps, including antigen presentation/processing by DCs (the sentinels of the immune system), DC maturation (a process that allows T cell activation after interaction with DCs), T cell expansion, T cell cytokine release, and antibody class switching. The removal of age-associated senescent cells could have positive and/or negatively effects on certain obligate steps in the immune response. We developed two different adjuvant-based protocols in order to investigate this question. Whatever the protocol used, we observed a slight decrease in antibody production by old mice after ABT-263 treatment. Our findings contrast with literature reports in which the reduction of intrinsic levels of inflammatory cytokines (such as TNF-α) led to an improvement in B cell function and an increase in antibody production (Frasca et al. 2014). Interestingly, a recent study showed that the treatment of old mice with the senolytic fisetin was associated with greater antibody production in response to a viral infection (Camell et al. 2021). It is noteworthy that after the administration of a vaccine containing CpG ODN, antibody production was enhanced - rather than reduced - in young ABT-263-treated mice (OVA expressed by B16 cells for the boost). This marked, age-specific difference is difficult to interpret. Differences in exposure to OVA (due to differences in tumor growth) between young and old animals might have a role in the response to the boost. Depending on the vaccination protocol, the effect of ABT-263 on the T cell response in old mice was different. In the classical OVA prime/boost regimen, no significant effects were observed. In contrast, when tumor cells served as booster of the immune response, ABT-263 treatment reduced, albeit not significantly, the T cell response in old mice. As similar data were obtained with whole OVA and the MHC class-I restricted OVA peptide, this suggests that $CD8^+$ T cells were affected. These data are in line with a recent report showing reduced $CD8^+$ T cell response in aged mice upon senescent cell depletion (dasatinib/quercetin cocktail) (Torrance et al. 2023). The global effect of ABT-

263 on the T cell response was not age-dependent, as the results were similar in young and old mice (albeit differences occurred between LNs and spleen (Figure. 4C). In line with the reduced ($CD8^+$) T cell response, tumor development was increased in ABT263-treated vaccinated mice in general and in older mice in particular. Although senolytics have been shown to increase tumor growth in some settings (Kovacovicova et al. 2018), this effect is unlikely to occur in our setting because tumor cells were inoculated 13 days after discontinuation of the ABT-263 treatment. We are now seeking to determine whether this effect is due to enhanced immunosuppression.

Rejuvenation of the immune system in older individuals is an attractive approach for improving vaccine effectiveness; various targets have been suggested, including mammalian target of rapamycin (Mannick et al. 2018). Our data show that ABT-263 treatment before vaccine inoculation (classical vaccine prime/boost system) has little effect of antigen-specific B cell and T cell responses. In contrast, in the vaccine prime and tumor boost model, ABT-263 treatment impairs the humoral and cellular immune responses and worsens tumor development outcomes in vaccinated, old mice. Some of these effects were more marked in old mice (e.g. tumor growth) or only observed in old mice (e.g. less antibody production) which indicates that the removal of pre-existing senescent cells had a causal role. However, it is clear from our data that ABT-263 also acts independently of age-related senescent cells. This is particularly true for the enhanced B cell response, which was observed only in young animals. The mechanisms underpinning this enhanced response have not been identified and warrant future study. Small numbers of senescent cells are found in young individuals (Jeon et al. 2022). In our experiments, the effects of ABT-263 in young mice might be due to the removal of these senescent cells. The drug might also have effects on non-senescent cells. It remains to be established, both in young and aged mice, how ABT-263 affects immune cells, the senescent cell populations within immune cell subgroups, as well as the senescent cell populations in the parenchymal tissues relevant to immune functions and tumor growth.

Although our results provide novel insights into the effect of senolytics on vaccine efficacy, the present study had some limitations. Indeed, mouse data should be interpreted with caution and might not necessarily be extrapolatable to the situation in humans. Another limitation of the present study relates to our focus on a restricted set of adjuvants and on ABT-263, a drug that mainly targets Bcl2 family members. The specific targeting of age-related resident senescent cells should now be studied by applying other Bcl-2 inhibitors (such as ABT-199

and ABT-737) or other classes of senolytics that do not target Bcl-2. The senotherapeutic potential of fisetin or the dasatinib/quercetin cocktail - treatments that have different mechanisms - should be investigated. Along with pharmacological strategies, the selective depletion of p16-expressing or p21-expressing cells in transgenic mouse models might be instructive. Studies with senomorphics, which modulate senescent cell functions and interfere with the SASP to prevent inflammaging, might also provide additional information. Lastly, in the current study, we designed a short term senolytic treatment with continuous (daily) inoculation of the drug in aged mice. It would be interesting to design a prolonged treatment (e.g. once or twice monthly) in middle aged mice (e.g. (Farr et al. 2017) and investigate the effects of chronic drug administration in vaccinated aged mice. In conclusion, and bearing in mind the above-mentioned limitations, our data indicate that the senolytic drug ABT-263 cannot – at least in the mouse model – be considered for use as an adjuvant for enhancing antitumor immune responses upon vaccination in the aged individual. In the context of cancer vaccines, ABT-263 should be used with caution. Further preclinical and clinical research is needed to determine the age-related influence of senescent cells on vaccine efficacy.

Acknowledgments

We thank members of the animal facility for technical assistance.

Funding

This project has received funding from the European Union's Horizon 2020 research and innovation program under the Marie Sklodowska Curie grant agreement No 861190 (PAVE)". OC received a PhD fellowship from the European Union.

Author Contributions

FT conceived and supervised the study. OC designed the experiments, and performed the animal experiments. OC and DF analyzed the humoral response, LD performed immunohistochemistry, SH performed the RT-PCR, AH and ANM performed the cell cultures. OC, LD, IW, DB, PB, FA and FT analyzed the resulting data. OC and FT designed the figures

and drafted the manuscript. All the authors revised the manuscript and provided critical comments. FT, LJC, PB, FA and FT obtained funding.

Disclosure Of Potential Conflicts Of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Data Availablity Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

4 – Experimental Procedures

4-1 Mice and ethics statement

Specific pathogen-free C57BL/6J mice (2 months-old and 22-months-old, male) were purchased from Janvier (Le Genest-St-Isle, France). All experiments complied with the current national and institutional regulations and ethical guidelines (Institut Pasteur de Lille/B59-350009). Protocols were approved by the regional Animal Experimentation Ethics Committee (CEEA 75) and the French Ministry of Higher Education and Research (authorization numbers: 00357.03 and APAFIS#23718-2020012112087749 v4).

4-2 *In vivo* ABT-263 treatment

ABT-263 was purchased from Clinisciences (Nanterre, France) and dissolved in DMSO for preparation of stock solutions and kept at -20°C. For in vivo treatments 10% ABT-263 was formulated in 30% polyethylene glycol 400 and 60% Phosal 50 PG. Mice were treated by oral gavage (100 µl) with 50 mg/kg ABT-263 or vehicle control administered for four consecutive days in two treatments four days apart. Cytokines were quantified by ELISA (Invitrogen and Biolegend).

4-3 Stimulation of mouse splenocytes

Red blood cells from cell suspensions were removed with Red Blood Cell lysis buffer. After washing, cell pellets were resuspended in RPMI medium supplemented with 10% fecal bovine serum (FBS), 1% penicillin/streptomycin. Splenocytes from aged and young mice ($1x10^6$ cells/well) were treated in triplicates with ABT-263 (1µM, 0.1% DMSO) for 24h in RPMI medium, washed and then stimulated with LPS (1µg/ml). Cell viability was measured with XTT assay by following manufactures instructions (Cell Proliferation Kit II (XTT) Roche, MERCK). The colored formazan product was photometrically measured at 450 and 570 nm in a multiwell plate reader (Multiscan FC Microplate Reader). Analysis of annexin V expression was performed 4h post-ABT-263 treatment.

4-4 Flow cytometry

Spleens were disaggregated into single cell suspensions by using a plunger from a sterile syringe to mince and stained for flow cytometric analysis. Samples were blocked with anti-CD16/32 (Biolegend) for 30 minutes at 4°C and the following anti-mouse primary antibodies incubated for 1h at 4°C. All antibodies were from Biolegend: CD3:Pacific Blue, CD4:FITC, CD44:AF700, CD62L.PerCP/Cy5.5, PD1:PE, CD153:APC, PE isotype Ctrl, PerCP/Cy5.5 isotype Ctrl, APC isotype Ctrl (Figure. 2D). Single stained samples were prepared as compensations. Cells were washed twice and resuspended in PBS/2% FCS for analysis on LSR Fortessa using FlowJo v10.8 software (BD Life Sciences). Apoptosis induction by ABT-263 was analyzed by flow cytometer using Annexin V-FITC/PI staining kit (Biolegend).

4-5 Detection of SA-β-galactosidase activity

Cellular senescence was quantified using CF12FDG [5-Dodecanoylaminofluorescein Di-b-D-Galactopyranoside]. Briefly, lysosomal alkalinization of splenocytes was induced by pre incubation of Bafilomycin A1 (100 nM) at 37° C for 1h. 33 µM C_{12}FDG was added in the culture media containing Baf A1 for another 2h. The cells were washed three times in cold PBS before staining for FACS analysis. Frozen sections of spleen (5 µm-thick) were used. Tissues were mounted in OCT embedding compound (Sigma Aldrich) and rapidly frozen in liquid nitrogen. Frozen sections were mounted on Superfrost adhesion microscope slides and washed twice in PBS. They were immersed in staining solution overnight at 37°C. Sections were rinsed, counterstained with eosin, rinsed, dehydrated, cleared.

