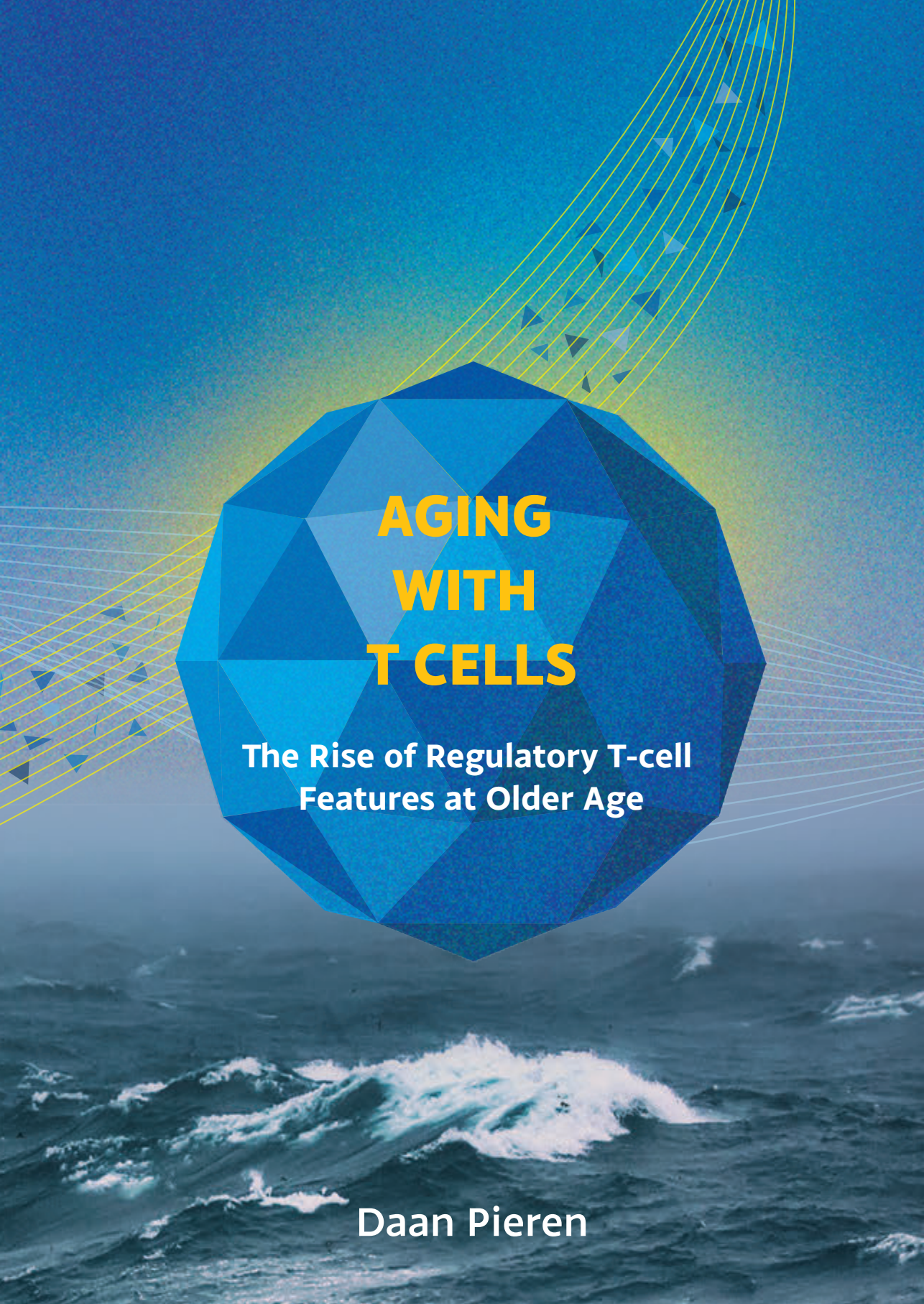
AGING
WITH
T CELLS
The Rise of Regulatory T-cell
Features at Older Age
Daan Pieren

# AGING WITH T CELLS

## The Rise of Regulatory T-cell Features at Older Age

**Daan Pieren**

The work presented in this thesis was carried out within the Centre for Infectious Disease Control of the National Institute for Public Health and the Environment (RIVM) in collaboration with the division of Infectious Diseases and Immunology (Section Immunology) of the Utrecht University.

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# AGING WITH T CELLS

The Rise of Regulatory T-cell Features at Older Age

**VEROUDEREN MET T-CELLEN**

De Toename van Regulatoire T-celkenmerken op Oudere Leeftijd
(met een samenvatting in het Nederlands)

**Proefschrift**

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# GENERAL INTRODUCTION AND SCOPE OF THIS THESIS

## AGING AND INFECTIOUS DISEASES

The proportion of people aged 60 years or older among the total world population has risen during the past years and is projected to further increase significantly during the upcoming years [1]. As stated by the World Health Organization: "the first time in history when there will be more older people than younger people is rapidly approaching" [2].

Aging highly increases the risk of acquiring infectious diseases and the course of disease is prolonged and more severe at old age. Especially respiratory infectious pathogens such as influenza virus, respiratory syncytial virus (RSV), and *Streptococcus pneumoniae* highly contribute to disease in older adults and often are the pathogens leading to hospitalization and death among aged individuals [3-9]. In addition to these well-known pathogens, West Nile Virus [10] and the currently ongoing pandemic of severe acute respiratory syndrome corona virus 2 (SARS-CoV-2), which causes coronavirus disease 2019 (COVID-19) [11,12], are examples of pathogens that emphasize the susceptibility of older adults. It has been shown that especially older adults are at higher risk of severe disease, hospitalization, and death following SARS-CoV-2 infection compared to younger individuals [11,13]. In addition, the rate of hospitalization as a consequence of community-acquired pneumonia after infection with a respiratory pathogen is especially high amongst the elderly [9]. Furthermore, hospitalization of older adults accelerates the occurrence of chronic conditions that are often the cause for disability in daily living activities [14]. Developing strategies to prevent or ameliorate infectious-disease related hospitalization is therefore important.

Vaccination against pathogens that cause disease in the elderly is used as a strategy to prevent or, more realistically, reduce the severity of clinical disease. Indeed, it has been shown that vaccination against influenza reduces the hospitalization rate amongst elderly [14,15]. However, vaccine-induced protection becomes impaired with progressing age. Studies investigating the efficacy of vaccination in elderly generally report diminished protective responses after vaccination against influenza [16-18], Japanese Encephalitis [19], Hepatitis B virus [20], and *S. pneumoniae* [21].

The immune system is one of the most important lines of defense against pathogens and can be regarded a highly specialized and balanced system: its task is to clear infections effectively enough to eradicate the pathogen, but at the same time to limit its own response to minimize collateral damage. To achieve this delicate balance, all immune processes are tightly regulated. However, during aging, the immune system undergoes changes that bring

the tightly regulated system out of balance. The age-related changes to the immune system have collectively been termed 'immunosenescence' [22]. As a consequence of immunosenescence, the effectivity of the immune system to respond and protect against pathogens declines and thereby the susceptibility to infectious diseases and disease severity increase [22].

As life-expectancy and the elderly population continues to grow, detailed insight into what happens to the immune system with age is key to understand susceptibility to disease in older adults. These fundamental insights may eventually be starting points to develop preventive strategies against infectious diseases, including vaccination, as well as to reduce the burden of disease in the elderly with the final goal to increase the amount of life years spent in good health.



## THE IMMUNE SYSTEM: T CELLS DURING VACCINATION AND INFECTION

The immune system consists of two arms: the innate immune system and the adaptive immune system. These two arms work in concert, complement each other, and require each other to exert their optimal function and to achieve optimal protection. When a pathogen is encountered for the first time, innate immune cells are triggered and specialized antigen presenting cells (APCs) take up (parts of) the pathogen. These APCs then migrate to the lymph node and present parts of the pathogen to the adaptive immune system, of which T lymphocytes (T cells) and B lymphocytes (B cells) are critical mediators [23]. T cells can roughly be divided into two subsets: $CD4^+$ T cells and $CD8^+$ T cells. Part of the $CD4^+$ T-cell population has been named 'helper T cells' as it has been shown that they interact with dendritic cells to improve the quality of a protective $CD8^+$ T-cell response against virus infected cells and tumors, as well as providing help to B cells for the production of antibodies [24,25]. Additionally, $CD4^+$ T cells can also limit disease severity after experimental influenza infection in humans, indicating a direct anti-viral role for these cells [26]. $CD8^+$ T cells are generally regarded as a T-cell subset that is capable of eliminating intracellular pathogens by killing infected cells through their cytotoxic capacity [23]. The cytotoxic capacity of $CD8^+$ T cells to kill infected cells is mediated by secretion of perforins and granzymes [27,28] and secretion of interferon-γ (IFN-γ) to enhance viral clearance [23]. The protective role of $CD8^+$ T cells has been shown in mice infected with RSV [29,30], as well as in experimentally RSV- and Influenza-virus infected humans [31,32]. During a primary response, interaction of APCs with naive T cells initiates proliferation, activation, and differentiation of

these naive T cells, which are crucial processes for the anti-viral response [33-36]. As a result, the few antigen-specific naive T cells expand into a specialized memory T-cell subset. Once the primary infection is cleared, memory T cells that recognize parts of the virus remain present, as well as antibodies that were produced by B cells [23]. Upon secondary encounter, preexisting antibodies may limit infection and memory T cells will respond quickly to prevent spread of the virus by eliminating infected cells and to protect against disease, as has been shown for influenza-virus infection [37].

The goal of vaccination is to mimic the primary response to a pathogen without causing disease, enticing the host to build up protection against subsequent encounters with the infectious pathogen. On a critical note, the main goal of the majority of vaccines against infectious diseases that have been developed or are under development is to elicit protective antibody levels [38]. Indeed, presence of functional antibodies can protect against viral infection. However, antibodies that are generated in response to infection are known to wane over time, especially in the elderly, leaving the individual susceptible to a recurrent infection. For recovery from infection, cellular responses are required [39]. T cells target internal proteins of viruses that generally are more conserved and these T-cell responses may therefore be better preserved. The role of T cells is somewhat neglected in development of vaccines, whereas it has been shown that $CD4^+$ follicular helper T cells boost generation of antibodies [40], and that $CD8^+$ T-cell responses are better correlates of vaccine protection compared to antibody levels, particularly in the elderly [41]. Moreover, waning of protective antibody levels opens a window for infection, which then needs to be resolved by generated memory T cells from previous exposure and/or vaccination. As an example, antibodies elicited after SARS-CoV-2 infection rapidly decline within two to three months after recuperation [42,43], highlighting the importance of T cells as a protective immune barrier against subsequent SARS-CoV-2 infections. Thus, a proper T-cell response induced by vaccination is important in the elderly, in the face of declining antibody levels.

## THE ACCUMULATION OF REGULATORY T CELLS DURING AGING

The composition of the T-cell population changes during aging in both the $CD4^+$ and $CD8^+$ T-cell fractions and these changes are thought to lead to susceptibility to disease and impaired vaccine-responses in the elderly. For example, one of such changes in T-cell population composition is the decline in proportion of naive T cells with age, whereas the proportion of memory T cells increases [44].

Additionally, both the $CD4^+$ and the $CD8^+$ T-cell populations comprise regulatory T cells (Tregs). Tregs are capable of suppressing the activation and proliferation of other T cells, as well as other cells of the immune system [45]. Tregs are highly important cells as they maintain homeostasis within the immune system: dampening anti-viral cytotoxic T-cell responses adequately enough to minimize immunopathology, while still allowing killing of infected cells by cytotoxic T cells. However, it has been reported that Tregs accumulate during aging, leading to the hypothesis that declined immune responses observed with age can be partly attributed to the accumulation of suppressive Tregs (**Figure 1**).

**$CD4^+$ Regulatory T cells**

The importance of $CD4^+$ Tregs was shown by one of the pioneering $T_{REG}$ studies reporting that $CD25^+$ $CD4^+$ T cells can prevent devastating autoimmune disease [46]. Later studies identified Forkhead Box P3 (FoxP3) as the transcription factor that characterizes suppressive Tregs in mice and humans [47-49]. Additionally, Tregs were also found to be highly important to prevent viral lung disease [50]. Importantly, it has been shown that aged mice accumulated Tregs [51-55] and this accumulation led to impaired protective immune responses in these mice [51,52,55]. However, the accumulation of Tregs with aging appears to depend on the site investigated: aged mice accumulate Tregs in their lymphoid organs (although not in the thymus). Observations on accumulation of Tregs in peripheral blood of aged mice are not all consistent, although most studies do not find increased $T_{REG}$ levels [51]. In line with this, studies remain inconclusive whether Tregs accumulate in the peripheral blood of older humans, showing both increased [51,56,57] and stable $T_{REG}$ levels [58,59]. A study that assessed the proportion of human Tregs outside of the blood indicate accumulation of Tregs with aging in human skin [60]. Thus, mostly mouse-derived evidence seems to point towards accumulation of Tregs outside of the peripheral blood, thereby maintaining the hypothesis that this accumulation may dampen protective responses in the elderly (**Figure 1**). However, how and why Tregs accumulate with age remains to be elucidated.

**$CD8^+$ Regulatory T cells**

Whereas the existence of $CD4^+$ Tregs has been widely described and accepted, the existence of $CD8^+$ Tregs is more unclear and controversial as $CD8^+$ T cells are mostly regarded as efficient killer cells targeting infected or abnormal cells. However, evidence for $CD8^+$ Tregs is re-emerging after the initial studies reporting on $CD8^+$ Tregs in the 1970's [61,62]. The phenotype of $CD8^+$ Tregs that distinguishes these cells from conventional $CD8^+$ T cells is currently still under

investigation and a clear-cut phenotype has not been established. In mice, a subset of $CD8^+$ Tregs has been characterized by expression of CD122 [63], the β-chain of the interleukin (IL)-2 and IL-15 receptors, and Programmed cell Death protein-1 (PD-1) [64]. These cells produced IL-10 as one of the mechanisms for their suppressive capacity [64,65], and showed increased suppression in the presence of IL-15 [64]. Additionally, the transcription factor Helios was reported to be required for the stabilization of suppressive functionality of $CD8^+$ Tregs [66], potentially being an important hallmark for the characterization of $CD8^+$ Tregs. In humans, several phenotypes have been reported to describe $CD8^+$ Tregs, including $CD8^+CXCR3^+$ T cells [67], and $CD25^+$ $FoxP3^+$ $CD8^+$ T cells [68-70]. To date, $CD8^+$ Tregs have been shown to prevent allograft rejection [64], accumulation of abnormally activated T cells [63], and experimental autoimmune encephalitis [71] in mice, the latter by suppression of autoreactive $CD4^+$ T cells [72]. $CD8^+$ Tregs have also been shown to increase with age in mice [52], although this is still unclear in humans, as studies show higher [69] and lower levels of $CD8^+$ Tregs [73]. These differences are most likely due to the use of different markers to identify $CD8^+$ Tregs. Thus, whether $CD8^+$ T cells with immunosuppressive capacity accumulate with age and dampen protective T-cell responses remains to be explored.

## AGING WITH T CELLS: HOW DO T CELLS CHANGE?

It is well known that aging results in a decline of immune functions throughout the immune system [22], collectively called 'immunosenescence'. T cells are also subject to immunosenescence, as they show changes in their phenotype, and proliferative and activation potential [44]. Immunosenescence of T cells is used as a general term to describe two hallmarks that occur to T cells during aging: T-cell senescence and T-cell exhaustion (**Figure 2**). Together, these changes are speculated to impair the T-cell response against infections, as has been shown in aged mice in primary [74-78] and secondary [79] T-cell responses against infection, and studies in humans showed similar results [80-82]. Thus, the development and accumulation of immunosenescent T cells during the process of aging is thought to be an important mediator for increased susceptibility to infectious disease in older adults. However, in-depth knowledge on how T cells alter during aging and whether this alteration causes susceptibility to disease is still lacking.

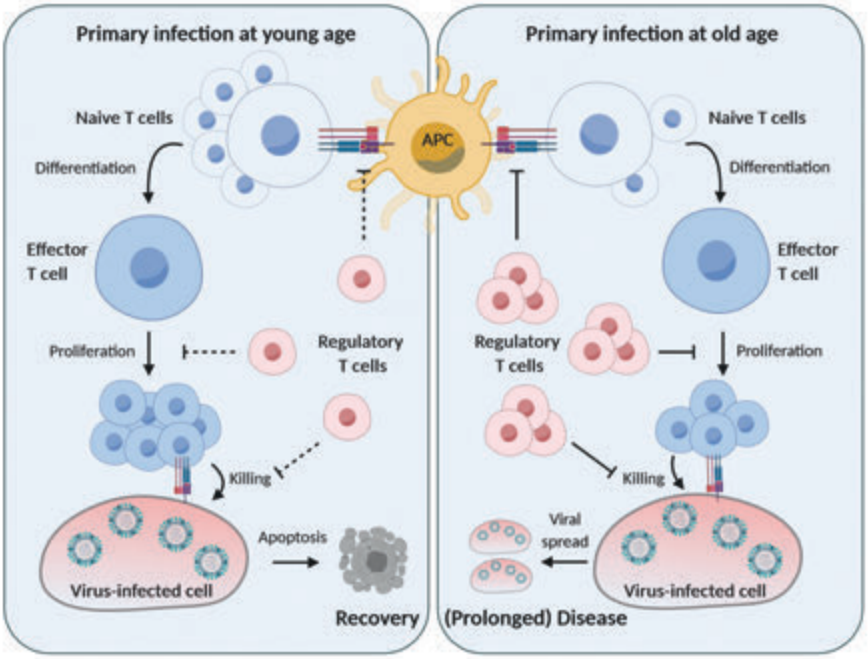


**Figure 1. Potential consequences of the accumulation of regulatory T cells during aging.** Schematic representation of a primary viral infection and part of the subsequent immune response at young and old age. After viral infection, antigen-presenting cells (APCs) present part of the pathogen to naive T cells within the lymph node. At younger age, naive T cells differentiate into effector T cells, which in turn expand by cellular proliferation. Effector T cells then target virus-infected cells and kill these cells, resulting in apoptosis of the virally infected cell to limit viral dissemination. Moreover, during these responses in young individuals, regulatory T cells (Tregs) maintain homeostasis by interfering at multiple steps in this process to prevent immunopathology. Tregs may limit APC-naive T-cell interactions, limit effector cell proliferation, and limit effector cell-mediated killing. However at old age, Tregs have accumulated and are therefore much more potent at dampening the three aforementioned steps towards killing of the infected cell. Suppression of killing may therefore result in viral dissemination, leading to more infected cells and prolonged and/or more severe disease.

### T-cell Senescence

Outside of the field of immunology, cellular senescence is used to describe a state of cells that are irreversibly stuck in their cell cycle and do not proliferate anymore due to irreparable cell damage [83]. By preventing proliferation of damaged cells, cellular senescence is viewed as a process that prevents or limits tumor growth [84]. One of the hallmarks that drives cellular senescence is the intracellular accumulation of DNA damage [83]. Senescent cells are known to slowly accumulate over a lifetime [85]. These cells have popularly been termed ‘zombie cells’, as it has been shown that these cells accumulate with age in a variety of organs, do not divide, nor undergo apoptosis [86]. However, these

cells secrete a range of inflammatory cytokines (e.g. tumor necrosis factor alpha (TNF-α) and IL-6) collectively called the senescence-associated secretory phenotype (SASP) [86,87].

Within the field of T-cell immunology and aging, accumulation of senescent T cells is thought to partly explain reduced T-cell protective responses in older adults. As a way to identify senescent T cells, both $CD4^+$ and $CD8^+$ T cells have previously been stratified into three differentiation stages based on their expression of co-stimulatory receptors CD27 and CD28 [88]. $CD8^+$ T cells lose CD27 and CD28 expression during aging as these cells convert from $CD27^+CD28^+$ early-differentiated cells to $CD27^+CD28^-$ intermediate-differentiated cells and, finally, into $CD27^-CD28^-$ late-differentiated T cells [88-91], which are thought to be senescent T cells. Late-differentiated $CD27^-CD28^-$ cells show reduced proliferation and responsiveness [88,92,93], which are two vital processes for the maintenance and protective ability of adaptive immune responses. Moreover, several alterations have been reported within these cells that can be linked to impaired functionality of these cells, such as increased expression of the senescence marker p16 [94], increased presence of DNA damage [93,95,96], and shortened telomeres [91].

**T-cell Exhaustion**

Expression of co-inhibitory receptors on $CD8^+$ T cells is extensively being studied in the T-cell aging field as it is thought that increased expression of these receptors may account for the decline of $CD8^+$ T-cell responses with age. T-cell 'exhaustion' was initially coined in the context of chronic viral infections in mice [97] and was later linked to sustained expression of co-inhibitory receptors [98]. Exhausted T cells show a gradual loss of function which is thought to develop through high- and/or chronically present antigen load to which T cells respond [85,99]. Discovery of novel (combination of) T-cell exhaustion and inhibitory markers and insight into their role during aging is important to explain the decline of T-cell responses with age.

PD-1 is one of the most well-known co-inhibitory markers that reduces T-cell proliferation and effector functionality [100]. The first study that strikingly showed the importance of PD-1 in mice showed that during an acute infection, PD-1 is highly upregulated on T cells and was required to prevent overt inflammatory response, after which PD-1 expression was downregulated [101]. However, chronic infection leads to sustained expression of PD-1, and thereby limits the protective response [101]. In mice it was discovered that aging promotes the accumulation of PD-1-expressing cells [102-105]. Most likely, existence of exhausted cells over the course of aging in mice is due to

sustained antigen exposure over a lifetime. Currently, multiple markers for cellular exhaustion in addition to PD-1 have been reported to increase in aged mice: Lymphocyte-activation gene 3 (LAG-3), T-cell immunoglobulin and mucin-domain containing-3 (TIM-3) expression by $CD8^+$ T cells have been shown to mark populations of exhausted cells that increase with age in mice [102,106,107].

In contrast to findings in mice, expression of co-inhibitory receptors by human T cells is less evident. Age-related increased expression of LAG-3 by $CD4^+$ and $CD8^+$ T cells in blood of individuals aged 61 years or older was shown, whereas expression of PD-1 and TIM-3 were not found to increase [108]. Recently, the proportion of $CD8^+$ T cells that expressed the co-inhibitory receptor T-cell immunoglobulin and ITIM domain (TIGIT) was shown to increase with age in humans [108]. To date, TIGIT is one of the few co-inhibitory markers of which it convincingly has been shown in humans that its expression increases with age. TIGIT-expression by antigen-specific $CD8^+$ T cells have been shown to mark exhausted $CD8^+$ T cells in the context of viral infection and cancer [109-111]. TIGIT competes with the co-stimulatory receptor CD226 for their shared ligands CD155 and CD112 expressed by APCs [109,112,113]. The balance between expression of TIGIT and CD226 on a single $CD8^+$ T cell is important, as signaling through TIGIT results in T-cell inhibition, whereas signaling through CD226 results in T-cell activation. In line with this, $TIGIT^+$ $CD8^+$ T cells have been reported to be functionally impaired compared to $TIGIT^-$ $CD8^+$ T cells [108,109]. The age-related increase of $TIGIT^+$ $CD8^+$ T cells is therefore thought to diminish $CD8^+$ T-cell responses in the elderly, potentially explaining their increased susceptibility to infectious diseases. This is currently also speculated to occur during severe COVID-19 disease, caused by SARS-CoV-2 infection [114] and may be applicable to other infectious diseases as well.

**Inflammaging**

In addition to T-cell senescence and exhaustion, another phenomenon that has been reported to occur at older age is so-called 'inflammaging' (**Figure 2**). Whereas intermittent increases in the level of pro-inflammatory cytokines are vital in responses against infections to survive, inflammaging is characterized by the presence of a constant low-grade inflammatory state [115-117]. This state is characterized by increased circulating levels of pro-inflammatory factors, including TNF-α, IL-6, and C-reactive protein (CRP). Inflammaging is thought to develop over time as a result of multiple factors, including lifestyle factors and environmental factors, such as exposure to antigens over a lifetime [116,118], which lead to increased susceptibility to communicable and non-

communicable diseases. For example, obesity is known to promote a pro-inflammatory cellular environment as it dysregulates the metabolic system and in turn this limits protective immune responses against infectious pathogens and vaccination [119,120]. Moreover, it is currently speculated that obesity may shift severe COVID-19 disease towards younger age groups in countries with high prevalence of obese younger individuals [121]. Additionally, inflammaging has been associated with increased prevalence of cardiovascular disease, diabetes mellitus, and autoimmune disease [122].

Recent studies suggest that senescent T cells may be a cause for the occurrence of inflammaging by secretion of SASP-related proteins [123]. $CD4^+$ T cells of older adults have been shown to produce higher levels of pro-inflammatory cytokines, thereby contributing to inflammaging [124]. Moreover, T cells have recently been found to be an important driving factor in the development of inflammaging in mice [125]. T cells with a mitochondrial dysfunction were found to induce a pro-inflammatory cytokine storm that consisted of increased serum levels of IFN-γ, TNF-α, and IL-6. In turn, these cytokines were found to drive cellular senescence of peripheral tissues which could in part be reversed by blocking of TNF-α. Thus, these studies suggest that T cells may be a driving factor of inflammaging, a process that is linked to susceptibility to both communicable and non-communicable diseases.

**To conclude, T cells have a central role in increased susceptibility to infectious disease and reduced responsiveness to vaccination found in older adults. Elucidating the underlying mechanisms for diminished T-cell responses with age in the context of infection and vaccination is one of the most important aims in the field of aging of the immune system. Identifying changes that occur to T cells with age may drive future research on potential interventions to promote T-cell responses in older adults.**

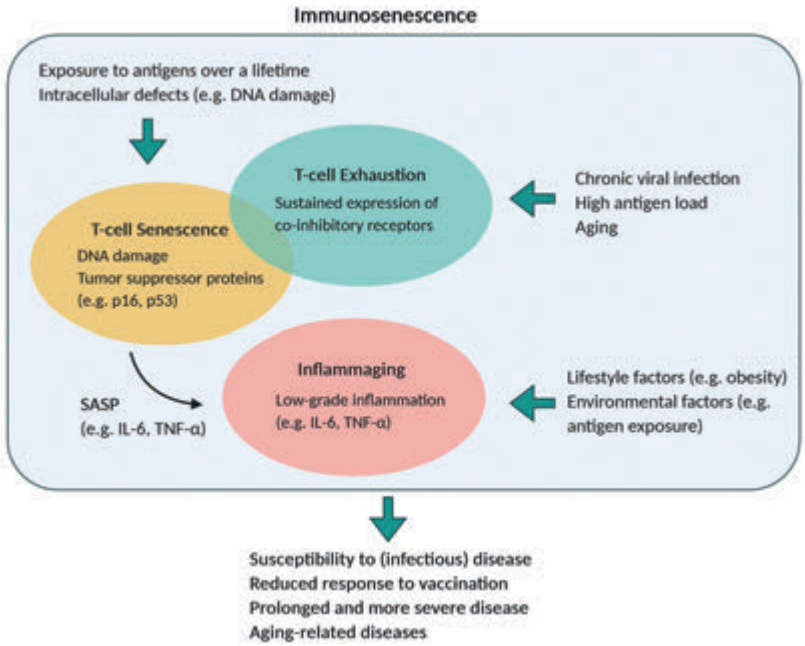


**Figure 2. Processes that contribute to immunosenescence.**
Schematic representation of the three processes that contribute to 'immunosenescence'. Both T-cell senescence and T-cell exhaustion are characterized by the loss of T-cell function, such as reduced proliferative capacity. However, T-cell senescence is thought to develop as a consequence of exposure to different antigens over a lifetime, leading to irreparable cell damage and expression of tumor suppressor proteins which in turn limit T-cell function. T-cell exhaustion is thought to develop as a consequence of chronic viral infection and/or presence of a high antigen load, but has also been shown to develop during aging in the absence of these causes. Inflammaging is characterized by the low-grade constant presence of pro-inflammatory proteins, such as IL-6 and TNF-α. The source of these pro-inflammatory mediators that lead to inflammaging lies at lifestyle (e.g. obesity) and environmental factors (e.g. antigen exposure), but also as a consequence of senescent cells secreting the senescence-associated secretory phenotype (SASP). Together, immunosenescence contributes to susceptibility to disease, reduced response to vaccination, prolonged and more severe disease, and aging-related diseases, such as cardiovascular disease and diabetes.

## AIM AND SCOPE OF THIS THESIS

T cells are imperative for protection against pathogens and various molecules on different subsets of T cells are involved in maintaining a balanced immune system. T cells express molecules that initiate cellular activation and proliferation, but there are also molecules that act as a breaking mechanism on these responses to prevent over-activation. During aging, this system becomes dysregulated. To explain reduced response to vaccination, susceptibility to infectious disease, and increased disease severity observed at older age, it is crucial to identify the underlying age-related changes of T cells. In this thesis, we set out to identify novel age-related changes of the T-cell population in mice and humans that may contribute to understanding the impact of aging on T cells. We investigate T-cell phenotype, responses, and proliferation in both mice and humans under healthy conditions and during respiratory viral infection in humans.

The kinetics of a T-cell response (i.e. T-cell activation and proliferation over time after stimulation) are often overlooked in literature. However, proliferation and activation kinetics of a T-cell response may precisely be the factors that determine the outcome of an immune response. In **chapter 2** of this thesis, we investigate the impact of aging on the phenotype of $CD4^+$ and $CD8^+$ T cells and their responsiveness to stimulation over time. Our findings on alterations of T-cell response kinetics as a consequence of aging may serve as a new hallmark of T-cell aging and contribute to understanding the reduced response of T cells after vaccination or infection.

One of the most important hallmarks of aging in many organisms is the accumulation of DNA damage over a lifetime. Accumulation of DNA damage within the nucleus of a cell causes the cell to become senescent. However, the impact of DNA damage on T cells during aging is not fully known. In **chapter 3**, we investigate the impact of compromised repair of DNA damage on the phenotype and responsiveness of T cells by using a specific mouse model for cellular senescence. Mice that have a deficiency in one of the DNA repair genes (*Ercc1*) show compromised DNA repair and therefore accumulate DNA damage. The *Ercc1* mouse model enables the investigation of senescent cellular environment on T cells that reside in this environment. In this chapter, we use the *Ercc1* mouse model to study the effect of compromised DNA repair on T-cell phenotype and responsiveness.

Chapters 4 and 5 of this thesis address the impact of aging on $CD8^+$ T cells in humans. In **chapter 4**, we aimed to improve the insight into the progression of immunosenescence in human $CD8^+$ T cells. We addressed this aim by

identifying novel combinations of age-related markers on $CD8^+$ T cells and their relationship to the three differentiation stages described for $CD8^+$ T cells, based on expression of co-stimulatory receptors CD28 and CD27.

In **chapter 5**, we continue our search for novel age-related effects on the human $CD8^+$ T-cell population. In this chapter, we identify a new human $CD8^+$ T-cell subset that remained hidden within the virtual memory T cell subset (an innate-like population of $CD8^+$ T cells that has recently been found to associate with aging). We named these $CD8^+$ cells $KIR^+RA^+$ T cells based on their expression of KIR and CD45RA. Here, we investigate the phenotype and function of $KIR^+RA^+$ $CD8^+$ T cells in young and older adults, and we address their relevance in a clinical setting in individuals that are suffering from respiratory viral infection, including Influenza A virus and the novel SARS-CoV-2.

Lastly, **chapter 6** of this thesis summarizes our findings, discusses our conclusions within the current literature and provides future perspectives based on our findings.

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# RESPONSE KINETICS REVEAL NOVEL FEATURES OF AGEING IN MURINE T CELLS

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## ABSTRACT

The impact of ageing on the immune system results in defects in T-cell responsiveness. The search for ageing hallmarks has been challenging due to the complex nature of immune responses in which the kinetics of T-cell responsiveness have largely been neglected.

We aimed to unravel hallmarks of ageing in the kinetics of the murine T-cell response. To this end, we assessed ageing-related T-cell response kinetics by studying the effect of the duration and strength of *in vitro* stimulation on activation, proliferation, and cytokine secretion by T cells of young and aged mice.

Collectively, our data show that stimulatory strength and time kinetics of cytokine secretion, activation markers, and proliferation of Th, Tc, and Treg cells are crucial in understanding the impact of ageing on T cells. Despite low proliferative capacity, T-cell subsets of aged mice do respond to stimulation by upregulation of activation markers and secretion of cytokines.

These findings therefore indicate that replicative senescence of aged T cells is not a measure of unresponsiveness per se, but rather stress that ageing influences the kinetics of proliferation, upregulation of activation markers and cytokine secretion each to a different extent.

## INTRODUCTION

The immune system reflects consequences of ageing by many alterations in the T-cell population that compromise T-cell responsiveness at old age [1,2]. Ageing-related changes have been widely reported in helper T cells (Th), cytotoxic T cells (Tc), and regulatory T cells (Treg) that act in concert to provide T cell-mediated immunity. Changes due to ageing occur among a wide variety of different immune parameters, such as the induction of cell surface activation markers, secretion of cytokines, and proliferative capacity [3-6]. The complexity to which ageing alters T-cell responses poses a major challenge in research on T-cell ageing. Whereas many studies address ageing-related T-cell phenotypes, only limited insight is available on the impact of ageing on the response kinetics over time [7]. In this study, we assessed ageing-related T-cell response kinetics by studying the effect of the duration and strength of *in vitro* stimulation on activation, proliferation, and cytokine secretion by T cells of young and aged mice.



T cells of humans and mice rapidly upregulate expression of classical activation markers CD69 and CD25 after stimulation [8,9]. Upregulation of these markers at older age in human and murine T cells is reduced [10-13]. Expression of Programmed cell death-1 (PD-1) and Cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) is upregulated after T-cell activation [14,15], yet a substantial proportion of T cells of aged mice show constitutive expression of these inhibitory markers [16-19]. Additionally, ageing diminishes the capacity of T cells to proliferate in humans and mice [7]. Reduced proliferation is a major characteristic of T-cell senescence [6]. As both proliferation and expression of activation and inhibition markers by T-cell subsets are highly dynamic during an immune response, elucidating the kinetics of these parameters may reveal ageing-related alterations of T-cell responsiveness.

Cytokine secretion after *in vitro* stimulation of T cells is also known to alter with ageing in both humans and mice [20]. However, findings are highly ambiguous in part due to the lack of studies addressing time kinetics of cytokine secretion, while these kinetics are vital for understanding ageing-related alterations [20]. For example, many studies in both humans and mice have shown contradicting results on the impact of ageing on IFN-γ [12,16,21-29] and IL-2 [20-23,27,30-35] secretion by cells. In addition, a suggested shift from a Type-1 towards a Type-2 cytokine secretion profile due to ageing [36,37] has also been counteracted in other studies [20,21,23,24]. The lack of consensus on the impact of ageing on secreted cytokines may be caused by a lack of

time kinetics in cytokine secretion assays as well as differences in strength of stimulation.

In this study, we aimed to reveal the impact of ageing on T-cell responsiveness by assessing the *in vitro* response kinetics of cytokine secretion, activation marker upregulation, and proliferation of T cells of young and aged mice in response to antigen-independent stimulation. We found that despite low proliferative capacity, T cell subsets of aged mice do respond to stimulation by upregulation of activation markers and secretion of cytokines. Furthermore, dimensionality reduction (viSNE) [38] analyses allowed us to assess the phenotypical changes occurring in T cells over time and revealed increased variation in the responsiveness of T-cell subsets of aged mice. Our findings stress the importance of addressing T-cell response kinetics and the strength of stimuli used to characterize the impact of ageing on the T-cell compartment.

## MATERIALS AND METHODS

### Mice

Young (2 months of age) and aged (age range groups: 17-18 months, 22-24 months, and 28 months of age) C57BL/6 mice were purchased from Envigo (Venray, Limburg, The Netherlands). Mice were maintained at the animal facilities of the Institute for Translational Vaccination (Bilthoven, Utrecht, The Netherlands).

### Ethics

Animal studies were approved by the Animal Ethical Committee of the National Institute for Public Health and the Environment (DEC no. 201400042). All procedures were carried out in accordance with Dutch national legislation.

### Preparation of single cell suspensions and proliferation labelling

Spleen single cell suspensions were prepared by homogenizing the spleen through a cell strainer. Red blood cells were lysed with ACK lysis buffer (0.155 M NH4Cl; 10 mM KHCO3; 0.1 mM Na2EDTA, pH 7.2-7.4). Labelling to track proliferation was performed as follows: splenocytes were resuspended in PBS to $10*10^6$ cells/mL and then labelled with 0.5 μM CellTrace™ Violet (Invitrogen, Carlsbad, CA, USA) in PBS per milliliter of splenocyte suspension for 20 minutes at $37^0$C. Cells were then washed twice with ice-cold RPMI (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) medium containing 10% fetal calf serum (FCS, Greiner Bio-One, Kremsmünster, Austria).

### *In vitro* antigen-independent stimulation and splenocyte culture

Soluble anti-CD3 (clone 145-2C11, eBioscience, San Diego, CA, USA) and anti-CD28 (aCD28, clone PV-1, SouthernBiotech, Birmingham, AL, USA) were used to stimulate cells to a low (0.019 µg/mL anti-CD3), intermediate (0.019 µg/mL anti-CD3 + 0.5 µg/mL anti-CD28), or high (0.5 µg/mL anti-CD3 + 0.5 µg/mL anti-CD28) extent over time. For proliferation assays in response to exogenous IL-2, 0.1 µg/mL recombinant murine IL-2 (eBioscience) was added with and without the presence of low anti-CD3 (0.019 µg/mL). Stimulatory conditions were prepared in RPMI medium containing 10% FCS and then added to the splenocyte suspensions ($4*10^5$ cells/well) before incubation in 96-well U-bottom plates (CELLSTAR, Greiner bio-One) at $37^0$C for up to four days.



### Immunofluorescence labelling and flow cytometric analyses

Single cell suspensions were washed and labelled at $4^0$C for a combination of cell surface markers with the following antibodies: anti-CD4-BUV395 (clone GK1.5), anti-CD8a-V450 (clone 53-6.7), anti-CD122-PE-CF594 (clone TMbeta1), anti-CD44-V450 (clone IM7) and anti-CD69-BV786 (clone H1.2F3) (BD Horizon, Franklin Lakes, NJ, USA); anti-CD69-PerCP-Cy5.5 (clone H1.2F3) and anti-PD-1-BV785 (clone 29F.1A12) (BioLegend, San Diego, CA, USA); anti-CD25-PECy7 (clone PC61.5) (eBioscience); and Live/Dead Fixable Aqua (Invitrogen). Cells were labelled intracellularly with the following antibodies according to the FoxP3 Transcription Factor staining buffer set protocol (eBioscience): anti-CD3zeta-FITC (clone H146-968) (Abcam, Cambridge, Cambridgeshire, UK); anti-CTLA-4-BV605 (clone UC10-4B9), and anti-TNF-α-BV785 (clone MP6-XT22) (BioLegend); anti-FoxP3-eFluor660 (clone 150D/E4), anti-GARP-PE (clone YGIC86), anti-IFN-γ-PE-Cy7 (clone XMG1.2), and IL-4-PE (clone 11B11) (eBioscience); anti-IL-5-PE (clone TRFK5) (BD Pharmingen). Labelled cells were detected on a BD LSRFortessa X-20 (BD Biosciences, Franklin Lakes, NJ, USA). Gating analyses were performed using FlowJo software (Tree Star, Ashland, OR, USA).

### Cell proliferation

Proliferation of cells was measured using detection of CellTrace by flow cytometry. As reported earlier [39], the fold change proliferation was calculated by dividing the CellTrace median fluorescent intensity (MFI) of the medium control by the stimulated cells of each individual animal.

### Dimensionality reduced analyses

Dimensionality reduced analyses (viSNE) of flow cytometry data were performed in Cytobank (www.cytobank.com) [38]. Cell density maps of dimensionally reduced single-cell viSNE data showing clustering of $CD4^+$ and $CD4^-$ naive and memory populations or Th, Tc, and Treg-cell subsets of six pooled young mice (2 months old) and four pooled aged mice (28 months old) before and after receiving an intermediate strength of stimulation for four days. Expression of the designated cellular markers in the heatmaps was based on their ArcSinh5-transformed median expression. Indicated cluster frequencies were based on the cells present within the gates of the viSNE plots. The total viSNE plot of each T cell subset for each indicated day comprised 100% of cells of that subset.

### Cytokine assays in supernatant

Supernatants of cell cultures were stored at -80$^0$C and thawed before measuring the cytokines present. Secreted cytokines in culture supernatants were measured by using a Milliplex MAP kit Mouse Cytokine/Chemokine Magnetic Bead Panel (Millipore, Burlington, MA, USA) according to the manufacturer's instructions. The following cytokines were measured: IL-2, IL-4, IL-5, IL-10, IL-17, IFN-γ, and TNF-α. Cytokines were detected on a Luminex (Bio-Rad, Hercules, CA, USA).

### Intracellular cytokine analysis

For intracellular cytokine labelling, splenocyte suspensions (2*10$^5$ cells/well) of young and aged mice were cultured with phorbol 12-myristate 13-acetate (PMA) (0.05 μg/mL) and ionomycin (0.5 μg/mL) (Sigma Aldrich) in medium for four hours in total. After one hour of culturing, GolgiPlug (containing Brefeldin A; 1:1000, BD Biosciences) was added to allow intracellular accumulation of produced cytokines. After labelling with anti-CD4, anti-CD8, and Live/Dead Fixable Aqua, cells were fixed and permeabilized and labelled intracellularly with anti-CD3 and for the presence of IFN-γ, TNF-α, IL-4, and IL-5 as described in our flow cytometric labelling protocol.

### Statistical analyses

Statistical analyses were performed using GraphPad Prism 7 software (La Jolla, CA, USA). Statistical significance was determined by using Mann-Whitney U test, Kruskal-Wallis test, or Two-way ANOVA. For all analyses, $p$ values < 0.05 were considered statistically significant.