4-6 Immunohistochemistry

Tissue sections were stained with rabbit polyclonal anti-cleaved Caspase-3 (Cell Signaling), rabbit monoclonal anti-p16, rabbit polyclonal anti-Bcl-2, or rabbit monoclonal anti-Bcl-XL (all from Abcam) antibodies. The slides were blocked for endogenous peroxidase with 3% H2O2 and boiled for antigen retrieval in citrate buffer (pH 6). Sections were incubated with the appropriate secondary antibody from Vector Laboratories (goat anti-rabbit IgG antibody (H+L), washed, and incubated with the VECTASTAIN®Elite ABC Peroxidase standard kit (Vector laboratories, Newark, NJ). After several washes, the chromogen 3,3′-diaminobenzidine from the Peroxidase Substrate Kit (Vector Laboratories) was added to each slide. The slides were counterstained with Mayer's Hemalun (Merck, Darmstadt, Germany). Lastly, the slides were mounted with glycerin mounting medium (Dako, Santa Clara, CA). Images were acquired using an Evos M5000 microscope.

4-7 Gene expression analysis by RT-qPCR

Quantitative RT-PCR was performed exactly as described in (Delval et al. 2023). The murine primers used were as follows : *p16* (5'-GCTCTGGCTTTCGTGAACATGT-3', 5'-TTGAGCAGAAGAGCTGCTACGT-3' and (*bcl2*) (5'-TCATGTGTGTGGAGAGCGTCA-3', 5'-GATCCAGGTGTGCAGATGCC-3') and (*Gapdh*) (5'-GCAAAGTGGAGATTGTTGCC-3', 5'- GCCTTGACTGTGCCGTTGA-3'). Data were normalized against expression of the *Gapdh*.

4-8 Immunization and serum preparation

C57BL/6J mice were immunized subcutaneously on the right flank with 50µg of endotoxin free OVA (Endofit, InvivoGen, Toulouse, France) plus 10µg Quil-A® (Invivogen) or plus 20µg CpG ODN 1826 Vaccigrade (InvivoGen). The same procedure was repeated for the boost but with 25µg OVA and 5µg Quil-A® or 10µg CPG ODN per animal. Control mice were immunized with PBS. Blood samples were collected at the prime (facial vein) and at the day of sacrifice under anesthesia from external jugular vein. Samples were further separated by centrifugation at 10000g for 10 min and serums were stored at -20°C until testing.

4-9 OVA-specific T cell response and antibody detection by ELISA

Spleen and LN cells ($1x10^6$ cells and $3x10^5$ cells/well, respectively) were restimulated with OVA (100µg/ml) or the H-2Kb restricted OVA-derived peptide SIINFEKL ($OVA_{257-264,}$ 2µg/ml) for 48h. IFN-γ in supernatants was quantified by ELISA (Thermoscientific). OVA-coated plates (96-well high-binding plates) were incubated with 50µl of samples diluted in PBS containing 0.5% (w/v) BSA at 37°C for 2h. After washes, anti-mouse IgM and IgG HRP conjugate (ThermoFisher and Biolegend, 1:2000 (v/v) in diluent solution) were incubated for 1 h at 37°C. The color reaction was developed by adding TMB solution and the enzymatic reaction was stopped by adding 100µL of 2 N H_2SO_4. Optical density was determined at 450 nm.

4-10 In vivo tumor growth

Fifty percent confluent B16-OVA tumor cells were harvested using trypsin and subsequently prepared for injection by resuspending the cells in HBSS after washing. Mice were injected subcutaneously in the flank wih 5×10^5 tumor cells in 100µl HBSS. Animals were monitored for body weights once daily starting at the day of the ABT-263 treatment and continuing until the day of euthanasia. Tumor growth was monitored over time using microcallipers. Tumor volume (mm^3) was calculated using the formula: 1/2 length (mm) X width $(mm)^2$.

4-11 Statistical analysis

Results are expressed as the mean ± SEM (standard error of the mean) unless otherwise stated. All statistical analysis was performed using GraphPad Prism v9.2.0 software. Unpaired T test was used to compare two groups unless otherwise stated. Comparisons of more than two groups with each other were analyzed with the Two-way ANOVA Tukey`s multiple comparison test. *$P < 0.05$; **$P < 0.01$, *** $P < 0.001$.

General Discussion

The decline in immune responsiveness due to aging is a crucial matter for vaccine-based prevention and treatment in older individuals. This decline is caused by multiple impairments in various pathways, including cellular senescence. (Van Deursen & M 2014; Palacio et al. 2019; Budamagunta et al. 2021; Yousefzadeh et al. 2021; Agrawal & Weinberger 2022; Lee, Flores, et al. 2022; Lorenzo et al. 2022). The significance of cellular senescence in the context of vaccination deserves attention, and it may be feasible to create preventive and/or therapeutic solutions for it. (Goronzy & Weyand 2013) Cellular senescence is a state in which cells permanently stop dividing as a result of persistent activation of stress signaling pathways, which is linked to age and is implicated in a variety of age-related illnesses, including diabetes, cardiovascular disease, Alzheimer's disease, osteoarthritis, and cancer. (Zhang et al. 2019; Farr et al. 2017; Musi et al. 2018; Palmer et al. 2015; Palmer et al. 2019; Schafer et al. 2020; Zhang et al. 2023) Cells have different reactions to stress, such as repair, cell death, or senescence, which are determined by the type of cell and the intensity and type of stress being encountered. (Demaria et al. 2014; Storer et al. 2013; Krizhanovsky et al. 2008) Senescent cells can cause an imbalance in the natural renewal of tissues by halting cell growth and negatively impacting the functions of neighboring cells through the Senescence-Associated Secretory Phenotype (SASP). (Wiley & Campisi 2016) The long-term effect of constant antigenic stress and burden decrease functional capacity and elevates inflammatory state known as inflammaging. It is highly likely that the persistent activation of the immune system and inflammation is attributed to the effects of both clinical and subclinical infections, as well as the constant exposure to various antigens, such as food and allergens. (Franceschi, Bonafè & Valensin 2000; De Martinis et al. 2004) Senescence serves as a natural response to prevent genomic instability caused by persistent antigens, which helps to minimize the accumulation of DNA damage. Although an abundance of cell aging, particularly in T cells, can ultimately lead to a notable decrement in immune function and diminished vaccine potency in older individuals. (Ovadya et al. 2018; Orillard et al. 2023; Bowyer et al. 2020; Shi et al. 2014) Currently, there are five vaccines recommended for individuals aged 60 and above to protect against COVID-19, influenza, pneumococcal infections, Tdap (tetanus, diphteria, and whooping cough), and zoster infections. Among the five, only the tetanus-reduced diphtheria-acellular pertussis vaccine elicits an acceptable, yet diminished protective antibody response in comparison to young adults. (Weston et al. 2012) Following exposure to a pathogen or administration of a vaccine, a small number of T cells that are specific to an antigen undergo significant expansion, increasing up to 10,000- to 50,000-fold and differentiating into effector T cells. One of the key features of age-related changes in the T cell compartment is the substantial reduction of naive

T cells. (Saule et al. 2006; Li et al. 2019; Goronzy et al. 2015) Consequently, naive T cells in individuals over 65 years of age lose their stemness and functional plasticity, transitioning towards a more memory-like state. (Hu et al. 2020) In both the CD4+ and CD8+ T cell compartments, the accumulation of T cells with a terminally differentiated memory-like phenotype occurs earlier in CD8+ than in CD4+ T cells in both humans and mice. (Joshi et al. 2011; Kaech et al. 2002; Soto-Heredero et al. 2023) It has been recently demonstrated that age-related TCR repertoire attrition occurs earlier in CD8+ than CD4+ T cells. (Sun et al. 2022) Among the various subpopulations of activated CD8+ T cells in humans, the proportion of terminal effector memory CCR7−CD45RA+ (TEMRA) T cells tends to increase with age. (Appay et al. 2002) The cells are identified based on their lack of costimulatory markers CD28 and CD27, with the re-emergence of CD45RA and the expression of exhaustion markers PD-1, CX3CR1, or CD57 NK receptor. (Brenchley et al. 2003; Kared et al. 2016) These CD8+ subpopulations have been deemed senescent due to their diminished proliferation and reduced TCR activation. (Larbi & Fulop 2014) Notably, sestrin expression is higher in CD8+ T cells of older individuals (over 65 years old). Genetic silencing of sestrins has been found to restore the proliferation capacity and function of these senescent T cells, as well as enhance the vaccination response in aged mice. (Lanna et al. 2017; Pereira, De Maeyer, et al. 2020; Pereira, Xu, et al. 2020) It has been found that exhausted T cells, which were previously studied in the context of cancer, viral infections, and autoimmunity, are also present in aging. (Zhao et al. 2020; Parks et al. 2023) It was found that the development of these PD-1+CD8+ T cells was motivated by external factors of an aged environment, and the creation of GzmK by these cells triggered senescence in neighboring fibroblasts, leading to an increase in inflammaging. (Mogilenko et al. 2021; Galletti et al. 2020) Currently, the mechanisms that contribute to poor vaccination efficacy in the elderly are not well understood, but available evidence indicates that a combination of intrinsic and extrinsic factors, such as T cell senescence and exhaustion, play a role. Although the mechanisms are not entirely clear, several strategies have demonstrated potential in postponing signs of senescence and may therefore enhance vaccination response in older individuals. The use of IL-7 or its analogues has been discovered to have the effect of augmenting thymic mass and elevating the levels of naive T cell precursors. (Okoye et al. 2015) Another example of inflammaging was the cutaneous VZV vaccination. The team led by Akbar successfully identified the significant activation of the p38 mitogen-activated protein (MAP) kinase pathway. The neutralization of this pathway through the administration of an oral p38 MAP kinase inhibitor called losmapimod significantly decreased serum levels of CRP, IL-6, and TNF-α and improved the response to VZV

vaccination. (Vukmanovic-Stejic et al. 2018) The discovery that mTOR inhibition can enhance the functioning of the elderly and increase their responsiveness to influenza vaccination by decreasing the expression of PD-1 on both CD4 and CD8 T cells. This led to the development of a new drug, RAD001, which has been found to improve the elderly recipients' ability to produce antibodies against the virus by around 20%. (Mannick et al. 2014) The impact of exercise on postponing immunosenescence has been thoroughly examined, and it has been found that higher levels of physical activity are connected to a decrease in the number of senescent lymphocytes, enhanced T cell function, and improved vaccine response in older individuals. (Minuzzi et al. 2018; Baker et al. 2023; Wong et al. 2019)

The impact of senescent cell depletion on vaccine response in older individuals or preclinical models of aging has not yet been studied. Therefore, with the aim of improving immune function in older adults, we used a senolytic approach and focused on the broad Bcl-2 family inhibitor ABT-263, which is currently used in clinical trials to target apoptosis-resistant tumor cells. Our objective was to investigate the potential of this new senotherapeutic strategy for enhancing vaccine responses in older individuals. We began this study without any preconceived notions. The removal of pre-existing senescent cells could potentially have both positive and negative effects on vaccine effectiveness, as it involves changes in immune cell counts and functions, cellular organization in lymphoid and non-lymphoid tissues, and circulating factors that regulate appropriate immune responses. (Nikolich-Žugich 2014; Frasca et al. 2017; Mittelbrunn & Kroemer 2021). Age-associated inflammation, which is due in part to a chronic SASP, blunts immunity in aged individuals (Freund et al. 2010; Goldberg et al. 2020; Pereira, Xu, et al. 2020) Hence, the SASP might be a key element in the weak immune responses to vaccines. (Faget et al. 2019; Vukmanovic-Stejic et al. 2018; Fourati et al. 2016) By removing senescent cells, which contribute to inflammaging by producing SASP factors, we can potentially enhance vaccine responses. For instance, SASP factors suppress T cell proliferation and B-cell class switching and hypermutation, which are necessary for generating high-affinity antibodies. (Frasca et al. 2014; Palacio et al. 2019). Decreasing the expression of immunosuppressive cytokines by senescent cells (IL-10, for instance (Acosta et al. 2013)) might also improve immune responses. Moreover, the depletion of pre-existing senescent cells in older individuals might enable danger-associated signals in the microenvironment to act as adjuvants and promote effective immune responses. (Lee et al. 2021) Hence, the use of ABT-263 to eliminate age-related senescent cells might conceivably have a beneficial effect on immune responses to vaccines. However, one must also bear in mind that in older individuals,

almost all immune cells express senescent markers (including the *bona fide* p16) – albeit at different levels (Hall et al. 2016; Grosse et al. 2020; Martínez-Zamudio et al. 2021; Yousefzadeh et al. 2021). These cells include macrophages, NK cells, T cells, and (memory) B cells. One should also consider that non-senescent immune cells including T and B cells express Bcl-2 members and that Bcl-2 expression changes in aging (Delbridge et al. 2016; Rohner et al. 2020). Hence, ABT-263 could eliminate critical (non-senescent and/or senescent) immune cells and have a catastrophic effect on immune responses to vaccines. Our experimental data, however, do not support this hypothesis because ABT-263 treatment did not abrogate immune responses, at least in the classical prime/boost protocol. One could also hypothesize that the removal of pre-existing senescent cells could reduce the production of components required for immune responses after vaccination. During an immune response, senescence and energy-sensing signaling pathways converge to regulate functional responses in immune cells – including B cells (Akbar 2017). Some SASP factors signal to different immune cells. For instance, IL-1□, IL-6, IL-7 and TGF are central players in CD4 T cell homeostasis, stimulation and differentiation and thus have an impact on adaptive immune responses (Ben-Sasson et al. 2009). The production of extracellular matrix proteins, proteases and growth factors (typically SASP factors) might also be important in immune responses triggered by the vaccine. In fact, the inflammation signatures of SASP comprise various immune cell attracting chemokines, activating cytokines, adhesion molecules, and immune modulators.(Freund et al. 2010) The production of these factors by pre-existing immune and non-immune senescent cells might be important for vaccine-associated immune responses.

To study the effect of senolysis on immune responses following vaccination, we designed an experimental protocol in which the effect of ABT-263 was restricted to pre-existing senescent cells and not new, stress-induced Bcl-2-expressing cells. Indeed, studies of transgenic mice and gene-targeted mice have revealed that Bcl-2 family members have roles in immune responses (Smith et al. 2000). Since ABT-263 has a short *in vivo* half-life (8-12h) and the treatment regimen ended three days before vaccine administration in our experiments, it is unlikely that the treatment impacted the future immune response triggered by stressed Bcl-2-expressing cells (*e.g*. cells stressed by the adjuvant in the vaccine formulation). ABT-263 treatment effectively removed senescent cells in old mice, as assessed by enhanced apoptosis, lower p16 and Bcl-2 expression, a lower level of β-Gal activity, and lower blood levels of SASP-related factors. Despite the age environment, senescent cells (or at least those involved in the production of circulating SASP factors Figure 3B) failed to repopulate mice 23 days after the discontinuation

of ABT-263 treatment, which indicates that the drug had a long-term effect. The sustained effect of ABT-263 in preventing the repopulation of senescent cells suggests a possible approach for improving long-term health and welfare in aging populations. Immune responses to vaccines comprise several steps, including antigen presentation/processing by dendritic cells (DCs, the sentinels of the immune system), DC maturation (a process that allows T cell activation after interaction with DCs), T cell expansion, T cell cytokine release, and antibody class switching. The removal of age-associated senescent cells could have positive and/or negatively effects on certain obligate steps in the immune response. We developed two different adjuvant-based protocols in order to investigate this question. Whatever the protocol used, we observed a slight decrease in antibody production by old mice after ABT-263 treatment. Our findings contrast with literature reports in which the reduction of intrinsic levels of inflammatory cytokines (such as TNF-α) led to an improvement in B cell function and an increase in antibody production (Frasca et al. 2014). Interestingly, a recent study showed that the treatment of old mice with the senolytic fisetin was associated with greater antibody production in response to a viral infection (Camell et al. 2021). It is noteworthy that after the administration of a vaccine containing CpG ODN, antibody production was enhanced - rather than reduced - in young ABT-263-treated mice (OVA expressed by B16 cells for the boost). This marked, age-specific difference is difficult to interpret. Differences in exposure to OVA (due to differences in tumor growth) between young and old animals might have a role in the response to the boost. Depending on the vaccination protocol, the effect of ABT-263 on the T cell response in old mice was different. In the classical OVA prime/boost regimen, no significant effects were observed. In contrast, when tumor cells served as booster of the immune response, ABT-263 treatment reduced, albeit not significantly, the T cell response in old mice. As similar data were obtained with whole OVA and the MHC class-I restricted OVA peptide, this suggests that $CD8^+$ T cells were affected. These data are in line with a recent report showing reduced $CD8^+$ T cell response in aged mice upon senescent cell depletion (dasatinib/quercetin cocktail) (Torrance et al. 2023) The global effect of ABT-263 on the T cell response was not age-dependent, as the results were similar in young and old mice (albeit differences occurred between spleen and LNs, Figure. 4C). In line with the reduced ($CD8^+$) T cell response, tumor development was increased in ABT263-treated vaccinated mice in general and in older mice in particular. Although senolytics have been shown to increase tumor growth in some settings (Kovacovicova et al. 2018), this effect is unlikely to occur in our setting because tumor cells were inoculated 13 days after discontinuation of the ABT-263 treatment. We are now seeking to determine whether this effect is due to enhanced immunosuppression.