## RESULTS

### Proportion of splenic regulatory T cells increases with progressing age

We assessed the composition of the total splenic CD3$^+$ T-cell pool of young (n=6 per experiment, 2 months old) and aged mice of various ages (n=4-6 per experiment, 17 to 18 months, 22 to 24 months, and 28 months old). Using flow cytometry, significantly lower frequencies of CD3$^+$ T cells were detected in the spleens of aged mice compared to young mice, except for the oldest group of mice (**Figure 1A, Supplementary Figure 1**). Within this T-cell pool, we found decreased proportions of Th cells (CD3$^+$CD4$^+$FoxP3$^-$) (**Figure 1B**), comparable proportions of Tc cells (CD3$^+$CD4$^-$FoxP3$^-$) (**Figure 1C**), and increased proportions of Treg cells (CD3$^+$CD4$^+$FoxP3$^+$) (**Figure 1D**) with progressing age. Defining Tc cells as CD3$^+$CD4$^-$FoxP3$^-$ may also include small proportions of non-Tc-cell subsets, such as CD4$^-$CD8$^-$ T cells or γδ T cells. However, an additional data set showed that the proportion of these cells did not differ between young and aged mice (**Supplementary Figure 1**).

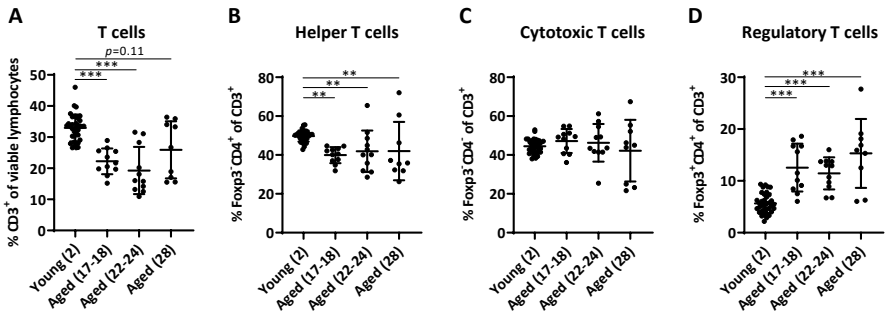


**Figure 1. Proportion of regulatory T cells increases with progressing age.**
The frequencies of T cells (CD3$^+$ of viable lymphocytes) (**A**), Th cells (FoxP3$^-$CD4$^+$ of CD3$^+$) (**B**), Tc cells (FoxP3$^-$ CD4$^-$ of CD3$^+$) (**C**), and Treg cells (Foxp3$^+$ CD4$^+$ of CD3$^+$) (**D**) were determined in spleens of young (2 months old, n=36) and aged mice of three age categories (17-18 months old, n=11; 22-24 months old, n=11; 28 months old, n=9). Numbers between brackets indicate the age in months. Mean ± SD; $^{**}p < 0.01$, $^{***}p < 0.001$ for difference between young and each group of aged mice using Kruskal-Wallis test.

### Proliferative T-cell responses decline with progressing age

Next, we exposed splenic single-cell suspensions of young and aged mice to a low (low anti-CD3), intermediate (low anti-CD3 + anti-CD28), or high (high anti-CD3 + anti-CD28) strength of stimulation to investigate the proliferative capacity of Th, Tc, and Treg cell subsets. Proliferation of Th, Tc, and Treg cells was measured by flow cytometry up to four days after stimulation (**Figure 2**). Proliferative capacity of all three T-cell subsets showed a gradual decline with

older age (**Figure 2A**). Stronger stimulation induced higher rates of proliferation in Th and Tc cells of young mice, which was most pronounced after four days (**Figure 2B**). In contrast, the Th and Tc cells of mice of 22 months and older did not show any proliferative response to these stimuli over time. Proliferation of Treg cells of young mice did not increase in response to higher strength of stimulation, but Treg cells of young mice proliferated significantly more compared to aged mice under any condition tested. These data indicate a loss of capacity to proliferate at old age across all T cell subsets that cannot be overcome by increasing the stimulatory strength.

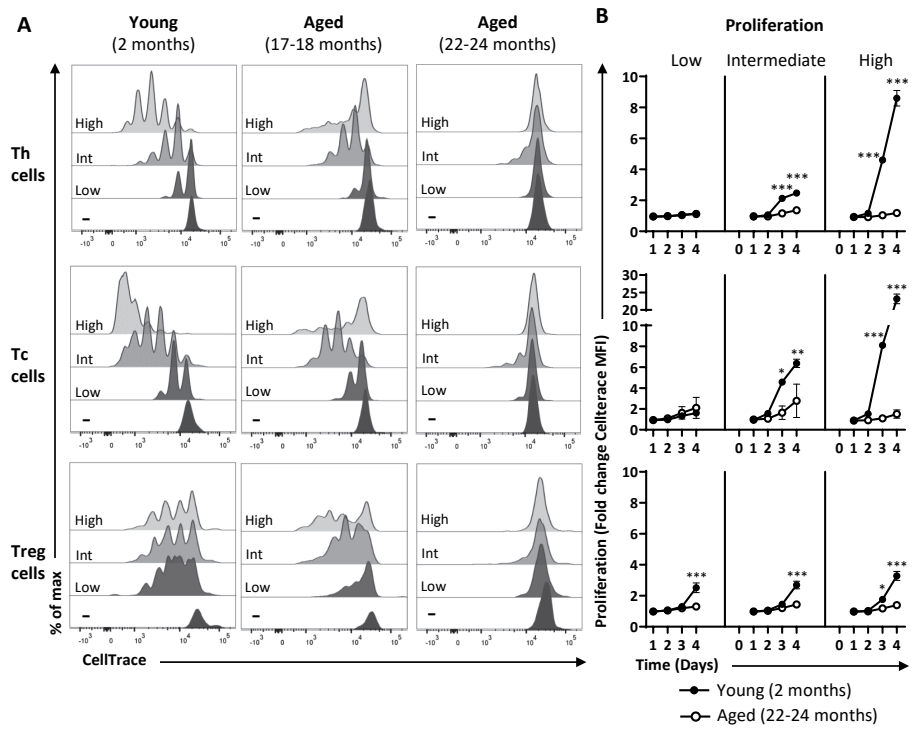


**Figure 2. Gradual loss of proliferative capacity in Th, Tc, and Treg cells with ageing.**
(**A**) The proliferative capacity of Th, Tc, and Treg cells of young (2 months old) and aged (17-18 and 22-24 months old) mice was measured four days after exposure to a low, intermediate, or high stimulatory strength. For each condition graphs show data of one mouse that is representative of n=6 per age group. (**B**) Graphs show proliferation of Th, Tc, and Treg cells by fold change of proliferation marker Celltrace in young (n=6, 2 months old) and aged (n=6, 22-24 months old) mice after four days cultured in the presence of a low, intermediate, or high stimulatory strength. Mean ± SEM; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ for difference between young and aged mice using Two-way ANOVA.

**Ageing-related alterations in cytokine profiles depend on response kinetics and strength of stimulation**

Cytokine production by stimulated T cells changes with ageing, which has widely been shown by measurements of cytokines secreted in supernatants. However, such findings are highly ambiguous due to the lack of studying the kinetics of cytokine responses, as studies often address cytokine secretion with a single stimulatory dose and at one point in time [20]. We examined the impact of ageing on the induction of effector cytokines during the response of T cells to differing stimulatory strengths after two and four days of culturing (**Figure 3**).

Stimulation of T cells with a low concentration of anti-CD3 did not result in detectable cytokine levels after two and four days in both young and old mice (data not shown). Using intermediate or high strength of stimulation, cells of young mice produced significantly higher levels of IL-2 at day two compared to cells of aged mice (**Figure 3A**). Cells of aged mice showed stronger Th2-related responses, with significantly higher IL-4 (**Figure 3B**) and IL-5 (**Figure 3C**) production compared to cells of young mice four days after exposure to intermediate or high strength stimuli. In addition, secretion of IL-10 was found only at day four, and this cytokine was produced at higher levels by cells of aged mice (**Figure 3D**). Interestingly, cells of aged mice but not young mice, produced high amounts of IL-10 in response to intermediate strength of stimulation, which indicates that aged mice require less stimulation to trigger IL-10 production compared to young mice.

Detection of ageing-related differences in secretion of pro-inflammatory cytokines IL-17, IFN-γ, and TNF-α also depended on the time and strength of stimulation. Aged mice showed a trend towards higher IL-17 secretion, but only after four days and by high strength stimulation (**Figure 3E**). Aged mice also showed higher IFN-γ secretion, which became apparent only after four days of stimulation with both intermediate and high strength stimulation (**Figure 3F**). TNF-α secretion by cells of aged mice was consistently lower compared to secretion by cells of young mice (**Figure 3G**). Taken together, these results indicate that both time and the stimulatory strength are important in assessing ageing-related cytokine profiles.

Measuring cytokines in supernatant does not indicate which T cell subset accounts for the cytokines produced. Therefore, we investigated the maximum potential of $CD4^+$ and $CD8^+$ cells to intracellularly express Th1-related (IFN-γ and TNF-α) and Th2-related (IL-4 and IL-5) cytokines during a four-hour stimulation with PMA/ionomycin (**Supplementary Figure 2**). Aged mice showed significantly higher frequencies of IFN-γ-producing cells in both $CD4^+$ and $CD8^+$

T cells, suggesting that Th and Tc cells both contribute to the higher IFN-γ we measured in supernatants (**Figure 3F**). In contrast to higher TNF-α in the supernatant (**Figure 3G**), CD4⁺ and CD8⁺ T cells of aged mice produced higher levels of TNF-α than those of young mice. This difference may be due to the different modes of stimulation or by different rates of TNF-α consumption. Additionally, the higher levels of IL-4 and IL-5 we detected in the supernatant of aged mice was paralleled by a significantly higher frequency of aged IL-4/IL-5⁺ CD4⁺ T cells (**Supplementary Figure 2**). This suggests that predominantly CD4⁺ T cells account for the elevated levels of IL-4/5 found in supernatants of spleen cells cultured from aged mice.

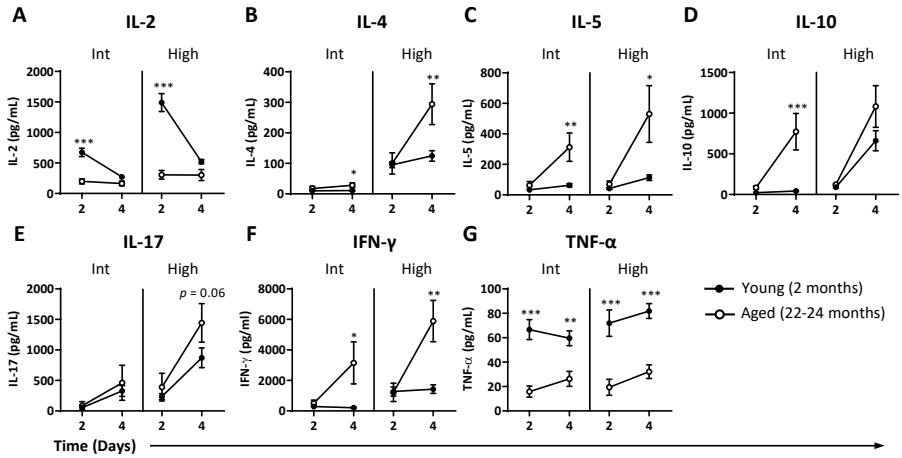


**Figure 3. Duration and strength of stimulation determine ageing-related cytokine profiles.** Cytokine levels of IL-2 (**A**), IL-4 (**B**), IL-5 (**C**), IL-10 (**D**), IL-17 (**E**), IFN-γ (**F**), and TNF-α (**G**) were measured in supernatant of splenocytes exposed for two or four days to an intermediate (int) or high stimulatory strength. Data represent one experiment with young (n=6, 2 months old) and aged (n=6, 22-24 months old) mice. Mean ± SEM; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ for difference between young and old mice at time points indicated using Two-way ANOVA.

## Reduced induction of T cell activation in aged mice can partially be restored by stronger stimulation

We next analyzed the expression of the classical activation markers CD69 and CD25 to investigate to what extent ageing influences the activation kinetics of T cells (**Figure 4**). After low strength stimulation, the frequency of CD25⁺ Th and Tc cells of young mice increased and peaked at day one (**Figure 4A**). With higher strength of stimulation, the maximum frequencies of CD25⁺ cells induced were higher and maintained over time, irrespective of age. In Th and Tc cells of aged mice, maximum frequencies of CD25⁺ cells however never reached those of young mice. Treg cells of aged mice showed significantly

lower frequencies of CD25⁺ cells compared to young mice in response to both low and intermediate strength of stimulation. However, high dose stimulation resulted in comparable CD25⁺ Treg frequencies between young and aged mice (**Figure 4A**), although the expression of CD25 per cell was lower on aged Treg cells (data not shown).

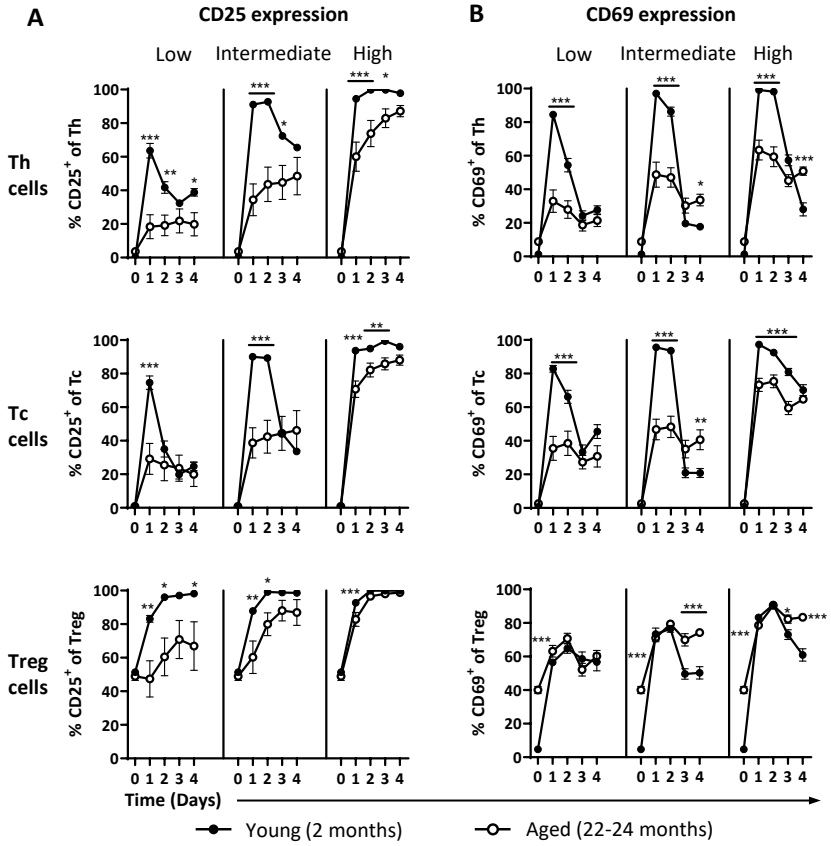


**Figure 4. Reduced induction of T-cell activation in aged mice can partially be restored by stronger stimulation.**

The proportion of CD25⁺ (**A**) and CD69⁺ (**B**) Th, Treg, and Tc cells of young (n=6, 2 months old) and aged (n=6, 22-24 months old) mice cultured up to four days with a low, intermediate, or high stimulatory strength. Mean ± SEM; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ for difference between young and aged mice at time points indicated using Two-way ANOVA.

Frequencies of cells expressing the early activation marker CD69 on Th and Tc cells were comparable between young and aged mice before stimulation, while the frequency of CD69⁺ Treg cells of aged mice was significantly higher (**Figure 4B**). At young age, all three T-cell subsets showed a rapid incline in the frequency of CD69⁺ cells within one day. At old age, Th and Tc cells also

quickly upregulated CD69 with a stimulatory dose-dependent increase, but never reached the maximum $CD69^+$ cell frequencies of young cells. In contrast, Treg cells of old mice expressed similar or higher frequencies of $CD69^+$ cells than Tregs of young mice after stimulation. Moreover, after one to two days of culture, all three subsets of young mice showed a decline in the frequency of $CD69^+$ cells. This decline was less pronounced on the T cells of aged mice, as shown by persistent $CD69^+$ cell frequencies, leaving stimulation-induced frequencies of $CD69^+$ cells observed at later time points of the culture higher in the aged mice than in the young mice. Thus, these data indicate that activation kinetics of T cells at old age can in part be improved by increasing the stimulatory strength. However, a substantial fraction of the Th and Tc cells of aged mice still refrain from expressing CD25 or CD69.

**T cells of aged mice do not proliferate in response to exogenous IL-2.**

T cells of aged mice showed diminished proliferation, lower IL-2 secretion, and altered CD25 expression kinetics in response to stimulation (**Figure 2, 3, and 4**). As IL-2 is an important driver of T cell proliferation, we hypothesized that the deficiency of IL-2 in culture may contribute to the diminished T cell proliferation observed in aged mice. Therefore, we assessed whether exogenous IL-2 could overcome the IL-2 deficiency and partially restore defective proliferation of T cells at old age. We stimulated cells of young and aged mice with a low stimulatory strength of anti-CD3 +/- exogenous recombinant murine IL-2 and monitored Th, Tc, and Treg cell proliferation (**Supplementary Figure 3**). Addition of exogenous IL-2 did not enhance proliferation in T cell subsets of aged mice, whereas it enhanced proliferation of Th and Tc cells of young mice (**Supplementary Figure 3**).

**Ageing influences naive and memory $CD4^+$ and $CD4^-$ T-cell heterogeneity by enrichment for regulatory cell types**

Age-related differences found in T cell responses have largely been ascribed to the shifted balance of naive T cells towards memory T cells during ageing rather than an ageing-related effect within these cell subsets [2]. We and others observed changes in the composition of the T cell pool at old age other than only a shifted naive/memory balance, such as increase of the Treg cell frequency [40] (**Figure 1D**). We therefore assessed if the impact of ageing may be reflected beyond the mere shift in balance of naive/memory T cells and may be further defined by changes of the heterogeneity within naive and memory T-cell populations. To address this question, we first investigated the proportion of naive and memory cells in young and aged mice based on expression of

CD44 (**Figure 5A**). Aged mice indeed showed decreased naive and increased memory proportions in the $CD4^+$ and $CD4^-$ T-cell compartments (**Figure 5B**).
We next aimed to assess T-cell heterogeneity within naive and memory T-cell subsets. We characterized phenotypically distinct clusters of T cells within the naive and memory cells among the $CD4^+$ and $CD4^-$ compartments of the T-cell pool by application of dimensionality reduction (viSNE) (**Supplementary Figure 4**). This panel included the ageing-related markers PD-1 and CTLA-4, as well as the activation markers CD25, CD69, CD122, and Glycoprotein A repetitions predominant (GARP).

Cluster analysis of $CD4^+$ and $CD4^-$ cells revealed heterogeneity within naive and memory cells, which differed between young and aged mice (**Figure 5C-F**). FoxP3, PD-1, CD25, and CD122 accounted for the heterogeneity within these subsets. Cluster 1 of naive and memory $CD4^+$ T cells shows high expression of FoxP3, indicating that this cluster is highly enriched for Treg cells (**Figure 5C, D**). The proportion of cluster 1 in naive $CD4^+$ T cells is larger in aged mice (40.9%) compared to young mice (10.4%) (**Figure 5C**), while in memory $CD4^+$ T cells the proportion of cluster 1 is comparable between young (22.0%) and aged mice (24.9%) (**Figure 5D**). The increased proportion of $CD4^+$ Treg cells among the naive $CD4^+$ cells is reflected in individual aged mice (**Figure 5G**), while there is no ageing-related difference in Treg cell proportions among the memory T-cell pool.

Previous studies have described a population of regulatory Tc cells (Tc reg cells) expressing CD122 and PD-1 [41,42]. Interestingly, cluster 1 of naive $CD4^-$ Tc cells is $CD122^+$ $PD\text{-}1^+$ with a higher proportion of this cluster in the pool of aged mice (**Figure 5E**), which is reflected in individual mice (**Figure 5G, Supplementary Figure 5**). The proportion of these Tc reg cells among the memory $CD4^-$ pool is comparable between young and aged mice (**Figure 5F, G**).

Finally, expression of PD-1 contributed to heterogeneity within naive and memory cell subsets (**Figure 5C-F**). This heterogeneity differed with age as aged mice showed higher frequencies of $PD\text{-}1^+$ in naive Th, Tc, and Treg cells, and in memory Th and Treg cell subsets compared to young mice (**Supplementary Figure 6**). Additionally, aged mice showed lower frequencies of $CD25^+$ naive Treg cells and lower frequencies of $GARP^+$ naive and memory Treg cells (**Supplementary Figure 6**).

Thus, naive and memory T cells both are highly heterogeneous and this heterogeneity is changed by ageing. Importantly, ageing influences constitution of these subsets by increasing the expression of PD-1 and by enriching naive $CD4^+$ and $CD4^-$ cell subsets with cells expressing a regulatory phenotype.

Therefore, ageing-related changes may be found beyond the shift from naive towards memory cell pools.

**Increased variation in the responsiveness of T-cell subsets of aged mice**

We next investigated the phenotypical changes that occur within Th, Tc, and Treg cell subsets after exposure to intermediate strength of stimulation (**Figure 6A, B**). viSNE analysis of Th, Tc, and Treg cell subsets before stimulation indicated ageing-related differences similar to analyses presented in **Figure 5**: Cells of aged mice showed increased expression of PD-1, presence of $CD122^{+}PD\text{-}1^{+}$ Tc cells, and diminished CD25 expression by Tregs (Day 0, **Figure 6A, C, D, Supplementary Figure 7**).

After stimulation, the number of phenotypically distinct clusters identified in Th cells and Tc cells was higher among cells from aged mice (six Th cell clusters, nine Tc cell clusters) compared to the number of clusters found in Th cells and Tc cells from young mice (four Th clusters, four Tc clusters) (**Figure 6B**). The number of Treg cell clusters was comparable between young and aged mice (six clusters) (**Figure 6B**).

Differences within each T cell subset of young mice were mainly found in the expression levels of CD69, CD25, CD122, PD-1, and CTLA-4 after stimulation (**Figure 6C, Supplementary Figure 7**). Th, Tc, and Treg cells of young mice showed activation-induced PD-1 and CD25 expression which remained present over time, while their CTLA-4, GARP, CD69 expression was high at day two and had declined by day four (**Figure 6C, Supplementary Figure 7**).

Compared to young mice, Th and Tc clusters of aged mice showed greater phenotypical diversity. Almost all Tc cells of aged mice constitutively expressed CD122 but lost this expression after stimulation, while CD122 on Tc cells of young mice increased over time after stimulation. Moreover, among both Th and Tc-cell subsets, distinct activated ($CD25^{+}CD69^{+}$) and non-activated ($CD25^{-}CD69^{-}$) clusters were identified. Distinct activated cell clusters ($CD25^{+}CD69^{+}$) expressing PD-1 were identified within both the Th and Tc subsets of aged mice after stimulation (Th and Tc, **Figure 6C**, blue arrows; **Figure 6D**, blue bars). In contrast, also $CD25^{-}CD69^{-}$ clusters of $PD\text{-}1^{+}$ Th and Tc cells were present (Th and Tc, **Figure 6C**, red arrows; **Figure 6D**, red bars). For Treg cells, aged mice also showed a cluster of $CD25^{-}CD69^{-}$ cells expressing PD-1 after two days of stimulation, but this cluster was not identified at day four (Treg, **Figure 6C**).

These data demonstrate that the group of aged mice shows more variation in response to stimulation compared to the group of young mice. These differences are not likely to be due to differences in abundance of naive and memory cells, as PD-1 expression increased with age within both these compartments.

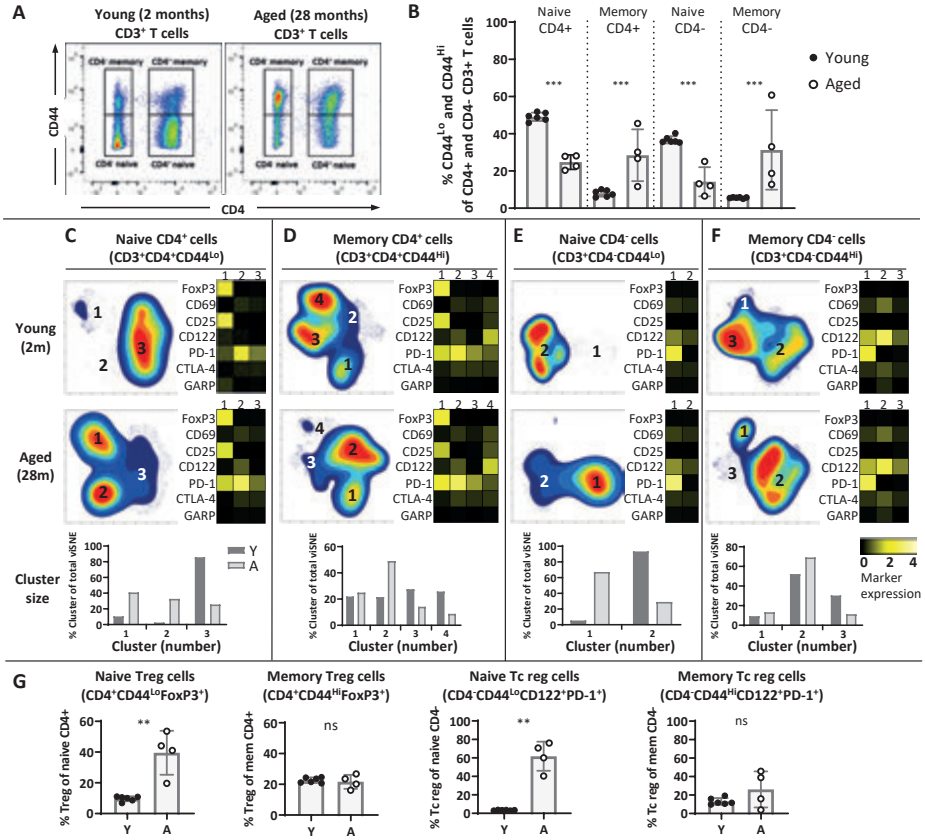


**Figure 5. Aging impacts the heterogeneity found within naive and memory T cells and enriches the naive compartment with cells expressing a Treg phenotype.**

Naive ($CD44^{Lo}$) and memory ($CD44^{Hi}$) cells were identified in the $CD4^+$ and $CD4^-$ T-cell populations by expression of CD44 (**A**). Frequencies of naive and memory cells in the $CD4^+$ and $CD4^-$ T cell populations were determined in young (n=6, 2 months old) and aged (n=4, 28 months old) mice (**B**). Cell density maps of dimensionality reduced single-cell data show clustering within either naive or memory populations of $CD4^+$ (**C,D**) and $CD4^-$ T cells (**E,F**) from pooled samples of young or aged mice. Numbers in the clusters on the density maps correspond to the cluster numbers in the heatmaps shown to the right of each cell density plot (**C-F**). Bar graphs below the density and heatmaps indicate the proportions of each cluster defined within the total viSNE (**C-F**). Heatmaps depict the ArcSinh5-transformed median expression of the designated markers of the respective clusters. The frequency of Treg cells of $CD4^+$ naive and memory cells (FoxP3$^+$ of $CD3^+CD4^+CD44^{Lo/Hi}$) and Tc reg cells of $CD4^-$ naive and memory cells (PD-1$^+$ CD122$^+$ of $CD3^+CD4^-CD44^{Lo/Hi}$) was analyzed by flow cytometry in young (Y) and aged (A) mice (**G**). Mean ± SD; $^{**}p < 0.01$, $^{***}p < 0.001$ for difference between young and aged mice using Mann-Whitney test.

**PD-1$^+$ Th and Tc populations contain less activated cells at old age**

To verify the findings of our viSNE analyses on an individual mouse level rather than a pooled group of mice, we addressed the frequency of activated ($CD25^+CD69^+$) PD-$1^+$ and PD-$1^-$ Th and Tc cells in young and aged mice before and after two days of exposure to an intermediate strength of stimulation (**Figure 7A, B, Supplementary Figure 8**). The frequency of PD-$1^+$ Th and Tc cells of young mice significantly increased after stimulation, whereas cells of aged mice did not (**Figure 7B**). Th and Tc cells of aged mice have increased expression of CD25 and CD69 within both PD-$1^+$ and PD-$1^-$ subsets observed at time points indicated, but to a lesser extent than cells of young mice (**Figure 7C**).

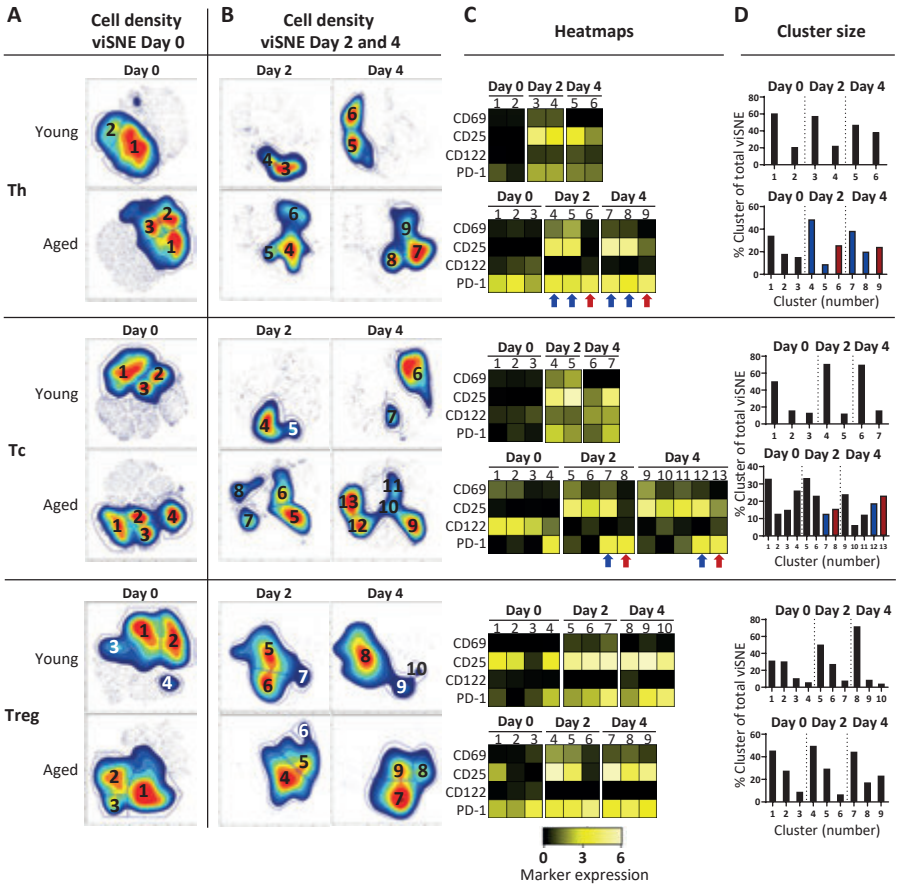


**Figure 6. Specific T-cell fingerprints show phenotypical alterations associated with ageing.** Splenocytes of young (n=6, 2 months old) and aged mice (n=4, 28 months old) were analyzed using flow cytometry before and after stimulation for up to four days with an intermediate stimulatory strength. Cell density maps of dimensionally reduced single-cell data show clustering within either Th, Tc, or Treg cell populations of the pooled young and aged mice before stimulation (Day 0) (**A**), and cell density maps at day 2 and 4 after stimulation (**B**). Numbers within the gates correspond to the cluster numbers in the heatmaps (**C**) and bar graphs (**D**). Heatmaps depict the ArcSinh5-transformed median expression of the designated markers in Th, Tc, and Treg cell clusters at the indicated days of young and aged mice. Bar graphs indicate the cell proportions of the clusters identified by viSNE analysis within each T cell subset. Red arrows and bars indicate the non-activated ($CD25^{-}CD69^{-}$) $PD\text{-}1^{+}$ Th and Tc cell clusters in aged mice (**C,D**). Blue arrows and bars indicate the activated ($CD25^{+}CD69^{+}$) $PD\text{-}1^{+}$ Th and Tc cell clusters in aged mice (**C,D**).

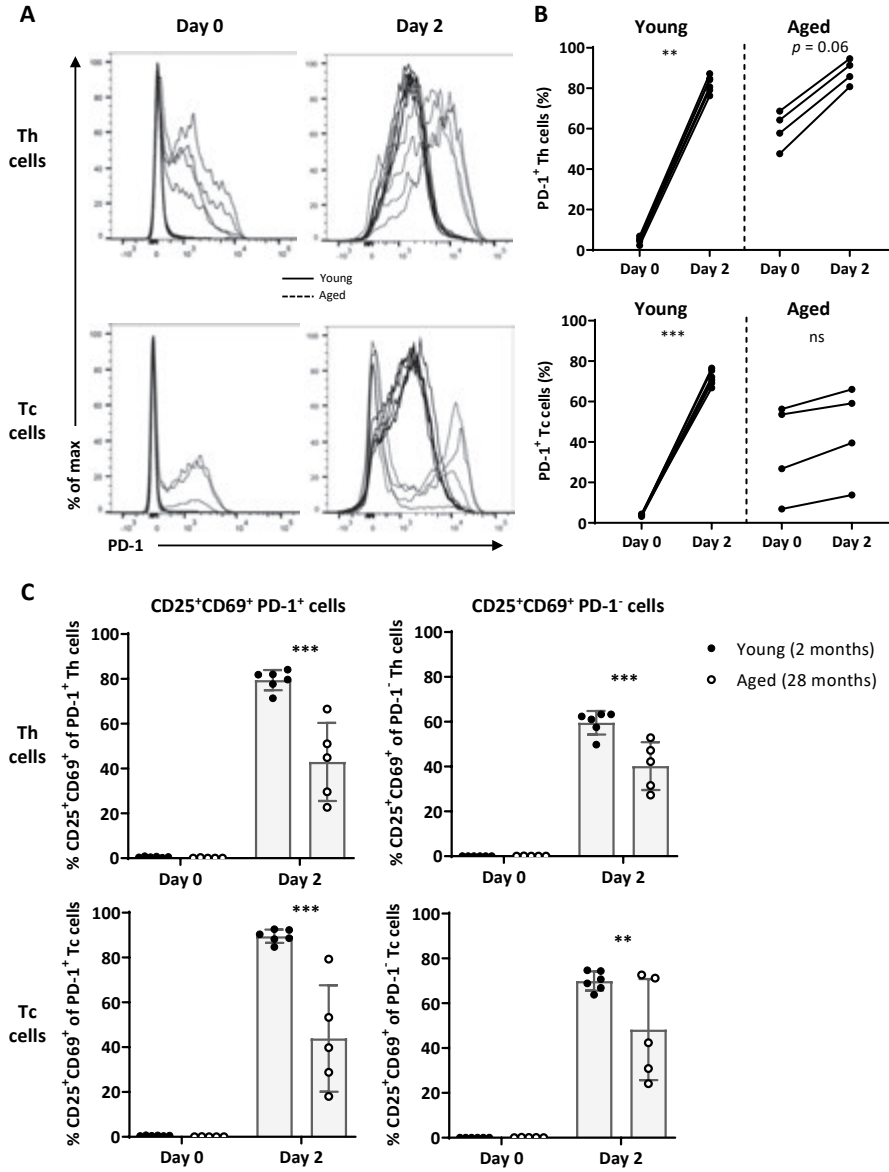


**Figure 7. PD-1⁺ Th and Tc populations contain less activated cells at old age.**
(**A**) Histograms depict expression of PD-1 by Th and Tc cells of young (n=6, 2 months old) and aged (n=4, 28 months old) mice at day 0 and at day 2 after culturing with an intermediate stimulatory strength. (**B**) Graphs show the induction of PD-1⁺ Th and Tc cells of young and aged mice after stimulation (day 2). (**C**) The activation status of PD-1⁺ and PD-1⁻ Th and Tc cells from young and aged mice was analyzed before and after stimulation. The proportion of activated cells was characterized by the simultaneous expression of CD25 and CD69. Mean ± SD; $^{**}p < 0.01$, $^{***}p < 0.001$ for difference between young and aged mice using Kruskal-Wallis test.

## DISCUSSION

In this study, we investigated the impact of ageing on T-cell response kinetics by studying the effect of the duration and strength of *in vitro* stimulation on T-cell activation markers, proliferation, and cytokine secretion in young and aged mice. Our study shows that T-cell response kinetics are a valuable tool to better understand the impact of ageing on T cells. Moreover, our findings indicate that PD-1 expression and impaired proliferation at old age may not imply unresponsive T cells per se.



Decreased induction of CD69 and CD25 expression has been used as a measure for lower rate of activation in $CD4^+$ and $CD8^+$ T cells of aged humans and mice [7,10-12], but only few studies have addressed CD25 and CD69 expression kinetics [7]. The stimulation-induced frequency of $CD25^+$ and $CD69^+$ Th and Tc cells was lower in aged mice compared to young mice during the early phase of the response. Levels found in T cells of aged mice eventually reached levels close to those found in young mice. Moreover, the presence of $CD69^+$ cells was more persistent in all T cell subsets of aged mice after their stimulation and became even higher than the declining number of $CD69^+$ cells in the T-cell pool of young mice at later time points. These findings indicate that early activation of Th and Tc cells of aged mice is diminished and that subsequent downregulation of CD69 is delayed. Persistent $CD69^+$ Tc cell frequencies after stimulation have previously been reported in aged mice [7], and we now show that Th and Treg cell subsets of aged mice also contain cells with persistent stimulation-induced CD69 expression. Persistent frequencies of $CD69^+$ Tregs in aged mice may harbor a Treg subset with enhanced suppressor activity [43,44]. Whether such $CD69^+$ Tregs may account for reduced Th and Tc cell activation at old age remains to be addressed in future studies.

Treg cells of aged mice could still be adequately activated as they were capable of reaching $CD25^+$ cell frequencies similar to those observed in young mice. However, these comparable levels only occurred after increasing the stimulatory strength. This finding may explain earlier reports showing unaltered suppressive functionality of Treg cells at old age despite their reduced CD25 expression at baseline [40,45,46]. Thus, ageing has a differential impact on CD25 kinetics of Treg cells compared to Th and Tc cells.

We observed declined proliferative capacity of Th, Tc, and Tregs cells with progressing age, and increasing the stimulatory strength did not resolve diminished proliferation. Declined proliferation of T cells is a major hallmark of replicative senescence [6]. Moreover, expression of PD-1 has been postulated as a marker of cell populations with diminished proliferative capacity [16,47].

We here showed that none of the PD-1$^+$ or PD-1$^-$ Tc cells of aged mice older than 24 months proliferated in response to stimulation. Since PD-1$^-$ Tc cells do not proliferate at old age, the expression of PD-1 cannot be used as sole predictor for diminished proliferation of Tc cells. Moreover, replicative senescence does not imply that T cell subsets of aged mice are unresponsive, as T cells of aged mice did show upregulation of activation markers and cytokine secretion.

Reports on the impact of ageing on secretion of cytokines are highly ambiguous due to the diversity in stimuli used and the lack of measurements on cytokine response kinetics [20]. Our data highlight the importance of studying both the time and strength of stimulation to address cytokine production. In addition, a shift from a Type-1 to a Type-2 cytokine response with ageing has been reported, but is still under debate [20,21,23,24,36,37]. Our data show no prominent shift from a Type-1 to a Type-2 cytokine profile as the Type-2 cytokines IL-4, IL-5, IL-10, but also Type-1 cytokine IFN-γ were more abundantly secreted by cells from aged mice compared to young mice after four days of culturing. Collectively, our data indicate that aged mice require lower strength of stimulation compared to young mice to produce Type-1 and Type-2 cytokines. This was found in supernatant for most cytokines, and our analyses of intracellular cytokines suggested increased numbers of T cells being able to produce both Th1- and Th2-related cytokines.

In addition to its role as an exhaustion marker, PD-1 can also be considered as an activation marker since it can be induced upon stimulation [16,47,48]. Indeed, in T cells from young mice we found significant upregulation of PD-1 upon stimulation. In contrast to young mice, the frequency of PD-1$^+$ cells did not increase in Tc cells of aged mice upon stimulation, which indicates that PD-1 on Tc cells may not be activation-induced at old age. Moreover, in parallel to the absence of significantly increased PD-1 expression upon stimulation, Tc cells did show induced expression of CD25 and CD69. Therefore, these findings may suggest that induced expression of CD69 and CD25 we found on PD-1$^+$ Tc cells of aged mice represent activated PD-1$^+$ Tc cells. The limited number of cells we obtained from our mice were all used for the assays presented and did not allow extensive sorting of different subsets. Conclusive evidence for the rate of activation of PD-1$^+$ versus PD-1$^-$ cells upon stimulation requires stimulation of sorted PD-1$^+$ and PD-1$^-$ Tc cells to provide insight into whether PD-1$^+$ cells can be activated.

Age-related differences found in T cells have largely been ascribed to the shifted balance of naive T cells towards memory T cells during ageing [2]. Therefore, many of the aging-related differences have been explained by differences between naïve versus memory cells rather than to aging-related

differences within naive or memory T cell populations. We indeed found inflation of the frequency of memory cells by aging. However, analysis of naive and the memory T cell pools revealed that attributing ageing-related differences to different T cell subsets should go beyond mere abundance of naive versus memory cells. Our phenotypic analyses show that the heterogeneity of both the naive and memory pools change with age. Among this heterogeneity, our *ex vivo* multi-dimensional analyses indicate that the naive $CD4^+$ and Tc cell pools of aged mice are enriched for cells with a regulatory phenotype. $CD122^+PD\text{-}1^+$ Tc cells have been reported to be Tc reg cells [41,42]. The high proportion of naive $CD122^+PD\text{-}1^+$ Tc cells observed in aged mice indicates an accumulation of this regulatory cell type with ageing, which has, to the best of our knowledge, not been reported earlier. Additionally, the naive $CD4^+$ T cell pool of aged mice was enriched for $Foxp3^+$ Treg cells. In addition, aged Treg cells have previously been shown to contain higher proportions of $PD\text{-}1^+$ cells. We now show that these $PD\text{-}1^+$ Treg cells are present in both naive and memory Treg cell subsets. Moreover, higher proportions of $CD25^{low}$ Treg cells at old age have been reported [40], and our data show that these cells are primarily present in the naive Treg cell subset. Our data analysis of combined expression of PD-1 and CD25 indicates a Treg subpopulation of $PD\text{-}1^+CD25^{Low}$ $CD4^+$ Tregs that increases with ageing.