A possible explanation for the increased resistance of tumors to ABT-263 treatment could be the upregulation of MCL-1 in order to avoid apoptosis mediated by ABT-263. (Kohli et al. 2022) The combination of BCL-2 and MCL-1 inhibitors has been demonstrated to be selectively toxic to melanoma cells. (Mukherjee et al. 2020; Lee et al. 2019) To address this issue, it is suggested that other drugs be combined with ABT-263. The regulation of this phenomenon is mediated by mTOR, and a combination with an mTOR inhibitor is also being considered (Kohli et al. 2022). In our setting, we used B16 melanoma to investigate the effect of senolysis on anti-tumor vaccine. We are currently investigating the effect of prior SC depletion before vaccination in a model of pancreatic ductal adenocarcinoma (PDAC). For this, we collaborated with partners in the Netherlands and Germany (the PAVE consortium, Marie Curie network training). A mesothelin peptide-based vaccine was formulated in poly(lactic-*co*-glycolic acid, PLGA)-based nanoparticles (100-200 nm in size) (Luis Ricondo Cruz, leiden). R848 was incorporated in the nanoparticles. We have shown that a prime/boost (2 boosts) strategy can protect young mice against the growth of KPC, a clinically relevant model of PDAC (subcutaneous inoculation) (Ferari et al. in preparation). The effect of the vaccine is presently investigated in a model of orthotopic cancer (inoculation in the pancreas). In our settings, the NP-based vaccine will be inoculated in young and aged mice previously treated or not with ABT-263. The humoral and cellular responses and the tumor growth will be compared. Depending on the data, other strategies will be set up including the duration of the treatment (middle-aged animals) and/or the nature of the senolytics.

Rejuvenation of the immune system in older individuals is an attractive approach for improving vaccine effectiveness; various targets have been suggested, including mammalian target of rapamycin (Mannick et al. 2018). Later, it was discovered that rapamycin hindered the growth of mammalian cells and possessed immunosuppressive properties. The properties of this compound made it acceptable for cancer therapy, specifically for renal cell carcinoma, according to the Food and Drug Administration in the United States. For cancer patients, the typical oral dose of rapamycin is around 10 mg per day. In subsequent mouse studies, it was found that rapamycin may improve the response to influenza antigen at lower doses by inhibiting the formation of germinal centers, which reduce the production of high-affinity antibodies. However, this allows the production of lower-affinity antibodies and increases the presence of influenza-specific IgM antibodies. (Keating et al. 2013) One another example is restoration through nutrition. (Pae et al. 2011) Naïve T cells that are normally in a state of dormancy must become activated by altering their metabolism following the encounters with

antigens. The shift in demand suggests that more energy and cell components are needed for proliferation. (Johnson et al. 2016) In studies where older individuals' diets were supplemented with an energy source and trace elements, it was observed that those individuals showed a stronger response to influenza and pneumococcal vaccinations. (Akatsu et al. 2016; Vidal et al. 2012; Moriguti et al. 2005) Cellular senescence, another potentially druggable mechanism, has been the focus of much attention in an effort to prevent or treat many age-related diseases, including cardiovascular, neurodegenerative, metabolic, and malignant diseases. Our data show that ABT-263 treatment before vaccine inoculation (classical vaccine prime/boost system) has little effect of antigen-specific B cell and T cell responses. In contrast, in the vaccine prime and tumor boost model, ABT-263 treatment impairs the humoral and cellular immune responses and worsens tumor development outcomes in vaccinated, old mice. Some of these effects were more marked in old mice (e.g. tumor growth) or only observed in old mice (e.g. less antibody production) which indicates that the removal of pre-existing senescent cells had a causal role. However, it is clear from our data that ABT-263 also acts independently of age-related senescent cells. This is particularly true for the enhanced B cell response, which was observed only in young animals. The mechanisms underpinning this enhanced response have not been identified and warrant future study. Small numbers of senescent cells are found in young individuals (Jeon et al. 2022). In our experiments, the effects of ABT-263 in young mice might be due to the removal of these senescent cells. The drug might also have effects on non-senescent cells. It remains to be established how ABT-263 affects immune cells, the senescent cell populations within immune cell subgroups, as well as the senescent cell populations in the parenchymal tissues relevant to immune functions and tumor growth.

Although our results provide novel insights into the effect of senolytics on vaccine efficacy, the present study had some limitations. Indeed, mouse data should be interpreted with caution and might not necessarily be extrapolatable to the situation in humans. Another limitation of the present study relates to our focus on a restricted set of adjuvants and on ABT-263, a drug that mainly targets Bcl2 family members. The specific targeting of age-related resident senescent cells should now be studied by applying other Bcl-2 inhibitors (such as ABT-199 and ABT-737) or other classes of senolytics that do not target Bcl-2. The senotherapeutic potential of fisetin or the dasatinib/quercetin cocktail - treatments that have different mechanisms - should be investigated. Although Quil-A, known for its ability to activate DAMPs and CpG to activate TLRs, is a popular choice for adjuvant, other adjuvants such as Alum or MF59 may also be considered for use in combination with senolytic treatments. Intra

dermal injections are a preferred method for accessing dermal dendritic cells (DCs) and Langerhans cells (LCs). By directing these dermal sentinels to antigens, it is possible to trigger highly effective antigen presentation to both skin-resident memory T cells and lymph-node-resident memory T cells by encouraging migration to lymphoid organs. (Lee et al. 2023) In our research, we have utilized B16 melanoma cells for the induction of tumors. Given the presence of diverse microenvironments in other tumor models, it would be intriguing to examine the senolytic effect when combined with other cancer models (e.g. PDAC). In our study, we focused on ABT-263, a drug that mainly targets Bcl2 family members. The specific targeting of age-related resident senescent cells should now be studied by applying other Bcl-2 inhibitors or other classes of senolytics that do not target Bcl-2. The senotherapeutic potential of fisetin or the dasatinib/quercetin cocktail - treatments that have different mechanisms - should be investigated. One could also envisage to deplete senescent cells by vaccination. For instance, Amor and colleagues identified a specific antigen expressed by senescent cells, namely the urokinase-type plasminogen activator receptor (uPAR) (Amor et al. 2020). Using a CAR T cells strategy, they were able to deplete senescent cells and protect against senescent-dependent pathologies.

Along with pharmacological strategies, the selective depletion of p16-expressing or p21-expressing cells in transgenic mouse models might be instructive. Studies with senomorphics, which modulate senescent cell functions and interfere with the SASP to prevent inflammaging, might also provide additional information. Finally, in the current study, we designed a short-term senolytic treatment with continuous (daily) inoculation of the drug in aged mice. It would be interesting to design a prolonged treatment (e.g. once or twice monthly) in middle aged mice (e.g. Farr et al. 2017) and investigate the effects of chronic drug administration in vaccinated aged mice. Therefore we have initiated a long term study to investigate the immune response starting the senolytic treatment at early ages.

In conclusion, and bearing in mind the above-mentioned limitations, our data indicate that the senolytic drug ABT-263 cannot – at least in the mouse model – be considered for use as an adjuvant for enhancanti-tumorumor immune responses upon vaccination in the aged individual. In the context of cancer vaccines, ABT-263 should be used with caution. While these results are promising, it is important to approach them with caution as they only provide early proof-of-concept evidence for the potential of senolytics in different settings, such as immunization. Despite this, it is clear that using senolytics to either remove senescent immune

cells directly or improve the immune system's clearance of SnCs carries an inherent risk of limiting the beneficial effects of senescence. Recent research has indicated that SCs may have a role in regenerative processes, despite being an understudied topic. In 2015, a team led by Jeremy Brockes from UCL, London, discovered that senescence is recurrently reversed during limb regeneration in salamanders. (Yun et al. 2015). Following an injury in both zebrafish and neonatal mouse hearts , a temporary increase in the number of SA-β-gal+ cardiac fibroblasts was observed. (Sarig et al. 2019) Another study conducted in 2019 provided evidence of a temporary accumulation of senescence cells (SCs) following the amputation of zebrafish caudal fins. (Da Silva-Álvarez et al. 2020) and tissue regeneration was impaired by removal of SCs with ABT-263. To avoid any negative consequences that might arise from removing SCs from specific tissues, it is crucial to weigh the potential disadvantages of such an action. Despite the proposition that eliminating SCs could potentially extend lifespan, the complex and unpredictable nature of senescence suggests that it may still harbor deeper intricacies. It is uncertain whether the elimination of cells that were previously integral to the organism's homeostasis is truly desired, especially in tissues that cannot regenerate lost cells in aged individuals, such as the immune system. Further research is required to determine the impact of senescent cells on vaccine efficacy and to explore the age-related factors that influence this relationship.