T cells at old age showed reduced IL-2 production and lack of proliferation. Since IL-2 is known for its capacity to promote T-cell proliferation, this finding suggested that the lack of IL-2 production may contribute to the lack of proliferation we observed at old age. However, addition of exogenous IL-2 did not result in restoration of T-cell proliferation in aged mice, indicating that reduced production of IL-2 is not likely to contribute to the reduced proliferation found at old age. Hence, explanations for the defective responsiveness should be searched for in other factors, such as the distorted expression of IL-2 receptor chains CD25 and CD122 [9,49,50] or increased co-inhibitory signals and increased Treg populations we observed at old age. Furthermore, aging-related mediators produced by non-T cells that may function as antigen presenting cells present in our cultures may have contributed to the aging-related defects of T-cell proliferation and activation we observed. Although altered co-stimulation by antigen-presenting cells reported with age may interfere with our findings [2,51], polyclonal stimulation of T cells may largely overrule aging-related effects on APC-mediated co-stimulation and antigen presentation in our assays.

The strength of our analyses lies at examining the T-cell pool as a whole within its natural cell composition, which reflects the overall response of all splenocyte subpopulations. Many studies separate $CD4^+$ and $CD8^+$ naive and

memory cells [2,21]. Based on our study, it would be a contribution to the field of ageing for future studies to reveal how purified Th, Tc, and Treg cell subsets respond separately, as well as naive and memory cells within these cell subsets.

Collectively, our data show that stimulatory strength and time kinetics of cytokine secretion, activation markers, and proliferation of Th, Tc, and Treg cells are crucial in understanding the impact of ageing on T cells. Despite low proliferative capacity, T cell subsets of aged mice do respond to stimulation by upregulation of activation markers and secretion of cytokines. These findings therefore indicate that replicative senescence of aged T cells is not a measure of unresponsiveness per se, but rather stress that ageing influences the kinetics of proliferation, upregulation of activation markers and cytokine secretion each to a different extent. Moreover, our multi-dimensional analyses revealed phenotypical T-cell subpopulations that further delineate the impact of ageing on T cells.

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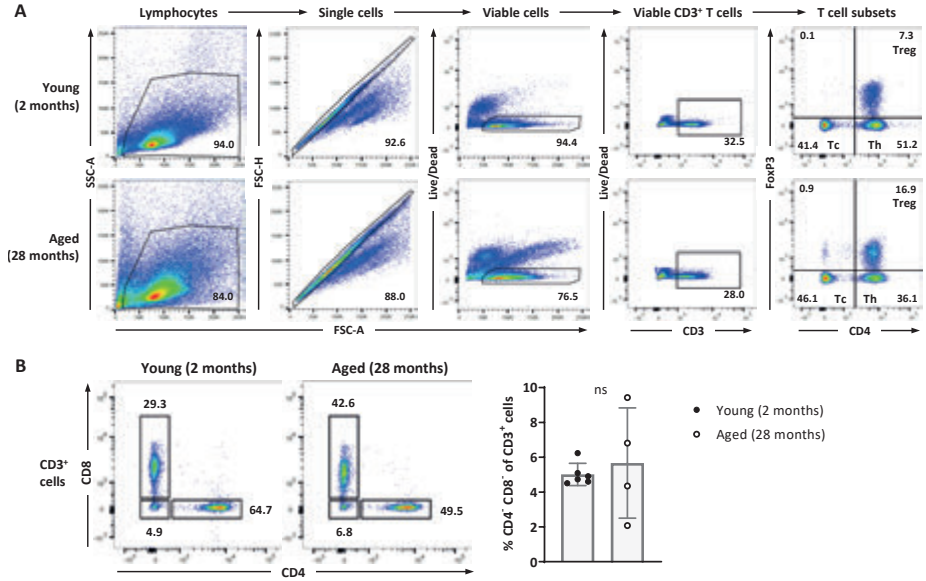
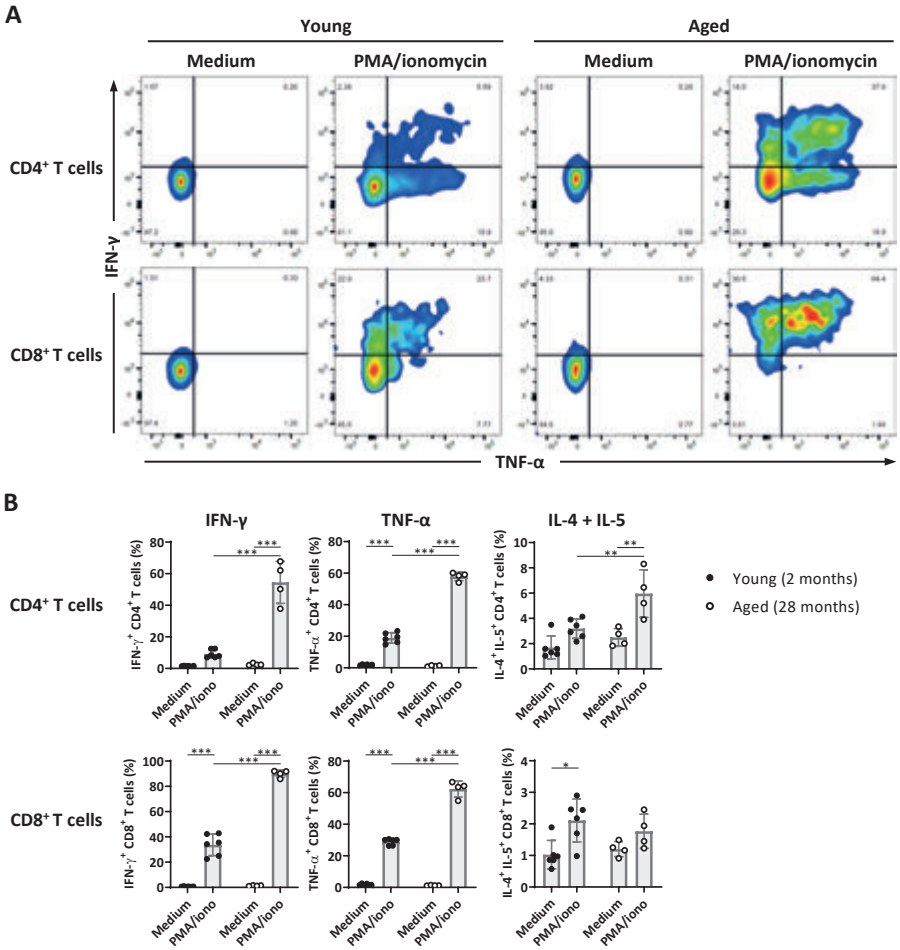
# SUPPLEMENTARY FIGURES



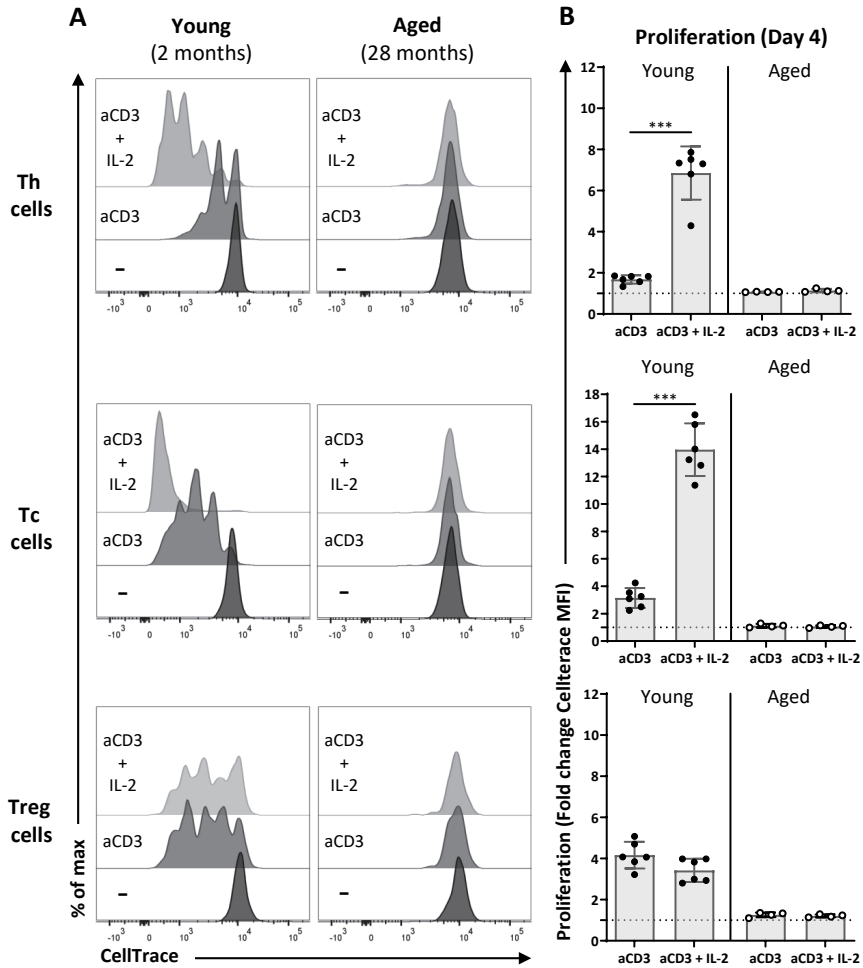

**Supplementary Figure 1. Gating strategy towards Th, Tc, and Treg cell subsets and the proportion of CD4$^-$CD8$^-$ T cells in young and aged mice.**

(**A**) Plots show the gating strategy from splenocyte lymphocytes, single cells, viable cells, viable CD3$^+$ cells towards the three T cell subsets; Th cells (FoxP3$^-$ CD4$^+$ of CD3$^+$), Tc cells (FoxP3$^-$ CD4$^-$ of CD3$^+$), and Treg cells (Foxp3$^+$ CD4$^+$ of CD3$^+$) in a young (2 months old) and an aged (28 months old) mouse representing their age group. (**B**) Plots and graphs show the proportion of CD3$^+$ T cells that do not express CD4 and CD8 in young and aged mice. Mean ± SD; ns = not statistically significant for difference between young and aged mice using Mann-Whitney test.



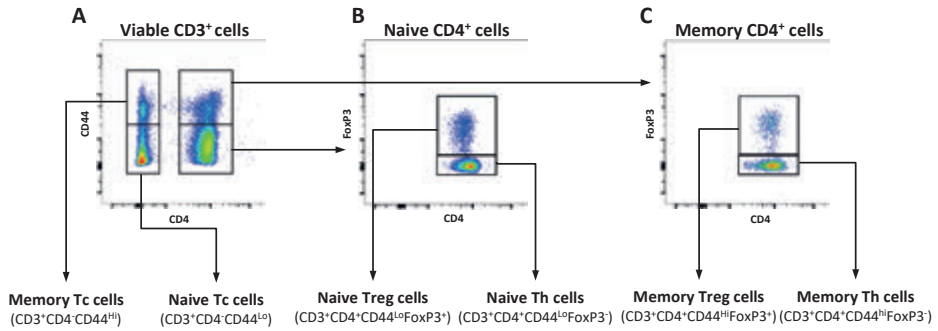

**Supplementary Figure 2. Flow cytometry of intracellular cytokines in CD4⁺ and CD8⁺ T cells of young and aged mice.**

(**A**) Plots show the intracellular expression of IFN-γ and TNF-α in CD4⁺ and CD8⁺ T cells of a young (2 months old) and an aged (28 months old) mouse representative of their age group after stimulation with PMA/ionomycin or medium as unstimulated control for four hours. (**B**) Graphs show the proportions of IFN-γ⁺, TNF-α⁺, or IL-4⁺ + IL-5⁺ in CD4⁺ and CD8⁺ T cells of young (n=6, 2 months old) and aged (n=4, 28 months old) mice. Mean ± SD; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ for difference between young and aged mice using Two-way ANOVA.



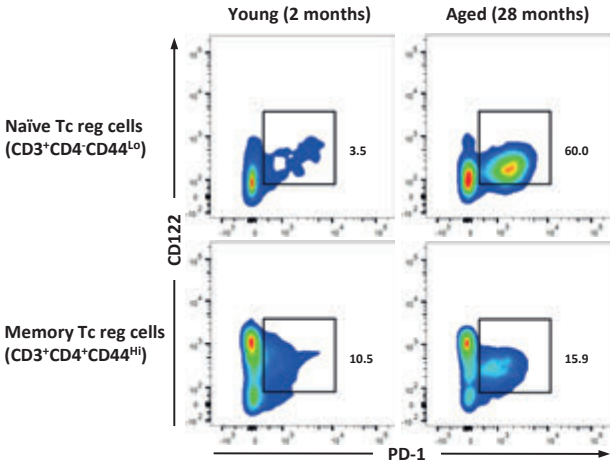

**Supplementary Figure 3. T cells of aged mice do not proliferate in response to exogenous IL-2.**

(**A**) Histograms depict the proliferative response of Th, Tc, and Treg cells of young (2 months old) and aged (28 months old) mice measured after four days of exposure to a low anti-CD3 stimulatory strength or low anti-CD3 + exogenous IL-2. Histograms show one mouse that is representative of its age group. (**B**) Graphs show proliferation by fold change in CellTrace MFI of Th, Tc, and Treg cells of young (n=6, 2 months old) and aged (n=6, 28 months old) mice in response to anti-CD3 +/- exogenous IL-2. Mean ± SD; ***$p < 0.001$ for difference between young and aged mice using Two-way ANOVA.



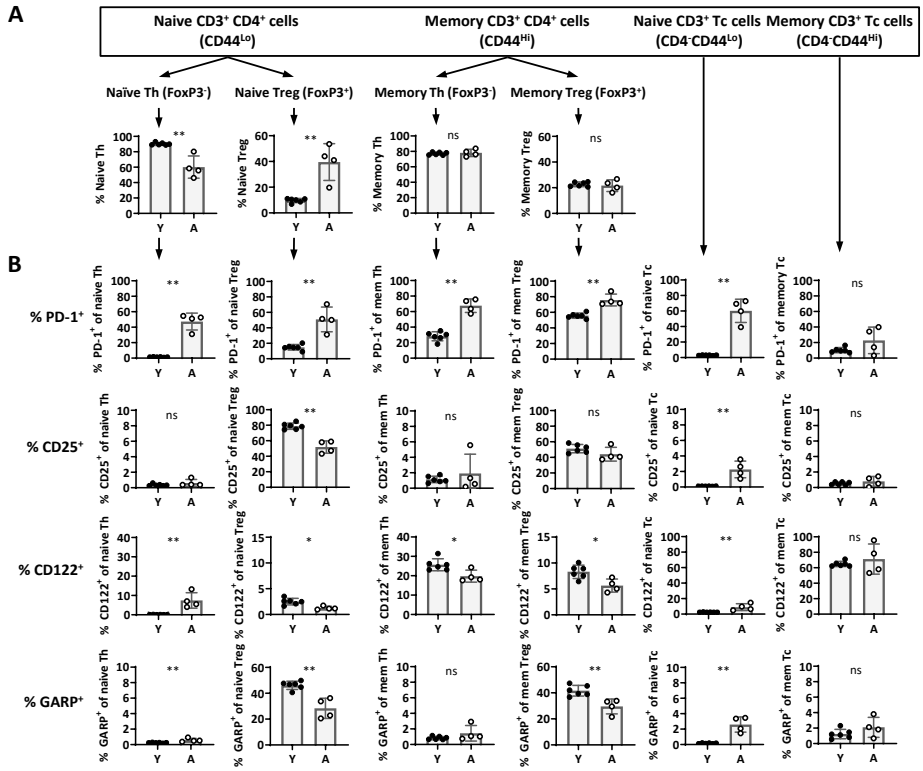

**Supplementary Figure 4. Gating strategy towards naive and memory Th, Tc, and Treg cell subsets for viSNE analyses.**

For our viSNE analysis we gated on naive and memory T cell subsets based on CD44 expression, here shown for one young mouse. Viable $CD3^+$ T cells were gated on (**A**) naive Tc cells ($CD3^+CD4^-CD44^{Lo}$), memory Tc cells ($CD3^+CD4^-CD44^{Hi}$), naive $CD4^+$ T cells ($CD3^+CD4^+CD44^{Lo}$), and memory $CD4^+$ T cells ($CD3^+CD4^+CD44^{Hi}$). On these four subsets, we performed the viSNE analyses shown in Figure 5. Based on the viSNE analyses, we further divided (**B**) naive $CD4^+$ T cells ($CD3^+CD4^+CD44^{Lo}$) and (**c**) memory $CD4^+$ T cells ($CD3^+CD4^+CD44^{Hi}$) into naive Treg cells ($CD3^+CD4^+CD44^{Lo}FoxP3^+$), memory Treg cells ($CD3^+CD4^+CD44^{Hi}FoxP3^+$), naive Th cells ($CD3^+CD4^+CD44^{Lo}FoxP3^-$), and memory Th cells ($CD3^+CD4^+CD44^{Hi}FoxP3^-$).



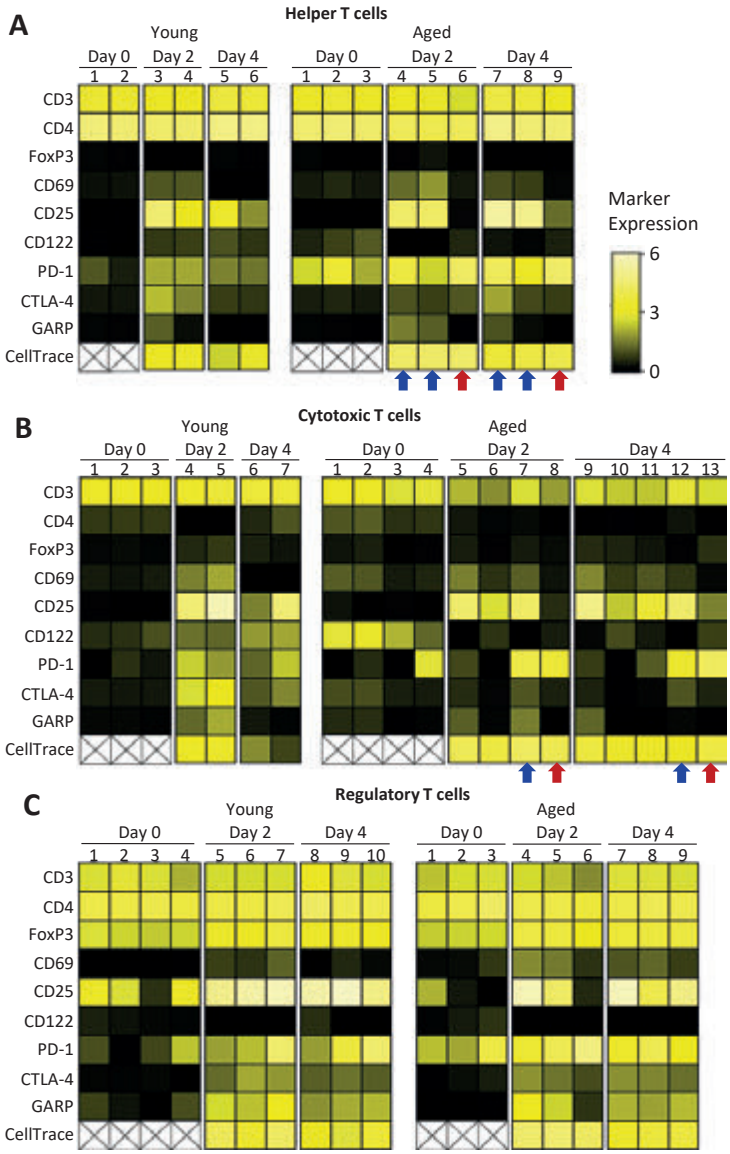

**Supplementary Figure 5. Gating strategy of regulatory Tc cells.**

Plots show gating of naive and memory Tc reg cells by expression of CD122 and PD-1 in a young (2 months old) and an aged (28 months old) mouse that represent their age group. Based on simultaneous expression of CD122 and PD-1 to identify Tc reg as previously reported, naive Tc reg cells were defined as $CD3^+CD4^-CD44^{Lo}CD122^+PD\text{-}1^+$ and memory Tc reg cells were defined as $CD3^+CD4^-CD44^{Hi}CD122^+PD\text{-}1^+$.

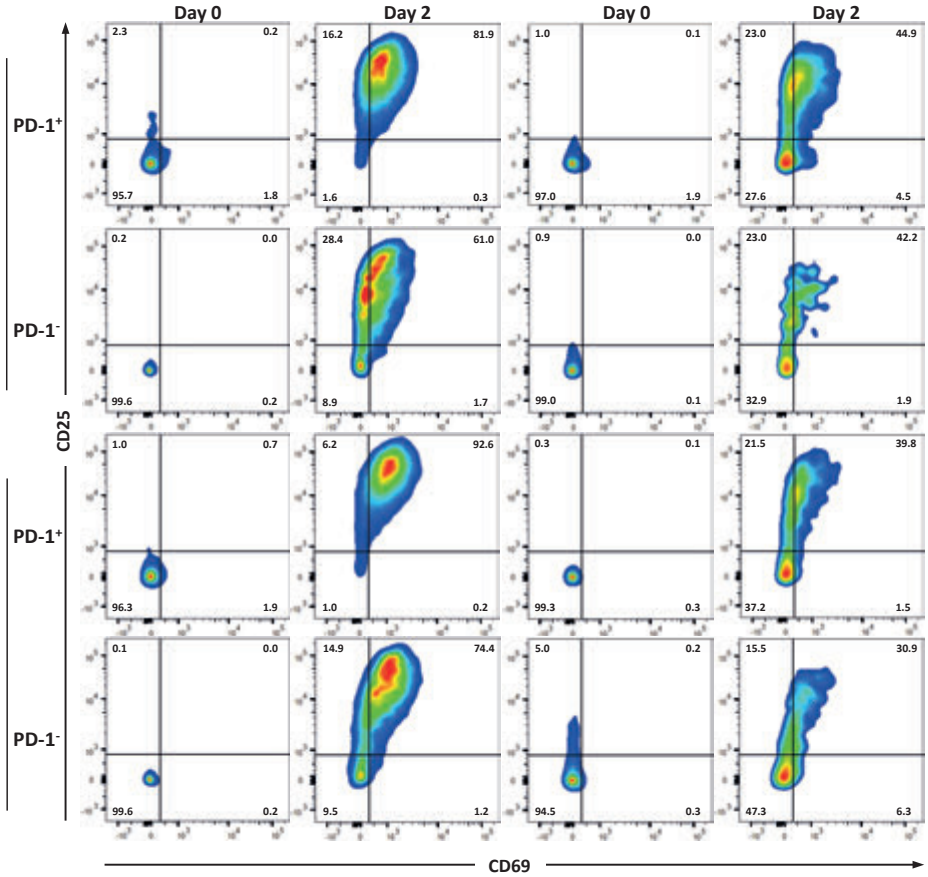



**Supplementary Figure 6. Frequencies of PD-1$^+$, CD25$^+$, CD122$^+$, and GARP$^+$ naive and memory T cell subsets.**

Graphs show the frequencies of PD-1$^+$, CD25$^+$, CD122$^+$, and GARP$^+$ T-cell subsets used to verify results generated by viSNE. (**A**) Upper (boxed) naive and memory T-cell subsets are those that have been analyzed by viSNE. Based on FoxP3 expression, naive CD3$^+$CD4$^+$ cells were subdivided into naive Th cells (CD44$^{Lo}$Foxp3$^-$) and naive Treg cells (CD44$^{Lo}$Foxp3$^+$), and memory CD3$^+$CD4$^+$ cells were subdivided into memory Th cells (CD44$^{Hi}$Foxp3$^-$) and memory Treg cells (CD44$^{Hi}$Foxp3$^+$). (**B**) Frequencies of PD-1$^+$, CD25$^+$, CD122$^+$, and GARP$^+$ cells were determined in the naive and memory subsets of Th, Treg, and Tc cells in spleens from young (n=6, 2 months old) and aged (n=4, 28 months old) mice. Mean ± SD; *$p < 0.05$, **$p < 0.01$, ns = not statistically significant for difference between young and aged mice using Mann-Whitney test.




**Supplementary Figure 7. Heatmaps of indicated markers included in viSNE analyses performed on Th, Tc, and Treg cells of young and aged mice before and after stimulation.**
Expression of T-cell markers before and after exposure of splenocytes of young (n=6, 2 months old) and aged (n=4, 28 months old) mice to an intermediate stimulatory strength for two and four days. Heatmaps depict the ArcSinh5-transformed median expression of the designated markers in Th (**A**), Tc (**B**), and Treg (**C**) cell clusters at the indicated days of young and aged mice. Numbers above each heatmap correspond to the cluster numbers identified by viSNE as shown in Figure 6. Red arrows indicate the non-activated ($CD25^{-}CD69^{-}$) $PD\text{-}1^{+}$ Th and Tc cell clusters in aged mice (**A,B**). Blue arrows indicate the activated ($CD25^{+}CD69^{+}$) $PD\text{-}1^{+}$ Th and Tc cell clusters in aged mice (**A,B**).




**Supplementary Figure 8. Gating of PD-1$^+$ or PD-1$^-$ Th and Tc cells expressing CD25$^+$ and CD69$^+$ before and after stimulation.**

Plots show the expression of CD25 and CD69 in PD-1$^+$ and PD-1$^-$ Th and Tc cells before and after two day exposure to an intermediate stimulatory strength. Plots depict analyses of one young (2 months old) and one aged (28 months old) mouse that represent their age group.


3

# COMPROMISED DNA REPAIR PROMOTES THE ACCUMULATION OF REGULATORY T CELLS WITH AN AGING-RELATED PHENOTYPE AND RESPONSIVENESS

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## ABSTRACT

Decline of immune function during aging has in part been ascribed to the accumulation of regulatory T cells (Tregs) and decreased T-cell responses with age. Aside from changes to T cells that occur over a lifetime, the impact of intracellular aging processes such as compromised DNA repair on T cells remains incompletely defined.

Here we aimed to define the impact of compromised DNA repair on T-cell phenotype and responsiveness by studying T cells from mice with a deficiency in their DNA excision-repair gene *Ercc1*. These *Ercc1* mutant (*Ercc1*$^{-/\Delta 7}$) mice show accumulation of nuclear DNA damage resulting in accelerated aging.

Similarly to wild-type aged mice, *Ercc1*$^{-/\Delta 7}$ mice accumulated naive Tregs with reduced CD25 and increased PD-1 expression in their spleen. *Ercc1*-deficiency limited the capacity of Tregs, helper T cells, and cytotoxic T cells to proliferate and upregulate CD25 in response to T-cell receptor- and IL-2-mediated stimulation. The recent demonstration that the mammalian target of rapamycin (mTOR) may impair DNA repair lead us to hypothesize that changes induced in the T-cell population by compromised DNA repair may be slowed down or reversed by blocking mTOR with rapamycin. *In vivo* dietary treatment of *Ercc1*$^{-/\Delta 7}$ mice with rapamycin did not reduce Treg levels, but highly increased the proportion of memory CD25$^{+}$ and PD-1$^{+}$ Tregs instead.

Our study elucidates that compromised DNA repair promotes the accumulation of naive Tregs with an aging-related phenotype and causes reduced T-cell responsiveness, which may be independent of mTOR activation.

## INTRODUCTION

The phenotype and functionality of T cells change during the course of aging, which contributes to aging-related pathology, increased susceptibility to infectious diseases, and reduced vaccine efficacy in the elderly [1]. The decline in T-cell function with age can in part be explained by changes to their phenotype and proliferative capacity, often referred to as T-cell exhaustion and/or T-cell senescence [2]. Additionally, we and others have shown that FoxP3$^+$ regulatory T cells accumulate with age [3-8] and it is thought that these cells impair protective immune responses by their suppressive capacity [4,5,9]. Insight into biological processes that contribute to decreased T-cell function and the accumulation of regulatory T cells (Tregs) with age is required to better understand the process of T-cell aging.



Characteristics of T-cell aging are mostly investigated on a chronological scale, i.e. changes that occur among T cells in relation to progressing time. Indeed, the exposure of antigens over a lifetime causes major alterations to the T-cell compartment [10]. Aside from this standard pathway, aging is also driven by cell intrinsic processes such as the accumulation of nuclear DNA damage; a hallmark of aging and recently defined as the factor driving all other hallmarks of aging [11,12]. Accumulation of DNA damage with age is a result of suboptimal DNA repair and is thought to be one of the drivers of cellular senescence [13-15]. This may also apply to aging of T cells, as T cells with a highly differentiated phenotype accumulate with age [16] and express higher levels of DNA damage [17]. However, to what extent characteristics of T-cell aging are explained by suboptimal DNA repair remains unclear. In this study, we defined the impact of compromised DNA repair on hallmarks of T-cell aging.

The endonuclease complex ERCC1-XPF mediates the repair of a broad variety of DNA lesions: I) bulky helix-distorting lesions are removed by global-genome nucleotide excision repair; II) lesions blocking transcription are removed by transcription-coupled repair; III) DNA crosslinks are removed via interstrand crosslink repair; and IV) a subset of persisting double-strand DNA breaks are removed by the single-strand annealing pathway [18,19]. Mice with a deficiency in the DNA excision-repair gene *Ercc1* (*Ercc1*$^{-/\Delta 7}$) have one knock-out and one truncated allele of *Ercc1* and therefore show impaired DNA repair within the four aforementioned pathways, which results in the accumulation of nuclear DNA damage [20-22]. As a consequence, *Ercc1*$^{-/\Delta 7}$ mice show numerous age-related pathologies and signs of accelerated aging with a reduced average lifespan of only 20 weeks [12,23,24]. *Ercc1*$^{-/\Delta 7}$ mice therefore provide an aging model

that enables investigation of the impact of compromised DNA repair on T-cell phenotype and responsiveness.

Another important driver of cellular aging is the activation of the mammalian target of rapamycin (mTOR) [25]. mTOR is a well-known target in anti-aging research as inhibition of mTOR by rapamycin has been widely reported to slow down the process of aging in terms of life- and health span [26-28]. Moreover, dietary supplementation of rapamycin in an encapsulated form (eRapa) to aged mice has been shown to reduce age-related changes observed in T-cells [29]. Interestingly, activation of the mTOR kinase mTORC1 has been reported to impair DNA damage response signaling, leading to accumulation of unrepaired DNA lesions [30]. Moreover, mTORC1 activation may negatively interfere with the ataxia telangiectasia mutated (ATM) checkpoint that promotes DNA repair [31]. We therefore hypothesized that changes to the T-cell compartment induced by compromised DNA repair may be slowed down by *in vivo* blocking of mTOR by eRapa.

In this study, we defined the impact of compromised DNA repair on T-cell phenotype and T-cell responsiveness in *Ercc1*$^{-/\Delta 7}$ mice. Additionally, we assessed whether the impact of compromised DNA repair on T cells could be avoided by inhibition of mTOR by *in vivo* dietary treatment with eRapa. We present evidence suggesting that compromised DNA repair promotes the aging-related accumulation of Tregs and reduced T-cell responsiveness that we find in wild-type (WT) aged mice, which appears to be independent of mTOR activation.

## MATERIALS AND METHODS

### Mice

Young (2 months of age) and aged (22 months of age) wild type C57BL/6 female mice were purchased from Envigo (Venray, Limburg, The Netherlands). Young and aged mice were maintained at the animal facilities of the Institute for Translational Vaccinology (Bilthoven, Utrecht, The Netherlands). Generation and characterization of *Ercc1*$^{-/\Delta 7}$ mice has been previously described [24]. Breeding stocks of the parental strains, i.e. Ercc1$^{+/-}$ mice in a pure C57BL6J background and Ercc1$^{+/\Delta 7}$ mice in a pure FVB background were generated and maintened as described [23,24]. Genetically uniform F1-hybrid C57BL6-FVB Ercc1$^{-/\Delta 7}$ mice were generated by combining both parental strains. Typical unfavorable characteristics, such as blindness in the FVB background or deafness in the C57BL6J background, do not occur in this hybrid background. Animals were housed in individual ventilated cages under specific pathogen-free conditions (20–22°C; 12 hr. light: 12 hr. dark cycle) and provided food and water *ad libitum*.

Since *Ercc1*$^{-/\Delta 7}$ mice are smaller, food was administered within the cages and water bottles with long nozzles were used from ~2 weeks of age. Wild-type F1 *Ercc1*$^{+/+}$ littermates at the indicated ages were used as controls. Male and female *Ercc1*$^{-/\Delta 7}$ and *Ercc1*$^{+/+}$ mice were maintained at the animal facilities of the Erasmus Medical Centre (Rotterdam, Zuid-Holland, The Netherlands). Distribution of males and females per group; n=3 males and n=3 females in the group of *Ercc1*$^{+/+}$ mice, n=3 males and n=4 females in the group of *Ercc1*$^{-/\Delta 7}$ mice, and n=3 males and n=3 females in the group of *Ercc1*$^{-/\Delta 7}$ mice fed with eRapa.

**Dietary treatment with rapamycin**

Diets were based on AIN93G, using 2.3 g/kg choline chloride instead of choline bitartrate (Research Diet Services, Wijk bij Duurstede, the Netherlands). Microencapsulated rapamycin (eRapa) and empty microcapsules (Eudragit S100) were obtained from Southwestern Research Institute (San Antonio, TX, USA). 42mg eRapa, containing 10% Rapamycin, was added per kg AIN93G food mix, resulting in a 42ppm rapamycin supplemented diet. For the control diet, 38mg empty microcapsules were added per kg AIN93G food mix. The diets were processed into pellets which were radiated with 9 kGy (Isotron, Ede, the Netherlands). Supplemented eRapa and control diets were supplied *ad libitum* to the mice at 8 weeks of age for the remainer of their life.

**Ethics**

Animal studies were approved by the Animal Ethical Committee of the National Institute for Public Health and the Environment (DEC no. 201400042) or Erasmus Medical Centre (DEC no. 139-12-13). All procedures were carried out in accordance with Dutch national legislation.

**Preparation of single cell suspensions and proliferation labeling**

Spleens of all mice were homogenized through a cell strainer to prepare single cell suspensions and red blood cells were lysed on ice with ACK lysis buffer (0.155M NH4Cl; 10mM KHCO3; 0.1mM Na2EDTA, pH 7.2-7.4). Subsequently, splenocytes were resuspended in PBS to $10*10^6$ cells/mL and labeled with 0.5 μM CellTrace™ Violet (Invitrogen, Carlsbad, CA, USA) in PBS per milliliter of splenocyte suspension for 20 minutes at 37°C to track T-cell proliferation. Cells were washed in ice-cold RPMI-1640 medium (GIBCO, Thermo Fisher Scientific, Waltham, MA, USA) containing 10% fetal calf serum (FCS) (Greiner Bio-One, Kremsmünster, Austria).

**Splenocyte culture and in vitro stimulation**

To investigate T-cell proliferation and upregulation of the activation marker CD25, splenic single cell suspensions were exposed to soluble anti-CD3 (0.019 µg/mL; clone 145-2C11, eBioscience, San Diego, CA, USA) alone or in the presence of soluble anti-CD28 (0.5 µg/mL; clone PV-1, Southern Biotech, Birmingham, AL, USA) or recombinant murine IL-2 (0.1 µg/mL; eBioscience). Stimuli were prepared in RPMI-1640 medium containing 10% FCS and then added to splenocyte suspensions ($4*10^5$ cells/well). Cells were cultured in 96-well U-bottom plates (CELLSTAR, Greiner Bio-One) at 37°C and 5% $CO_2$ for four days.

**Immunofluorescence labeling and flow cytometric analyses**

Splenic single cell suspensions were washed with PBS containing 2% FCS and labeled for 30 minutes at 4°C for a combination of cell surface markers with the following fluorescently labeled anti-mouse antibodies: anti-CD4-BUV395 (clone GK1.5), anti-CD44-V450 (clone IM7) (BD Horizon, Franklin Lakes, NJ, USA); anti-CD25-PE-Cy7 (clone PC61.5) (eBioscience); CD122-PE-Dazzle 594 (clone TM-beta1), and anti-PD-1-BV785 (clone 29F.1A12) (BioLegend, San Diego, CA, USA). Live/Dead™ Fixable Aqua Dead Cell Stain Kit (Invitrogen) was included in the cell surface labeling to assess cell viability. Cells were subsequently labeled intracellularly according to the FoxP3 Transcription Factor staining buffer set protocol (eBioscience) with anti-CD3zeta-FITC (clone H146-968) (Abcam, Cambridge, Cambridgeshire, UK) and anti-FoxP3-eFluor660 (clone 150D/E4) (eBioscience). Labeled cells were detected on a BD LSRFortessa X-20 (BD Biosciences, Franklin Lakes, NJ, USA). Data analysis was performed using FlowJo software (Tree Star, Ashland, OR, USA).

**Dimensionality reduced analyses**

Dimensionality reduced analysis (viSNE) of flow cytometry data was performed in Cytobank (www.cytobank.com) [32]. Cell density maps show clustering of $CD4^+$ and $CD4^-$ naive and memory T-cell populations that were generated from pooled flow cytometry datafiles of *Ercc1*$^{-/\Delta 7}$ mice (n=7) and pooled flow cytometry datafiles of the different *Ercc1*$^{+/+}$ mice (n=6). The number of cells included in the viSNE analysis were equal between *Ercc1*$^{-/\Delta 7}$ and *Ercc1*$^{+/+}$ mice; $2.3*10^5$ naive $CD4^+$ T cells, $1.4*10^5$ naive $CD4^-$ T cells, $3.3*10^4$ memory $CD4^+$ T cells, and $2.2*10^4$ memory $CD4^-$ T cells. Clustering was based on expression of FoxP3, CD25, CD122, and PD-1. Expression of the designated cellular markers in the heatmaps was based on their ArcSinh5-transformed median expression.

### Statistics

Statistical analyses were performed using GraphPad Prism 7 software (La Jolla, CA, USA). The appropriate parametric or non-parametric tests were used based on the tested normality of distribution of the data by Kolmogrov-Smirnov test and Shapiro-Wilk test. Dependent on the number of comparisons and the normality of distribution of the data, unpaired Student *t* Test, Mann-Whitney U test, or One-way ANOVA test followed by Holm-Sidak multiple comparisons test were performed as indicated in the figure legends. Data presented in bar graphs are expressed as the mean ± standard deviation (SD). For all analyses, $p$ values $< 0.05$ were considered statistically significant.

## RESULTS



### Compromised DNA repair contributes to increased proportions of memory T cells.

With age, the total T-cell population among lymphocytes decreases, whereas the proportion of memory cells within the T-cell population increases [3,10,33]. We first assessed whether compromised DNA repair contributes to these aging-related changes. We compared the T-cell populations in spleens of accelerated aging *Ercc1*$^{-/\Delta 7}$ mice (n=7, 4 months of age) with those in spleens of littermate control *Ercc1*$^{+/+}$ mice (n=6, 4 months of age) (**Figure 1A-C**). As a reference we did similar analyses in WT young (n=6, 2 months of age) and WT aged (n=6, 22 months of age) mice (**Figure 1D-F**).

*Ercc1*-deficient mice did not significantly show a decrease of CD3$^+$ T-cell frequency among live spleen lymphocytes (**Figure 1A**) that occured in normal WT aging (**Figure 1D**). Subsequently, we analyzed the frequency of memory cells by expression of the memory marker CD44 among CD4$^+$ T cells and CD4$^-$ T cells. Of note, CD3$^+$ CD4$^-$ T cells can be considered CD8$^+$ T cells as this subset mainly comprises CD8$^+$ T cells and a very low proportion of CD4$^-$ CD8$^-$ cells in *Ercc1*$^{-/\Delta 7}$ mice that we analysed in an additional data set (3% on average) (**Supplementary Figure 1**), similar to previous observations in WT mice (4% on average) [3]. Although *Ercc1*$^{-/\Delta 7}$ mice did not reach the major increase of memory CD4$^+$ and CD4$^-$ T cell frequencies found in WT aged mice (**Figure 1E,F**), the proportion of memory T cells was higher in *Ercc1*$^{-/\Delta 7}$ mice compared to control *Ercc1*$^{+/+}$ mice (**Figure 1B,C**). Thus, our data indicate that the rise in proportion of memory T cells among CD4$^+$ and CD4$^-$ T cells found in WT aged mice can in part be attributed to compromised DNA repair.

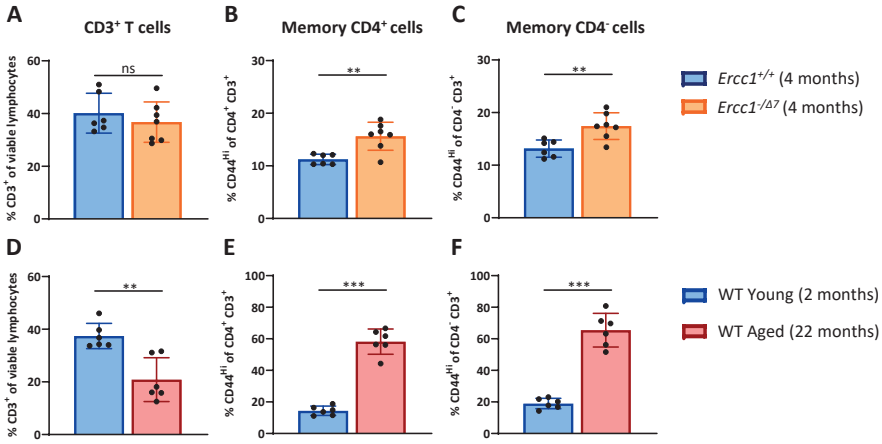


**Figure 1. Compromised DNA repair contributes to increased proportions of memory T cells.** The frequencies of (**A**) total T cells (CD3⁺ cells of viable lymphocytes), (**B**) memory CD4⁺ T cells (CD44$^{Hi}$ of CD4⁺CD3⁺ cells), and (**C**) memory CD4⁻ T cells (CD44$^{Hi}$ of CD4⁻CD3⁺ cells) were determined in the spleen of *Ercc1*$^{+/+}$ (n=6, 4 months of age) and *Ercc1*$^{-/\Delta7}$ (n=7, 4 months of age) mice, as well as in (**D-F**) WT young (n=6, 2 months old) and aged mice (n=6, 22 months old). Bar graphs show mean ± SD; $^{**}p < 0.01$, $^{***}$ $p < 0.001$, ns = not statistically significant for the difference between groups using unpaired Student's *t* test, two-tailed.