Figures

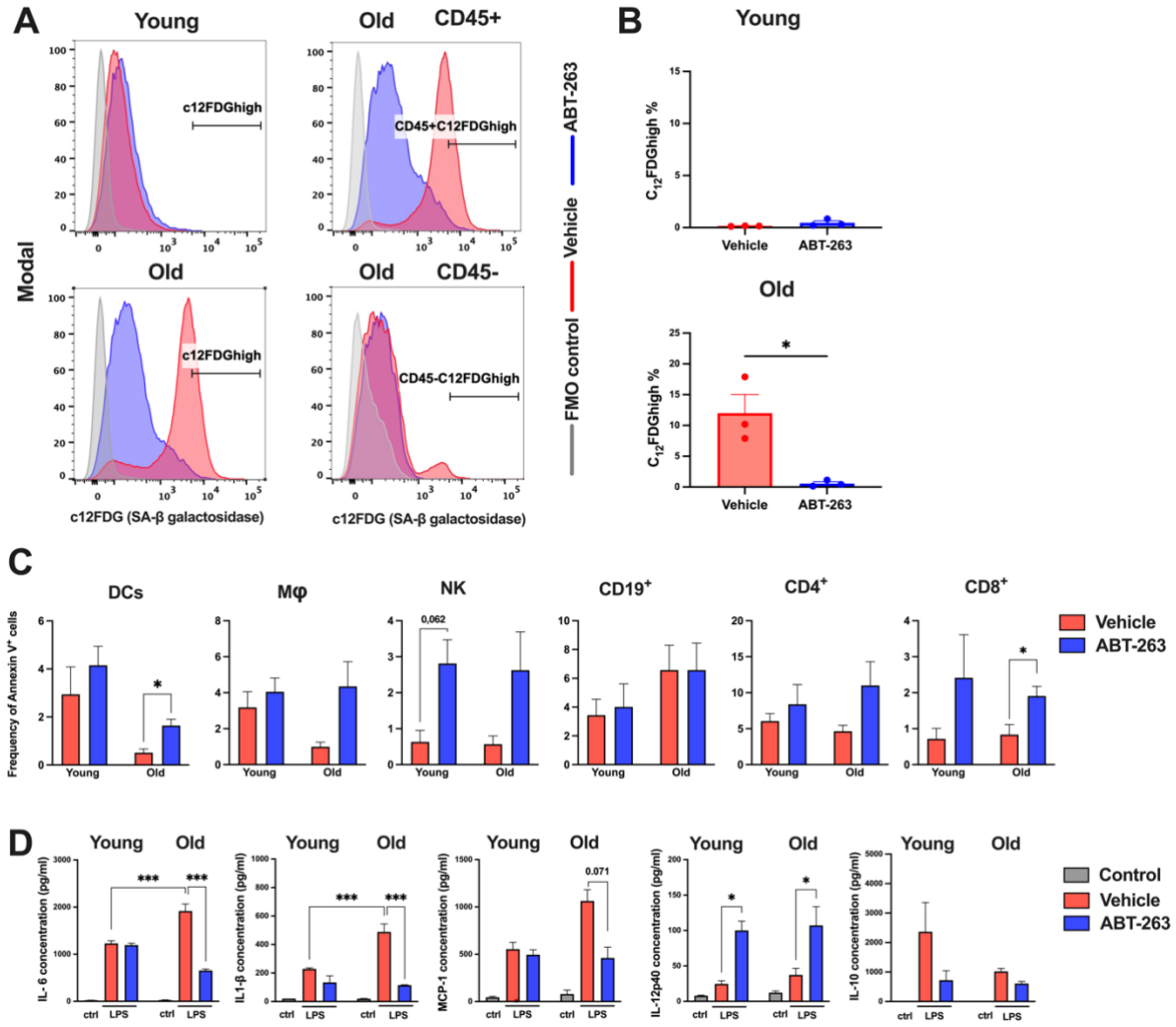
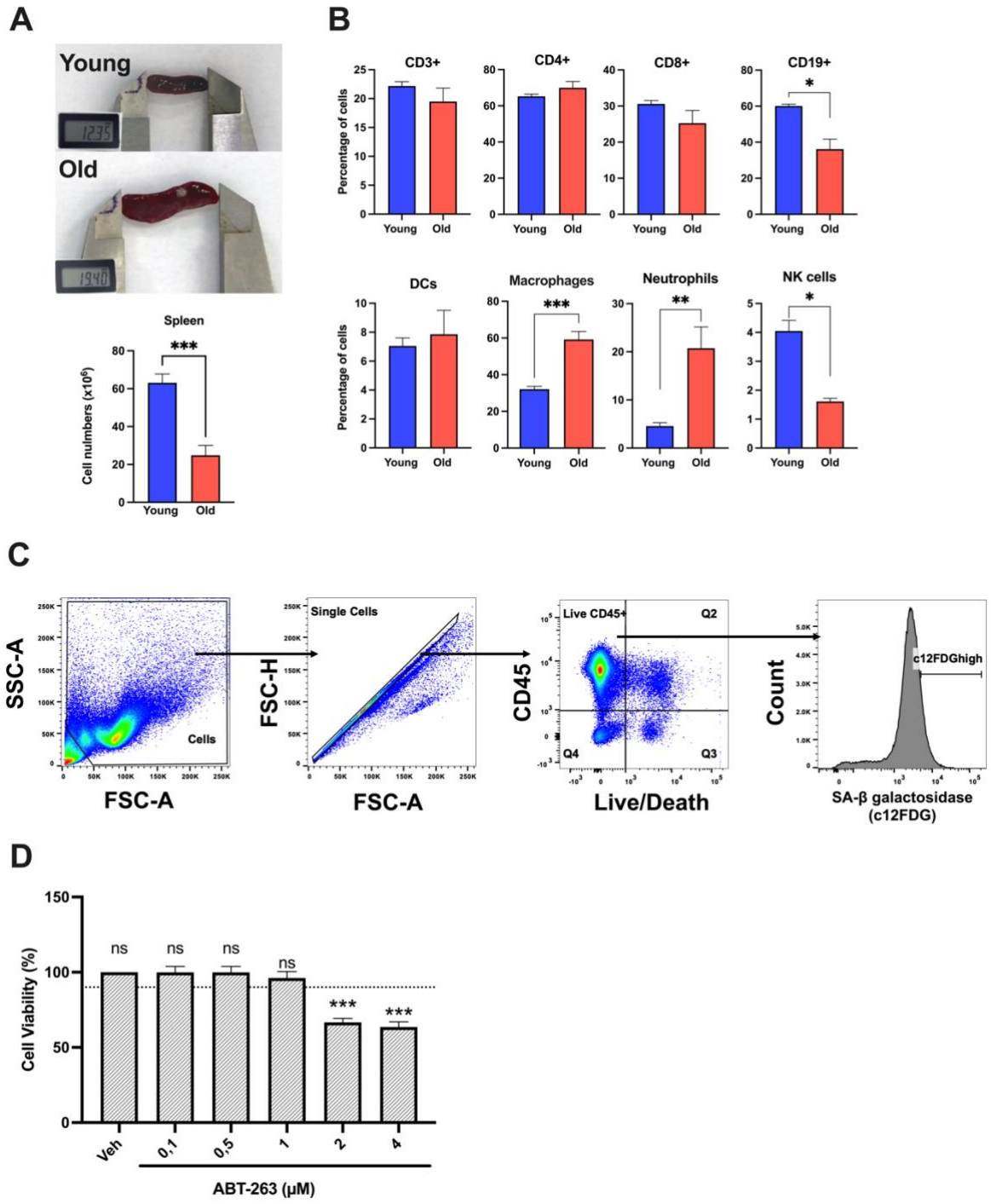


FIGURE 1 Depletion of senescent cells by ABT-263 and consequences on LPS-induced production of SASP-related cytokines by old splenocytes. **A**, Splenocytes collected from young and old mice were incubated with the fluorescent β galactosidase substrate $CF_{12}FDG$ and detection of the fluorescent substrate cleaved by β-galactosidase was performed by flow cytometry. Representative profiles are depicted (*upper* panel: the CD45-positive fraction and *lower* panel: the CD45-negative fraction). **B**, *Left* panel, Old splenocytes were cultured in the presence of ABT-263 (1µM) for 24h and SA-β-galactosidase activity was assessed by flow cytometry. The percentages of labelled cells are indicated. **C**, Frequencies of annexin V positive cells among each cell population (4h post ABT-263 treatment). **D**, Splenocytes were treated with vehicle or ABT-263 (1µM) for 24h and then stimulated with LPS at 1µg/ml. After 24h, supernatants were collected and cytokine production was determined by ELISA. For all graphs, errors indicate mean (n=3-4). One representative experiment out of two (**A-C**) or three (**D**) are depicted. Significant differences were determined using the unpaired T test (**B** and **C**) and the two-way ANOVA Tukey`s multiple comparisons test (**D**) (*$P < 0.05$, **$P < 0.01$,***, $P < 0.01$).



Supplementary FIGURE 1. A, Representative spleens from young and old mice and splenic cell counts. **B**, The percentages of the different cell populations as determined by flow cytometry are depicted. **C,** Gating strategy showing the analysis of $C_{12}FDG^{high}$ (SA-β gal positive) immune cells in the spleens of young and old mice. **D,** Splenocytes collected from young mice were exposed to grading doses of ABT-263. After 24h, cell viability was measured. **A**, **B** and **D**, A pool of two experiments is depicted (n=6). Significant differences were determined using the unpaired T test (**A** and **B**) and the two-way ANOVA Tukey`s multiple comparisons test (**C**) (*$P < 0.05$, **, $P < 0.01$; ***, $P < 0.01$).

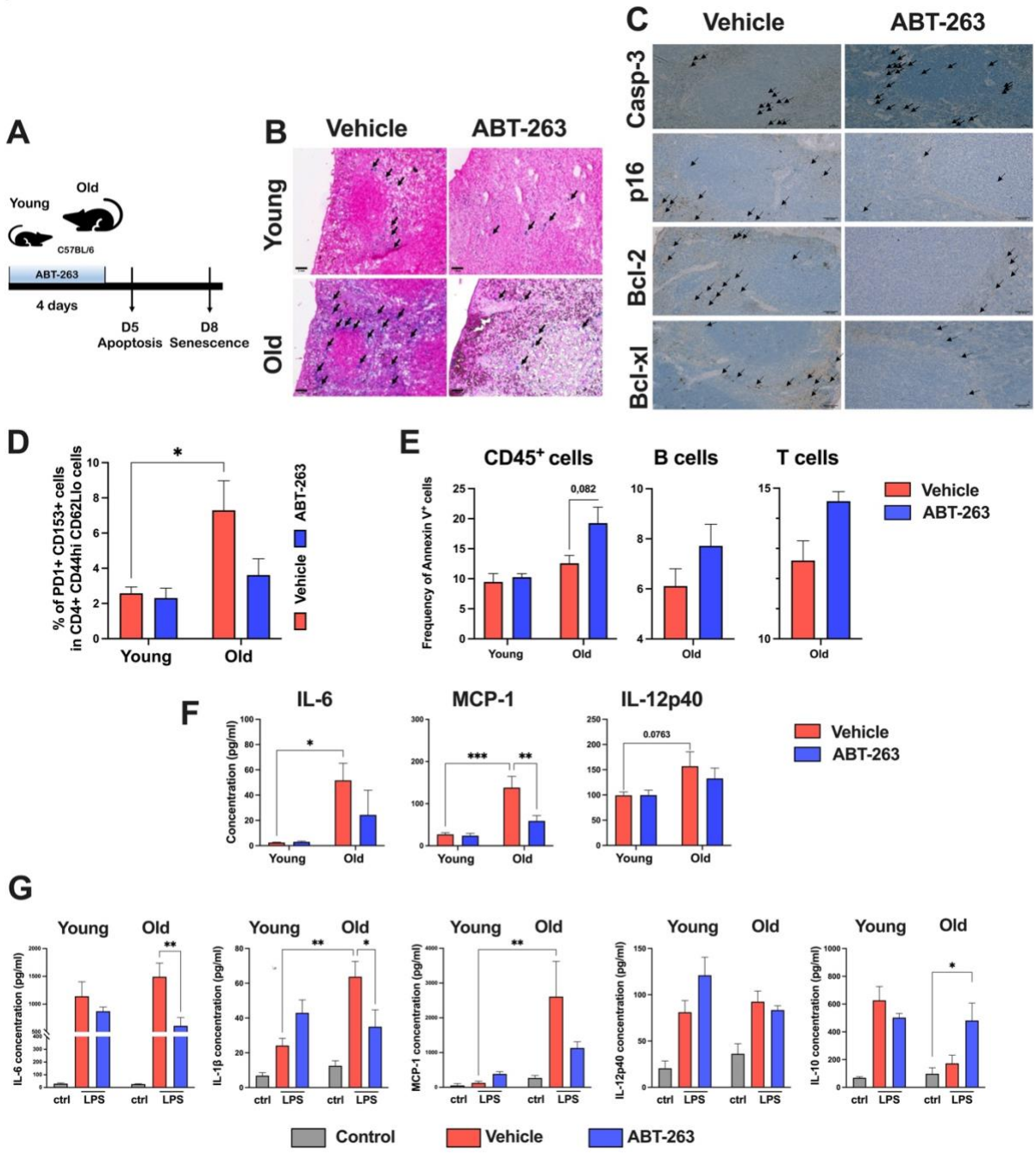
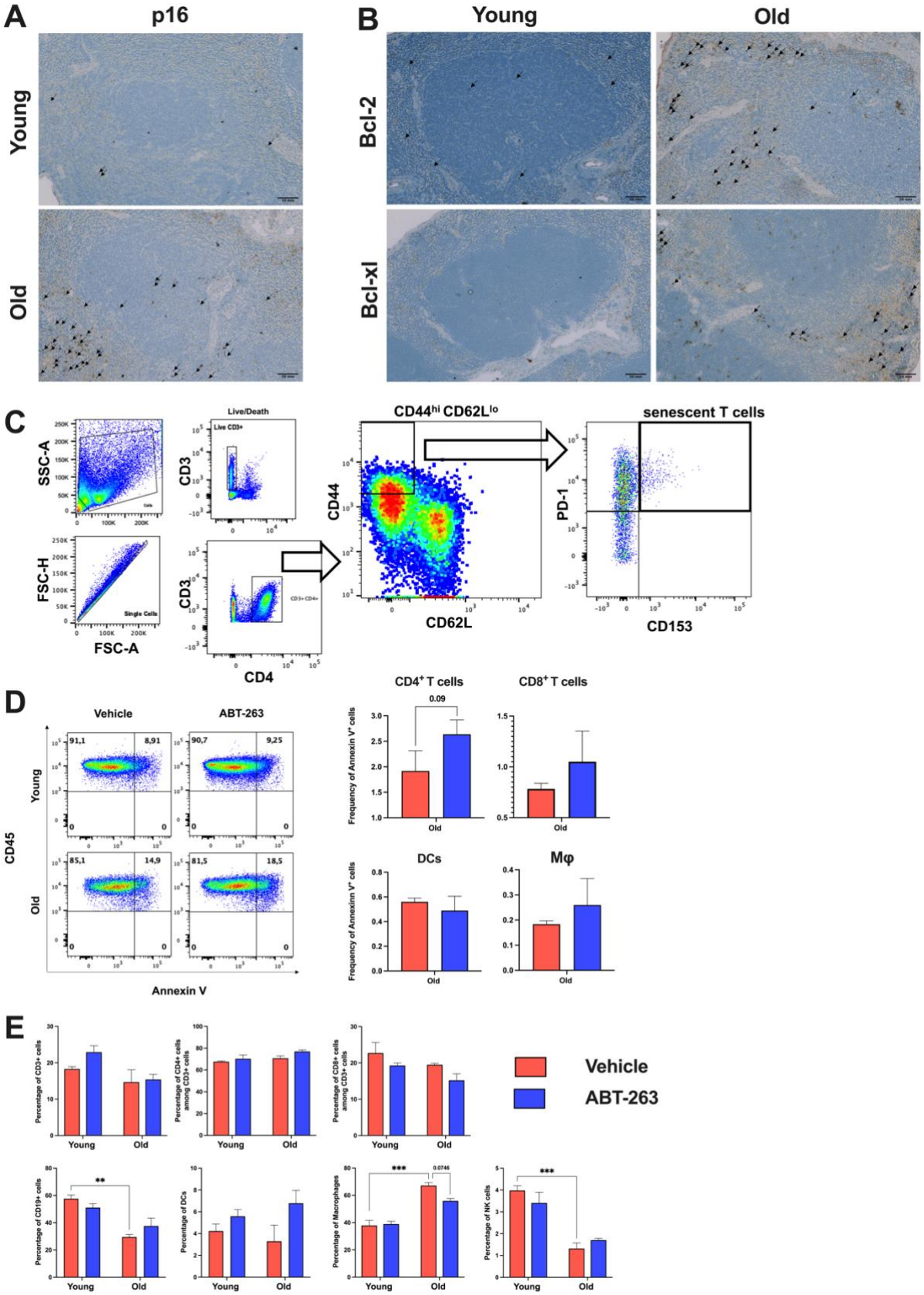


FIGURE 2 Effect of ABT-263 treatment in the SASP signature in old mice. **A**, Schematic procedure. Young and old mice were orally treated with ABT-263 for 4 days. One day (day 5) and 4 days later (day 8), cell apoptosis and cellular senescence were respectively analyzed. **B**, Representative images of SA-□-Gal staining of spleen sections. Arrows depict positive cells. **C**, Representative images of cleaved caspase-3 (day 5) and p16, Bcl-2, and Bcl-xl (day 8) staining of spleen sections. Arrows depict positive cells. **D,** The percentages of $PD1^{+}$ and $CD153^{+}$ cells within $CD4^{+}$ $CD44^{high}$ $CD62L^{low}$ cells are depicted for the four animal groups (n=6-12) (day 8). **E**, Frequencies of annexin V positive cells among CD45+ cells? (n=3) (day 5). **F**, Cytokines in blood were quantified by ELISA (n=3-5) (day 8). **G**, Splenocytes (day 8) were stimulated with LPS (1µg/ml). Supernatants were collected and cytokine production was

determined by ELISA (n=7). Pooled results from two independent experiments (**D** and **G)** and one of two representative experiments (**B, C, E** and **F**) are shown. Significant differences were determined using two-way ANOVA Tukey`s multiple comparisons test (*$P < 0.05$, **, $P < 0.01$; ***, $P < 0.01$).



Supplementary FIGURE 2. A, Representative images of p16 (**A**), Bcl-2 (**B**, upper panel) and Bcl-xl (**B**, lower panel) staining of spleen sections from young and old mice. Arrows depict positive cells. C, Analysis of CD44high CD62Llow PD1+ CD153+ senescent T cells by flow

cytometry. FSC, SSC and Zombie Red were chosen to exclude cell debris, doublets and dead cells. **D** and **E**, Young and old mice were treated with ABT-263 during four consecutive days. **D**, Mice were sacrificed one day after the arrest of the treatment and the frequencies of annexin V+ CD4+ and CD8+ T cells, DCs and macrophages were analyzed by flow cytometry. **E**, Mice were sacrificed 4 days after the arrest of the treatment. The percentages of the different cell populations as determined by flow cytometry are depicted. **A**-**E**, One experiment is depicted out of two performed (n=3-5). Significant differences were determined using the two-way ANOVA Tukey`s multiple comparisons test (**, $P < 0.01$; ***, $P < 0.01$).