## Compromised DNA repair promotes accumulation of FoxP3⁺ Tregs within the naive CD4⁺ T-cell subset.

We previously observed that naive CD4⁺ T cells of WT aged mice are enriched with regulatory cells, as defined by expression of Foxp3, that express an aging-related phenotype characterized by increased expression of PD-1 and lower expression of CD25 [3]. Here we asked whether this major hallmark of aging in WT aged mice can be attributed to compromised DNA repair. We applied dimensionality reduction (viSNE) to form phenotypically distinct clusters based on simultaneous expression of different aging-related molecules within CD4⁺ naive (CD44$^{Lo}$) and memory (CD44$^{Hi}$) T cells of *Ercc1*$^{-/\Delta7}$ mice and *Ercc1*$^{+/+}$ mice (**Supplementary Figure 2**, gating strategy). Cluster formation was based on the combined expression of FoxP3, CD25, CD122, and PD-1 as aging-related markers previously used to reveal aging-related clusters of T cells [3].

viSNE analysis of T cells of *Ercc1*$^{+/+}$ (n=6 pooled) and *Ercc1*$^{-/\Delta7}$ mice (n=7 pooled) showed four phenotypically distinct clusters within the naive CD4⁺ T-cell subset (**Figure 2A**). Cluster 1 highly expressed the Treg marker FoxP3 (**Figure 2B**) and the proportion of this cluster was higher in *Ercc1*$^{-/\Delta7}$ mice compared to *Ercc1*$^{+/+}$ mice (16.2% versus 10.8%) (**Figure 2C**). Accumulation of Tregs within the naive CD4⁺ T-cell subset was indeed significantly higher in

individual *Ercc1*$^{-/\Delta 7}$ mice compared to control *Ercc1*$^{+/+}$ mice (**Figure 2D**), which closely resembled findings in WT aged mice (**Supplementary Figure 3**). Additionally, accumulated Tregs of *Ercc1*$^{-/\Delta 7}$ mice comprised significantly lower CD25$^{+}$ (**Figure 2E**) and higher PD-1$^{+}$ cell frequencies (**Figure 2F**) and expression levels per cell (MFI) (**Supplementary Figure 4**) compared to *Ercc1*$^{+/+}$ mice, which is similar to observations in WT aged mice [3]. Thus, compromised DNA repair appears to contribute to the accumulation of Tregs within the naive CD4$^{+}$ T-cell compartment with an aging-related phenotype that resembles findings in WT aged mice.

**FoxP3$^{+}$ Tregs within the memory CD4$^{+}$ T-cell subset in mice with compromised DNA repair.**

WT aged mice do not accumulate Tregs within the memory CD4$^{+}$ T-cell pool ([3] and **Supplementary Figure 3**) and we assessed whether *Ercc1*$^{-/\Delta 7}$ mice resemble these findings. viSNE analysis of memory (CD44$^{Hi}$) CD4$^{+}$ T cells of *Ercc1*$^{-/\Delta 7}$ and *Ercc1*$^{+/+}$ mice showed three phenotypically distinct clusters (**Figure 2G**) with cluster 1 indicating FoxP3-expressing Tregs (**Figure 2H**). Whereas the proportion of cluster 1 containing Foxp3$^{+}$ cells was higher in *Ercc1*$^{-/\Delta 7}$ mice compared to *Ercc1*$^{+/+}$ mice (37.4% versus 32.9%) (**Figure 2I**), the frequency of Foxp3$^{+}$ Tregs within the memory CD4$^{+}$ T-cell subset did not show a difference between individual *Ercc1*$^{-/\Delta 7}$ and *Ercc1*$^{+/+}$ mice (**Figure 2J**). In contrast to previous findings in WT aged mice [3], *Ercc1*-deficiency did not show altered frequency and expression of CD25 and PD-1 on Tregs within the memory CD4$^{+}$ T-cell subsets (**Figure 2K,L; Supplementary Figure 4**). These findings suggest that compromised DNA repair may not significantly contribute to phenotypical changes of Tregs within the memory CD4$^{+}$ T-cell pool.

**The aging-related phenotype of naive Th cells in mice with compromised DNA repair.**

We next investigated whether compromised DNA repair contributes to phenotypical changes within naive (CD44$^{Lo}$) helper T cells (Th cells). viSNE-clusters 2, 3, and 4 identified within the naive CD4$^{+}$ T-cell pool do not express FoxP3 (**Figure 2A,B**), indicating that these cells are naive Th cells. The minimal difference in proportion of clusters 2, 3, and 4 between *Ercc1*$^{-/\Delta 7}$ and *Ercc1*$^{+/+}$ mice (**Figure 2C**) indicates that *Ercc1*-deficiency did not profoundly affect the phenotype of naive Th cells based on the markers we included. Indeed, *Ercc1*$^{-/\Delta 7}$ mice did not show the increased frequencies or expression of PD-1$^{+}$ cells among naive Th cells (**Supplementary Figure 4**) that we previously found in WT aged mice [3]. Thus, these findings suggest that *Ercc1*-deficiency may not

have a significant impact on expression of CD122, CD25, and PD-1 by the naive Th population and therefore might not explain the aging-related changes based on these markers within the naive Th-cell subset in WT aged mice.

**Compromised DNA repair promotes the aging-related phenotype of memory Th cells.**

Next, we assessed the impact of compromised DNA repair on the phenotype of memory ($CD44^{Hi}$) Th cells. These cells were represented by the FoxP3-negative clusters 2 and 3 within the memory $CD4^+$ T cells in our viSNE analyses (**Figure 2G,H**). Cluster 2 was more abundant in *Ercc1*$^{-/\Delta 7}$ compared to *Ercc1*$^{+/+}$ mice (40.4% versus 34.0%), which points towards more *Ercc1*$^{-/\Delta 7}$ memory Th cells expressing PD-1 as reported previously in WT aged mice [3]. Indeed, a trend towards higher PD-$1^+$ memory Th-cell frequency and higher expression of PD-1 on *Ercc1*$^{-/\Delta 7}$ memory Th cells compared to *Ercc1*$^{+/+}$ cells was found (**Supplementary Figure 4**). Further, we observed a lower proportion of cluster 3 (18.6% versus 29.2%) in *Ercc1*$^{-/\Delta 7}$ compared to *Ercc1*$^{+/+}$ mice (**Figure 2I**), which is reflected in lower CD122 expression and $CD122^+$ memory Th-cell frequency in *Ercc1*$^{-/\Delta 7}$ mice (**Supplementary Figure 4**) and corresponds to earlier findings in WT aged mice [3]. Together, these data indicate that *Ercc1*-deficiency imposes an aging-related phenotype on memory Th cells that has been reported based on the expression of PD-1 and CD122.[3]

**Aging-related changes in naive and memory Tc cells in mice with compromised DNA repair.**

$CD3^+$ cells that do not express CD4 can be considered $CD8^+$ cytotoxic T cells (Tc cells), as this subset mainly comprises $CD8^+$ T cells ([3], **Supplementary Figure 1**). Clustering of naive ($CD44^{Lo}$) Tc cells by viSNE showed relatively comparable cell-density plots, expression of phenotypical markers, and cluster proportions (**Figure 3A-C**) between *Ercc1*$^{-/\Delta 7}$ and *Ercc1*$^{+/+}$ mice, which suggests a low impact of *Ercc1*-deficiency on naive Tc cells. Indeed, we observed no phenotypical difference in expression and cell frequencies of naive Tc cells (**Figure 3D,E; Supplementary Figure 4**) apart from a slight decrease in the frequency of PD-$1^+$ Tc cells of *Ercc1*$^{-/\Delta 7}$ mice (**Figure 3F**). These findings are in contrast with increased PD-$1^+$ cell-frequencies found among naive Tc cells of WT aged mice [3].

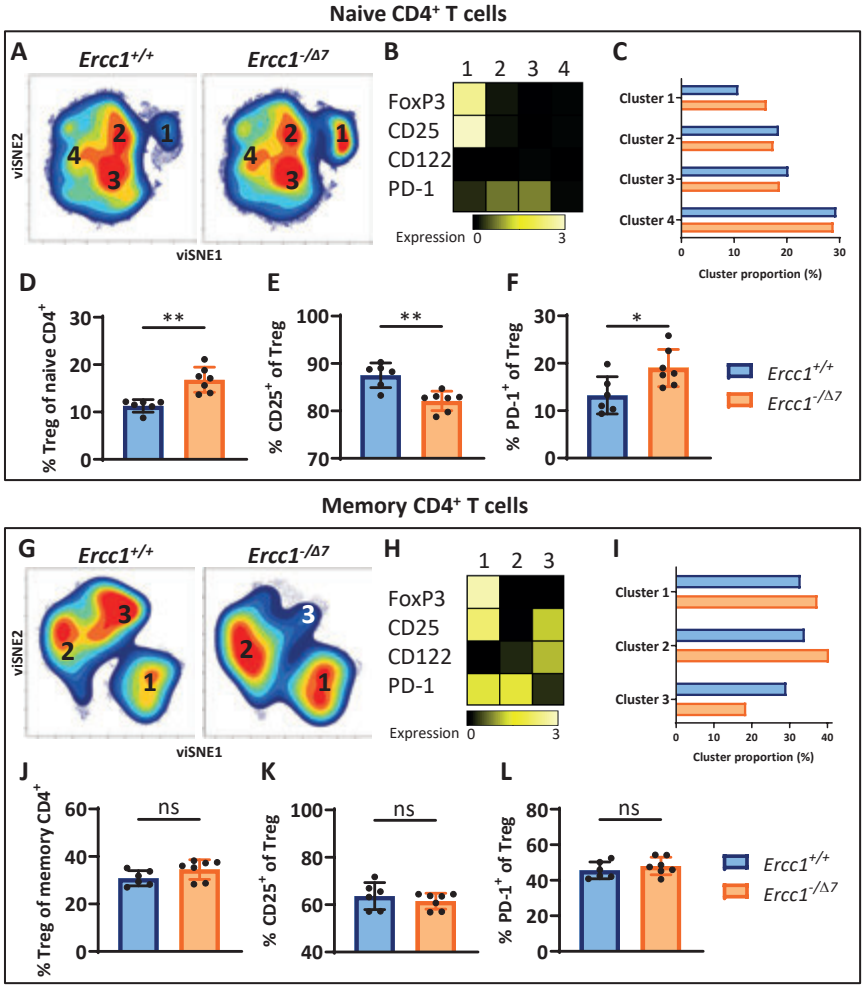


**Figure 2. Compromised DNA repair promotes accumulation of FoxP3⁺ Tregs within the naive CD4⁺ T-cell subset.**

Naive (CD44$^{Lo}$) and memory (CD44$^{Hi}$) cells were identified within the CD4⁺ T-cell population. Cell density maps of dimensionality reduced single-cell data by viSNE show clustering within (**A**) naive CD4⁺ T cells and (**G**) memory CD4⁺ T cells of pooled flow cytometry datafiles of *Ercc1*$^{+/+}$ mice (n=6) and pooled datafiles of *Ercc1*$^{-/\Delta 7}$ mice (n=7). Numbers in the density maps correspond to the cluster numbers above the heat maps (**B,H**). These heat maps depict the Arcsinh-transformed median expression of the indicated markers. Bar graphs (**C,I**) indicate the proportion of each cluster within the total viSNE for *Ercc1*$^{+/+}$ (blue) and *Ercc1*$^{-/\Delta 7}$ (orange) mice. Bar graphs (**D-F, J-L**) show the frequency of (**D**) naive and (**J**) memory FoxP3⁺ regulatory T cells in individual *Ercc1*$^{+/+}$ and *Ercc1*$^{-/\Delta 7}$ mice, as well as the frequency of (**E,K**) CD25⁺ and (**F,L**) PD-1⁺ cells within these naive and memory Treg subsets. Bar graphs show mean ± SD; * $p < 0.05$, ** $p < 0.01$, ns = not statistically significant for the difference between *Ercc1*$^{+/+}$ and *Ercc1*$^{-/\Delta 7}$ mice using parametric unpaired Student's *t* test or non-parametric Mann-Whitney test, two-tailed, dependent on the tested normality of distribution of the data.

Memory ($CD44^{Hi}$) Tc cells of WT aged mice do not differ from those of WT young mice [3]. In contrast, viSNE analysis of *Ercc1*$^{-/\Delta 7}$ memory Tc cells showed an increase in cluster 1 (21.5% versus 12.7%) and 2 (35.7% versus 25.6%), and a decrease in cluster 3 (38.4% versus 56.9%) compared to *Ercc1*$^{+/+}$ mice (**Figure 3E-G**). These differences were reflected in a reduced frequency of $CD122^{+}$ cells and an increased frequency of PD-$1^{+}$ cells among the memory Tc cells of *Ercc1*$^{-/\Delta 7}$ mice compared to *Ercc1*$^{+/+}$ mice (**Figure 3I,J**), which is also shown in the expression of these markers on a per cell basis (**Supplementary Figure 4**). Together, these data indicate that the impact of compromised DNA damage repair on naive and memory Tc cells may not explain findings on aging of Tc cells in WT aged mice.

**Compromised DNA repair promotes the accumulation of regulatory cells within the memory Tc-cell subset.**

PD-$1^{+}CD122^{+}$ Tc cells are regulatory Tc cells (Tc reg) [34,35] and these cells mainly accumulate within the naive Tc-cell subet of WT aged mice [3]. In contrast, PD-$1^{+}CD122^{+}$ Tc reg cells did not accumulate within the naive Tc-cell subset of *Ercc1*$^{-/\Delta 7}$ mice (**Supplementary Figure 5**), but rather within their memory Tc-cell subset. Therefore, compromised DNA repair does not explain findings on Tc reg cells in WT aged mice.

**eRapa reduces memory T cells that are induced by compromised DNA repair.**

Inhibition of mTOR by rapamycin reduces aging-related T-cell changes in WT mice [29]. Moreover, the mTOR pathway is thought to impair the DNA damage response [30]. We therefore hypothesized that *in vivo* dietary treatment of *Ercc1*$^{-/\Delta 7}$ mice with eRapa may slow down changes to the T-cell compartment induced by compromised DNA repair. To this end, 8-week old *Ercc1*$^{-/\Delta 7}$ mice were fed eRapa for the remainder of their life (42 ppm, reported previously as a life- and healthspan extending dose [36]).

eRapa-fed *Ercc1*$^{-/\Delta 7}$ mice showed decreased proportions of $CD3^{+}$ T cells among live spleen-derived lymphocytes compared to control-fed *Ercc1*$^{+/+}$ and *Ercc1*$^{-/\Delta 7}$ mice (**Figure 4A**), consistent with previous reports in WT mice [29]. Interestingly, eRapa reduced the proportion of memory $CD4^{+}$ and $CD4^{-}$ T cells towards levels found in control-fed *Ercc1*$^{+/+}$ mice (**Figure 4B,C**), suggesting that the increase of memory T cells induced by compromised DNA repair may in part be dependent on mTOR activation.

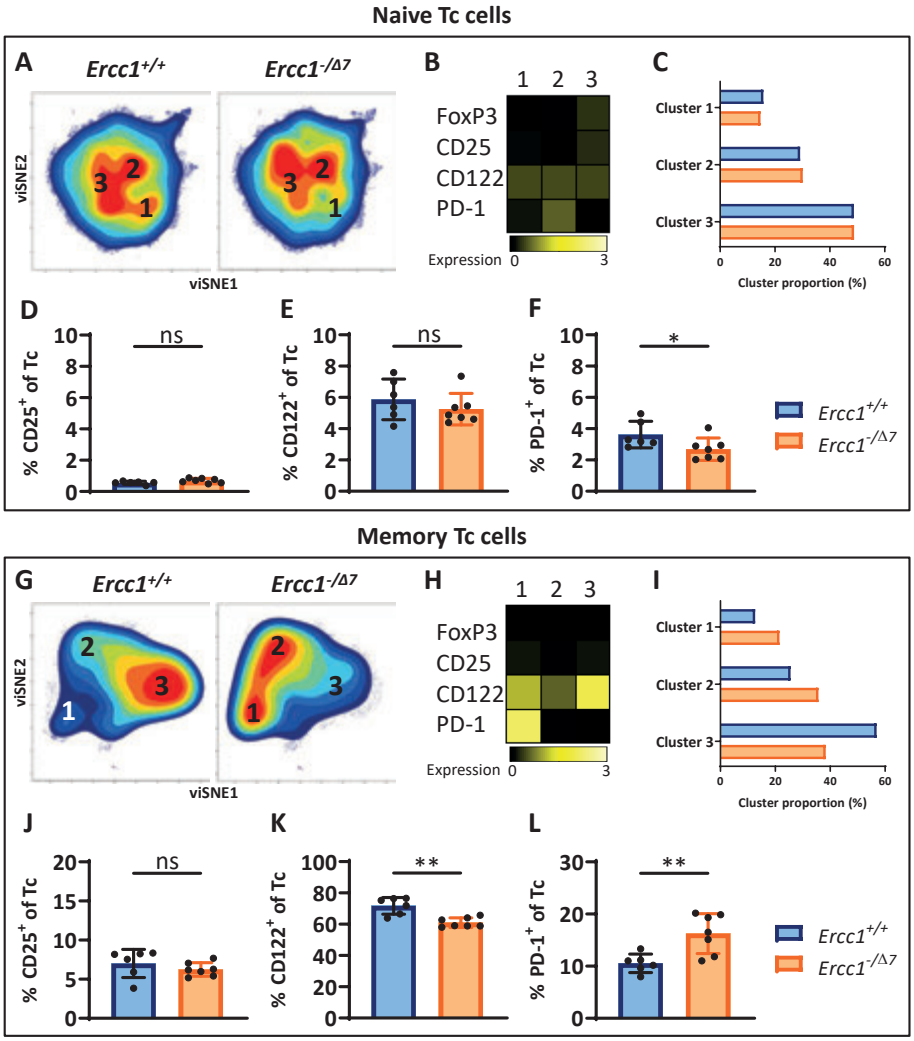


**Figure 3. Aging-related changes in naive and memory Tc cells in mice with compromised DNA repair.**

Naive (CD44$^{Lo}$) and memory (CD44$^{Hi}$) cells were identified within the CD4$^-$ T-cell population (Tc cells)**.** Cell density maps of dimensionality reduced single-cell data by viSNE show clustering within (**A**) naive Tc cells and (**G**) memory Tc cells of pooled flow cytometry datafiles of *Ercc1*$^{+/+}$ mice (n=6) and pooled datafiles of *Ercc1*$^{-/\Delta7}$ mice (n=7). Numbers in (**B,H**) the density maps correspond to the cluster numbers above the heat maps. These heat maps depict the Arcsinh-transformed median expression of the indicated markers. Bar graphs (**C,I**) indicate the proportion of each cluster within the total viSNE for *Ercc1*$^{+/+}$ (blue) and *Ercc1*$^{-/\Delta7}$ (orange) mice. Bar graphs show the frequencies of (**D,J**) CD25$^+$, (**E,K**) CD122$^+$, and (**F,L**) PD-1$^+$ cells among naive and memory Tc cells in individual *Ercc1*$^{+/+}$ and *Ercc1*$^{-/\Delta7}$ mice. Bar graphs show mean ± SD; *$p < 0.05$, **$p < 0.01$, ns = not statistically significant for the difference between *Ercc1*$^{+/+}$ and *Ercc1*$^{-/\Delta7}$ mice using parametric unpaired Student's *t* test or non-parametric Mann-Whitney test, two-tailed, dependent on the tested normality of distribution of the data.

**eRapa increases the proportion of memory Tregs but does not reduce the accumulation of total Tregs**

eRapa did not prevent the rise in proportion of FoxP3$^+$ Tregs within the overall CD3$^+$ T-cell pool in *Ercc1*$^{-/\Delta 7}$ mice (**Figure 4D**). However, we observed that eRapa decreased the proportion of Tregs within the naive CD4$^+$ T-cell pool of *Ercc1*$^{-/\Delta 7}$ mice and strongly increased the proportion of Tregs within the memory CD4$^+$ T-cell pool of these mice (**Figure 4E,H**). eRapa did not prominently reverse the aging-related lower CD25$^+$ and higher PD-1$^+$ cell frequencies found among naive Tregs of *Ercc1*$^{-/\Delta 7}$ mice (**Figure 4F,G**). In contrast, eRapa highly increased the frequency of CD25$^+$ and PD-1$^+$ cells among memory Tregs, which was not observed in *Ercc1*$^{-/\Delta 7}$ mice without eRapa (**Figure 4I,J**). Together, these data suggest that accumulation of Tregs with an aging-related phenotype by compromised DNA repair may be independent of mTOR activation.

**Aging-related reduction of CD122$^+$ memory Th-cell frequencies can be restored by eRapa.**

One of the few observations in the Th-cell subset of *Ercc1*$^{-/\Delta 7}$ mice that reflected findings in WT aged mice [3] was a decreased frequency of CD122$^+$ cells among memory Th cells (**Supplementary Figure 4**). eRapa restored the proportion of CD122$^+$ memory Th cells to levels found in *Ercc1*$^{+/+}$ mice (**Supplementary Figure 6**). This finding shows that eRapa may reverse this aging-related aspect of memory Th cells and suggests that CD122 expression by memory Th cells is dependent on mTOR activation.

**Compromised DNA repair limits T-cell receptor/Interleukin-2-mediated Treg proliferation and activation.**

Reduced proliferation and reduced upregulation of the activation marker CD25 in response to cellular stimulation are hallmarks of declined T-cell responsiveness at older age [3,37]. To investigate the consequences of compromised DNA repair on T-cell responses, we exposed total spleen cells of *Ercc1*$^{+/+}$ and *Ercc1*$^{-/\Delta 7}$ mice, or young and aged WT mice to anti-CD3 alone to mimic stimulation of the T-cell receptor (TCR), or in combination with anti-CD28 or interleukin-2 (IL-2) as a co-stimulator. After four days, we measured T-cell responsiveness by proliferation and upregulation of the activation marker CD25. Additionally, we stimulated T cells of eRapa-fed *Ercc1*$^{-/\Delta 7-}$ mice to assess whether eRapa would prevent consequences of compromised DNA repair on T-cell responsiveness.

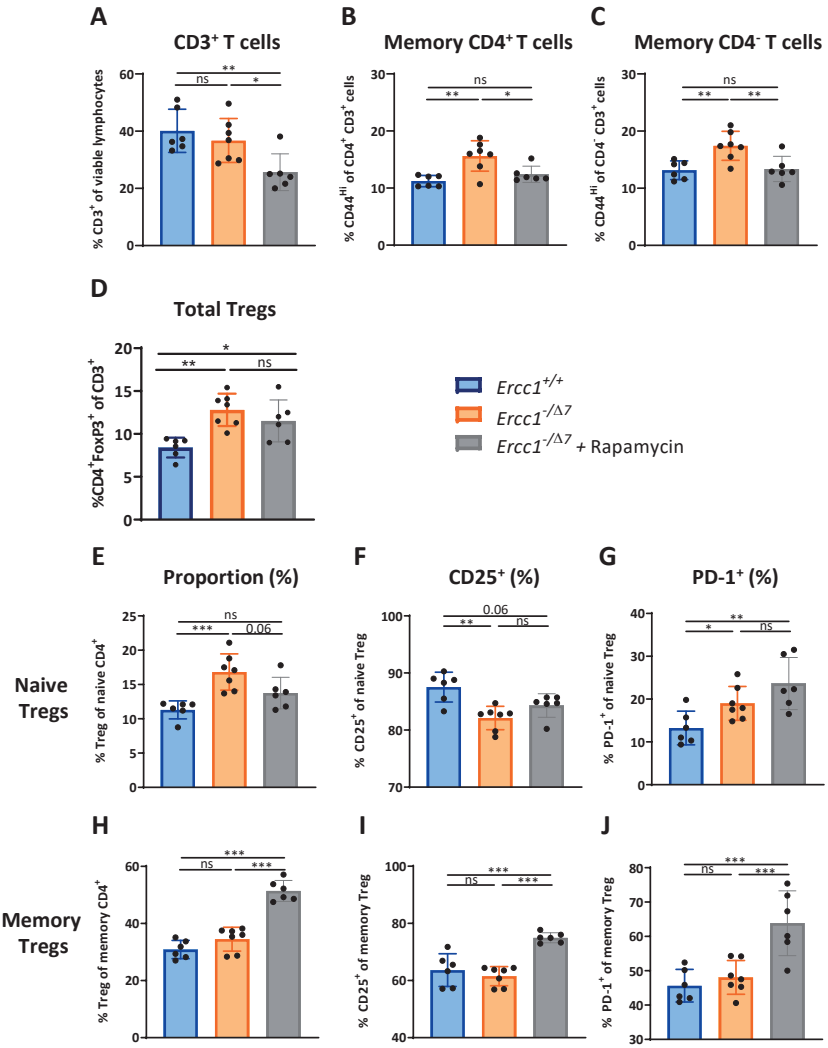


**Figure 4. eRapa increases the proportion of Tregs within the memory CD4⁺ T-cell subset.** The frequencies of (**A**) total T cells (CD3⁺ cells of viable lymphocytes), (**B**) memory CD4⁺ T cells (CD44$^{Hi}$ of CD4⁺CD3⁺ cells), (**C**) memory CD4⁻ T cells (CD44$^{Hi}$ of CD4⁻CD3⁺ cells), and (**D**) total Tregs (CD4⁺FoxP3⁺ of CD3⁺) were determined in the spleens of *Ercc1*$^{+/+}$ (n=6, blue bars), *Ercc1*$^{-/\Delta7}$ (n=7, orange bars), and *Ercc1*$^{-/\Delta7}$ treated with eRapa (n=6, grey bars). The frequencies of (**E**) naive Tregs (FoxP3⁺ cells of CD44$^{Lo}$CD4⁺CD3⁺), and (**h**) memory Tregs (FoxP3⁺ cells of CD44$^{Hi}$CD4⁺CD3⁺), and their respective (**F,I**) CD25⁺ and (**G,J**) PD-1⁺ cell frequencies were also determined in these mice. Bar graphs show mean ± SD; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ns = not statistically significant for the difference between groups using one-way ANOVA corrected with Holm-Sidak test for multiple comparisons.

We observed that compromised DNA repair in part explains diminished Treg proliferation in WT aged mice, as Tregs of *Ercc1*$^{-/\Delta 7}$ mice showed a trend towards reduced proliferation in response to anti-CD3 alone and significantly reduced proliferation in response to anti-CD3 with IL-2 (**Figure 5A,B**). However, Tregs of WT aged mice also showed reduced proliferation in response to anti-CD3 combined with co-stimulation by anti-CD28 (**Figure 5C**), whereas CD28-mediated co-stimulation appeared to remain intact in Tregs from *Ercc1*$^{-/\Delta 7}$ mice. eRapa did not restore the reduction of Treg proliferation observed in *Ercc1*$^{-/\Delta 7}$ mice (**Figure 5A,B**). Additionally, Tregs of *Ercc1*$^{-/\Delta 7}$ mice showed a trend towards reduced CD25 upregulation in response to anti-CD3 with IL-2, although the variation within the group of *Ercc1*$^{-/\Delta 7}$ mice was relatively high (**Figure 5D**). These findings in part reflect observations in WT aged mice, as WT aged mice also showed reduced CD25 expression in response to anti-CD3 in the absence of exogenous IL-2 (**Figure 5E**). eRapa did not restore Treg CD25 expression levels in *Ercc1*$^{-/\Delta 7}$ compared to *Ercc1*$^{+/+}$ mice (**Figure 5D**). It has to be noted that the number of eRapa-fed *Ercc1*$^{-/\Delta 7-}$ mice stimulated with anti-CD3 with IL-2 was low (n=3) due to a limited number of splenocytes available for the different assays and these results should therefore be approached with caution. Together, our findings indicate that compromised DNA repair contributes to reduced Treg responsiveness to anti-CD3 and IL-2 observed with WT aging, which may be independent of mTOR activation.

**Compromised DNA repair limits T-cell receptor/Interleukin-2 mediated Th- and Tc-cell responsiveness.**

Th and Tc cells of *Ercc1*$^{-/\Delta 7}$ mice showed a trend towards reduced proliferation in response to anti-CD3 with IL-2, whereas Th and Tc cells of WT aged mice showed reduced proliferation also in response to anti-CD3 with anti-CD28 (**Supplementary Figure 7**). Despite the small number of mice, eRapa did not appear to restore proliferation of Th and Tc cells from *Ercc1*$^{-/\Delta 7}$ mice in response to anti-CD3 with IL-2 (**Supplementary Figure 7**). Tc cells but not Th cells of *Ercc1*$^{-/\Delta 7}$ mice showed reduced CD25 expression induced by response to anti-CD3 with IL-2, despite the individual response within each group being highly variable. In contrast, both cell types showed reduced CD25 induction in WT aged mice (**Supplementary Figure 7**). *In vivo* treatment of *Ercc1*$^{-/\Delta 7}$ mice with eRapa did not affect stimulation-induced CD25 expression by their Th cells (**Supplementary Figure 7**). Thus, compromised DNA repair may partly explain impaired Th- and Tc-cell responses found in WT aged mice.

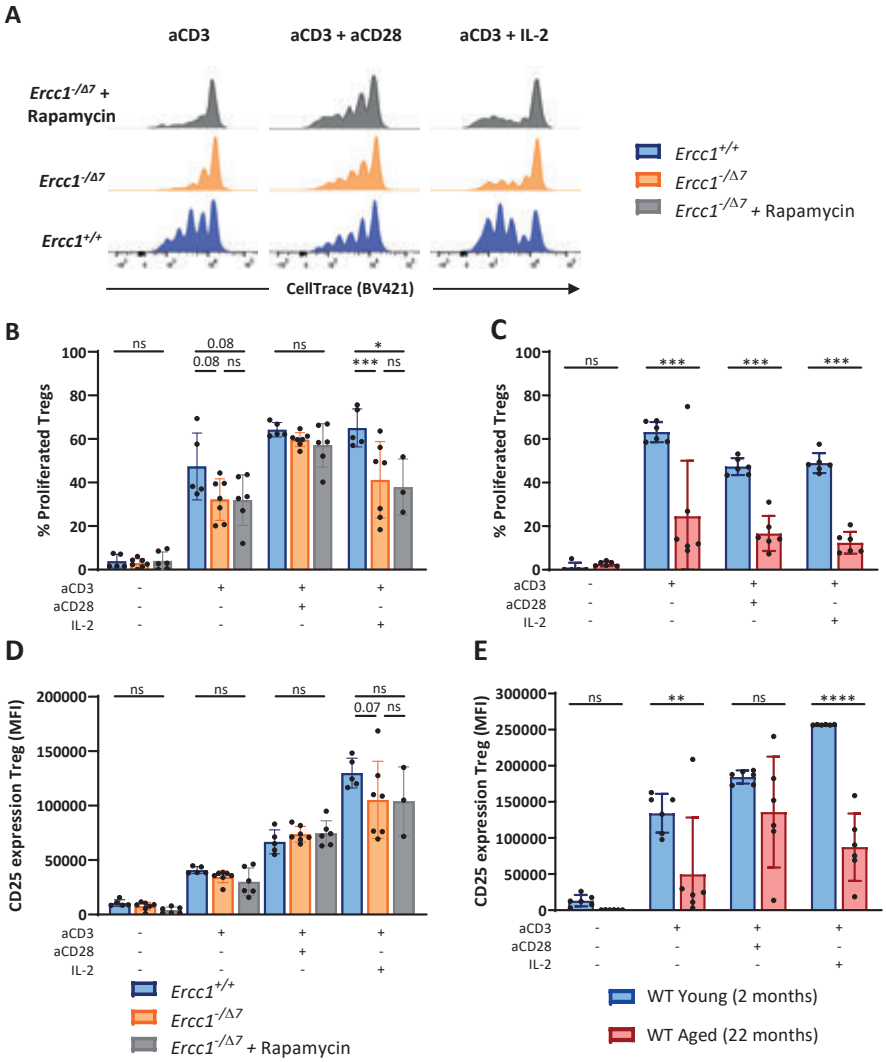


**Figure 5. Compromised DNA repair limits T-cell receptor/Interleukin-2-mediated Treg proliferation and activation.**

Total splenocytes of WT young (n=6) and aged (n=6) mice, and *Ercc1*$^{+/+}$ (n=5), *Ercc1*$^{-/\Delta7}$ (n=7), and *Ercc1*$^{-/\Delta7}$ mice treated with rapamycin (n=3-6) were exposed to anti-CD3 alone or in combination with anti-CD28 or IL-2 for four days. Treg proliferation was traced by CellTrace labeling (**A**); dilution of CellTrace label intensity indicates cellular proliferation. Bar graphs show (**B**) Treg proliferation of *Ercc1*$^{-/\Delta7}$ and *Ercc1*$^{-/\Delta7}$ mice and of (**C**) WT young and aged mice. Bar graphs show CD25 expression by Tregs of (**D**) *Ercc1*$^{-/\Delta7}$ and *Ercc1*$^{-/\Delta7}$ mice and (**E**) WT young and aged mice**.** Bar graphs show mean ± SD; *$p$ < 0.05, **$p$ < 0.01, ***$p$ <0.001, ****$p$ < 0.0001 ns = not statistically significant for the difference between groups using parametric one-way ANOVA corrected with Holm-Sidak correction for multiple comparisons. Due to the low number of eRapa-fed *Ercc1*$^{-/\Delta7}$ mice (n=3), statistical significance of this group was determined by non-parametric Mann-Whitney U Test.

## DISCUSSION

Aging has a significant effect on T-cell phenotype and responsiveness, which contributes to pathology, increased susceptibility to infectious diseases, and reduced vaccine efficacy in the elderly [1]. Aside from well described T-cell aging phenomena that occur over time, the contribution of intracellular processes that drive T-cell aging such as compromised DNA repair remain incompletely defined. Here we provide novel insights into T-cell aging as we show which parts of T-cell aging can be attributed to compromised DNA repair. Moreover, we show that the majority of the changes to T cells induced by compromised DNA-repair could not be reversed by inhibition of mTOR activation.

Accumulation of Tregs during aging has been described in mice and humans [3-8] and is thought to limit protective immune responses. However, the underlying cause of Treg accumulation with age remains unclear. Here we discovered that compromised DNA repair contributes to the accumulation of Tregs observed during aging, as we show that Tregs accumulate in *Ercc1*$^{-/\Delta 7}$ and WT aged mice. Similar to WT aged mice, Treg accumulation in *Ercc1*$^{-/\Delta 7}$ mice was explained by an increase in the proportion of Tregs within the naive CD4$^{+}$ T-cell subset. Consistent with previous findings in WT mice [3,8], these Tregs showed an age-related phenotype characterized by increased expression of PD-1 and decreased expression of CD25. Thus, our data indicate that compromised DNA repair may be a contributing factor in promoting Treg accumulation previously observed to occur during the process of aging.

An important question that now remains is how compromised DNA repair results in the accumulation of Tregs. We predict that compromised DNA-damage repair in cells that make up the micro-environment of T cells significantly contributes to the phenotypical and functional changes found within the T cell pool with age. Senescent cells develop during the process of aging in multiple organs partly due to intracellular accumulation of DNA damage [38,39]. These cells are known to secrete a collection of pro-inflammatory factors collectively known as the senescence-associated secretory phenotype (SASP). Accumulation of senescent cells therefore results in an inflammatory micro-environment [38]. Interestingly, it has been reported that the cytokine IL-6 is part of the pro-inflammatory SASP and promotes the accumulation of Tregs in WT aged mice [40]. Moreover, it is known that *Ercc1*$^{-/\Delta 7}$ mice also accumulate senescent cells and show increased levels of the SASP, including higher levels of IL-6 in several organs and serum [21,41-43]. Based on these and our findings, we speculate that accumulation of Tregs with age is mediated by pro-inflammatory factors like IL-6 secreted by senescent cells that result from

accumulation of DNA damage over life. Accumulation of Tregs in response to IL-6 induced by DNA-damage may be an attempt by the immune system to counteract pro-inflammatory conditions that occur during aging.

Diminished T-cell proliferation is a hallmark of T-cell aging [3,37]. Our data show that compromised DNA repair hampers TCR- and IL-2-mediated T-cell proliferation. Reduced expression of the IL-2 receptor (IL-2R) chains CD25 and CD122 may explain reduced IL-2-mediated proliferation [44,45]. Indeed, memory Th and Tc cells of *Ercc1*$^{-/\Delta 7}$ mice showed reduced CD122$^{+}$ cell frequencies and expression levels. Moreover, *Ercc1*$^{-/\Delta 7}$ Tregs and Tc cells showed reduced upregulation of CD25 expression after stimulation. Compromised DNA repair did not impact all T-cell stimulatory pathways since co-stimulation via CD28 in the presence of CD3 stimulation could trigger proliferation. This was in contrast to findings in WT aged mice as these mice show impaired proliferation in response to CD28 and CD3 stimulation. Together, our findings indicate that defects in IL-2-mediated T-cell proliferation observed with age can be attributed to compromised DNA repair. Conversely, compromised DNA repair did not hamper CD28-mediated T-cell proliferation. Therefore, impaired CD28-mediated proliferation likely develops via a mechanism other than *Ercc1*$^{-/\Delta 7}$-mediated compromised DNA repair.

As mTORC1 negatively interferes with the ATM checkpoint that promotes DNA damage repair [31], the mTOR pathway may be linked to DNA damage response signaling [30], and eRapa reduces aging-related phenotypical T-cell changes in WT mice [29], we investigated whether rapamycin could slow down the aging-related T-cell changes imposed by compromised DNA repair. *In vivo* treatment with eRapa did not slow down the accumulation of Tregs in *Ercc1*$^{-/\Delta 7}$ mice, suggesting that compromised DNA repair promotes the accumulation of Tregs independent of mTOR. Our findings concur with a previous study in eRapa-fed WT mice that shows no change to Treg numbers [29]. In contrast, Neff *et al.* report a decrease in Treg numbers after eRapa supplementation [46]. These conflicting results may be explained by the difference in Treg characterization. Whereas we and others [29] characterize Tregs by expression of the Treg master transcription factor FoxP3 [47], Neff *et al.* assessed Tregs as CD25$^{+}$ cells without including FoxP3 [46]. WT aging lowers the frequency and expression of CD25 on FoxP3$^{+}$ Tregs [3,7], which we also observed in *Ercc1*$^{-/\Delta 7}$ mice. Therefore, characterization of Tregs based on CD25 only may underestimate the total amount of Tregs present.

eRapa could not prevent decreased proliferation of T cells we observed in *Ercc1*$^{-/\Delta 7}$ mice. These findings are in contrast to previously observed findings in eRapa-fed WT mice, where eRapa improved T-cell proliferation [29]. An

explanation might be that we used whole spleen cultures that allow interactions between different subsets of T cells, whereas Hurez *et al.* studied an isolated subfraction of $CD4^+$ T cells. Our spleen cell cultures included eRapa-induced $CD25^+$ and $PD\text{-}1^+$ memory Tregs. $CD25^+$ and $PD\text{-}1^+$ memory Tregs of WT aged mice have been shown to comprise a Treg subset with enhanced suppressive capacity [8], which may suggest suppression of Th- and Tc-cell proliferation in our cultures by these eRapa-induced memory Tregs. Generation of $CD25^+$ and $PD\text{-}1^+$ memory Tregs by eRapa may be explained by upregulation of the memory marker CD44 on naive Tregs, since *in vivo* treatment with rapamycin can induce expression of CD44 and PD-1 $FoxP3^+$ Tregs [48]. Alternatively, memory $FoxP3^-$ $CD4^+$ T cells may have upregulated FoxP3 following eRapa. However, this is less likely as $Foxp3^-$ effector T cells and $CD4^+CD25^-$ cells exposed *in vitro* to rapamycin do not develop into $FoxP3^+$ Tregs [49,50]. Future studies on the effects of eRapa on proliferation of isolated T-cell fractions and whole-cell cultures will be important to provide full insight in the immune modulatory properties of eRapa.

Our data show that compromised DNA repair contributes to increased frequencies of memory T cells, but not to the extent found in WT aged mice. Since antigens are a major driving force behind the formation of memory T cells [10], the higher level of inflation of memory T cells found in WT aged mice is likely explained by the higher antigenic exposure over their longer lifetime. These findings indicate that compromised DNA repair partly contributes to aging-related phenotypic T-cell alterations and that aging of the T-cell pool is a diverse process that is driven by multiple factors in addition to DNA-damage. Interestingly, eRapa decreased the frequency of memory T cells found in *Ercc1*$^{-/\Delta 7}$ mice at the benefit of a rise in the proportion of naive T cells, which is consistent with a previous report in WT mice [29]. Since higher frequencies of naive cells at young age are linked to better T-cell mediated protection, we predict that the eRapa-induced rise of the naive T-cell frequency may contribute to improved T-cell mediated protection. This is supported by studies showing rapamycin improved T-cell function against pathogens [51] and antigen-specific T-cell responses [52].

Although our data indicate that compromised DNA damage repair contributes to aging of the immune system, there are limitations regarding our study. First, although *Ercc1*-deficient mice show compromised DNA repair in at least four repair pathways [12], it does not account for deficiencies in other DNA-repair pathways, such as nonhomologous end-joining or base-excision repair [53]. It is therefore possible that defects in DNA-repair mechanisms other than those mediated by *Ercc1*-deficiency have their own characteristic impact

on aging-related T-cell changes. Second, although endogenous nuclear DNA damage accumulation in *Ercc1*$^{-/\Delta 7}$ mice has been confirmed in liver and kidney cells [22]. it is unknown whether T cells of *Ercc1*$^{-/\Delta 7}$ mice also accumulate DNA damage. Therefore, it remains to be established whether T-cell changes we ascribe to *Ercc1*-deficiency are due to T-cell intrinsic accumulation of DNA damage, or due to DNA damage present in the microenvironment these T cells reside in. Additionally, the inflammatory profile affecting T cells might differ between normal aging by response to antigen exposure over time, senescence-driven aging, and DNA damage-accelerated aging. Finally, we used a 42 ppm eRapa dose in our *in vivo* experiments as this dose was previously reported to further expand the health- and lifespan of WT mice compared to lower eRapa dosages [36]. Compared to other studies investigating the effect of eRapa on T-cell phenotype and function in WT mice [29,46], our dose is 3-fold higher. As different doses of rapamycin show different outcomes of lifespan in WT mice [27,46], it is likely that changes within the T-cell population are also dose-dependent. Additionally, to what extent the 42 ppm dose of eRapa inhibits mTORC1 and mTORC2 signals remains unknown and warrants future immune studies.

Collectively, this study reveals a novel and pivotal role for compromised DNA repair in promoting accumulation of Tregs with an aging-related phenotype. Although smaller mTOR-mediated effects by eRapa may be missed by the relatively small group sizes in our study, compromised DNA repair appears to impose accumulation of naive Tregs through a mechanism that may be independent of mTOR activation. Our study indicates that preventing DNA-damage over the course of life may help to prevent accumulation of immunosuppressive Tregs hampering protective immunity at old age. Moreover, our study warrants further studies of biological processes that may underlie aging-related immune defects in order to better understand the process of aging.



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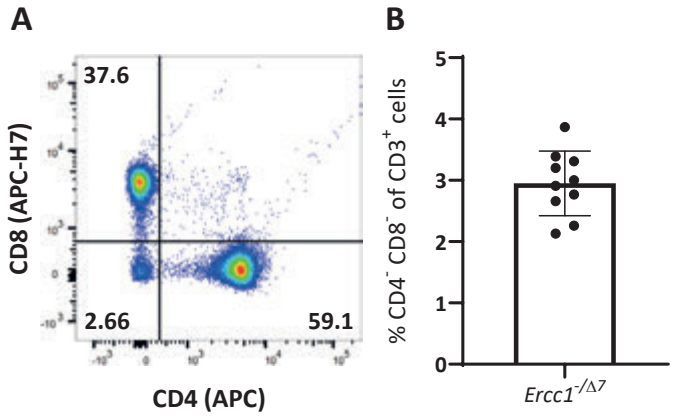
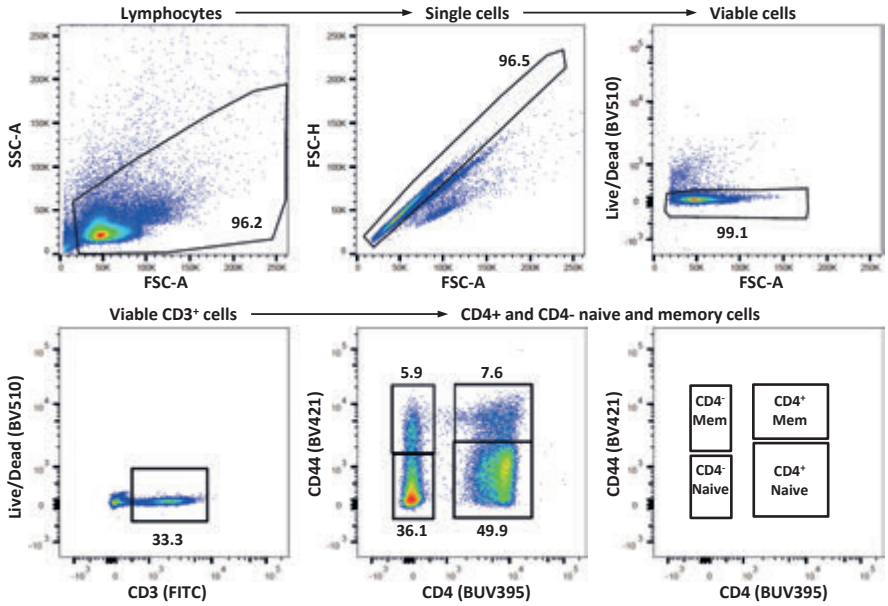
## SUPPLEMENTARY FIGURES

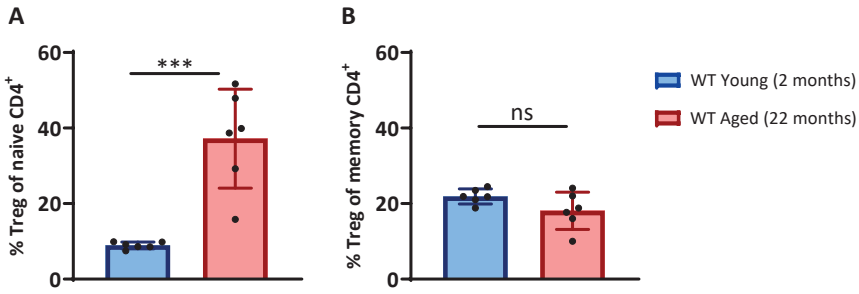



**Supplementary Figure 1. Gating and proportion of CD3⁺CD4⁻CD8⁻ cells in *Ercc1*$^{-/\Delta 7}$ mice.** Representative FACS plot (**A**) shows expression of CD8 and CD4 within the live CD3⁺ T cells of *Ercc1*$^{-/\Delta 7}$ mice. Numbers indicate the percentage of events in each gate as part of all events in the FACS plot. Bar graph (**B**) shows the frequency of CD4⁻CD8⁻ cells within the live CD3⁺ T-cell subset that was assessed in the spleen of *Ercc1*$^{-/\Delta 7}$ mice (n=10) by flow cytometry. Bar graph shows the mean ± SD.



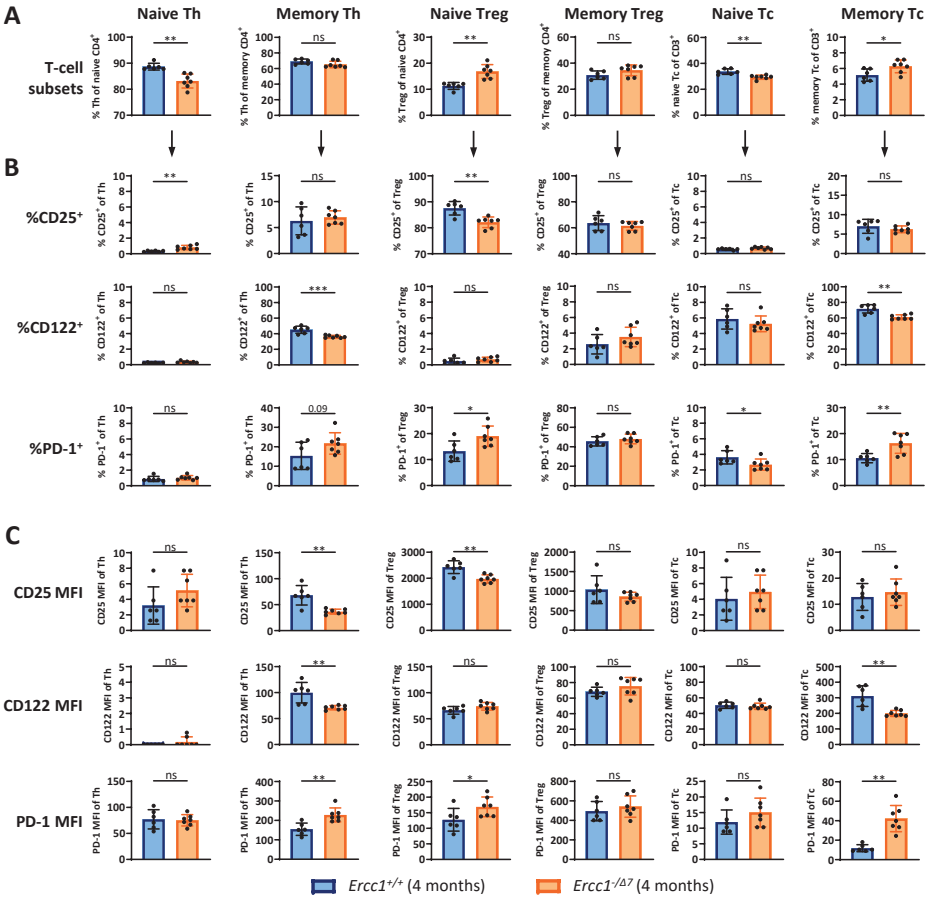

**Supplementary Figure 2. Gating strategy towards naive and memory CD4⁺ and CD4⁻ T cells that were analyzed by viSNE.**

Representative FACS plots show the gating from spleen lymphocytes to single cells, viable cells, and viable CD3⁺ cells. These CD3⁺ T cells were further subdivided into four T-cell subsets: naive CD4⁺ T cells ($CD44^{Lo}CD4^{+}CD3^{+}$), memory CD4⁺ T cells ($CD44^{Hi}CD4^{+}CD3^{+}$), naive CD4⁻ T cells (Tc cells) ($CD44^{Lo}CD4^{-}CD3^{+}$), memory CD4⁻ T cells (Tc cells) ($CD44^{Hi}CD4^{-}CD3^{+}$). Numbers indicate the percentage of events in each gate as part of all events in the FACS plot. The gated naive and memory cell subsets were subsequently analyzed by dimensionality reduction (viSNE).



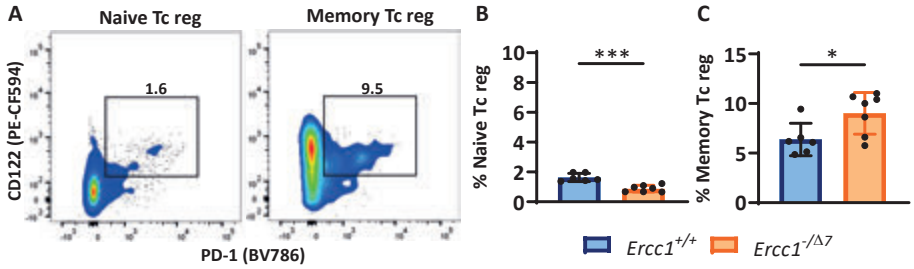

**Supplementary Figure 3. FoxP3⁺ Tregs accumulate within the naive CD4⁺ T cell subset of wild-type aged mice.**

The frequencies of (**A**) naive Tregs (FoxP3⁺ of $CD44^{Lo}CD4^{+}CD3^{+}$ cells) and (**B**) memory Tregs (FoxP3⁺ of $CD44^{Hi}CD4^{+}CD3^{+}$ cells) were determined in the spleens of WT young (n=6, 2 months old) and aged mice (n=6, 22 months old). Bar graphs show mean ± SD; $^{**}p < 0.01$, $^{***}p < 0.001$, ns = not statistically significant for the difference between groups using unpaired Student's *t* test, two-tailed.



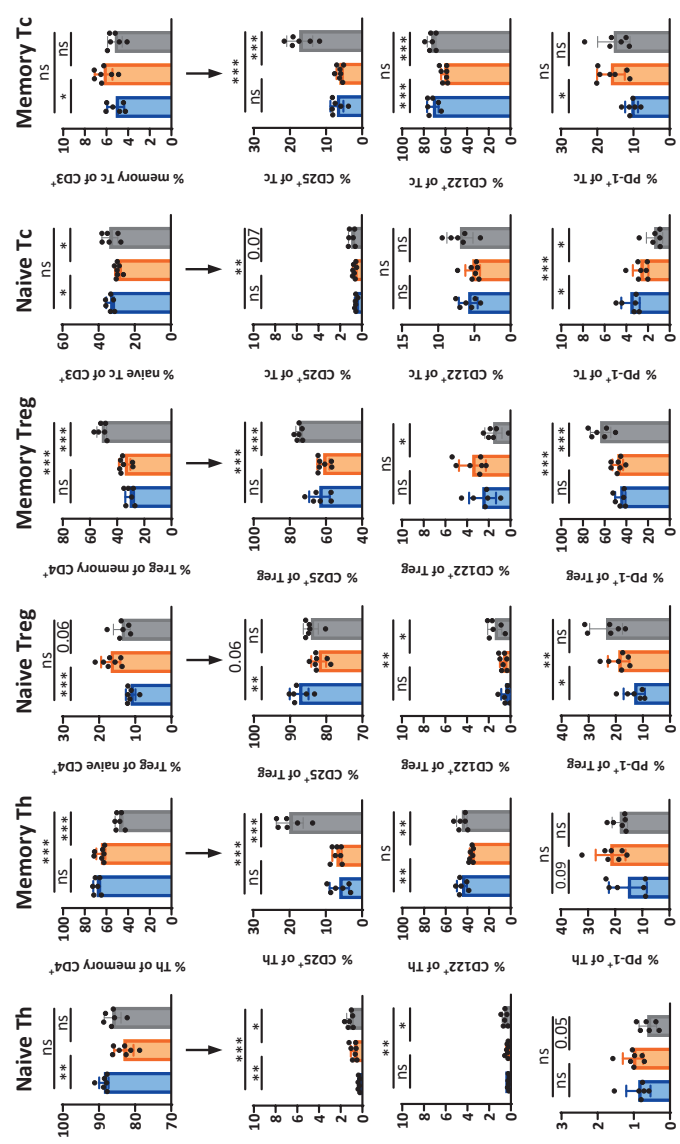

**Supplementary Figure 4. CD25⁺, CD122⁺, and PD-1⁺ cell frequencies and expression within naive and memory T-cell subsets.**

Bar graphs (**A**) indicate the frequencies of the following T-cell subsets: naive Th cells (FoxP3⁻ cells of CD44$^{Lo}$CD4⁺CD3⁺), memory Th cells (FoxP3⁻ cells of CD44$^{Hi}$CD4⁺CD3⁺), naive Treg cells (FoxP3⁺ cells of CD44$^{Lo}$CD4⁺CD3⁺), memory Treg cells (FoxP3⁺ cells of CD44$^{Hi}$CD4⁺CD3⁺), naive Tc cells (CD44$^{Lo}$CD4⁻ of CD3⁺), and memory Tc cells (CD44$^{Hi}$CD4⁻ of CD3⁺). Within each of these T-cell subsets (**B**) the frequencies of CD25⁺, CD122⁺, and PD-1⁺ cells were determined, as well as (**C**) the median expression intensity (MFI) of CD25, CD122, and PD-1. Bar graphs show mean ± SD; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ns = not statistically significant for the difference between groups using parametric unpaired Student's *t* test or non-parametric Mann-Whitney test, two-tailed, dependent on the tested normality of distribution of the data.

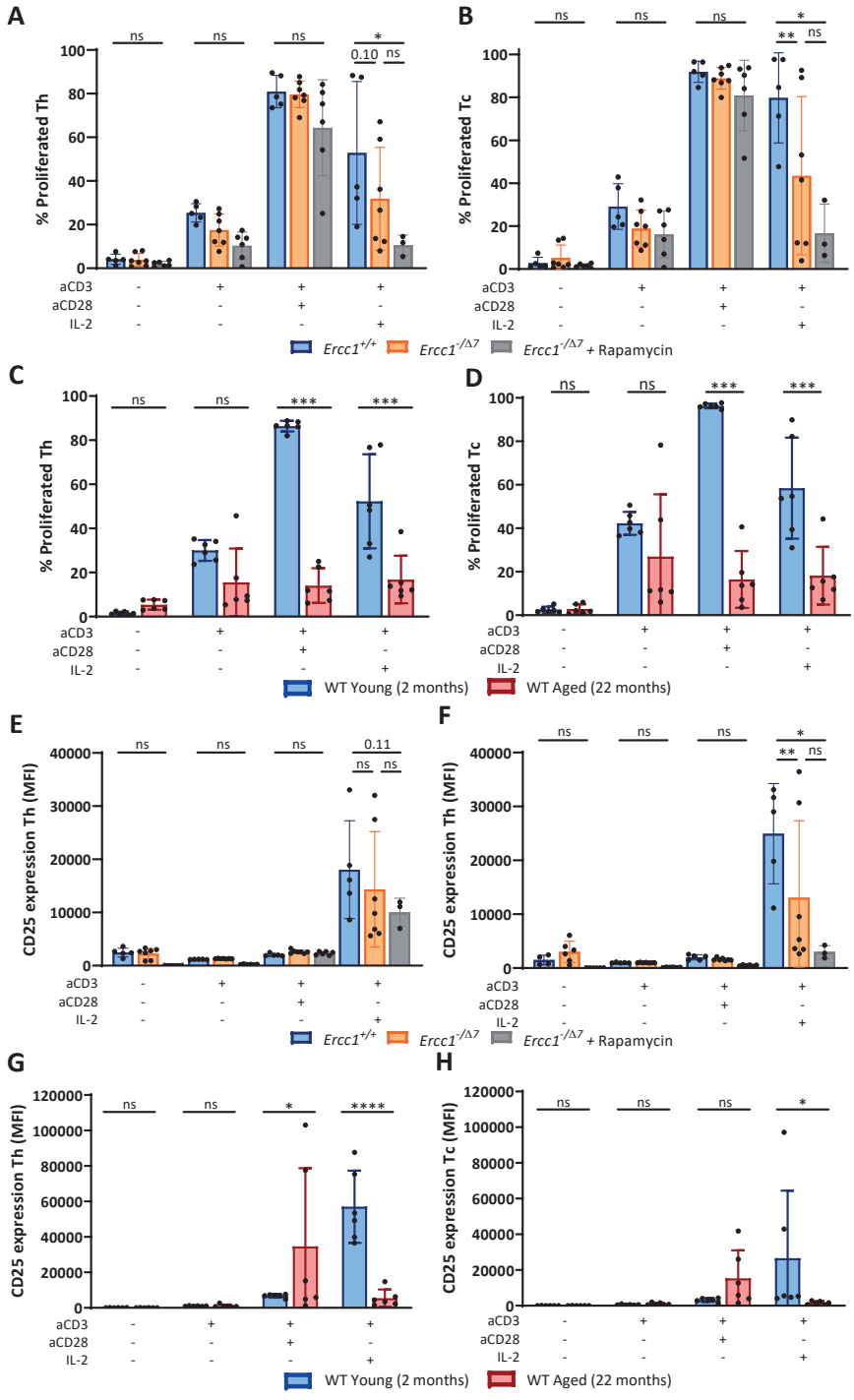



**Supplementary Figure 5. Tc reg cells accumulate within the memory Tc-cell subset of *Ercc1*-deficient mice.**

Representative FACS plots (**A**) show the gating of naive and memory Tc reg cells based on the expression of CD122 and PD-1. The frequencies of (**B**) Treg cells among naive Tc cells (PD-1$^{+}$ CD122$^{+}$ of CD44$^{Lo}$CD4$^{-}$CD3$^{+}$ cells) and (**C**) Treg cells among memory Tc cells (PD-1$^{+}$ CD122$^{+}$ of CD44$^{Hi}$CD4$^{-}$CD3$^{+}$ cells) were determined in the spleen of *Ercc1*$^{+/+}$ (n=6, 4 months of age) and *Ercc1*$^{-/\Delta 7}$ (n=7, 4 months of age) mice. Bar graphs show mean ± SD; *$p < 0.05$, ***$p < 0.001$ for the difference between groups using unpaired Student's *t* test, two-tailed.




**Supplementary Figure 6. The effect of eRapa on CD25$^+$, CD122$^+$, and PD-1$^+$ cell frequencies within naive and memory T-cell subsets.**
Bar graphs (**A**) indicate the frequencies of the indicated naive and memory T-cell subsets. Within each of these T-cell subsets the frequencies of CD25$^+$, CD122$^+$, and PD-1$^+$ cells were determined (**B**) in the spleen of *Ercc1*$^{+/+}$ (n=6, blue bars), *Ercc1*$^{-/\Delta7}$ (n=7, orange bars), and *Ercc1*$^{-/\Delta7}$ treated with eRapa (n=6, grey bars). Bar graphs show mean ± SD; $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, ns = not statistically significant for the difference between groups using one-way ANOVA corrected with Holm-Sidak test for multiple comparisons.




**Supplementary Figure 7. Compromised DNA repair limits T-cell receptor/Interleukin-2 mediated Th- and Tc-cell responsiveness.**

Total splenocytes of WT young (n=6) and aged (n=6) mice, and *Ercc1*$^{+/+}$ (n=5), *Ercc1*$^{-/\Delta 7}$ (n=7), and *Ercc1*$^{-/\Delta 7}$ mice treated with rapamycin (n=3-6) were exposed to anti-CD3 alone or in combination with anti-CD28 or IL-2 for four days. Bar graphs show Th- and Tc-cell proliferation of (**A,B**) *Ercc1*$^{-/\Delta 7}$ and *Ercc1*$^{-/\Delta 7}$ mice and of (**C,D**) WT young and aged mice. Bar graphs show CD25 expression by Th and Tc cells of (**E,F**) *Ercc1*$^{-/\Delta 7}$ and *Ercc1*$^{-/\Delta 7}$ mice and (**G,H**) WT young and aged mice**.** Bar graphs show mean ± SD; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ ns = not statistically significant for the difference between groups using parametric one-way ANOVA corrected with Holm-Sidak correction for multiple comparisons. Due to the low number of eRapa-fed *Ercc1*$^{-/\Delta 7}$ mice (n=3), statistical significance of this group was determined by non-parametric Mann-Whitney U Test.


4

# CO-EXPRESSION OF TIGIT AND HELIOS REFINES THE DEFINITION OF IMMUNOSENESCENT CD8$^+$ T CELLS DURING AGING

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**Manuscript in preparation**

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## ABSTRACT

Aging leads to alterations in the immune system resulting in diminished responsiveness against pathogens, collectively defined as immunosenescence. Features of immunosenescence accumulate with age prominently in the $CD8^+$ T-cell population. Progression of immunosenescence is complex and not fully understood, but can be ascribed to differentiation of T cells during the course of life.

We aimed to gain novel insight in the progression of immunosenescence in human $CD8^+$ T cells. To this end, we identified novel markers of immunosenescence in $CD8^+$ T-cells and assessed how these markers relate to the aging-related accumulation of highly differentiated $CD27^-CD28^-$ cells that is often used as proxy for immunosenescence.

We found that co-expression of the transcription factor Helios and the aging-related marker TIGIT marked $CD8^+$ T cells that failed to proliferate and to express activation markers CD69 and CD25 in response to stimulation *in vitro*. In blood from healthy individuals (21-82 years), we found that $TIGIT^+Helios^+$ T cells accumulate by aging-related increase of the highly differentiated $CD27^-CD28^-$ population. Interestingly, $TIGIT^+Helios^+$ T cells also accumulated with age among the less differentiated $CD27^+CD28^-$ T cells before their transit into the highly differentiated $CD27^-CD28^-$ stage. This finding suggests that T cells with immunosenescent features become prominent at old age within earlier differentiation states as well.

Our findings show that co-expression of TIGIT and Helios refines the definition of immunosenescent $CD8^+$ T cells and challenge the current dogma of late differentiation stage as proxy for T-cell immunosenescence, since immunosenescence may also accumulate by aging within an earlier differentiation stage.

## INTRODUCTION

Viral respiratory diseases such as influenza and COVID-19 severely strike the elderly due to the debilitating changes aging can impose on the immune system [1-3]. Immunological dysfunctions that evolve during aging occur throughout the immune system and are collectively defined as immunosenescence [3]. This is illustrated for $CD8^+$ T cells that are required to control viral infections and malignant cells, but lose proper responsiveness at old age, as is reflected by their reduced capacity to proliferate and become activated in response to stimulus at old age [4-7].

The concept of immunosenescence is approached from many different angles and immune markers, and it is difficult to achieve a clear view of how T cells change during aging. We aimed to improve insight into the progression of immunosenescence in $CD8^+$ T cells by identifying novel markers of aging-related dysfunction and stratified these markers to T-cell differentiation as classically defined by expression of co-stimulatory markers CD27 and CD28 [8,9].

Differentiation of $CD8^+$ T cells during the course of life results in gain of features that are associated with immunosenescence and the accumulation of highly differentiated T cells at old age [3,8,10,11]. Progression towards senescence in $CD8^+$ T cells can be stratified by three successive differentiation states based on expression of CD27 and CD28 [8,9]. This approach describes a gradual path from the early differentiation state ($CD27^+CD28^+$) via an intermediate differentiation state ($CD27^+CD28^-$) into the late differentiation state ($CD27^-CD28^-$) that expresses many features of immunosenescence, such as reduced capacity to proliferate [8]. Due to these features, the accumulation of $CD27^-CD28^-$ cells is often used as a proxy for the level of immunosenescence in the $CD8^+$ T-cell pool [8-10,12].

Although accumulation of late-differentiated T cells may be useful as surrogate marker of immunosenescence progression, it does not fully pinpoint functional changes in the $CD8^+$ T-cell population that accumulate by aging, such as reduced proliferation and activation. To better understand progression of immunosenescence by aging, it would be helpful to define markers that more precisely pinpoint functionally immunosenescent cells and complement the current view on differentiation as surrogate marker of immunosenescence. The co-inhibitory receptor TIGIT has recently been identified as a potent marker of immunosenescence as it is expressed by dysfunctional T cells that accumulate at older age [13]. Its clear functional role was shown by blocking of TIGIT resulting in improved functionality of immunosenescent T cells [13-15]. The transcription factor Helios is a marker that may further define different functional subsets

among TIGIT$^+$ T cells. For example, Helios co-expression with TIGIT has been described in CD4$^+$ T cells, in which it defines a functionally distinct cell subset that is characterized by regulatory properties [16,17]. Additionally, a significant proportion of CD8$^+$ T cells also expresses Helios and a recent study hinted towards Helios as marker that might indicate immunosenescence of CD8$^+$ T cells [18]. However, the value of Helios as marker of CD8$^+$ T-cell immunosenescence remains to be defined. Here, we aimed to define the potency of Helios combined with TIGIT as marker to more precisely pinpoint CD8$^+$ T-cell immunosenescence and how these markers relate to CD8$^+$ T-cell differentiation status during aging.

## MATERIALS AND METHODS

### Study design

Blood was obtained from healthy individuals (n=50, 21-82 years of age) from three different sources: blood donors (Sanquin Blood Supply Foundation), healthy participants of the NVI-255 study at the RIVM (Netherlands Trial Register NTR2070) [19], or the ILI-3 study at the RIVM (Netherlands Trial Register NTR4818) ([20], Van Kaaijk *et al.* submitted). Only CMV-seronegative participants were included in the study, as determined by ELISA [21] or our in-house Multiplex Immunoassay [22].

### PBMC isolation and flow cytometry

Peripheral Blood Mononuclear cells (PBMCs) were isolated by density gradient (Ficoll-Hypaque, Amersham Biosciences) from heparinized blood or buffy coats and stored at -135 °C in 10% dimethyl sulfoxide (DMSO, Sigma Aldrich) and 10% fetal calf serum (FCS) until further use. For flow cytometric analyses, frozen PBMCs were thawed at 37°C and were transferred to RPMI-1640 medium (GIBCO, Thermo Fisher Scientific) supplemented with 10% FCS and Penicillin-Streptomycin-Glutamine (P/S/G) and washed twice. Thawed cells were then rested in medium for 30 minutes at room temperature after which the number of viable cells was determined on a Coulter Counter (Beckman). $4*10^5$ PBMCs of each individual were labeled for surface markers at 4°C for 30 minutes in FACS buffer (1x PBS +0.5% BSA +2mM EDTA) with saturating concentrations of the following fluorescent labeled antibodies: CD3-FITC (clone UCHT1), CD4-PerCP-Cy5.5 (clone RPA-T4), CD28-BV711 (clone CD28.2), CD27-BV510 (clone O323), and CD57 (clone HCD57) all from Biolegend, CD8-BUV395 (clone RPA-T8, BD Horizon), TIGIT-PE-eFluor610 (clone MBSA43, eBioscience), CD226-BV785 (clone DX11, BD Optibuild), and KLRG1-PerCP-eFluor710 (clone 13F12F2,

Invitrogen). A Fixable Viability Stain 780 (BD Horizon) was added to the labeling to identify viable cells. Cells were then fixed and permeabilized with buffers for intracellular labeling (eBioscience) at 4°C for 30 minutes with fluorescent labeled antibodies targeting: Helios-PE-Cy7 (clone 22F6, Biolegend) and γH2AX (clone N1-431, BD Horizon). Samples were measured on an LSRFortessa™ X-20 (BD Biosciences) and data were analyzed using FlowJo software (v10.6.1, TreeStar). The gating strategy used to define T-cell subsets is shown in **Supplementary Figure 2b**.

**T-cell stimulation assays**

The number of viable cells in thawed PBMC samples was determined manually using trypan blue staining and Bürker-Türk. Cells were then washed with PBS and labeled with 0.5 µM CellTrace™ Violet (Invitrogen) in PBS per milliliter of cell suspension ($10^6$ cells/mL) for 20 minutes at 37 °C to track their proliferation. Ice-cold RPMI-1640 medium (+10% FCS, +P/S/G) was added and cells were rested at room temperature for 5 minutes. Cells were centrifuged at 400*g* for 5 minutes and washed with RPMI-1640 medium (+10% FCS, +P/S/G) three times. Part of the CellTrace-labeled PBMCs were cultured in the presence of 0.005 µg/mL plate-bound purified mouse anti-human CD3 (clone HIT3α, BD Biosciences) in RPMI-1640 medium in U-bottom plates ($2*10^5$ cells/well), whereas another part was directly labeled with fluorescent labeled antibodies (day zero). For each donor, expression of activation markers was assessed at day zero, day one, and day three after culturing. T-cell proliferation was assessed by dilution of CellTrace after three days of culturing. At each time point, cells were labeled for surface markers with the following fluorescent labeled antibodies: CD3-FITC (clone UCHT1, at day zero), CD4-PerCP-Cy5.5 (clone RPA-T4), CD69-BV785 (clone FN50), CD25-PE (clone M-A251) all from Biolegend, CD8-BUV395 (clone RPA-T8, BD Horizon), TIGIT-PE-eFluor610 (clone MBSA43, eBioscience), and CD226-BV785 (clone DX11, BD Optibuild). A Fixable Viability Stain 780 (BD Horizon) was added to the labeling to identify viable cells. Cells were labeled for intracellular markers with the following fluorescent labeled antibodies: CD3-FITC (clone UCHT1, Biolegend, at days one and three) and Helios-PE-Cy7 (clone 22F6, Biolegend).

**Dimensionality reduced analyses (viSNE)**

Dimensionality reduced analysis (viSNE) of flow cytometry data was performed in Cytobank (www.cytobank.com) [23]. Fingerprint color dot plots generated by viSNE indicate expression of each of the indicated markers within the $CD8^+$ T-cell population as measured by flow cytometry. These plots were generated

based on measurements in one donor to illustrate the overlap of markers within $CD8^+$ T cells. A number of 6,325 $CD8^+$ T cells was included in this analysis.

### Statistics

Statistical analysis was performed using GraphPad Prism version 8.4.1. The appropriate parametric or non-parametric tests were used based on the tested normality of distribution of the data. Paired analyses were performed with non-parametric Wilcoxon test or Friedman Test with Dunn's post-test. Correlations between variables were analyzed using Spearman's rank correlation coefficient (*r*). Linear regression analysis was performed to generate lines of best fit. Statistical significance was considered when $p < 0.05$, with $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, and $****p < 0.0001$. All data presented in bar graphs are depicted as mean ± s.d.

## RESULTS

### Co-expression of TIGIT and Helios defines immunosenescent T cells

We first questioned if co-expression of TIGIT and Helios links to dysfunctional immunosenescent $CD8^+$ T cells. We therefore explored if co-expression of TIGIT and Helios (**Figure 1A**) would relate to reduced capacity of $CD8^+$ T cells to get activated and proliferate in response to *in vitro* stimulation of PBMCs with anti-CD3. The proportion of $TIGIT^+Helios^+$ T cells within the $CD8^+$ T-cell population remained stable after one day of culturing with anti-CD3, although this proportion slightly declined after three days (**Figure 1A**). $TIGIT^+Helios^+$ cells were activated within the first day of culture (shown by induction of classic activation markers CD69 and CD25), but to a lesser extent than the $TIGIT^+Helios^-$ and $TIGIT^-Helios^-$ cell subsets (**Figure 1B, C, and Supplementary Figure 1**). Two days later, expression of the early activation marker CD69 had significantly dropped in the cells that were single positive or double negative for TIGIT and Helios. Such rapid return towards lower CD69 expression during a response *in vitro* is typically observed after activation of T cells [5]. However, $TIGIT^+Helios^+$ cells did not show a decline of CD69 expression, but the induced CD69 expression appeared to persist on these cells instead (**Figure 1B**)**.** From day 1 to day 3, expression of the activation marker CD25 increased further in all four subsets, but this upregulation was lower in $TIGIT^+Helios^+$ cells (**Figure 1C**). Together, these data indicate slower and reduced activation potential of $CD8^+$ T cells co-expressing TIGIT and Helios.

Although all $TIGIT^+$ subsets showed reduced proliferation, $TIGIT^+$ cells co-expressing Helios proliferated less compared to all other subsets (**Figure 1D**)**.**

In addition, the proportion of the TIGIT$^+$Helios$^+$ subset within the CD8$^+$ T-cell population negatively correlated with proliferation of the total CD8$^+$ T-cell population (**Figure 1E**), indicating a significant contribution of this subset to decline in proliferative capacity of the overall CD8$^+$ T-cell population.

Finally, TIGIT$^+$Helios$^+$ cells characteristically showed reduced expression of the co-stimulatory receptor CD226 (**Figure 2A-C**), which is the functional counterpart of the co-inhibitory TIGIT and has recently been shown to mark senescent T cells in mice [24]. Moreover, TIGIT$^+$Helios$^+$ cells showed a trend towards slightly higher levels of CD57 and killer cell lectin-like receptor G1 (KLRG1), which are markers that have previously been associated with cellular senescence of T cells [25,26] (**Figure 2D, E**).

Overall, these findings show that Helios refines the description of immunosenescence among the previously reported TIGIT$^+$ population that increases with age [13], as Helios more accurately defines immunosenescent cells among the TIGIT$^+$ CD8$^+$ T-cell population. Therefore, co-expression of TIGIT and Helios can be used to accurately define functionally senescent CD8$^+$ T cells.



**Late-differentiated CD8$^+$ T cells accumulate with age at the cost of early-differentiated T cells**

CD8$^+$ T cells downregulate CD27 and CD28 expression during aging by conversion from CD27$^+$CD28$^+$ early-differentiated cells to CD27$^+$CD28$^-$ intermediate-differentiated cells and, finally, into CD27$^-$CD28$^-$ late-differentiated T cells leading to accumulation of the latter subset during aging [8,9,12,27]. Accumulation of CD27$^-$CD28$^-$ cells has often been ascribed to latent infection by cytomegalovirus (CMV) as a result of repetitive T-cell stimulation [9,28,29]. However, we hypothesized that such T-cell differentiation would be a more general phenomenon and also occurs in the absence of CMV infection. We therefore assessed the association of differentiation status of CD8$^+$ T cells based on expression of CD27 and CD28 with age in PBMC of CMV seronegative individuals (n=50, 21-82 years). We found that early-differentiated cells were most abundant amongst CD8$^+$ T cells of these donors, compared to the presence of intermediate- and late-differentiated cells (**Figure 3A, B**). In relationship to age, the proportion of late-differentiated cells increased, whereas the proportion of early-differentiated cells declined (**Figure 3C**)**.** The proportion of intermediate-differentiated cells did not change with age. These findings show that late-differentiated cells accumulate with age at the expense of early-differentiated cells and indicate that such accumulation does not depend on CMV.

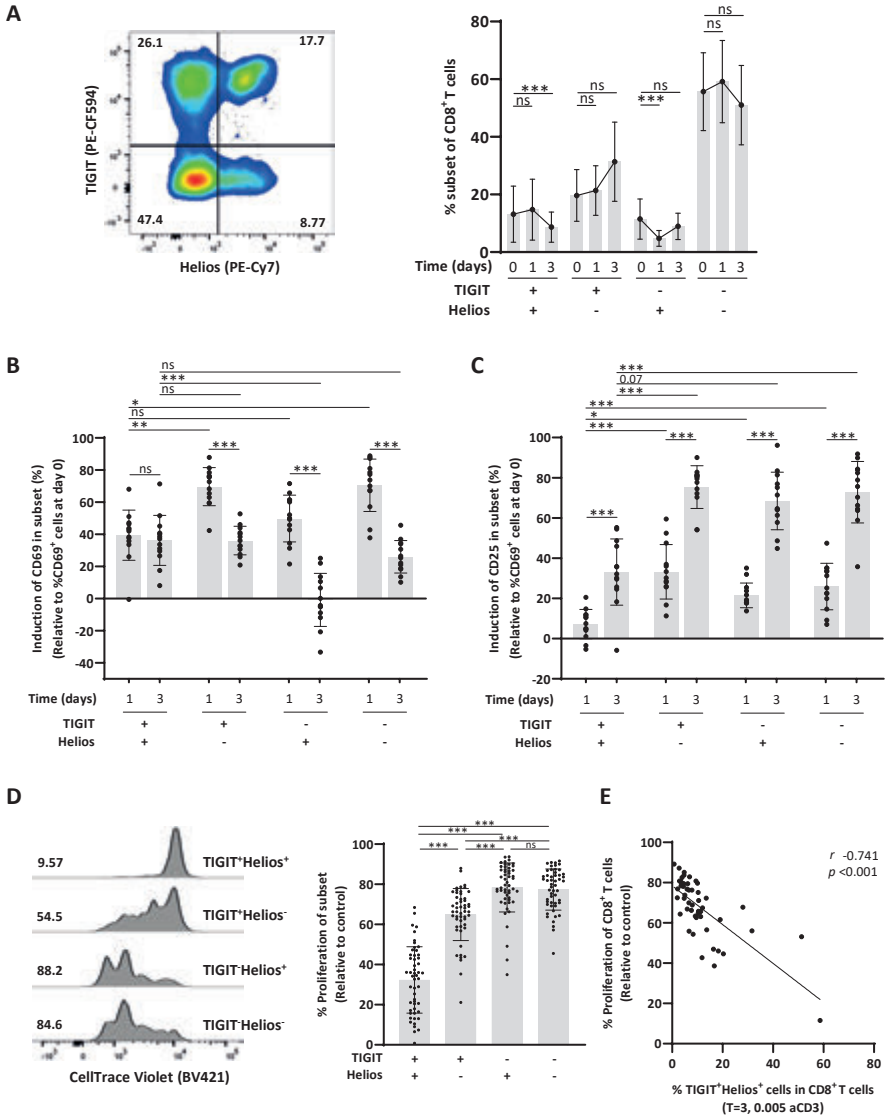


**Figure 1. Co-expression of TIGIT and Helios defines functionally senescent T cells**

(**A**) Representative flow-cytometry plot with expression of TIGIT and Helios by CD8⁺ T cells and the frequency of TIGIT/Helios cell subsets among CD8⁺ T cells over time after stimulation with anti-CD3 (day zero, one, and three) are shown. Induction of T-cell activation was assessed by measuring the frequency of (**B**) CD69⁺ cells and (**C**) CD25⁺ cells over time within the indicated TIGIT/Helios cell subsets relative to the frequency of CD69⁺ and CD25⁺ cells determined at day zero (% CD69⁺ or CD25⁺ cells at indicated day minus their % at day 0) (n=13). T-cell proliferation was assessed by dilution of CellTrace after three days of stimulation with anti-CD3. (**D**) Representative histogram shows the proliferation of the indicated TIGIT/Helios cell subsets by dilution of CellTrace (number indicates the frequency of proliferated cells of the subset). Bar graph shows the frequency of proliferated cells within each TIGIT/Helios cell subset

(n=50). (**E**) Relationship between the frequency of proliferated CD8⁺ T cells and the frequency of TIGIT⁺Helios⁺ cells within the CD8⁺ T-cell population after three days of stimulation. All frequencies of proliferating cells were relative to their unstimulated control sample. Correlations (*r* and *p* values) were assessed by Spearman test. Statistical significance of data presented in the bar graphs (means ± s.d.) was determined using Friedman test (with Dunn's post-test). Wilcoxon test was used in panels **B** and **C** for the comparison between the time points within each subset. (**p* <0.05, ***p* <0.01, ****p* <0.001, ns=not significant).

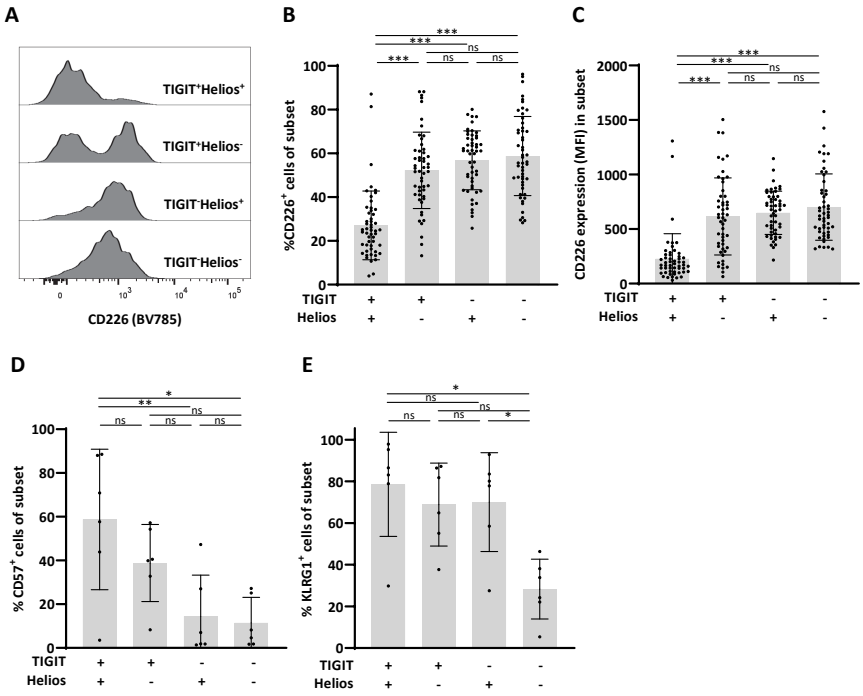


**Figure 2. Low expression of the co-stimulatory receptor CD226 by immunosenescent CD8⁺ T cells.**

(**A**) Representative histogram of CD226 expression within each of the TIGIT/Helios cell subsets. Bar graphs show (**B**) the frequency of CD226⁺ cells and (**C**) expression (median fluorescent intensity, MFI) of CD226 per cell within each of the four TIGIT/Helios cell subsets (n=50). Bar graphs show the frequency of (**D**) CD57⁺ and (**E**) KLRG1⁺ cells within each of the four TIGIT/Helios cell subsets (n=6). Statistical significance of data presented in the bar graphs (means ± s.d.) was determined using Friedman test (with Dunn's post-test). (**p* <0.05, ***p* <0.01, ****p* <0.001, ns=not significant).