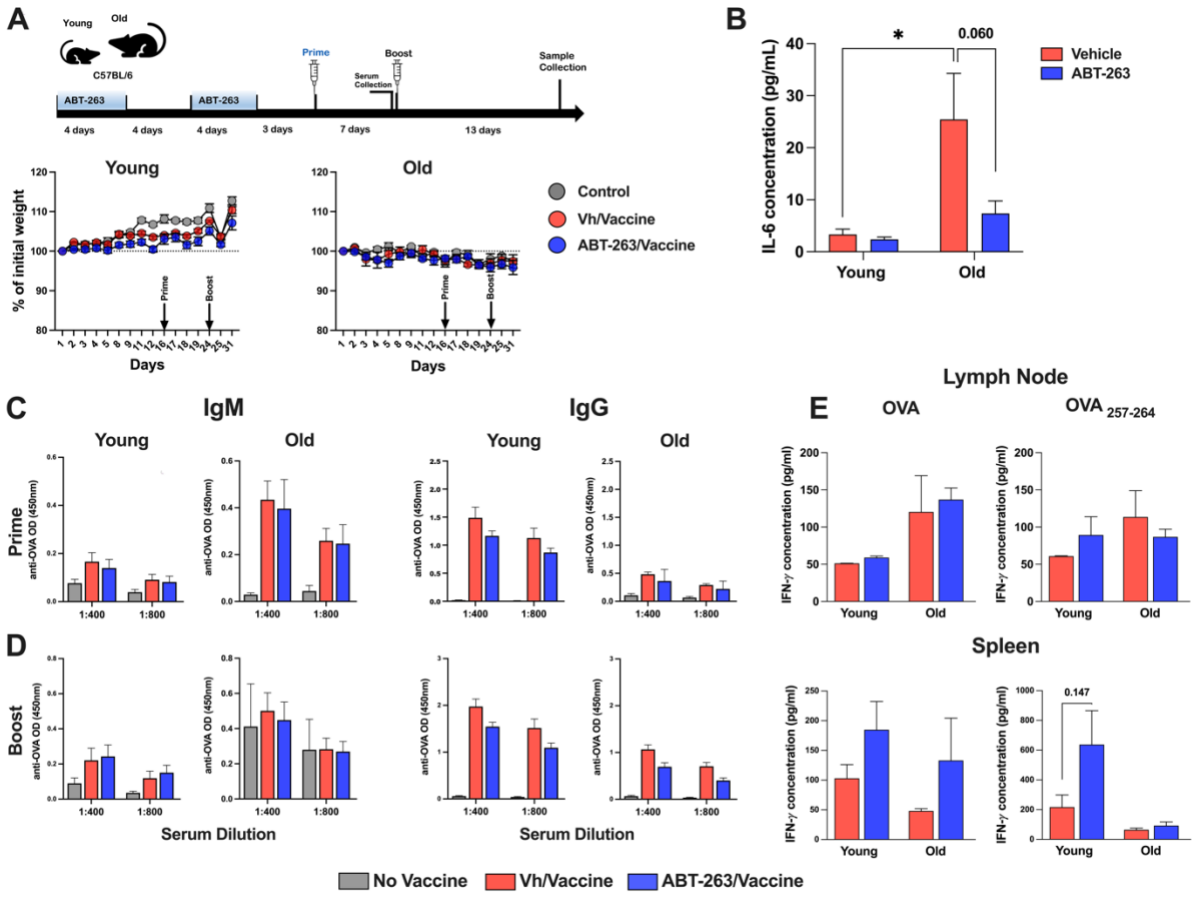
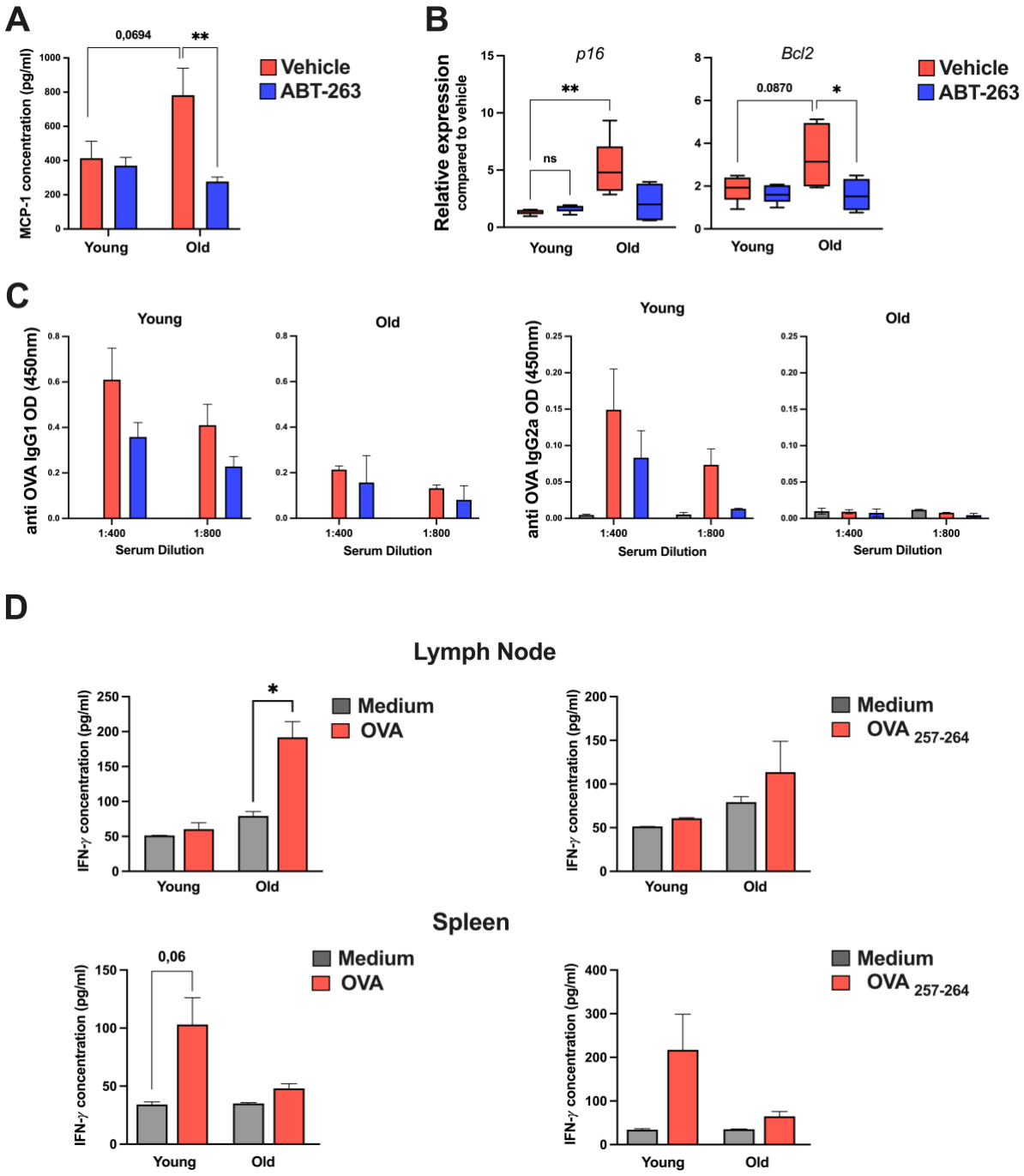


FIGURE 3 Effects of ABT-263 treatment on humoral and cellular OVA-specific immune responses in old and young mice. **A**, *Upper* panel, schematic procedure (prime-boost setting). Mice were orally treated with ABT-263 for 4 days. After a 4-day interval, the procedure was repeated. Three days after the last ABT-263 inoculation, mice were immunized with the vaccine (prime). Animals were boosted 7 days later and sacrificed 13 days after the boost. *Lower* panel, Body weight was measured over the course of treatment and vaccine procedure (n=5). **B**, IL-6 concentration in blood was quantified by ELISA (n=5). **C** and **D**, IgM and IgG titers were determined by indirect ELISA. Serum samples were collected after the prime (**C**) and after the boost (**D**) (n=5). **E**, LN cells and spleen cells from vaccinated mice were restimulated with whole OVA or $OVA_{257-264}$, for 48h. IFN-□ production was assessed by ELISA (n=5). **A**-**E**, One representative experiment out of two performed are depicted. Significant differences were determined using the two-way ANOVA Tukey`s multiple comparisons test (*$P < 0.05$).



Supplementary FIGURE 3. Young and old mice were treated or not with ABT-263 and then vaccinated (prime/boost). 13 days after the boost, animals were sacrificed. **A,** Serum MCP-1 was quantified by ELISA (n=5). **B,** *p16* and *Bcl2* gene expression in liver was measured by quantitative RT-PCR (n=4-5). **C,** OVA-specific IgG1 and IgG2a production was measured by indirect ELISA (n=3-5). **D**, LN cells and spleen cells from vaccinated young and old mice

(vehicle treated) were restimulated with OVA and the OVA peptide for 48h. Supernatants were collected and IFN-γ was quantified by ELISA (n=5). IFN-γ was not detected in unvaccinated animals (not shown). A-D, One representative experiment out of two performed is depicted. Significant differences were determined using the two-way ANOVA Tukey`s multiple comparisons test (*$P < 0.05$, **, $P < 0.01$).