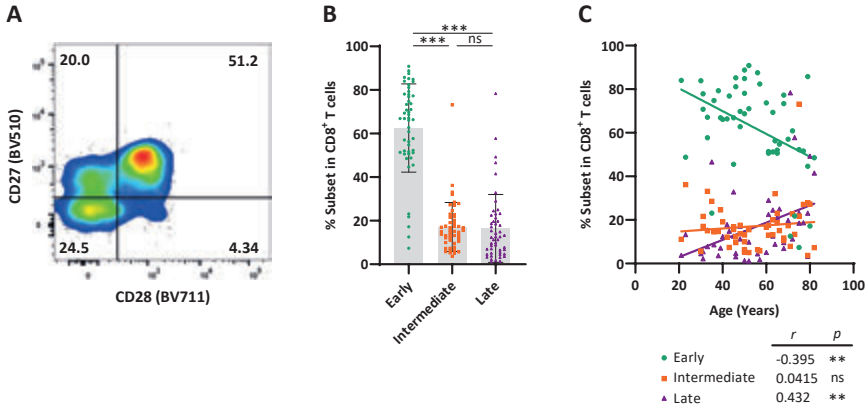


**Figure 3. Late-differentiated T cells accumulate with age at the expense of early-differentiated T cells.**

Expression of CD27 and CD28 by CD8⁺ T cells was analyzed in healthy individuals ranging from 21-82 years of age (n=50) using flow cytometry. (**A**) Representative flow-cytometry plot illustrating expression of CD27 and CD28 by CD8⁺ T cells. (**B**) Bar graphs show the frequency of early ($CD27^+CD28^+$), intermediate ($CD27^+CD28^-$), and late ($CD27^-CD28^-$) differentiated cells within the total CD8⁺ T-cell population of each individual. (**C**) Frequency of each of the three differentiation subsets among CD8⁺ T cells and their relationship with age. Correlations (*r* and *p* values) were assessed by Spearman test. Statistical significance of data presented in the bar graph (means ± s.d.) was determined using Friedman test (with Dunn's post-test). (**$p$ <0.01, ***$p$ <0.001, ns=not significant).

## TIGIT⁺Helios⁺ cells are enriched in the intermediate- and late-differentiated CD8⁺ T-cell populations

We next explored how expression of TIGIT and Helios relates to age and to the differentiation status of CD8⁺ T cells. Clustering of CD8⁺ T cells based on their expression of CD27, CD28, TIGIT, and Helios by viSNE analysis indicated that only part of the CD8⁺ T-cell pool co-expresses TIGIT and Helios and that expression of these molecules may relate to distinctive expression of the differentiation markers CD27 and CD28 (**Supplementary Figure 2A**). The proportion of TIGIT⁺ cells amongst CD8⁺ T cells increased at older age, whereas the proportion of Helios⁺ cells did not considerably change with age (**Figure 4A**). Interestingly, the subset of CD8⁺ T cells that co-expressed TIGIT and Helios increased with age (**Figure 4A**). Notably, these TIGIT⁺Helios⁺ cells appeared to be present mostly in the intermediate- and late-differentiated cell subsets (**Figure 4B**)**.** These findings indicate that co-expression of TIGIT and Helios defines an aging-related population of cells that accumulate in the intermediate- and late-differentiated CD8⁺ T-cell subsets.

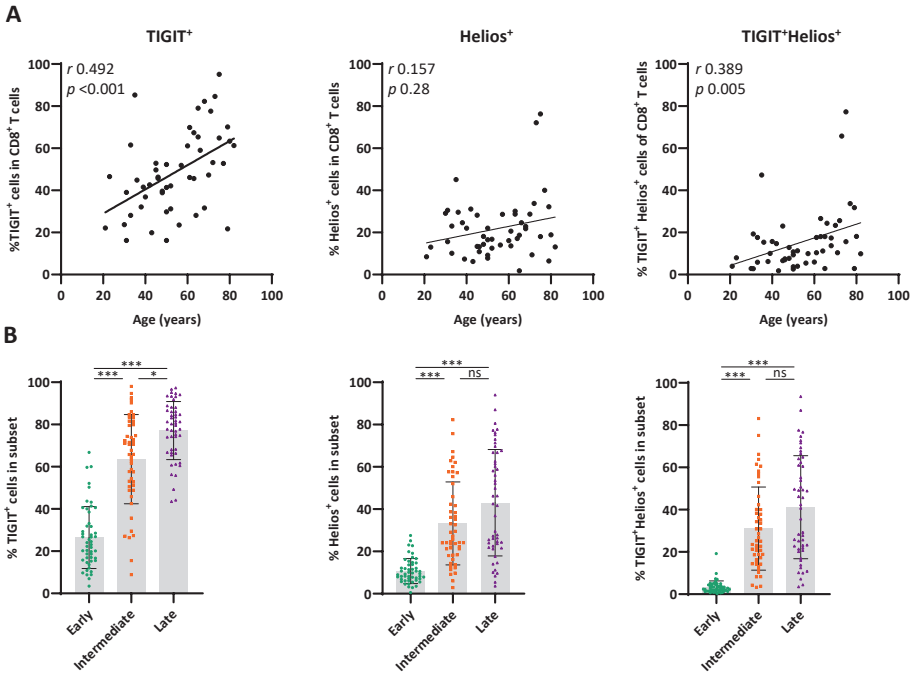


**Figure 4. TIGIT⁺Helios⁺ cells are enriched in the intermediate- and late-differentiated CD8⁺ T-cell populations.**

(**A**) Relationship between the frequency of TIGIT⁺, Helios⁺, or TIGIT⁺Helios⁺ cells within the CD8⁺ T-cell population and age. (**B**) Bar graphs show the frequency of TIGIT⁺, Helios⁺, and TIGIT⁺Helios⁺ cells within the early-, intermediate-, and late-differentiated cell subsets. Correlations (*r* and *p* values) were assessed by Spearman test. Statistical significance of data presented in the bar graph (means ± s.d.) was determined using Friedman test (with Dunn's post-test). (*$p$ <0.05, ***$p$ <0.001, ns=not significant).

## Progression of CD8⁺ T-cell senescence (TIGIT⁺Helios⁺) during aging occurs in the intermediate phase of differentiation

We next assessed whether expression of TIGIT and Helios within early-, intermediate-, and late-differentiated CD8⁺ T-cell subsets changes at older age. The frequency of TIGIT⁺ cells amongst CD8⁺ T cells significantly increases with age in both the early- and intermediate-differentiated cell subsets (**Figure 5A**). The proportion of Helios⁺ cells (irrespective of their TIGIT expression) amongst CD8⁺ T cells showed an increase with age specifically within the intermediate-differentiated cell subset (**Figure 5B**). Interestingly, the frequency of TIGIT⁺Helios⁺ in the CD8⁺ T-cell population increased within the early- and intermediate-differentiated cells subsets with age, but not within the late-differentiated subset (**Figure 5C**). These findings show that a significant fraction

of immunosenescent cells as defined by TIGIT⁺Helios⁺ already accumulates in the intermediate-differentiated CD8⁺ T-cell population at older age and that these cells are not only present in the late-differentiated stage. Moreover, despite age-related decline of the fraction of early-differentiated cells (**Figure 3C**), the minor fraction of TIGIT⁺Helios⁺ cells among the early-differentiated population increases with age (**Figure 5C**). These findings suggest that CD8⁺ T cells develop into immunosenescent cells during the course of aging already before reaching the late state of differentiation.

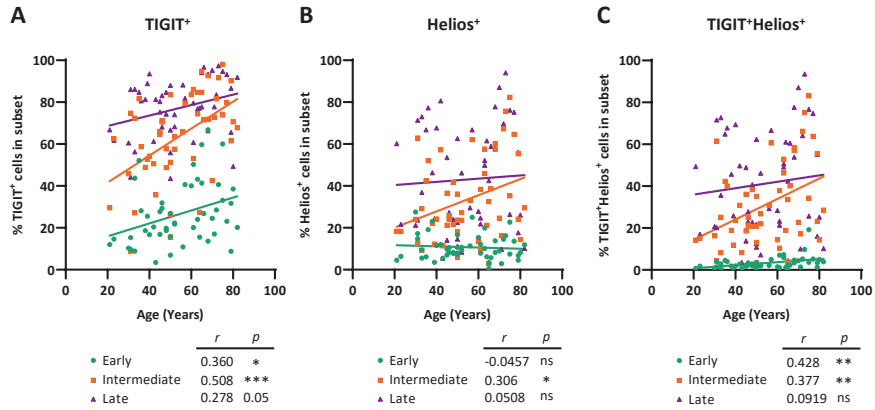


**Figure 5. Progression of CD8⁺ T-cell senescence (TIGIT⁺Helios⁺) during aging occurs in the intermediate phase of differentiation.**
Frequency of (**A**) TIGIT⁺, (**B**) Helios⁺, and (**C**) TIGIT⁺Helios⁺ cells within the early-, intermediate-, and late-differentiated cell subsets and their relationship with age (n=50). Correlations (*r* and *p* values) were assessed by Spearman test with $p < 0.05$ considered as statistically significant. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ns=not significant).

### TIGIT⁺Helios⁺ intermediate- and TIGIT⁺Helios⁺ late-differentiated cells accumulate by aging within the CD8⁺ T-cell population

Lastly, we addressed whether early-, intermediate-, and late-differentiated cells expressing TIGIT and/or Helios increase by aging within the CD8⁺ T-cell population. The frequency of TIGIT⁺ late-differentiated cells amongst CD8⁺ T cells increased with age, and also the TIGIT⁺ intermediate-differentiated cells amongst the CD8⁺ T-cell population showed a trend towards higher levels with age (**Figure 6A**). The frequency of Helios⁺ early- and Helios⁺ late-differentiated cells amongst total CD8⁺ T cells significantly declined and increased, respectively (**Figure 6B**). Interestingly, the frequency TIGIT⁺Helios⁺ intermediate- and late-differentiated cells amongst total CD8⁺ T cells increased with age (**Figure 5C**)

and indicate that TIGIT⁺Helios⁺ T cells accumulate in older adults not only as late-differentiated cells but also as intermediate-differentiated cells.

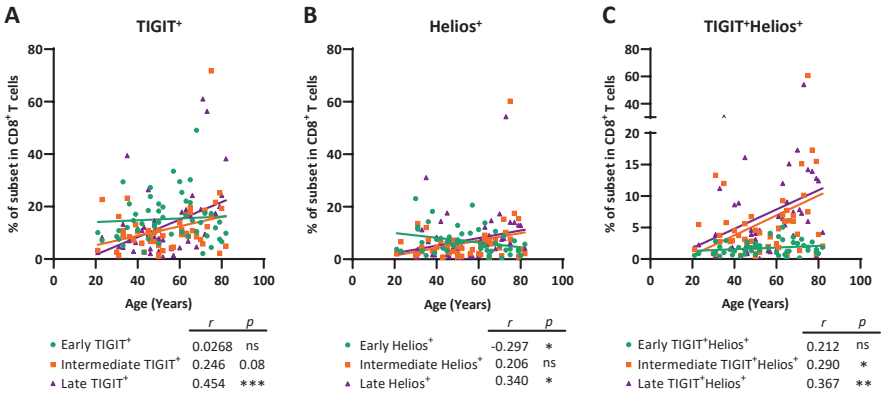


**Figure 6. TIGIT⁺Helios⁺ intermediate- and TIGIT⁺Helios⁺ late-differentiated cells accumulate by aging within the CD8⁺ T-cell population.**

Frequency of (**A**) early-, intermediate-, or late-differentiated TIGIT⁺ cells, (**B**) early-, intermediate-, or late-differentiated Helios⁺ cells, (**C**) early-, intermediate-, or late-differentiated TIGIT⁺Helios⁺ cells within the CD8⁺ T-cell population and their relationship with age (n=50). Correlations (*r* and *p* values) were assessed by Spearman test with $p < 0.05$ considered as statistically significant. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ns=not significant).

## DISCUSSION

The accumulation of late-differentiated CD8⁺ T cells at older age is a widely used marker of immunological aging due to features of immunosenescence [10,12]. We here identified co-expression of TIGIT and Helios as an accurate hallmark of immunosenescent CD8⁺ T cells. We show that these immunosenescent cells accumulate with age not only by the increase of the late-differentiated population, but also by aging-related development of immunosenescence already within earlier stages of differentiation. Therefore, accumulation of functionally immunosenescent cells at old age is partly independent of the final progression into late stage of differentiation. These findings indicate that aging-related development of immunosenescence should be analyzed by hallmarks of immunosenescence that complement classic analyses of late differentiation state using markers such as CD27 and CD28.

TIGIT has recently been identified as a marker for immunosenescent CD8⁺ T cells that accumulate at older age [13-15]. By including Helios in our analyses, we found that the TIGIT⁺ T-cell population is heterogeneous and that only a part of the TIGIT⁺ T cells co-expressed Helios. Particularly the TIGIT⁺Helios⁺ T-cell subset

showed features of immunosenescence identified by low capacity to proliferate and become activated, low expression of the co-stimulatory receptor CD226, and a trend towards higher frequencies of senescence-associated markers CD57 and KLRG1. These findings indicate that Helios more accurately defines immunosenescent cells among the TIGIT$^+$ CD8$^+$ T-cell population reported on previously [13]. Including analysis of Helios expression may therefore advance future studies on TIGIT$^+$ CD8$^+$ T cells in immunosenescence. Our finding that predominantly the Helios$^+$ subset of TIGIT$^+$ cells comprises the immunosenescent cells also implies that interventions to overcome immunosenescence by targeting the co-inhibitory receptor TIGIT [14,30] may be refined by particularly targeting this most immunosenescent subset rather than all TIGIT$^+$ CD8$^+$ T cells. Moreover, our findings warrant exploration of TIGIT$^+$Helios$^+$ CD8$^+$ T cells with respect to other known markers of aging and immunosenescence in CD8$^+$ T cells, such as NK cell-related receptors, low capacity to produce IL-2, and low telomerase expression [8,31].

We found that the proportion of late-differentiated T cells rises with age and is highly enriched with immunosenescent cells as defined by TIGIT$^+$Helios$^+$. This indicates that the CD8$^+$ T-cell pool becomes enriched with these immunosenescent cells over the years by accumulation of cells with a late-differentiated phenotype that express immunosenescent features. Previous work has reported that CD8$^+$ T cells follow a linear differentiation pathway from early- to late-differentiated state through the intermediate- differentiated phase [9]. Interestingly, although the proportion of cells in the intermediate-differentiation state did not alter with age, we found that this intermediate CD8$^+$ T-cell population becomes enriched with immunosenescent TIGIT$^+$Helios$^+$ cells at older age. Moreover, the proportion of TIGIT$^+$Helios$^+$ intermediate-differentiated cells among the CD8$^+$ T-cell population increased by aging. These findings indicate that CD8$^+$ T cells may become immunosenescent before entering the late-differentiation state and that aging-related increase of immunosenescence cannot merely be classified by accumulation of highly differentiated CD27$^-$CD28$^-$ cells. Interestingly, it has been shown that elevated numbers of intermediate-differentiated CD8$^+$ T cells is a predictor of frailty [32], emphasizing the significance of this subset during aging. The applicability of differentiation state based on CD27 and CD28 expression as proxy for immunosenescence may thus depend on age. Our findings suggest that especially at older age, part of the intermediate-differentiated population may be regarded immunosenescent in addition to late-differentiated T cells. Hence, this indicates that differentiation and development of immunosenescence with age do not fully progress in parallel and warrants further research on factors that drive these two processes.

Loss of CD27 and CD28 expression and concomitant gain of senescence features by $CD8^+$ T cells has often been linked to CMV seropositivity [9]. Our results show that $CD8^+$ T cells significantly lose expression of CD27 and CD28 and gain features of immunosenescence over the years in CMV seronegative individuals, indicating that expansion of highly differentiated T cells is not solely due to CMV. However, since latent CMV infection can promote differentiation of T cells it would be interesting to explore to what extent such induction of differentiation contributes also to the induction of immunosenescent $TIGIT^+Helios^+$ at old age.

A recent study suggested that Helios may be part of immunosenescence in T cells [18] and our data now show that Helios expression clearly marks immunosenescence in $CD8^+$ T cells along with co-expression of TIGIT. How Helios may contribute to altered function of $CD8^+$ T cells at old age and how it becomes induced in the process of aging is unclear. Helios is known as a transcription factor that is expressed by regulatory T cells and needed for their suppressive function [16,17]. Since immunosenescent T cells have gained several features known from regulatory T cells such as Helios and TIGIT, it is tempting to hypothesize that altered functions of a T cell gained at old age may be represented by gain of immunosuppressive functions. Targeting Helios may be a way to investigate the importance of Helios expression in functionality of these $TIGIT^+Helios^+$ $CD8^+$ T cells. As Helios is an intracellular transcription factor which impedes sorting of $TIGIT^+Helios^+$ T cells, targeting Helios in future studies with small molecule inhibitors or protein mimetics would be an alternative way to study the role of Helios expression. Additionally, it would be helpful to find markers on the cell surface that may either specifically result from Helios activity or be a specific inducer of Helios.

Together, our study indicates that co-expression of TIGIT and Helios provides a novel functional marker for immunosenescent $CD8^+$ T cells. Moreover, we show that these cells already accumulate in the intermediate-differentiation stage in older individuals. These findings challenge the current dogma of late-differentiation stage as proxy for T-cell immunosenescence by showing that immunosenescent T cells accumulate also within earlier differentiation stages. These insights may refine future interpretation of studies on impaired $CD8^+$ T-cell mediated immunity to viral infections at old age.

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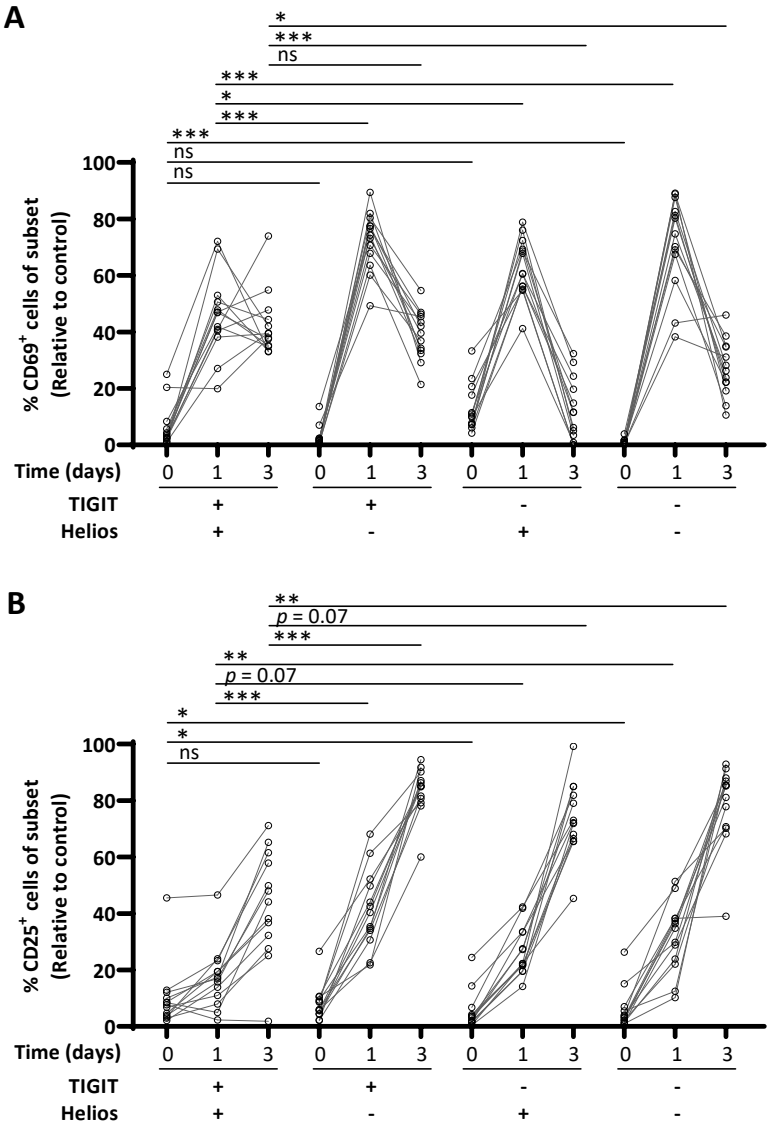
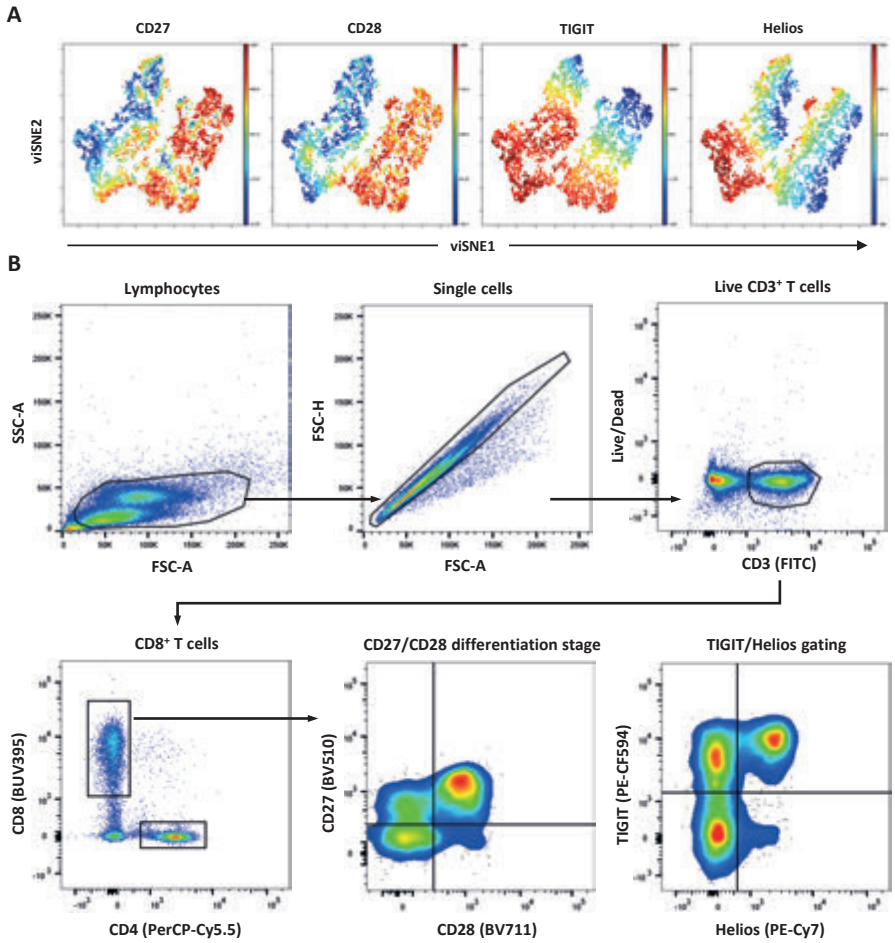
## SUPPLEMENTARY FIGURES




**Supplementary Figure 1. Kinetics of activation (CD69 and CD25) in TIGIT/Helios cell subsets.** Total PBMCs were cultured in the presence of anti-CD3 for one or three days. Frequencies of (**A**) $CD69^+$ cells and (**B**) $CD25^+$ cells within the indicated TIGIT/Helios cell subsets (n=13) were determined before (day zero) and after stimulation (day one and three). For day one and three after stimulation, frequencies are depicted as relative to their unstimulated control sample. Statistical significance of data presented in the line graphs was determined using Friedman test (with Dunn's post-test). (*p <0.05, **p <0.01, ***p <0.001, ns=not significant).




**Supplementary Figure 2. Gating strategy towards CD8⁺ T cells and their separation into TIGIT/Helios subsets and CD27/CD28 subsets.**

(**A**) Fingerprint color dot plots generated by viSNE indicate expression of each of the indicated markers within the CD8⁺ T-cell population of a representative donor as measured by flow cytometry. (**B**) Gating strategy towards stratification of CD8⁺ T cells into TIGIT/Helios cell or CD27/CD28 subsets as performed in healthy individuals using flow cytometry. CD8⁺ T cells identified by gating of total lymphocytes, single cells, and live CD3⁺ T cells. Additionally, CD8⁺ T cells were gated into early ($CD27^+CD28^+$), intermediate ($CD27^+CD28^-$), and late-differentiated ($CD27^-CD28^-$) cells based on their expression of CD27 and CD28. Total CD8⁺ T cells as well as CD8⁺ T cells in one of the three differentiation stages were also separated into TIGIT/Helios cell subsets.


5

# REGULATORY KIR$^{+}$RA$^{+}$ CD8$^{+}$ T CELLS ACCUMULATE WITH AGE AND MAY AGGRAVATE VIRAL RESPIRATORY DISEASE IN OLDER ADULTS

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**Manuscript under review**

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## ABSTRACT

Aging results in decreased $CD8^+$ T-cell responses leading to increased risk and duration of respiratory infections in the elderly. The great phenotypical diversity of the $CD8^+$ T-cell population has made it difficult to identify the impact of aging on $CD8^+$ T-cell subsets associated with diminished $CD8^+$ T-cell responses.

Here we identify a novel human $CD8^+$ T-cell subset characterized by expression of Killer-cell Immunoglobulin-like Receptors ($KIR^+$) and CD45RA ($RA^+$). These $KIR^+RA^+$ T cells accumulated with age in the blood of healthy individuals (20-82 years of age, n=50), expressed high levels of the age-related exhaustion marker TIGIT, and were functionally capable of suppressing proliferation of other $CD8^+$ T cells. Moreover, $KIR^+RA^+$ T cells were found highly activated in older adults suffering from an acute respiratory viral infection (n=36), including coronavirus and influenza-virus infection. Older adults with influenza-A infection showed that the activation status of the $KIR^+RA^+$ T cells positively correlated with duration of symptoms in (n=15, $r$=0.6, $p$=0.03).

Together, our data indicate that regulatory $KIR^+RA^+$ T cells are a previously unrecognized hallmark of aging that may aggravate disease during respiratory viral infections in older adults.

## INTRODUCTION

Aging dramatically increases the risk for developing prolonged and more severe disease after acute viral respiratory infections [1-5]. This has been shown for viruses such as influenza virus, respiratory syncytial virus (RSV), and coronaviruses [1,2,5,6]. Moreover, susceptibility of older adults for respiratory infections is currently being highlighted by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic [7,8]. The risk of severe disease is in part explained by decreasing functionality of $CD8^+$ T cells during the process of aging [9-12] resulting in impaired viral clearance [13-16]. To better understand the role of the impaired $CD8^+$ T-cell response during viral infection in the development of prolonged and severe disease in older adults, it is important to identify $CD8^+$ T-cell subsets that contribute to the decline of T-cell mediated responses.

The decline of $CD8^+$ T-cell-mediated immune responses with age is in part associated with changes in $CD8^+$ T-cell phenotype and defects in proliferation, often referred to as T-cell exhaustion and/or senescence (reviewed in [17,18]). T-cell exhaustion is characterized by increased expression of inhibitory receptors by $CD8^+$ T cells [19]. In humans, expression of the co-inhibitory receptor T-cell immunoglobulin and ITIM domain (TIGIT) by $CD8^+$ T cells has been shown to increase with age and is associated with diminished $CD8^+$ T-cell responses in the elderly [20]. Recent work demonstrated that effector memory ($T_{EM}$) and terminally differentiated effector cells ($T_{EMRA}$) were the most predominant $CD8^+$ T-cell subsets expressing TIGIT during aging [20].

Recently, so-called "virtual memory cells" have been identified in mice and humans [21-23] as a new $CD8^+$ T-cell subset that accumulates with age [22-24]. It is currently assumed that virtual memory cells are antigen-naïve T cells that acquire a memory phenotype through homeostatic proliferation [22,25,26] and may act as bystander cells during infection [22,26]. In aged mice, virtual memory cells express a senescent phenotype with diminished capacity to participate in primary immune responses [23]. In humans, virtual memory $CD8^+$ T cells are $CD45RA^+$ cells that have been defined by expression of killer-cell immunoglobulin-like receptors (KIR) and/or the NK-cell receptor NKG2A. However, further phenotypical and functional characterization as well as the clinical relevance of these cells in humans is currently unknown. We therefore aimed to address the significance of $KIR^+$ and/or $NKG2A^+$ $CD45RA^+$ $CD8^+$ T cells in aging by defining their phenotype and functionality, and their relevance for respiratory viral infections in older adults.

Here we identified a novel human $CD8^+$ T-cell subset that is characterized by expression of KIR and CD45RA, and the lack of NKG2A. We provide evidence that these "$KIR^+RA^+$ $CD8^+$ T cells" ($KIR^+$ $CD45RA^+$ $NKG2A^-$ $CD8^+$ T cells) accumulate with age and are therefore a previously unrecognized hallmark of aging. In contrast to previously described virtual memory cells, we show that $KIR^+RA^+$ and $NKG2A^+RA^+$ $CD8^+$ T cells are distinct subsets. $KIR^+RA^+$ T cells express high levels of the age-related exhaustion marker TIGIT, and appear to be a regulatory $CD8^+$ T-cell subset as we observed that these cells suppress the proliferation of other $CD8^+$ T cells. Importantly, we show that $KIR^+RA^+$ T cells are activated during respiratory disease caused by viruses such as influenza viruses and coronaviruses, including SARS-CoV-2. Our data collectively show that these $KIR^+RA^+$ cells are a major activated T-cell population in older adults suffering from respiratory viral infection and provide evidence that activation of this T-cell subset is a correlate of prolonged respiratory disease in influenza A virus infected older adults.

## MATERIALS AND METHODS

### Study design

*Older adults with Influenza-Like-Illness and matched controls*

Part of the samples investigated in the current study were embedded in a trial that monitored Influenza-Like-Illness (ILI) in community-dwelling older adults (ILI cohort; Netherlands Trial Register NTR4818) ([27], Van Kaaijk *et al.* submitted). The study was performed according to Good Clinical Practice, the Declaration of Helsinki. The study was approved by the ethical committee METC Noord-Holland and written informed consent was obtained from all participants. There were no exclusion criteria for this study. Participants were 60 years and older, and were instructed to report ILI-associated symptoms according to the Dutch Pel criteria [28] (fever > 37.8 °C with at least coughing, myalgia, nasal congestion, sore throat, difficulty breathing, or headache) as soon as possible after the symptoms started. Blood samples were drawn within 72 hours after the symptom report (acute phase of infection), as well as approximately two and eight weeks after the initial report. The presence or absence of ILI-associated symptoms were monitored during each visit. Nasopharyngeal and oropharyngeal swabs were taken at all three visits to identify the pathogen causing the ILI symptoms by qPCR as described below. Symptomatic participants reported on in this study are a subset of the ILI cohort. The selection was based on the presence of the type of respiratory virus as a single infection at the acute phase of infection and absence of all ILI-causing pathogens at two and eight weeks

after the initial report. Respiratory viruses included in our selection were real time PCR-based Multiplex Ligation-dependent Probe Amplification (MPLA)-confirmed cases of Influenza A virus (n=15), Influenza B virus (n=5), Respiratory Syncytial Virus (RSV) (n=2), Human Metapneumovirus (hMPV) (n=4), seasonal Coronavirus (n=5), or Rhinovirus (n=5) infection (RespiFinder® Smart 22 kit, Pathofinder) (**Supplementary Table 1**). Of these participants, the self-reported occurrence and duration of ILI-associated symptoms were used to calculate an overall symptom score by totaling the duration of all symptoms per individual divided by the number of symptoms of the individual. Participants who were asymptomatic and laboratory-confirmed respiratory infection negative were sampled and used as healthy controls in this ILI-cohort.

*Healthy individuals*

Blood samples obtained from healthy (asymptomatic) individuals (n=50, 21-82 years) investigated in the current study were either derived from the aforementioned ILI-cohort, or from healthy blood donors (Sanquin Blood Supply Foundation) or from healthy participants of a trial performed at the RIVM (Netherlands Trial Register NTR2070) [29]. Overall, the samples investigated in this study were relatively evenly distributed over age and sex (**Supplementary Table 2**). All healthy and symptomatic participants from the aforementioned cohorts that were included in this study were tested for cytomegalovirus (CMV) antibodies by an in-house Multiplex Immunoassay [30]. Only participants who tested negative were included in the study, to prevent bias towards CMV-induced T-cell alterations.

*SARS-CoV-2 infected adults with COVID-19*

With the recent COVID-19 pandemic caused by the novel severe acute respiratory syndrome corona virus 2 (SARS-CoV-2), we expanded our analyses of $KIR^+RA^+$ T cells by investigating the presence and phenotype of these cells in the blood of adults with COVID-19. Whole blood of PCR-confirmed SARS-CoV-2 infected participants (32-52 years of age, n=9) and that of asymptomatic household members who tested negative for SARS-CoV2 (indicated as healthy controls) (16-51 years of age, n=9) was obtained as part of the First Few X (FFX) cases of SARS-CoV-2 infection in the Netherlands (**Supplementary Table 3**) [31]. Blood samples were drawn within the first two weeks after COVID-19-like symptoms were reported (acute phase of infection), as well as two and four weeks thereafter. The study was approved by the Utrecht ethical committee (METC Utrecht; NL13529.041.06).

**PBMC isolation and flow cytometry**

Peripheral Blood Mononuclear cells (PBMCs) were isolated by density gradient (Ficoll-Hypaque, Amersham Biosciences) from heparinized blood or buffy coats and stored at -135 °C in 10% dimethyl sulfoxide (DMSO, Sigma Aldrich) and 10% fetal calf serum (FCS) until further use. For *ex vivo* flow cytometric analyses, frozen PBMCs were thawed at 37°C and were transferred to RPMI-1640 medium (GIBCO, Thermo Fisher Scientific) supplemented with 10% FCS and Penicillin-Streptomycin-Glutamine (P/S/G) and washed twice. Thawed cells were then rested in medium for 30 minutes at room temperature after which the number of viable cells was determined on a Coulter Counter (Beckman). For *ex vivo* analyses on all healthy individuals, $4*10^5$ PBMCs of each individual were labeled for surface markers at 4°C for 30 minutes with saturating concentrations of antibodies targeting: CD3, CD4, CD8, CD27, CD45RA, CD122, CD226, KIR2D, KIR3DL1, NKG2A, and TIGIT (**listed in Supplementary Table 4**) in FACS buffer (1x PBS +0.5% BSA +2mM EDTA). A Fixable Viability Stain 780 (BD Horizon) was added to the labeling to identify viable cells. Cells were then fixed and permeabilized with buffers for subsequent intracellular labeling (eBioscience) at 4°C for 30 minutes with an antibody targeting Helios (**Supplementary Table 4**). For *ex vivo* analyses on symptomatic individuals and their asymptomatic/healthy controls, $2\text{-}6*10^6$ PBMCs were first labeled with an HLA-A2 GILG-dextramer (A*0201/GILGFVFTL dextramer, FITC-conjugated, Immundex) for 20 minutes at room temperature. Cells were then labeled for surface markers at 4 °C for 30 minutes with saturating concentrations of antibodies targeting: CD3, CD4, CD8, CD27, CD45RA, CD69, KIR2D, KIR3DL1, NKG2A, and TIGIT in FACS buffer, before intracellular labeling of Helios as described above (**listed in Supplementary Table 4**). A Fixable Viability Stain 780 (BD Horizon) was used to identify viable cells. Samples were measured on an LSRFortessa™ X-20 (BD Biosciences) and data were analyzed using FlowJo software (v10.6.1, TreeStar).

**T-cell culture assays**

*Cell sorting for T-cell cultures*

For all culture assays we used PBMC that were collected from healthy blood donors (Sanquin Blood Supply Foundation) and thawed upon storage at -135 degrees Celsius. The number of viable cells in thawed PBMC samples was determined manually using trypan blue staining and Bürker-Türk. For T-cell suppression and T-cell proliferation/activation assays, (part of) the cells were washed with 1xPBS and labelled with 0.5 µM CellTrace™ Violet (Invitrogen) in 1x PBS per milliliter of cell suspension ($10^6$ cells/mL) for 20 minutes at 37 °C to

track their proliferation. Ice-cold RPMI-1640 medium (+10% FCS, +P/S/G) was added and cells were rested at room temperature for 5 minutes. Cells were centrifuged at 400*g* for 5 minutes and washed with RPMI-1640 medium (+10% FCS, +P/S/G) three times. Cells were then labeled for surface markers at 4 °C for 30 minutes with saturating concentrations of antibodies targeting: CD4, CD8, CD45RA, CD56, KIR2D, KIR3DL1, and NKG2A (**listed in Supplementary Table 4**), before suspension in FACS buffer +25mM HEPES. Cell sorting was performed using the FACSMelody™ (BD Biosciences). $CD4^-CD56^-CD8^+$ single cells were sorted into the following subsets: $KIR^+RA^+$ T cells ($CD8^+CD45RA^+KIR^+NKG2A^-$), $NKG2A^+RA^+$ T cells ($CD8^+CD45RA^+KIR^-NKG2A^+$), and $KIR^-NKG2A^-$ T cells ($CD8^+KIR^-NKG2A^-$).

*T-cell stimulation: proliferation and activation assays*

Total CellTrace™ Violet (Invitrogen) labeled PBMCs were cultured in the presence of 0.005 µg/mL plate-bound purified mouse anti-human CD3 (Clone HIT3α, BD Biosciences) in RPMI-1640 medium (+10% FCS, +P/S/G) in U-bottom plates ($2*10^5$ cells/well). FACS-sorted and CellTrace™ Violet-labeled $KIR^+RA^+$, $NKG2A^+RA^+$ and $KIR^-NKG2A^-$ T-cell subsets were cultured in the presence of anti-CD3/CD28-coupled beads (Dynabeads™, Gibco) at a 1:12 bead/cell ratio in RPMI-1640 medium in U-bottom plates for three days. After three days of culturing, cells were first labeled for surface markers at 4 °C for 30 minutes with saturating concentrations of a combination of antibodies targeting: CD4, CD8, CD45RA, CD69, KIR2D, KIR3DL1, NKG2A, and TIGIT in FACS buffer, before intracellular labeling of CD3 and Helios (**listed in Supplementary Table 4**). A Fixable Viability Stain 780 (BD Horizon) was used to identify viable cells. Samples were measured on an LSRFortessa™ X-20 (BD Biosciences) and data were analyzed using FlowJo software (v10.6.1, TreeStar).

*T-cell suppression assays*

Suppression assays were performed by culturing FACS-sorted and CellTrace™ Violet-labeled $KIR^-NKG2A^-$ $CD8^+$ T cells (responder cells) with $KIR^+NKG2A^-$ $CD8^+$ T cells (suppressor cells) that were either CellTrace™ Violet-labeled or not. Cells were cultured together in a range of suppressor-to-responder ratios from 1:1 to 1:16 in a total amount of 10.000 cells/well. During culture, cells were stimulated with anti-CD3/CD28-coupled beads (Dynabeads™, Gibco) at a 1:12 bead/cell ratio in U-bottom plates. After three days of culturing, cells were labeled for surface markers at 4°C for 30 minutes with saturating concentrations of antibodies targeting: CD3, CD8, CD45RA, CD69, KIR2D, KIR3DL1, NKG2A, and TIGIT in FACS buffer (**listed in Supplementary Table 4**). A Fixable Viability Stain

780 (BD Horizon) was used to identify viable cells. Samples were measured on an LSRFortessa™ X-20 (BD Biosciences) and acquired flow cytometry data were analyzed using FlowJo software (TreeStar). Suppression of responder proliferation by suppressor cells was calculated as follows: ((% proliferation responder only - % proliferation with suppressor cells) / (% proliferation responder only))*100%.

**Intracellular cytokine assay**

PBMCs were added to U-bottom plates ($1*10^6$ cells/well) in RPMI-1640 medium (+10% FCS, +P/S/G) in the presence of anti-CD107a labeling (Biolegend), and exposed to phorbol-12-myristate-13-acetate (PMA, 25 ng/mL, Sigma-Aldrich) with ionomycin (250 ng/mL, Sigma-Aldrich) for 6 hours at 37 °C or to medium only as control. After two hours of incubation, Brefeldin A (1:100, Sigma-Aldrich) and Monensin (1:150, GolgiStop, BD Biosciences) were added and present during the final four-hour incubation period. After incubation, cells were labeled for surface makers for 30 minutes at 4 °C with saturating concentrations of antibodies targeting: CD8, CD27, CD45RA, KIR2D, KIR3DL1, and NKG2A in FACS buffer, followed by intracellular labeling of CD3 and the cytokines IL-2, IFN-γ, and TNF-α (**listed in Supplementary Table 4**). A Fixable Viability Stain 780 (BD Horizon) was used to identify viable cells. Samples were measured on an LSRFortessa™ X-20 (BD Biosciences) and data were analyzed using FlowJo software (v10.6.1, TreeStar).

**RNA sequencing**

*Cell sorting for RNA sequencing*

The number of viable cells in thawed PBMC samples was determined manually using trypan blue staining and Bürker-Türk as described above. Cells were labeled for surface markers at 4°C for 30 minutes with saturating concentrations of antibodies targeting: CD3, CD4, CD8, CD19, CD27, CD45RA, CD56, KIR2D, KIR3DL1, and NKG2A in FACS buffer (**listed in Supplementary Table 4**). Cells were suspended in FACS buffer +25mM HEPES. First, viable single $CD8^+$ T cells were selected as $CD56^-CD4^-CD19^-CD3^+CD8^+$ cells. These $CD8^+$ T cells were then subdivided into $T_{NAIVE}$ cells ($CD8^+CD45RA^+KIR^-NKG2A^-CD27^+$), $T_{EMRA}$ cells ($CD8^+CD45RA^+KIR^-NKG2A^-CD27^-$), $KIR^+RA^+$ T cells ($CD8^+CD45RA^+KIR^+NKG2A^-$), and $NKG2A^+RA^+$ T cells ($CD8^+CD45RA^+KIR^-NKG2A^+$), which were sorted separately into tubes containing RPMI-1640 medium (+20% FCS, +P/S/G). Cell sorting was performed using the FACSMelody™ (BD Biosciences).

*Library preparation*

FACS-sorted $T_{NAIVE}$, $T_{EMRA}$, $KIR^+RA^+$, and $NKG2A^+RA^+$ T-cell subsets of six healthy donors were centrifuged at 485*g* for 15 minutes and lysed in buffer RLT (Qiagen) containing 1% β-mercaptoethanol. Total RNA was extracted from the cell lysates by using the RNeasy kit (Qiagen) according to the manufacturer's protocol. RNA integrity was assessed by using the RNA 6000 Pico kit (Agilent Technologies) on a 2100 BioAnalyzer (Agilent Technologies). All RNA Integrity Number (RIN) scores were >7.0. cDNA synthesis and amplification was performed by using the SMART-Seq® v4 Ultra® Low Input RNA kit for sequencing (Takara) and AMPure XP beads (Beckman Coulter) were used to purify the samples. Proper size distribution of the fragments acquired was verified using the High Sensitivity DNA kit (Agilent Technologies). Next, 1 ng of cDNA was used to prepare a Nextera XT DNA library according to the manufacturer's instruction (Illumina). Libraries were subsequently validated for fragment size using QIAxcel DNA Screening Kit (Qiagen) and quantified using RT-qPCR with a KAPA Library Quantification kit (KK4824, Roche/KAPA Biosystems). 23 libraries were pooled at equimolar concentrations and sequenced using the Illumina NextSeq 500/550 High Output Kit v2.5 (single-end, 75-cycles). Basecalling and demultiplexing was performed using bcl2fastq2 Conversion Software v2.20, and demultiplexed FASTQ files which were generated based on sample-specific barcodes (>14 million reads/sample).



*RNA sequencing analysis*

We used an in-house pipeline to analyze the RNA sequencing data. First, reads were mapped to the human reference genome (GRCh38, release 12) using STAR (version 2.6.0) [32]. The number of mapped reads were counted for each gene and compiled into an expression matrix using featureCounts (version 1.6.1) [33]. The count table was used for statistical analysis and identification of Differentially expressed genes (DEG) using DESeq2 (v1.1) [34]. Genes were considered significantly different with a false discovery rate (FDR) < 0.1. The RNA-seq data discussed in this article will be deposited and made accessible in the National Center for Biotechnical Information Gene Expression Omnibus 23 upon acceptation for publication. Ingenuity Pathway Analysis (IPA, Qiagen) was used to investigate the relationships between selected pairs of T-cell subsets. Gene expression profiles of $T_{EMRA}$, $KIR^+RA^+$, and $NKG2A^+RA^+$ T-cell subsets were first separately compared to the gene expression profile of $T_{NAIVE}$ cells. These comparisons were then aligned in a heat map to identify shared and unique molecular and cellular functions of $T_{EMRA}$, $KIR^+RA^+$, and $NKG2A^+RA^+$ T cells. For this enrichment analysis, an absolute z-score of greater than 2.0, a False

Discovery Rate (FDR) of 0.10, and a -Log(*p*-value) of greater than 1.5 was used. *p*-values were calculated by Fisher's Exact Test.

**Whole blood flow cytometry in COVID-19 FFX study**

Whole blood of participants in the FFX study, investigating the pandemic of SARS-CoV-2 in the Netherlands, was obtained from adults suffering from COVID-19 with a PCR-confirmed SARS-CoV-2 infection and that of asymptomatic SARS-CoV-2-PCR-negative household members. 100 µL whole blood of each donor was labeled for surface markers at room temperature for 15 minutes with saturating concentrations of antibodies targeting: CD3, CD4, CD8, CD27, CD45, CD45RA, CD69, KIR2D, KIR3DL1, and NKG2A (**listed in Supplementary Table 4**) in Brilliant Stain Buffer (BD Biosciences). Erythrocytes were lysed with 1mL lysing solution (BD Biosciences) for 15 minutes at room temperature. To fasten measurement of stained PBMC, cell suspensions were concentrated by centrifugation at 400*g* for 5 minutes and resuspended in the 400 µL remaining after removal of 700 µL of fluid phase after centrifugation. Samples were measured on an LSRFortessa™ X-20 (BD Biosciences) and data were analyzed using FlowJo software (v10.6.1, TreeStar).

**Statistical analyses**

Statistical analysis was performed using GraphPad Prism version 8.4.1. The appropriate parametric or non-parametric tests were used based on the tested normality of distribution of the data. Two groups were compared by using Mann-Whitney U test (unpaired), or Wilcoxon Test (paired) with two-sided *p*-values. When comparing more than two groups, paired analyses were performed with either row-matched one-way ANOVA with Geisser-Greenhouse correction and Dunnett's post-test, or with Friedman Test with Dunn's post-test. Correlations between variables were analyzed using Spearman's rank correlation coefficient (*r*). Linear regression analysis was performed to generate lines of best fit. Statistical significance was considered when $p < 0.05$, with $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, and $****p < 0.0001$. All data presented in bar graphs are depicted as mean ± s.d.

## RESULTS

### KIR$^+$RA$^+$ T cells accumulate with age, whereas NKG2A$^+$RA$^+$ T cells decline with age

Virtual memory cells identified as CD45RA$^+$ CD8$^+$ T cells expressing KIR and/or NKG2A accumulate with age in PBMCs of healthy individuals (n=50) (**Figure 1A**), which is in line with previous reports [21-23]. However, the accumulation of CD45RA$^+$ CD8$^+$ T cells positive for KIR or NKG2A differed between young and aged individuals (**Figure 1B, Supplementary Figure 1A** for gating strategy). We observed an increase in the proportion of KIR$^+$ NKG2A$^-$ cells with age (**Figure 1C**), whereas the proportion of KIR$^-$ NKG2A$^+$ cells decreased with age (**Figure 1D**). CD45RA$^+$ KIR$^+$NKG2A$^-$ cells were abundantly present, on average 30%, within the classically defined terminally differentiated effector cell subset ($T_{EMRA}$) defined by CD27$^-$ and CD45RA$^+$ (**Figure 1E**). Based on these findings we hypothesize that the so-called "virtual memory cell" subset comprises two different subsets; KIR$^+$ NKG2A$^-$ CD45RA$^+$ T cells and KIR$^-$ NKG2A$^+$ CD45RA$^+$ T cells, which we will conveniently address as KIR$^+$RA$^+$ T cells and NKG2A$^+$RA$^+$ T cells, respectively.

By regarding KIR$^+$RA$^+$ T cells and NKG2A$^+$RA$^+$ T cells as separate CD8$^+$ T-cell subsets, the CD8$^+$ T-cell population can be divided into six different subsets (**Figure 1F, Supplementary Figure 1A**): naive T cells ($T_{NAIVE}$, CD45RA$^+$CD27$^+$KIR$^-$NKG2A$^-$), central memory T cells ($T_{CM}$, CD45RA$^-$CD27$^+$), effector memory T cells ($T_{EM}$, CD45RA$^-$CD27$^-$), $T_{EMRA}$ cells (CD45RA$^+$CD27$^-$KIR$^-$NKG2A$^-$), KIR$^+$RA$^+$ T cells (CD45RA$^+$KIR$^+$NKG2A$^-$), and NKG2A$^+$RA$^+$ T cells (CD45RA$^+$KIR$^-$NKG2A$^+$). Of these subsets, the proportions of $T_{NAIVE}$ and NKG2A$^+$RA$^+$ T cells decline with age, the proportion of $T_{CM}$ remains stable, whereas the $T_{EM}$, $T_{EMRA}$, and KIR$^+$RA$^+$ T cells increase with age (**Figure 1G, Supplementary Figure 1B**). Thus, different proportions of KIR$^+$RA$^+$ and NKG2A$^+$RA$^+$ T cells with progressing age may indicate that these are distinct cell subsets.

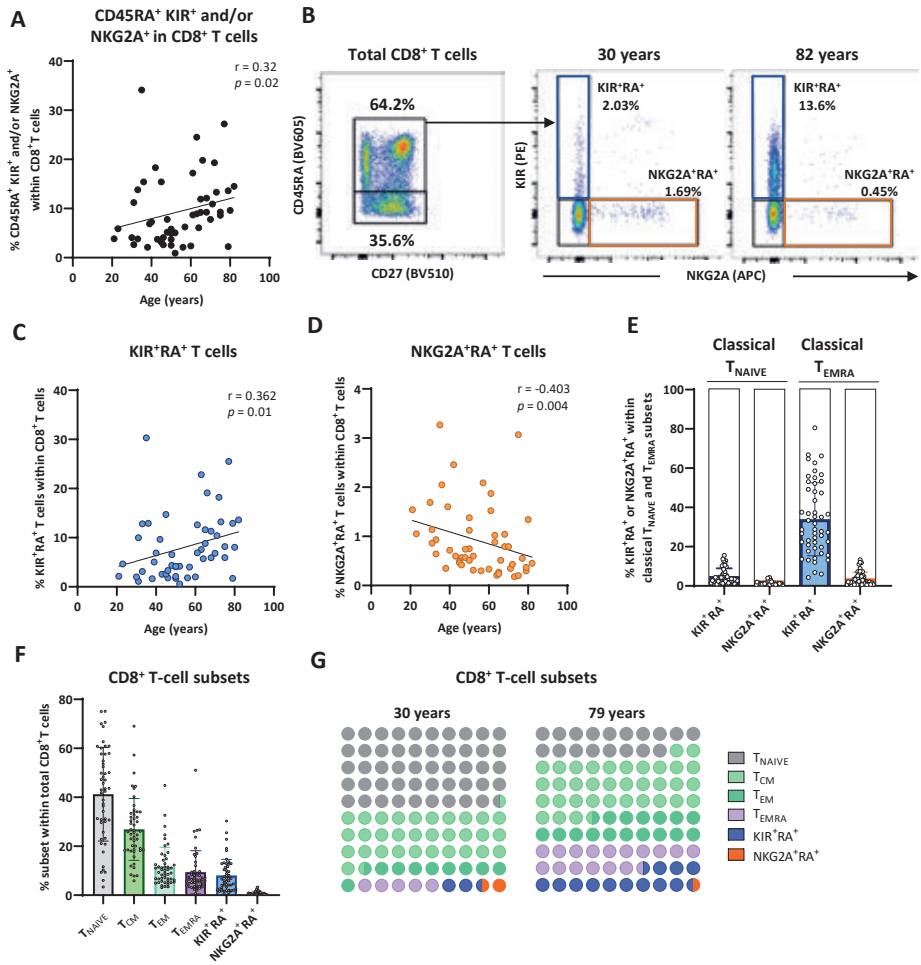


**Figure 1. KIR$^+$RA$^+$ T cells accumulate with age, whereas NKG2A$^+$RA$^+$ T cells decline with age.** KIR and NKG2A expression by CD45RA$^+$ CD8$^+$ T cells was analyzed in a cohort of healthy individuals ranging from 20-82 years of age (n=50) using flow cytometry. (**A**) Relationship between the proportion of the virtual memory cell subset (CD45RA$^+$ cells expressing KIR$^+$ and/or NKG2A$^+$) within the CD8$^+$ T-cell population and age. (**B**) Gating strategy to separate the virtual memory cell subset into KIR$^+$NKG2A$^-$ CD45RA$^+$ CD8$^+$ T cells (KIR$^+$RA$^+$ T cells, blue gate, % in total CD8$^+$ T cells) and KIR$^-$NKG2A$^+$ CD45RA$^+$ CD8$^+$ T cells (NKG2A$^+$RA$^+$ T cells, orange gate). Relationship between (**C**) KIR$^+$RA$^+$ and (**D**) NKG2A$^+$RA$^+$ cell proportions within the total CD8$^+$ T-cell population and age. (**E**) Proportions of KIR$^+$RA$^+$ and NKG2A$^+$RA$^+$ T cells within the classically defined $T_{NAIVE}$ (CD45RA$^+$CD27$^+$CD8$^+$ T cells) and $T_{EMRA}$ (CD45RA$^+$CD27$^-$CD8$^+$ T cells) subsets. (**F**) Proportions of the six CD8$^+$ T-cell subsets among the CD8$^+$ T-cell population. (**G**) The proportional change with age of each cell subset within the CD8$^+$ T-cell population of representative individuals at young and older age. Correlations (*r* values) were assessed by Spearman test. Bar graphs show means ± s.d.

**The gene expression profile of KIR$^+$RA$^+$ T cells is distinct from that of NK2A$^+$RA$^+$ T cells and shows features of aging and regulation**

To further understand differences between KIR$^+$RA$^+$ and NKG2A$^+$ T cells, we sorted these cell subsets (**Supplementary Figure 2A**) and analyzed their gene expression profiles by RNA sequencing (RNA-seq). As a reference, we included $T_{NAÏVE}$ and $T_{EMRA}$ cells in this analysis (**Supplementary Figure 2B**). Unsupervised principal component analysis (PCA) on the total transcriptome (34,745 genes) reveals that $T_{NAIVE}$ cells are distinct from the other three cell subsets (**Figure 2A**), but also reveals differences between KIR$^+$RA$^+$, NKG2A$^+$RA$^+$, and $T_{EMRA}$ cells. In addition, the PCA indicates that the variation among these memory subsets is highly donor-dependent (**Supplementary Figure 2C**).

To asses if KIR$^+$RA$^+$ and NKG2A$^+$RA$^+$ T cells may have acquired distinct molecular and cellular functions, we first identified differentially expressed genes (DEGs) between the four T-cell subsets using a paired analysis (matched donor pairs). These DEGs were used to identify enrichment of unique pathways of molecular and cellular functions in KIR$^+$RA$^+$, NKG2A$^+$RA$^+$, and $T_{EMRA}$ memory cells, as compared to $T_{NAIVE}$ cells (**Supplementary Figure 3**). Besides 83 pathways shared by these three memory subsets, sixteen pathways are shared between KIR$^+$RA$^+$ and NKG2A$^+$RA$^+$ cells but absent in $T_{EMRA}$ cells, which are mainly linked to chemotaxis and recruitment of cells (**Figure 2B**; **Supplementary Figure 3**). KIR$^+$RA$^+$ T cells are uniquely enriched for seven pathways (**Figure 2B**), including pathways related to killing of lymphocytes and mononuclear leukocytes (**Supplementary Figure 3**), whereas NKG2A$^+$RA$^+$ T cells are enriched for six unique pathways, associated with innate antigen presenting cell features (**Supplementary Figure 3**). These findings indicate that both KIR$^+$RA$^+$ and NKG2A$^+$RA$^+$ T cells have acquired distinct molecular and cellular functions.

As numbers of KIR$^+$RA$^+$ T cells increase with age, we focused our analyses on the characterization of this cell subset. Compared to NKG2A$^+$RA$^+$ T cells, genes involved in co-stimulation (*CD28*, *CCR7*) are downregulated in KIR$^+$RA$^+$ T cells (**Figure 2C**). In addition, KIR$^+$RA$^+$ T cells showed enrichment for transcripts related to a regulatory cell phenotype, such as *IKZF2* (encoding Helios) [35], *FCRL3* [36], and *KLRA1P* (Ly46 in mice) [37] (**Figure 2D,E**). Moreover, the transcriptome of KIR$^+$RA$^+$ T cells was enriched for several exhaustion-related transcripts (*TIGIT*, *TOX*, *KLRG1*, and decreased *CD28*) and transcripts of the senescence-related anti-apoptotic gene *FAIM* (**Figure 2D,E**), which may indicate cellular senescence [38], although other senescence-associated genes (e.g. *SESN2*, *SESN3*, *B3GAT1, ATM*, *RELA*, *RELB, BCL2*) were not differentially expressed. Thus, our transcriptome analysis shows that the transcriptome of KIR$^+$RA$^+$ T cells is enriched for aging- and regulatory-associated genes, which

distinguishes these cells from NKG2A⁺RA⁺ T cells that had previously been regarded to be part of the same CD8⁺ T-cell subset.

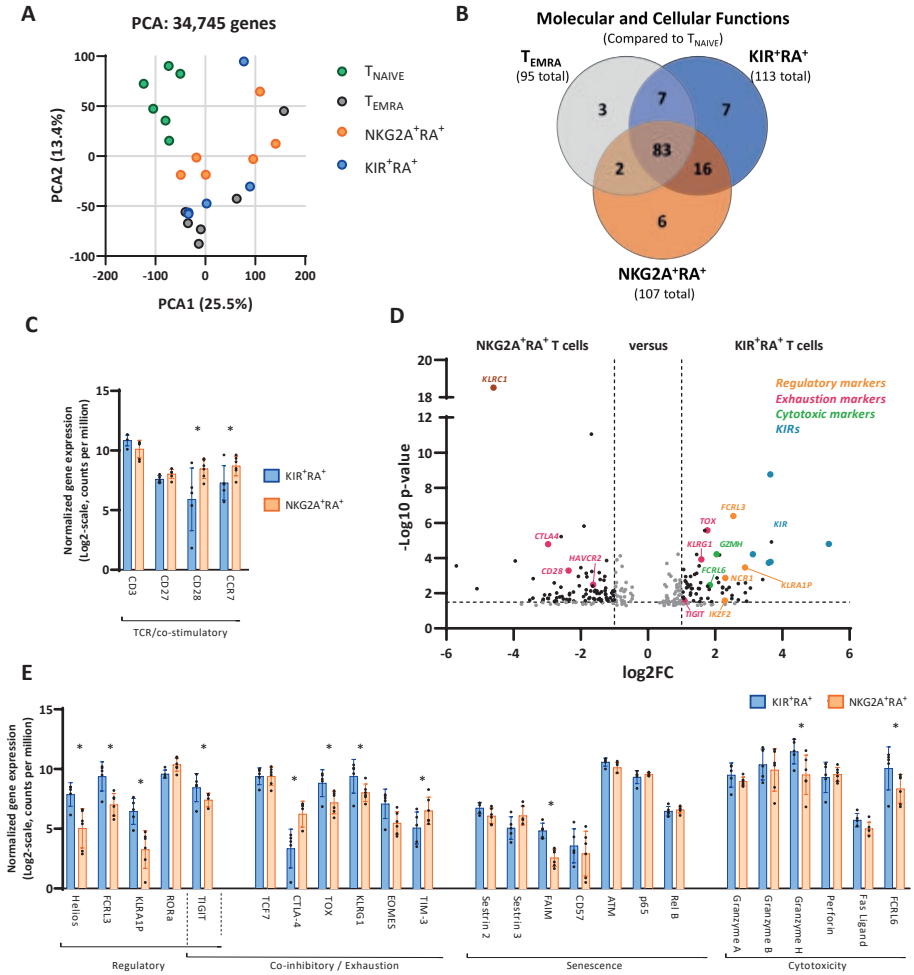


**Figure 2. The gene expression profile of KIR⁺RA⁺ T cells is distinct from that of NKG2A⁺RA⁺ T cells and shows features of aging and regulation.**

KIR⁺RA⁺, NKG2A⁺RA⁺, $T_{NAIVE}$, and $T_{EMRA}$ cell subsets were sorted from healthy donor PBMCs (n=6) and their gene expression profiles were analyzed by RNA sequencing. (**A**) Unsupervised principal component analysis (PCA) on the total transcriptome (34,745 genes) showing clustering of the four sorted cell subsets along components 1 and 2. (**B**) Venn diagram showing the unique and shared molecular and cellular functions for each of the three cell subsets compared to $T_{NAIVE}$ cells as identified by ingenuity pathway analysis (IPA). (**C**) Comparison of detected transcripts associated with TCR- and co-stimulation (shown by the names of the proteins they encode) between KIR⁺RA⁺ T cells and NKG2A⁺RA⁺ T cells. (**D**) Volcano plot indicating differences in gene expression between NKG2A⁺RA⁺ and KIR⁺RA⁺ T cells. The different colors indicate genes related to regulation, exhaustion, cytotoxicity,

and KIR receptors. (**E**) Comparison of expression of selected genes associated with cellular regulation, co-inhibition/exhaustion, senescence, and cytotoxicity between KIR$^{+}$RA$^{+}$ T cells and NKG2A$^{+}$RA$^{+}$ T cells. For each of these analyses, an absolute log2 Fold Change > 1.0; -Log10 *p* value of > 1.5 corresponding to $p < 0.05$ at a false discovery rate (FDR) of 0.10 was used.

### TIGIT$^{Hi}$CD226$^{Low}$ KIR$^{+}$RA$^{+}$ T cells contribute to age-related TIGIT expression in CD8$^{+}$ T cells

The co-inhibitory molecule TIGIT was one of the molecules that we identified in the transcriptome of KIR$^{+}$RA$^{+}$ T cells and has previously been described as a marker for regulation [39,40], exhaustion [41,42], and has recently been linked to aging [20]. We therefore questioned whether the accumulation of KIR$^{+}$RA$^{+}$ T cells may be linked to the aging-related increase in TIGIT$^{+}$ CD8$^{+}$ T cells. Consistent with previous findings [20], the frequency of TIGIT$^{+}$ cells of total CD8$^{+}$ T cells increased with age (**Figure 3A,B**). Strikingly, the proportion of KIR$^{+}$RA$^{+}$ T cells showed a strong positive correlation with the frequency of TIGIT$^{+}$ CD8$^{+}$ T cells (**Figure 3C**). Among all memory T-cell subsets in our study, this correlation was strongest for the KIR$^{+}$RA$^{+}$ T cells (**Supplementary Figure 4A**). KIR$^{+}$RA$^{+}$ T cells indeed showed the highest frequency of TIGIT$^{+}$ cells and median expression per cell (MFI) compared to all other CD8$^{+}$ T cell subsets (**Figure 3D, Supplementary Figure 4B**). These findings show that KIR$^{+}$RA$^{+}$ T cells contribute to the accumulation of TIGIT$^{+}$ CD8$^{+}$ T cells with age.

The co-stimulatory receptor CD226 competes with TIGIT for interaction with their shared ligands [42]. The ratio between CD226 and TIGIT on a CD8$^{+}$ T cell determines the threshold for its proliferative and activation potential [41-44]. Combining analyses of TIGIT and CD226 expression resulted in a separation of the six T-cell subsets (**Figure 3E**). KIR$^{+}$RA$^{+}$ T cells expressed the highest frequency of TIGIT$^{+}$ cells and the lowest frequency of CD226$^{+}$ cells, and clearly separated from NKG2A$^{+}$RA$^{+}$ T cells based on these two markers (**Figure 3F**). The ratio between expression of TIGIT and CD266 by KIR$^{+}$RA$^{+}$ T cells showed that the balance between these markers is strongly skewed towards TIGIT in this subset (**Figure 3G**). Thus, KIR$^{+}$RA$^{+}$ T cells are characterized by TIGIT$^{Hi}$CD226$^{Low}$ expression and contribute to age-related TIGIT expression by CD8$^{+}$ T cells.

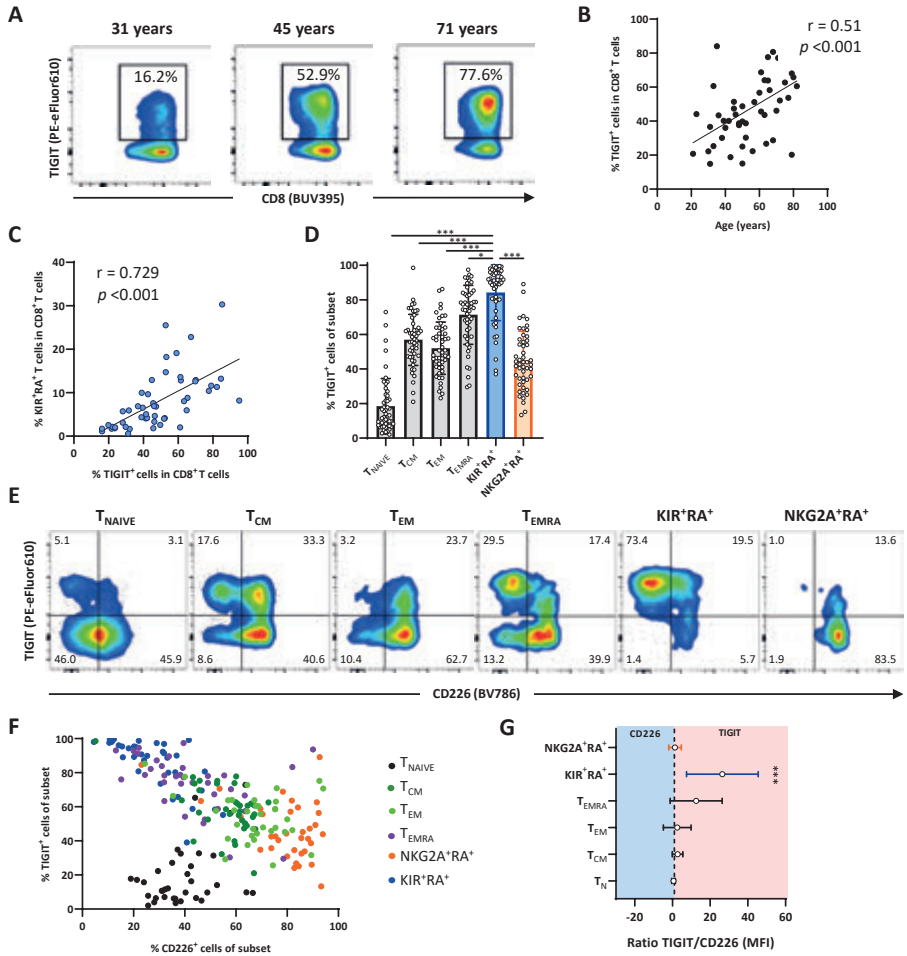


**Figure 3. TIGIT$^{Hi}$CD226$^{Low}$ KIR$^{+}$RA$^{+}$ T cells contribute to age-related TIGIT expression in CD8$^{+}$ T cells.**

(**A**) Flow cytometry plots show TIGIT expression by total CD8$^{+}$ T cells in selected individuals of different ages. (**B**) Relationship between the frequency of TIGIT$^{+}$ cells within the total CD8$^{+}$ T-cell population and age in healthy individuals (n=50). (**C**) Relationship between the frequency of KIR$^{+}$RA$^{+}$ cells and the frequency of TIGIT$^{+}$ cells within the CD8$^{+}$ T-cell population. (**D**) The frequency TIGIT$^{+}$ cells within the indicated CD8$^{+}$ T-cell subsets. (**E**) Representative flow cytometry plots of TIGIT and CD226 expression within each of the six CD8$^{+}$ T-cell subsets. (**F**) Relationship between TIGIT$^{+}$ and CD226$^{+}$ cell frequencies for each cell subset, as well as (**G**) the ratio between TIGIT and CD226 per cell subset based on the median expression per cell (MFI) of these markers. Correlations (*r* values) were assessed by Spearman test. Statistical significance of data presented in the bar graph (means ± s.d.) was determined using Friedman test (with Dunn's post-test). (*$p$ <0.05, ***$p$ <0.001).

### KIR$^+$RA$^+$ T cells are responsive to stimulation but have low proliferative capacity

We next characterized the functionality of KIR$^+$RA$^+$ T cells. Upon non-specific stimulation with PMA/ionomycin, KIR$^+$RA$^+$ T cells highly expressed CD107a compared to the other T-cell subsets, suggesting enhanced cellular degranulation (**Figure 4A**). The frequency of KIR$^+$RA$^+$ T cells producing IL-2 was similar to levels found in $T_{EMRA}$ and NKG2A$^+$RA$^+$ T cells (**Figure 4B**). Frequencies of KIR$^+$RA$^+$ T cells producing IFN-γ and TNF-α or combination of these cytokines, did not significantly differ from those found in the other subsets (**Figure 4C,D**, **Supplementary Figure 5A**). These findings show that the KIR$^+$RA$^+$ T-cell subset is not hampered in production of cytokines.

We further assessed responsiveness of the KIR$^+$RA$^+$ T cells by assessing their capacity to upregulate the activation marker CD69 and proliferate in response to anti-CD3 stimulation of PBMCs for three days. CD8$^+$ T cells expressing KIR showed the highest frequency of CD69$^+$ cells compared to cells that expressed NKG2A or cells that did not express KIR or NKG2A (**Figure 4E**). However, sorted KIR$^+$RA$^+$ T cells did not show increased frequencies of CD69$^+$ cells compared to sorted KIR$^-$NKG2A$^-$ and NKG2A$^+$RA$^+$ T cells (**Supplementary Figure 5B**). This suggests that KIR$^+$RA$^+$ T cells may depend on other cells and/or cell-derived cytokines for their activation. Furthermore, we observed that the proliferative capacity of the overall CD8$^+$ T-cell population declined with age (**Figure 4F**). When we analyzed correlations of CD8$^+$ T-cell proliferation with proportions of the different T-cell subsets (**Figure 4G** and **Supplementary Figure 5C**), we found that this overall decline of proliferation strongly correlated with increased proportions of KIR$^+$RA$^+$ T cells present *ex vivo* (**Figure 4G**). This negative correlation may be explained by non-proliferative KIR$^+$RA$^+$ T cells, despite their higher activation status. We therefore assessed proliferation in KIR$^+$NKG2A$^-$ and the KIR$^-$ T-cell populations after anti-CD3-mediated stimulation of PBMCs. CD8$^+$ T cells expressing KIR showed the lowest proliferation, compared to the populations that did not express KIR (**Figure 4H**), suggesting that the correlation between decreased CD8$^+$ T-cell proliferation and increased proportion of KIR$^+$RA$^+$ T cells can be explained by low proliferative capacity of KIR$^+$RA$^+$ T cells. Indeed, the proportion of T cells that were KIR$^+$ did not expand whereas the proportion of cells that expressed NKG2A did (**Figure 4I**). Together, these findings show that despite high activation potential of KIR$^+$RA$^+$ T cells, KIR$^+$RA$^+$ T cells have low proliferative capacity. Since the proportion of KIR$^+$RA$^+$ T cells among the total CD8$^+$ T-cell pool accumulates with age, their lack of proliferative capacity may contribute to the decreased proliferation of the total CD8$^+$ T-cell pool at older age.

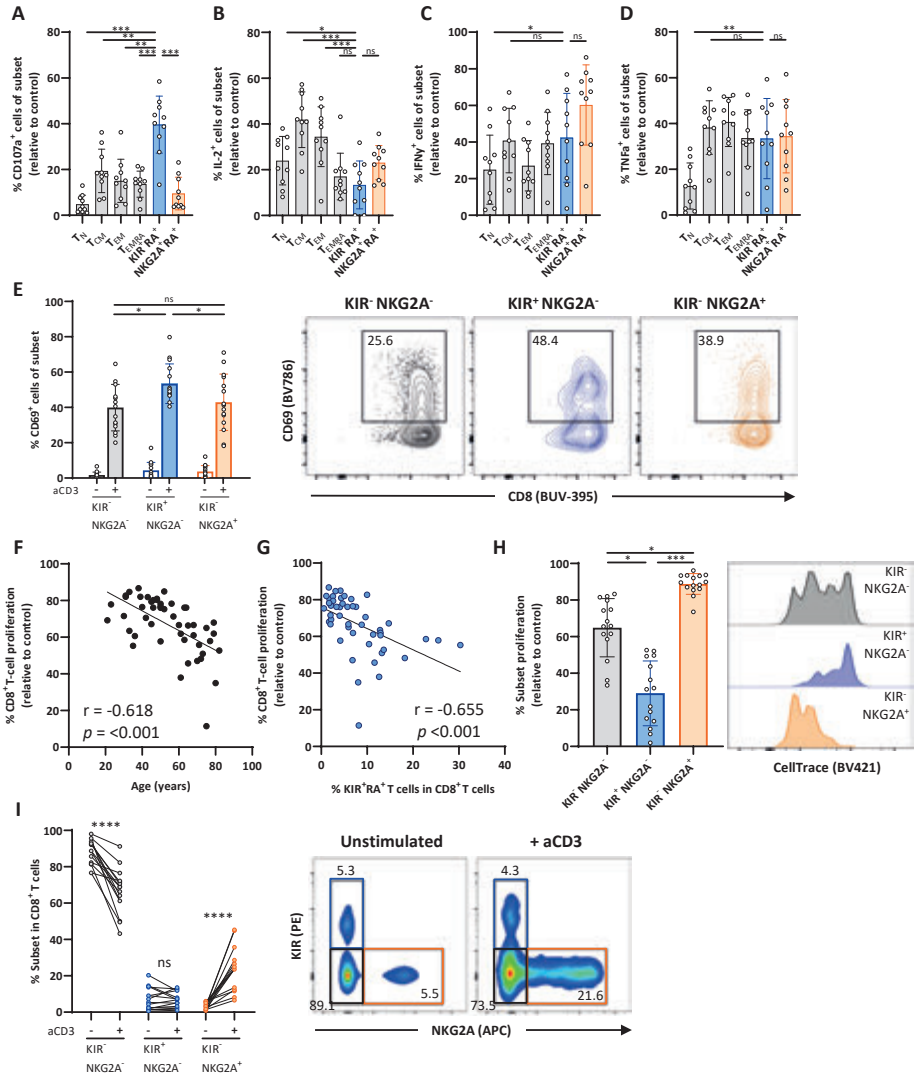


**Figure 4. KIR⁺RA⁺ T cells are responsive to stimulation but have low proliferative capacity.** Accumulation of intracellular cytokines was measured after exposure of total PBMCs of healthy individuals (n=10) to PMA/Ionomycin for six hours. Frequency of (**A**) CD107a⁺, (**B**) IL-2⁺, (**C**) IFN-γ⁺, and (**D**) TNF-α⁺ cells within the indicated CD8⁺ T-cell subsets (relative to medium control as calculated by % positive cells cultured with PMA/ionomycin minus % positive cells in medium control). (**E**) PBMCs of healthy individuals (n=15) were cultured for three days with (+) or without (-) stimulatory anti-CD3, after which the frequency of CD69⁺ cells was determined in CD8⁺ T cells that were KIR⁻NKG2A⁻, KIR⁺NKG2A⁻, or KIR⁻NKG2A⁺ as shown in the bar graph and representative flow cytometry plots. CellTrace-labeled PBMCs of healthy individuals (n=50) were cultured with stimulatory anti-CD3 for three days to detect proliferation of CD8⁺ T cells. Relationship between the frequency of proliferated CD8⁺ T cells (relative to medium control) and (**F**) age, and (**G**) *ex vivo* frequency of KIR⁺RA⁺ cells within the total CD8⁺ T-cell population.

(**H**) Proliferation of $CD8^+$ T cells that are $KIR^-NKG2A^-$, $KIR^+NKG2A^-$, or $KIR^-NKG2A^+$ and the representative flow cytometry plot (n=15). (**I**) Proportion and representative flow cytometry histograms of $KIR^-NKG2A^-$, $KIR^+NKG2A^-$, and $KIR^-NKG2A^+$ cells within the $CD8^+$ T-cell population cultured with (+) or without (-) stimulatory anti-CD3. Correlations (*r* values) were assessed by Spearman test. Statistical significance of data presented in bar graphs (means ± s.d.) was determined using row-matched one-way ANOVA (with Geisser-Greenhouse correction and Dunnett's post-test) (**A**-**D**), Friedman test (with Dunn's post-test) (**E, H**), or Wilcoxon matched-pairs test (**I**). (**p* <0.05, ***p* <0.01, ****p* <0.001, *****p* <0.0001, ns=not significant).

### $KIR^+RA^+$ T cells show a distinct regulatory $CD8^+$ T-cell phenotype and suppress proliferation of $KIR^-NKG2A^-$ $CD8^+$ T cells

The negative correlation between the proportion of $KIR^+RA^+$ T cells and $CD8^+$ T-cell proliferation may also imply that $KIR^+RA^+$ T cells regulate proliferation of other $CD8^+$ T cells. TIGIT and Helios are markers for $CD4^+$ regulatory T cells (Tregs) [35,40,45]. Since $KIR^+RA^+$ T cells highly express TIGIT at the cell surface and our RNA-seq data indicate enhanced TIGIT and expression of Helios transcripts, we explored the possibility that $KIR^+RA^+$ T cells might be regulatory $CD8^+$ T cells. We first confirmed the regulatory phenotype of $KIR^+RA^+$ T cells by high intracellular expression of Helios protein by flowcytometry (**Figure 5A**) as well as by high cell-surface expression of the $CD8^+$ Treg-associated marker CD122 [46] (**Figure 5B**). $KIR^+RA^+$ T cells showed the highest frequency of $Helios^+$ and $CD122^+$ cells compared to all other $CD8^+$ T-cell subsets (**Figure 5A,B**), as well as expression of these markers per cell (**Supplementary Figure 6A,B**). Combined expression of TIGIT and Helios even further delineated $KIR^+RA^+$ T cells from all other subsets (**Figure 5C**), indicating a distinct regulatory T-cell phenotype of $KIR^+RA^+$ T cells.

We next investigated whether $KIR^+RA^+$ T cells could functionally regulate T-cell proliferation in a suppression assay. Addition of $KIR^+RA^+$ T cells reduced proliferation of responder T cells ($KIR^-$ $NKG2A^-$ $CD8^+$ T cells) (**Figure 5D**) in a dose-dependent manner (**Figure 5E**). Adding responder cells instead of $KIR^+RA^+$ T cells to the responder cells did not reduce their proliferation, indicating suppression by $KIR^+RA^+$ T cells (**Figure 5D** left and right panels). Together, we show that $KIR^+RA^+$ T cells are a $CD8^+$ T-cell subset with regulatory phenotype and suppressive capacity. Therefore, accumulation of $KIR^+RA^+$ T cells with age may suggest that these cells contribute to suppressing immunity in older adults.

### $KIR^+RA^+$ T cells are highly activated during acute respiratory infection in older adults as bystander cells

To investigate whether $KIR^+RA^+$ T cells play a role in dampening protection in the elderly, we analyzed the presence and activation status of $KIR^+RA^+$ T cells

in peripheral blood of older adults (62-83 years of age, n=36) suffering from a common respiratory viral infection (**Supplementary Table 1**) ([27], Van Kaaijk *et al*. submitted). Blood samples were obtained at the acute phase of infection (within three days of fever onset) and during follow-up at 2 and 8 weeks.

The proportion of $KIR^+RA^+$ T cells within the total $CD8^+$ T-cell pool remained relatively stable from the acute phase throughout the following eight weeks, as exemplified in influenza A virus infected individuals (**Figure 6A**) and also by other viral infections in older adults (**Supplementary Figure 7A**). However, we observed a strikingly increased frequency of activated $CD69^+$ $KIR^+RA^+$ T cells in older adults during the acute phase of influenza A virus infection compared to the follow-up at two and eight weeks, and compared to healthy older adults (**Figure 6B, Supplementary Figure 7B**). At the acute phase of influenza A virus infection, $KIR^+RA^+$ T cells showed the highest frequencies of $CD69^+$ cells compared to all other $CD8^+$ T cells defined in our analyses (**Figure 6C, Supplementary Figure 7C**). Significant activation of $KIR^+RA^+$ T cells was also found for individuals infected with a seasonal coronavirus (**Figure 6D**) and similar trends were found for other respiratory viruses included in the study (**Figure 6E-H**).

To study whether $KIR^+RA^+$ T cells are virus-antigen specific T cells, we assessed influenza-specificity of these cells by measuring the number of $CD8^+$ T cells specific for the immunodominant influenza A-epitope GILGFVFTL [47] in influenza-A infected individuals (n=4). Whereas these specific cells could be detected in all other T cell subsets, most predominantly in the $T_{CM}$ and $T_{EM}$ subsets (**Figure 6I**), we observed no GILGFVFTL-specific cells within the $KIR^+RA^+$ T-cell subset. These findings suggest that $KIR^+RA^+$ T cells that are activated during an event of influenza are not influenza-specific T cells and therefore potentially are activated as bystander cells during infection.

### $KIR^+RA^+$ T cells are highly activated in SARS-CoV-2 infected adults suffering from COVID-19

Additionally, we had the unique opportunity to analyze the $KIR^+RA^+$ T-cell subset in adults infected with SARS-CoV-2 (32-52 years of age, n=9, **Supplementary Table 3**) [31]. In these individuals, we also observed significant activation of $KIR^+RA^+$ T cells during the early phase of COVID-19 compared to the follow-up at two and four weeks, and compared to SARS-CoV-2 negative controls (**Figure 6J**). Collectively, these findings show that activation of $KIR^+RA^+$ T cells is a major phenomenon in respiratory viral infectious diseases.