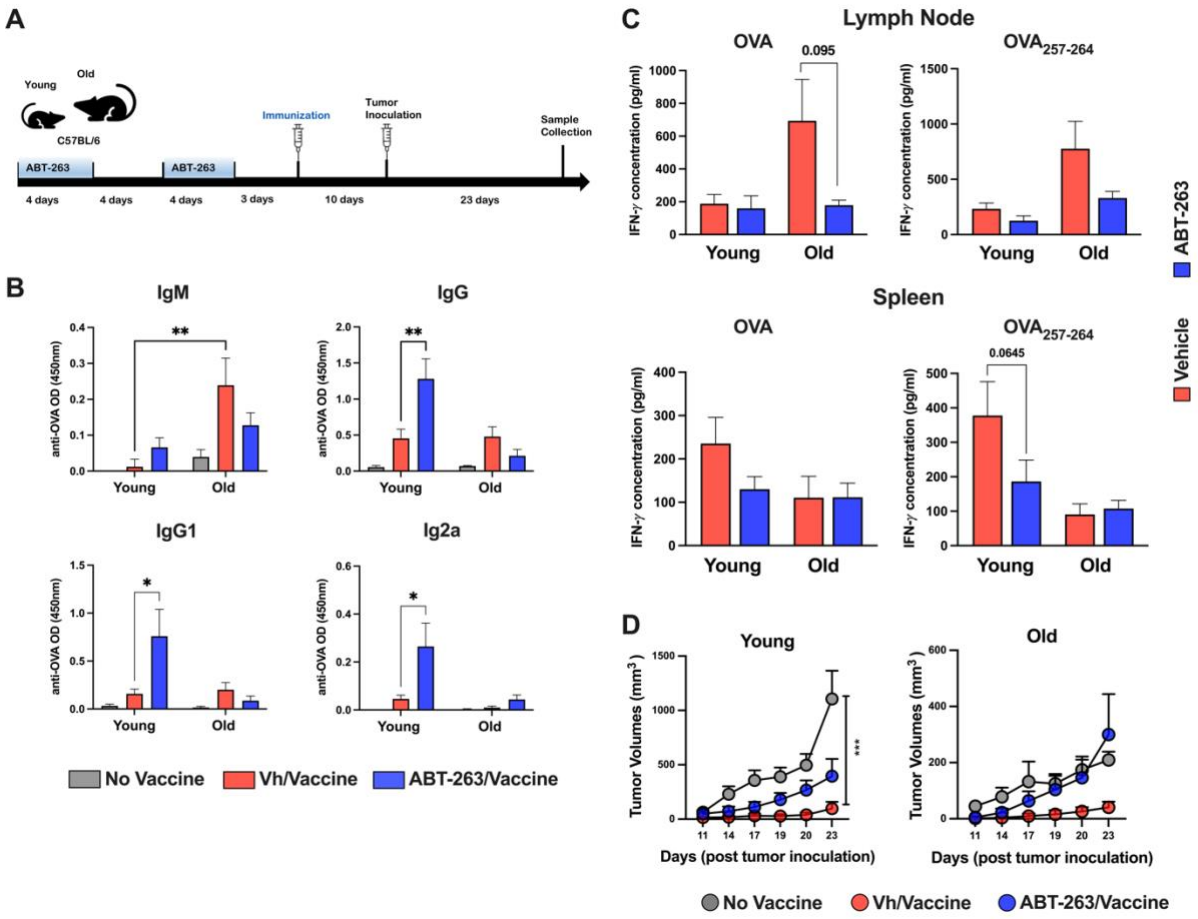
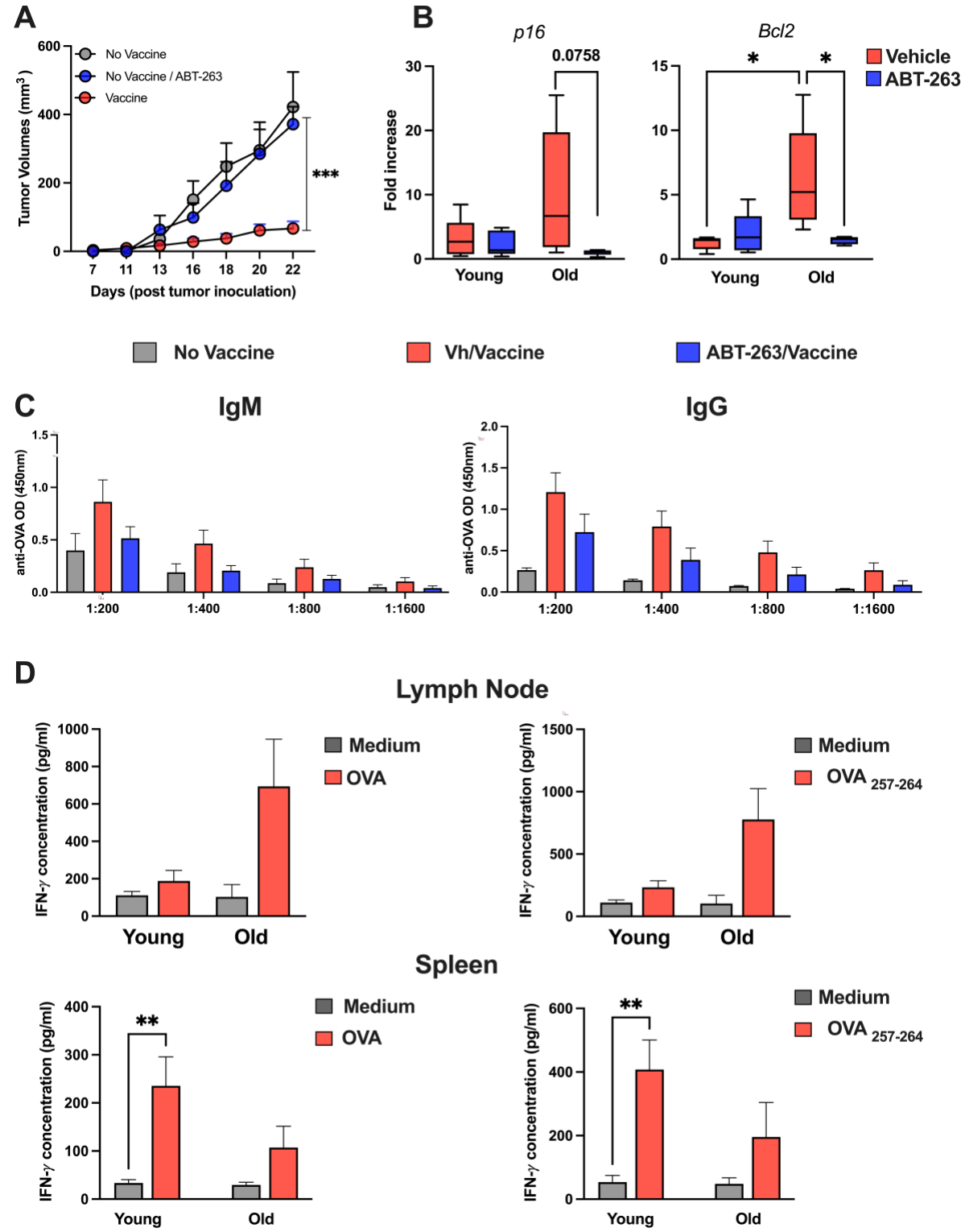


FIGURE 4 Effects of ABT-263 treatment on OVA-specific immune responses and B16-OVA growth in young and old mice. A schematic procedure (prime-only regimen and challenge with B16-OVA). Mice were orally treated with ABT-263 for 4 days. After a 4-day interval, the procedure was repeated. Three days after the last ABT-263 inoculation, mice were immunized with the vaccine. Tumor cells were inoculated 10 days later and sacrificed 23 days after the inoculation. **B**, IgM, IgG, IgG1 and IgG2a titers were determined by indirect ELISA (dilution: 1:800) (n=3 for unvaccinated mice and n=5 for vaccinated mice). **C**, LN cells and spleen cells from vaccinated young and old mice were restimulated with OVA or $OVA_{257-264}$ for 48h. Supernatants were collected and IFN-y were quantified by ELISA (n=10). **D**, B16-

OVA tumor volumes were measured over time (n=6 for unvaccinated mice and n=10 for vaccinated mice). **A** and **B**, A representative experiment out of two is depicted. **C** and **D**, A pool of two representative experiments is depicted. Significant differences were determined using the two-way ANOVA Tukey`s multiple comparisons test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.01$).



Supplementary FIGURE 4. **A,** B16-OVA cell growth was measured in non-vaccinated (vehicle and ABT-263 treated) and vaccinated young mice (n=5). **B,** *p16* and *Bcl2* gene expression in liver was measured by quantitative RT-PCR (n=4-5). **C**, OVA-specific IgM and IgG production was analyzed in vaccinated old mice treated or not with ABT-263 prior to vaccination. Serum from non-vaccinated mice were used as controls. Several dilutions of serum

were used for the ELISA assay (n=3 for unvaccinated mice and n=10 for vaccinated mice). **D**, LN cells and spleen cells from vaccinated young and old mice (vehicle treated) were restimulated with OVA and the OVA peptide for 48h. Supernatants were collected and IFN-□ was quantified by ELISA (n=10). IFN-□ was not detected in unvaccinated animals (not shown). **A** and **B**, A representative experiment out of two is depicted. **C** and **D**, A pool of two representative experiments is depicted. Significant differences were determined using the two-way ANOVA Tukey`s multiple comparisons test (*$P < 0.05$, **, $P < 0.01$,***, $P < 0.01$).

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