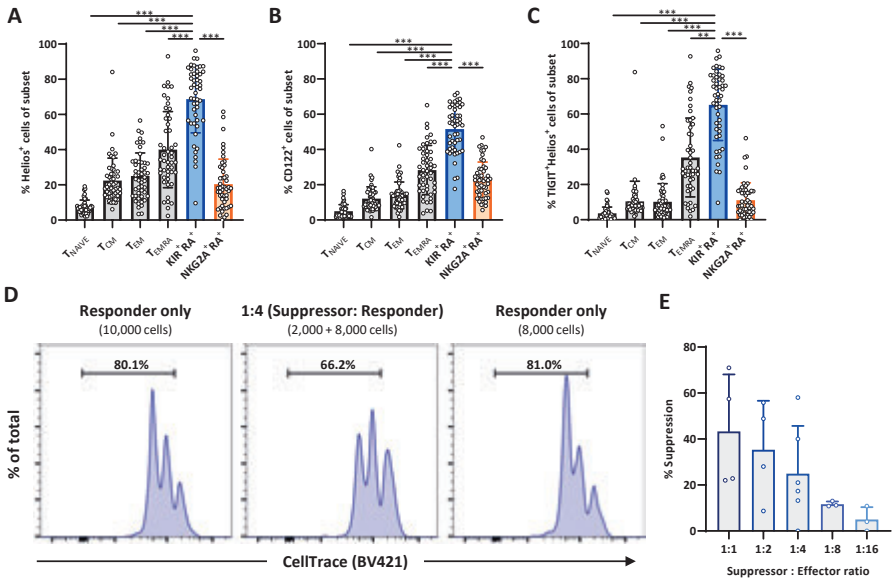


**Figure 5. KIR⁺RA⁺ T cells show a distinct regulatory CD8⁺ T-cell phenotype and suppress proliferation of KIR⁻NKG2A⁻ CD8⁺ T cells.**

Frequency of (**A**) Helios⁺, (**B**) CD122⁺, and (**C**) TIGIT⁺Helios⁺ cells within the indicated CD8⁺ T-cell subsets. For suppression assays, suppressor cells (KIR⁺RA⁺ T cells) and responder cells (KIR⁻NKG2A⁻ T cells) were sorted, added in different dose-range ratios ranging from 1:1 to 1:16 (suppressor:responder), and cultured with stimulatory anti-CD3 for three days. (**D**) Representative flow cytometry histograms show the frequency of proliferated KIR⁻NKG2A⁻ CD8⁺ T cells in responder only conditions (10,000 and 8,000 cells/well) and in a 1:4 suppressor:responder ratio. (**E**) Percentage of suppression derived from suppression assays performed in n=3-6 individual assays per indicated suppressor:responder ratio. Statistical significance of data presented in bar graphs (means ± s.d.) was determined using Friedman test (with Dunn's post-test) (**A-C**). (**$p$ <0.01, ***$p$ <0.001).

## Activation of KIR⁺RA⁺ T cells correlates with prolonged influenza A-induced symptoms

We reasoned that accumulation of suppressive KIR⁺RA⁺ T cells that become activated during respiratory viral infectious disease may suppress protective CD8⁺ T-cell responses and thereby prolong virus-induced respiratory disease in older adults. Therefore, we monitored the occurrence and duration of ILI-associated symptoms (cough, fever, myalgia, nasal congestion, sore throat, difficulty breathing, and headache) from which we calculated an overall symptom score in the influenza A-infected older individuals (**Supplementary Figure 7D**). The frequency of CD69⁺ KIR⁺RA⁺ T cells during ILI positively correlated with the overall symptom score, hinting towards a link between the average duration of symptoms and the presence of activated KIR⁺RA⁺ T cells (**Figure 6K**). Moreover, the presence of these activated cells positively correlated with the duration of

coughing (**Figure 6L**), which may suggest delayed viral clearance. However, the frequency of activated KIR$^+$RA$^+$ T cells did not correlate with the duration of fever (**Figure 6M**) or the other symptoms assessed separately (data not shown). Taken together, these data establish a potential clinical relevance of KIR$^+$RA$^+$ T cells as immunoregulatory cells that may negatively affect the resolution of infection and may prolong influenza A-induced symptoms in older adults.

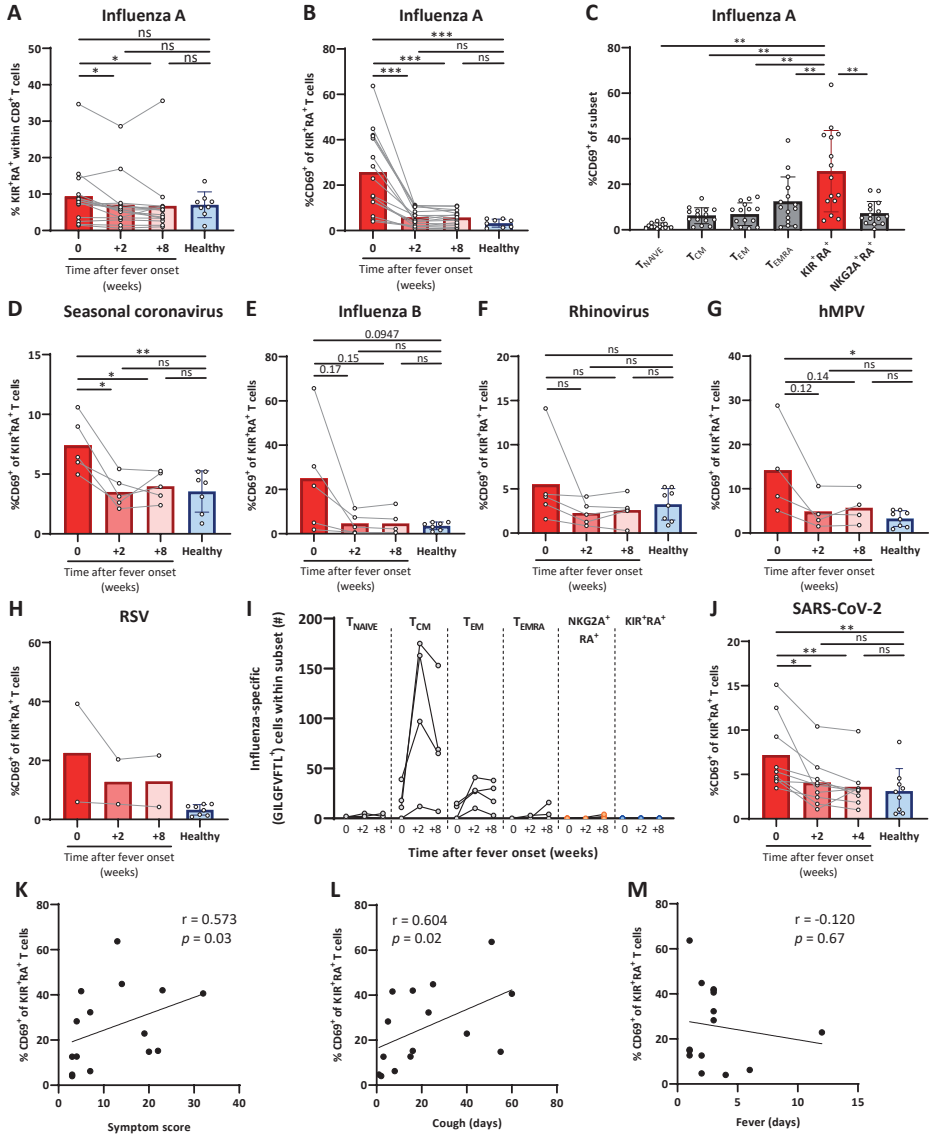


**Figure 6. KIR$^+$RA$^+$ T cells are highly activated during acute respiratory infection in older adults as bystander cells and correlate with prolonged disease.**

Blood samples from older adults suffering from a common respiratory virus infection (62-83 years of age, n=36) were analyzed at the acute phase (0) (within two days of fever onset), and during follow-up at +2 and +8 weeks. Healthy asymptomatic individuals (61-82 years of age, n=8) from the same cohort were used as control samples. (**A**) Frequency of $KIR^+RA^+$ T cells within the $CD8^+$ T-cell population and (**B**) the frequency of $CD69^+$ cells in $KIR^+RA^+$ T cells at the three time points in influenza-A infected older adults (n=15) and healthy asymptomatic controls (n=8). (**C**) The frequency of $CD69^+$ cells in six different $CD8^+$ T-cell subsets. The frequency of $CD69^+$ in $KIR^+RA^+$ T cells in the blood of older adults infected with (**D**) a seasonal coronavirus (n=5), (**E**) influenza B virus (n=5), (**F**) Rhinovirus (n=5), (**G**) human metapneumovirus (hMPV) (n=4), or (**H**) respiratory syncytial virus (RSV) (n=2). (**I**) The number of $CD8^+$ T cells specific for the immunodominant Influenza A matrix protein epitope GILGFVFTL detected by flow cytometry over time in the indicated $CD8^+$ T-cell subsets of HLA-A*02$^+$ influenza-A infected individuals (n=4). (**J**) The frequency of $CD69^+$ cells in $KIR^+RA^+$ T cells in the blood of adults infected with SARS-CoV-2 and suffering from COVID-19 (n=9). Relationship between the frequency of $CD69^+$ cells within the $KIR^+RA^+$ T-cell subset and (**K**) the calculated symptom score, (**L**) duration of cough, and (**M**) duration of fever in older adults suffering from influenza A virus infection (n=15). Correlations (*r* values) were assessed by Spearman test. Statistical significance of data presented in bar graphs (mean ± s.d.) was determined using row-matched one-way ANOVA (with Geisser-Greenhouse correction and Dunnett's post-test) for the difference between the time points and Mann-Whitney U test was used to determine the difference between infected and asymptomatic healthy individuals (**A, B, D-G, J**). Row-matched one-way ANOVA (with Geisser-Greenhouse correction and Dunnett's post-test) was used in (**C**). (*$p$ <0.05, **$p$ <0.01, ***$p$ <0.001, ns=not significant, or the exact $p$-value is shown.)



## DISCUSSION

Here we identify a novel human $CD8^+$ T-cell subset with regulatory properties, which we refer to as $KIR^+RA^+$ T cells based on their unique expression of KIR and CD45RA. We show that $KIR^+RA^+$ T cells accumulate during healthy aging and may contribute to diminished $CD8^+$ T-cell responses observed in older adults. Furthermore, our findings show that $KIR^+RA^+$ T cells become activated in older adults suffering from viral respiratory diseases and may negatively affect resolution of disease.

Human $CD8^+$ $CD45RA^+$ T cells that express KIR and/or NKG2A have been described as "virtual memory T cells" and these cells have been reported to increase with age [21-23]. We now show that the virtual memory T-cell subset comprises two distinct cell subsets: $KIR^+RA^+$ T cells that accumulate with age and $NKG2A^+RA^+$ T cells that decline with age. Our data show that $KIR^+RA^+$ T cells can be considered a distinct $CD8^+$ T-cell subset that increases with aging. Moreover, $KIR^+RA^+$ T cells constitute 30% of the T-cell subset that has been designated $T_{EMRA}$ cells [48-50]. Since $KIR^+RA^+$ T cells have remained hidden among the $T_{EMRA}$ subset, our findings indicate that part of previously reported age-related increase of the conventional $T_{EMRA}$ subset and expression of TIGIT

by these $T_{EMRA}$ cells [20] may actually be caused by the age-related increase in $KIR^+RA^+$ T cells.

The negative correlation of $KIR^+RA^+$ T cells with $CD8^+$ T-cell proliferation led us to hypothesize that $KIR^+RA^+$ T cells may regulate $CD8^+$ T-cell proliferation. Indeed, $KIR^+RA^+$ $CD8^+$ T cells appeared to express markers previously reported to be expressed by suppressive $CD4^+$ and $CD8^+$ Tregs such as TIGIT, Helios, and CD122 [35,40,46,51,52]. Moreover, we show that $KIR^+RA^+$ T cells suppress proliferation of other $CD8^+$ T cells, in agreement with the hypothesis of age-related accumulation of Tregs, which is thought to hamper protective T cell responses [53-56]. $KIR^+RA^+$ T cells have until now been regarded to be part of the virtual memory-cell subset. It has been speculated that murine virtual memory cells may acquire tolerogenic mechanisms [57], whereas another report showed that murine virtual memory cells can mediate protective immunity by killing [22]. An explanation for these contrasting results may be the existence of different subsets among murine virtual memory T cells like the $KIR^+RA^+$ and $NKG2A^+RA^+$ subsets we report among human virtual memory T cells.

A question that remains is by what mechanism $KIR^+RA^+$ T cells suppress proliferation of other $CD8^+$ T cells. First, we observed that $KIR^+RA^+$ T cells highly express the co-inhibitory receptor TIGIT. As TIGIT expression has been reported to directly and indirectly contribute to the stability and suppressive capacity of $CD4^+$ Tregs [39,40], these findings may indicate a similar role for TIGIT in suppressive $KIR^+RA^+$ T cells we present here. Second, Helios expression has been shown to be required for the survival and suppressive capacity of $CD8^+$ Tregs in mice [35]. As we observed that $KIR^+RA^+$ T cells highly express Helios, targeting Helios may be an additional candidate for unraveling the suppressive mechanism of $KIR^+RA^+$ T cells. Lastly, killing of other cells by release of granzymes and perforins has been described as one of the mechanisms by which Tregs may exert immune suppression [58,59]. This mechanism may also be involved in regulation by $KIR^+RA^+$ T cells since they express CD107a, indicative of enhanced release of granules containing cytotoxic factors, and their transcriptome is enriched with transcripts for pathways involved in killing of lymphocytes.

A study in mice has shown that virtual memory T cells act as bystander cells during infection [22]. The $KIR^+RA^+$ T cells we describe here can be considered as a subset of the T cells defined as virtual memory cells. We here show that $KIR^+RA^+$ $CD8^+$ T cells present in influenza A-infected older adults are highly activated during acute infection, but not specific for the highly conserved and immunodominant influenza A epitope GILGFVFTL. This provides evidence in favor of a potential bystander role of these cells. Interestingly, we also found a large proportion of activated $KIR^+RA^+$ T cells in adults suffering from a primary

event of COVID-19 caused by SARS-CoV-2 infection. Only a minority (up to 20%) of individuals are expected to have a small fraction of cross-reactive antigen-specific memory $CD8^+$ T cells directed against this virus, which further supports a bystander role for $KIR^+RA^+$ T cells [60]. The exact factors inducing bystander activation remain to be elucidated, but IL-15 has been implicated [22]. Together, our findings suggests that the accumulation of $KIR^+RA^+$ T cells with age may play a significant role in a wide range of respiratory viral infections.

To understand the *in vivo* clinical relevance of $KIR^+RA^+$ T cells, we analyzed the presence and dynamics of activation of these cells in older adults suffering from respiratory viral infection. Our findings show that $KIR^+RA^+$ T cells become a highly activated T-cell subset during respiratory infections caused by various viruses. It has been shown previously that coughing associates with increased viral load in the lungs [61]. We observed a positive correlation between the level of activated $KIR^+RA^+$ $CD8^+$ T cells and duration of coughing, which may suggest hampered clearance of infected lung cells. As cytotoxic $CD8^+$ T cells clear virally infected cells and thereby reduce the severity of disease [13-16], suppression of their responses by $KIR^+RA^+$ T cells may prolong illness. Indeed, $CD4^+$ Tregs have been reported to suppress $CD8^+$ effector cells in influenza A infected mice [62]. Recently, a function of TIGIT was identified in the prevention of pathological tissue damage in an infectious disease setting [63]. This suggests that $TIGIT^{Hi}$ $KIR^+RA^+$ T cells may also be advantageous in preventing excessive lung damage. Therefore, the role of $KIR^+RA^+$ T cells may be two-sided: they may be needed to limit lung damage caused by cytotoxic immune cells, whereas an overrepresented activation of $KIR^+RA^+$ T cells may hamper effective clearance of pathogens. Establishing correlates of respiratory disease is highly important for developing strategies to reduce the burden of disease following respiratory infections as elderly are at higher risk of severe disease, hospitalization, and death (reviewed in [4]). Our findings indicate that $KIR^+RA^+$ T cells may serve as such a correlate of respiratory disease. Moreover, our findings warrant future investigation on elucidating their immunoregulatory mechanisms as well as their role at the site of infection.

In summary, our findings support a model for declining $CD8^+$ T-cell responses with age where accumulation of regulatory $KIR^+RA^+$ T cells may in part determine the outcome of protective T-cell responses against respiratory infection. These cells may therefore serve as target for new preventive or therapeutic strategies.

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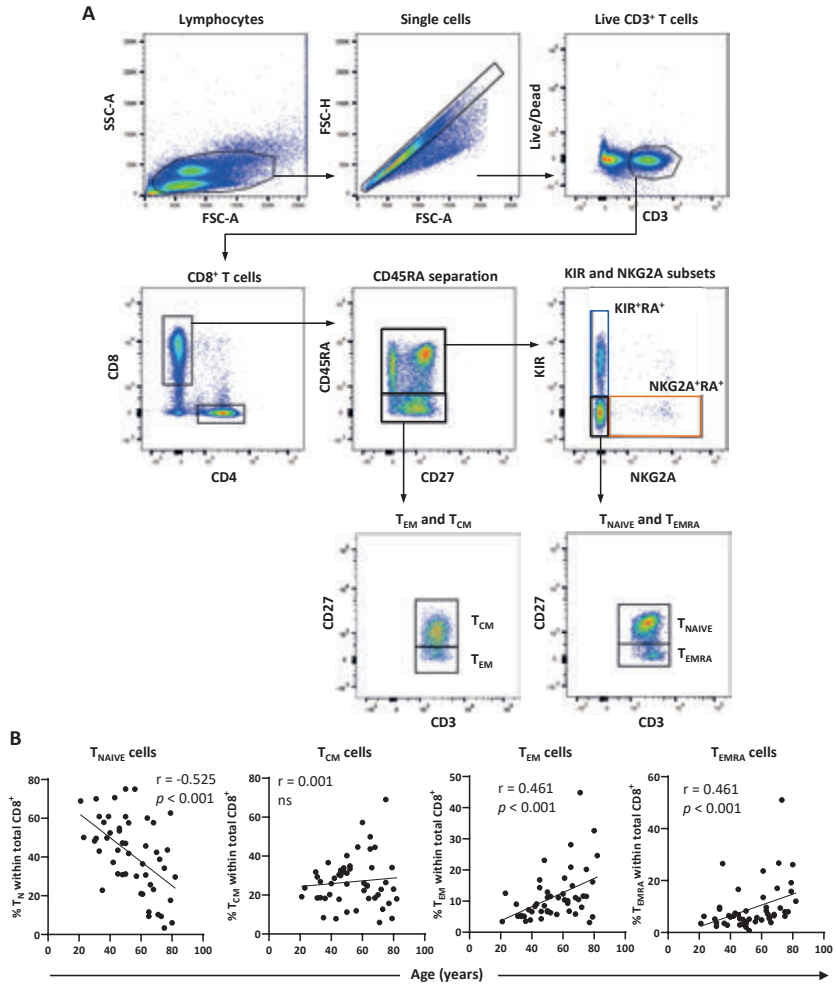
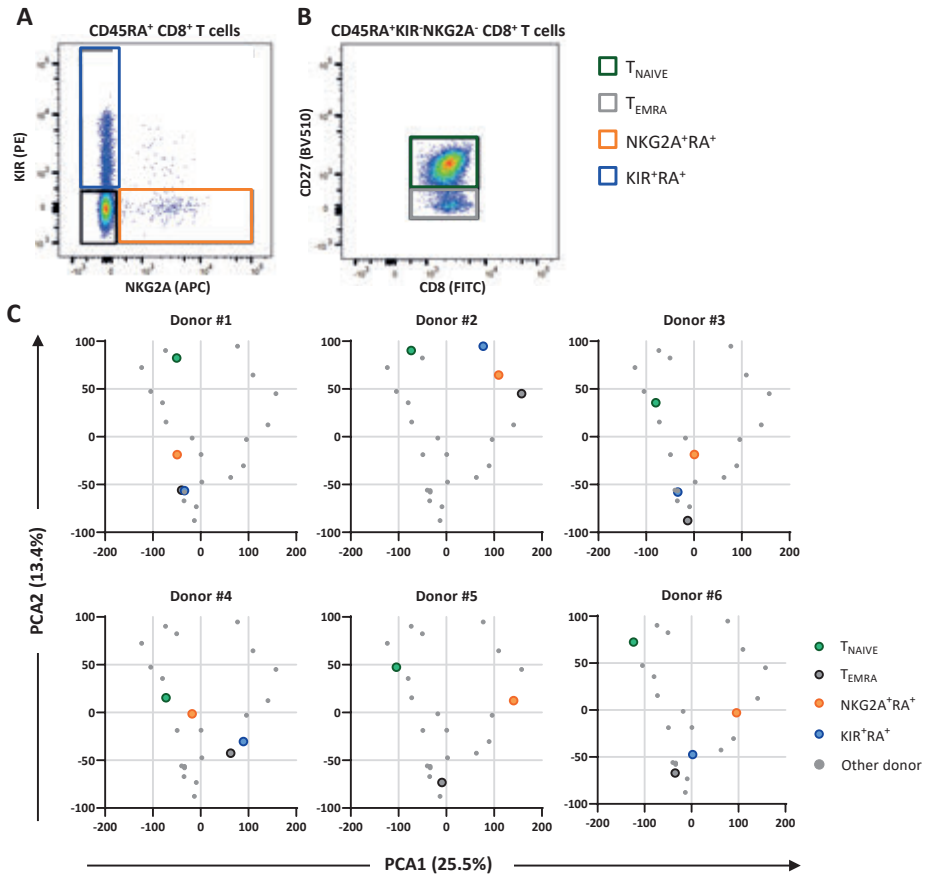
# SUPPLEMENTAL FIGURES




**Supplementary Figure 1. Gating strategy of CD8⁺ T-cell subsets and their proportion with age** (**A**) Gating strategy towards the six CD8⁺ T-cell subsets in healthy individuals (n=50, 20-82 years of age) using flow cytometry. CD8⁺ T cells were identified based on gating of total lymphocytes, single cells, and live CD3⁺ T cells. CD8⁺ T cells were then separated into CD45RA⁺ and CD45RA⁻ cells. Within the CD45RA⁺ T-cell population, KIR⁺RA⁺ T cells ($CD3^+CD8^+CD45RA^+KIR^+NKG2A^-$, blue gate) and NKG2A⁺RA⁺ T cells ($CD3^+CD8^+CD45RA^+KIR^-NKG2A^+$, orange gate) were identified. Remaining CD45RA⁺ KIR⁻ NKG2A⁻ cells were divided into naive T cells ($T_{NAIVE}$: $CD3^+CD8^+CD45RA^+KIR^-NKG2A^-CD27^+$) and terminally differentiated effector memory cells re-expressing RA ($T_{EMRA}$: $CD3^+CD8^+CD45RA^+KIR^-NKG2A^-CD27^-$). CD45RA⁻ CD8⁺ T cells were separated into central memory cells ($T_{CM}$: $CD3^+CD8^+CD45RA^-CD27^+$) and effector memory cells ($T_{EM}$: $CD3^+CD8^+CD45RA^-CD27^-$). (**B**) Relationship between the frequency of $T_{NAIVE}$, $T_{CM}$, $T_{EM}$, or $T_{EMRA}$ cells within the total CD8⁺ T-cell population and age. Correlations (*r* values) were assessed by Spearman test.

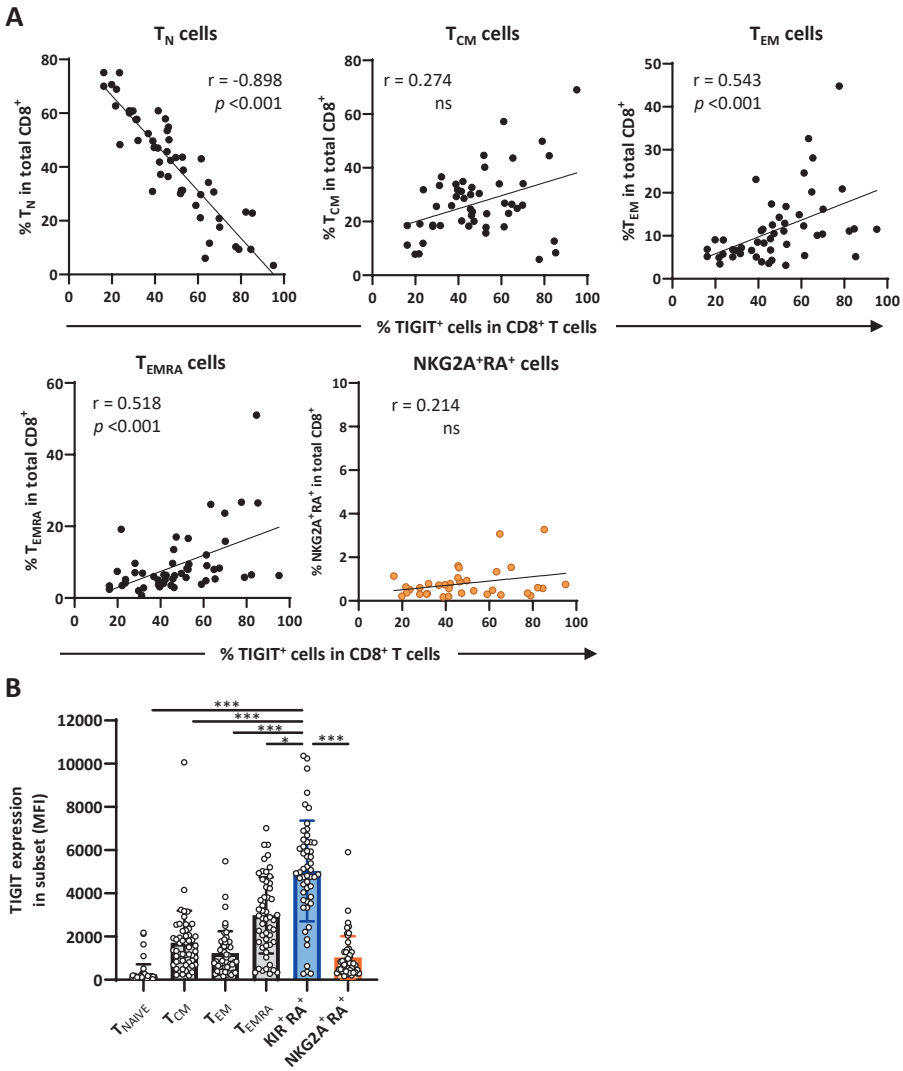



**Supplementary Figure 2. Sorted T-cell subsets and donor variation observed in unsupervised principal component analysis (PCA).**

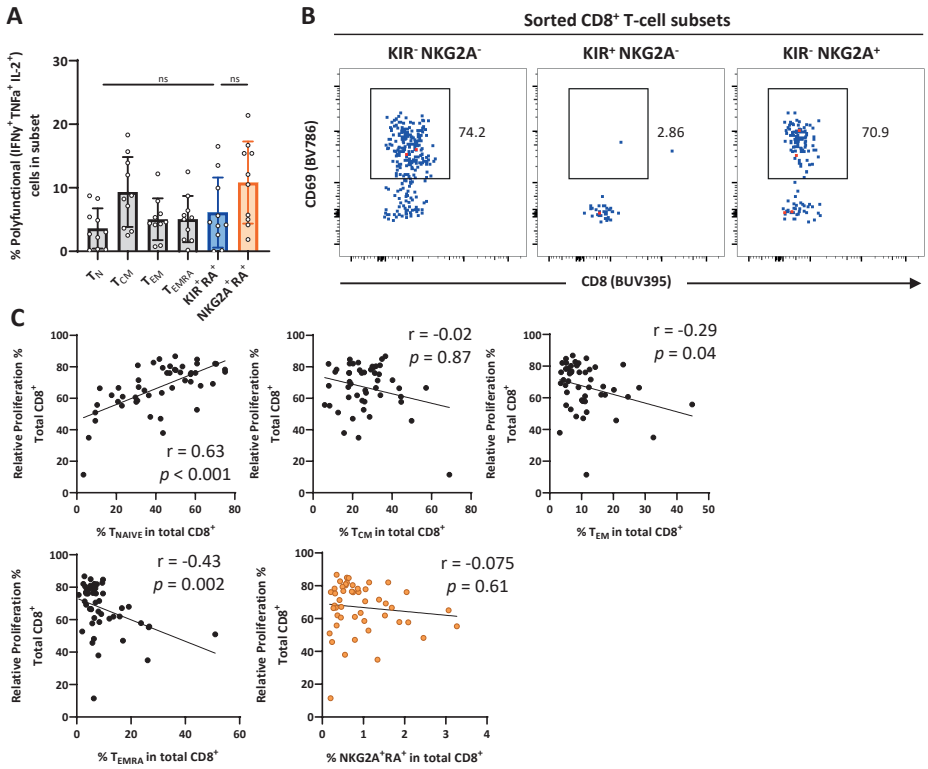
(**A**,**B**) Flow cytometry plots indicate the four FACS-sorted subsets, first gated on CD56$^-$CD4$^-$CD19$^-$CD3$^+$CD8$^+$ T cells: $T_{NAIVE}$ cells (CD8$^+$CD45RA$^+$KIR$^-$NKG2A$^-$CD27$^+$), $T_{EMRA}$ cells (CD8$^+$CD45RA$^+$KIR$^-$NKG2A$^-$CD27$^-$), KIR$^+$RA$^+$ T cells (CD8$^+$CD45RA$^+$KIR$^+$NKG2A$^-$), and NKG2A$^+$RA$^+$ T cells (CD8$^+$CD45RA$^+$KIR$^-$NKG2A$^+$). (**C**) Unsupervised principal component analysis (PCA) performed on the total transcriptome (34,745 genes) within each of the sorted cell subsets is shown for each of the six donors.


**Supplementary Figure 3. Ingenuity Pathway Analysis (IPA) performed on $T_{EMRA}$, $KIR^+RA^+$ and $NKG2A^+RA^+$ T cells compared to $T_{NAIVE}$ cells to identify molecular and cellular function pathways.**

Ingenuity Pathway Analysis (IPA) was performed by comparing the differentially expressed genes (DEGs) of $T_{EMRA}$, $KIR^+RA^+$, and $NKG2A^+RA^+$ T-cell subsets to $T_{NAIVE}$ cells to identify molecular and cellular functions of these cell subsets. Pathways identified in this analysis were then compared between $T_{EMRA}$, $KIR^+RA^+$, and $NKG2A^+RA^+$ T-cell subsets by aligning their hierarchical clustered heat maps to identify in which shared and unique pathways these subsets differed from each other and from $T_{NAIVE}$ cells. Colors in the heat map indicate the z-score calculated for each identified pathway, with cutoff values: -Log(*p*-value) >1.5, z-score with an absolute value > 2.0. Statistical significance ($p$ <0.05) was calculated by Fisher's Exact Test.

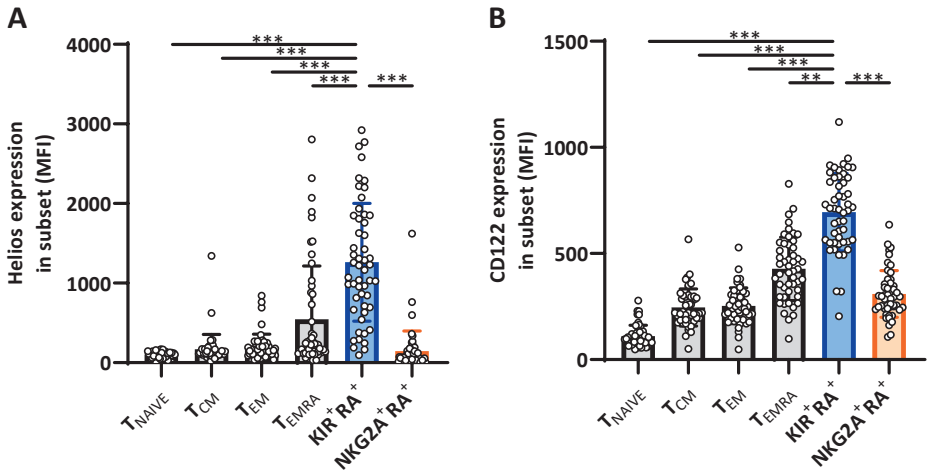



**Supplementary Figure 4. Proportion of TIGIT⁺ cells within CD8⁺ T-cell subsets.**
(**A**) Relationship between the proportion of $T_{NAIVE}$, $T_{CM}$, $T_{EM}$, $T_{EMRA}$, or NKG2A⁺RA⁺ cells within the CD8⁺ T-cell population and the proportion of TIGIT⁺ cells within the CD8⁺ T-cell population. (**B**) Expression (Median Fluorescent Intensity, MFI) of TIGIT within the indicated CD8⁺ T-cell subsets in healthy individuals (20-82 years of age, n=50). Correlations (*r* values) were assessed by Spearman test. Statistical significance of data presented in the bar graph (means ± s.d.) was determined using Friedman test (with Dunn's post-test). (**p* <0.05, ****p* <0.001).

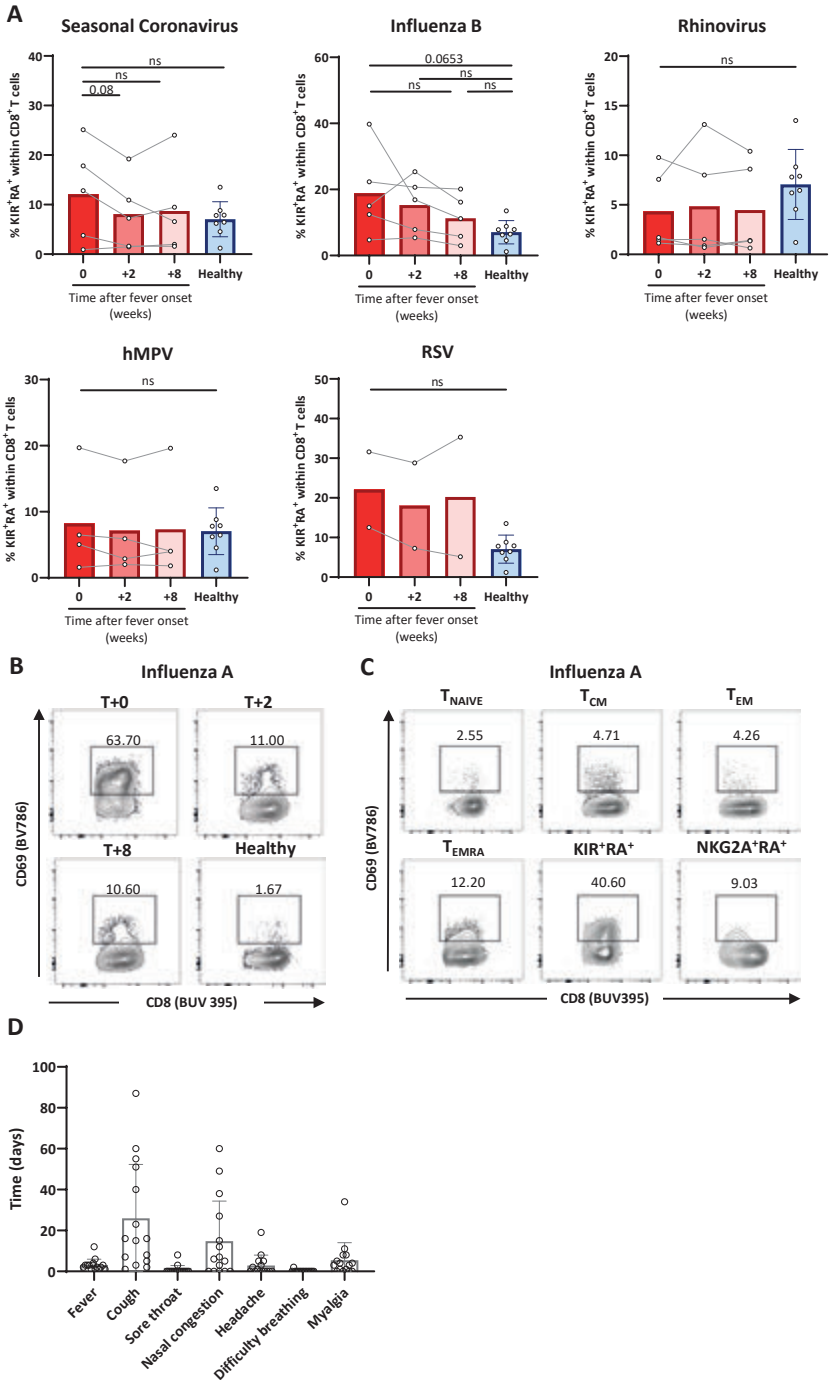



**Supplementary Figure 5. Analysis of the KIR$^+$RA$^+$ T-cell response.**

Accumulation of intracellular cytokines was measured after exposure of total PBMCs of healthy individuals (n=10) to PMA/Ionomycin for six hours. (**A**) Frequency of polyfunctional (IFN-γ$^+$ IL-2$^+$ TNF-α$^+$) cells within the indicated CD8$^+$ T-cell subsets (relative to medium control). (**B**) Frequency of CD69$^+$ cells in sorted CD8$^+$KIR$^-$NKG2A$^-$ T cells, CD8$^+$CD45RA$^+$KIR$^+$NKG2A$^+$ T cells, and CD8$^+$CD45RA$^+$KIR$^-$NKG2A$^+$ T cells after culturing with anti-CD3/anti-CD28 beads (1:12 bead-to-cell ratio) for three days (representing findings for n=4). (**C**) Relationships between the frequency of proliferated CD8$^+$ T cells (relative to medium control) and *ex vivo* frequency of $T_{NAIVE}$, $T_{CM}$, $T_{EM}$, $T_{EMRA}$, or NKG2A$^+$RA$^+$ cells within the total CD8$^+$ T-cell population of healthy individuals (20-82 years of age, n=50). Correlations (*r* values) were assessed by Spearman test. Statistical significance of data presented in the bar graph (means ± s.d.) was determined using row-matched one-way ANOVA (with Geisser-Greenhouse correction and Dunnett's post-test). (ns=not significant).




**Supplementary Figure 6. KIR⁺RA⁺ T cells highly express Helios and CD122.**
Expression (MFI) of (**A**) Helios and (**B**) CD122 within each of the indicated CD8⁺ T-cell subsets in healthy individuals (20-82 years of age, n=50). Statistical significance of data presented in bar graphs (means ± s.d.) was determined using Friedman test (with Dunn's post-test). (**$p$ <0.01, ***$p$ <0.001).




**Supplementary Figure 7. Presence of KIR⁺RA⁺ T cells during viral respiratory infection.**

Blood samples from older adults suffering from a common respiratory virus infection (62-83 years of age, n=36) were analyzed at the acute phase (0) (within two days of fever onset), and during follow-up at +2 and +8 weeks. Healthy asymptomatic individuals (61-82 years of age, n=8) from the same cohort were used as control samples. (**A**) Frequency of $KIR^+RA^+$ T cells amongst the $CD8^+$ T-cell population at the three time points in older adults infected with a seasonal coronavirus (n=5), influenza B virus (n=5), Rhinovirus (n=5), human metapneumovirus (hMPV) (n=4), or respiratory syncytial virus (RSV) (n=2). (**B**) Flow cytometry plots showing the proportion of $CD69^+$ cells in $KIR^+RA^+$ T cells in influenza A infected older adults at each of the indicated points in time. (**C**) Flow cytometry plots of the proportion of $CD69^+$ cells within each of the indicated $CD8^+$ T-cell subsets. (**D**) Bar graph indicates the symptom duration in days monitored in Influenza-A infected older adults (n=15) from which the symptom score was calculated. Statistical significance of data presented in bar graphs (mean ± s.d.) (**A**) was determined using row-matched one-way ANOVA (with Geisser-Greenhouse correction and Dunnett's post-test) for the difference between the time points and Mann-Whitney U test was used to determine the difference between infected and asymptomatic healthy individuals. (The exact *p*-value is shown or ns=not significant.)

# SUPPLEMENTARY TABLES

**Supplementary Table 1.** Samples of older adults with Influenza-like Illness

| Respiratory virus detected | Strain | Number | Median Age (years) |
|---|---|---|---|
| **Influenza A virus (n=15)** | H3N2 | 12 | 68 |
| | H1N1 | 3 | |
| **Influenza B virus (n=5)** | Yamagata/16/88 | 5 | 63 |
| **Coronavirus (n=5)** | C229E | 2 | |
| | HKU1 | 1 | 65 |
| | OC43 | 2 | |
| **Respiratory Syncytial Virus (n=2)** | RSV-A | 2 | 74 |
| **Human metapneumovirus (n=4)** | - | 4 | 71 |
| **Rhinovirus (n=5)** | - | 5 | 73 |
| **Asymptomatic Healthy Controls (n=8)** | None detected | 8 | 70,5 |

**Supplementary Table 2.** Healthy individuals

| **All individuals (n=50)** | | **Age (years)** |
|---|---|---|
| | Median Age | 52 |
| | Lowest | 21 |
| | Highest | 82 |
| **Female (n=21)** | | |
| | Median Age | 50 |
| | Lowest | 31 |
| | Highest | 82 |
| **Male (n=27)** | | |
| | Median Age | 61,5 |
| | Lowest | 21 |
| | Highest | 80 |



**Supplementary Table 3.** Samples of adults with COVID-19

| **Respiratory virus detected** | **Strain** | **Number** | **Median Age (years)** |
|---|---|---|---|
| **Coronavirus (n=9)** | SARS-CoV-2 | 9 | 43 |
| **Asymptomatic Healthy Controls (n=9)** | Not detected | 9 | 44 |

**Supplementary Table 4.** Antibodies used in this study

| | Fluorochrome | Clone | Isotype | Manufacturer | Catalogue nr. | Dilution | Whole blood Assay Dilution |
|---|---|---|---|---|---|---|---|
| **Surface markers** | | | | | | | |
| CD3 | BV421 | UCHT1 | Mouse IgG1, kappa | Biolegend | 300434 | 1:200 | - |
| CD3 | FITC | UCHT1 | Mouse IgG1, kappa | Biolegend | 300440 | 1:200 | 1:100 |
| CD4 | PE-Cy7 | RPA-T4 | Mouse IgG1, kappa | Biolegend | 300512 | 1:100 | - |
| CD4 | PerCP-Cy5.5 | RPA-T4 | Mouse IgG1, kappa | Biolegend | 300530 | 1:200 | 1:100 |
| CD8a | BB515 | RPA-T8 | Mouse IgG1, kappa | BD Horizon | 564526 | 1:100 | - |
| CD8a | BUV395 | RPA-T8 | Mouse IgG1, kappa | BD Horizon | 563795 | 1:100 | 1:200 |
| CD8a | FITC | RPA-T8 | Mouse IgG1, kappa | Biolegend | 301050 | 1:50 | - |
| CD19 | BV786 | HIB19 | Mouse IgG1, kappa | Biolegend | 302240 | 1:100 | - |
| CD27 | BV510 | O323 | Mouse IgG1, kappa | Biolegend | 302836 | 1:50 | 1:100 |
| CD45 | Pacific Blue | HI30 | Mouse IgG1, kappa | Biolegend | 304029 | - | 1:400 |
| CD45RA | BV605 | HI100 | Mouse IgG2b, kappa | Biolegend | 304134 | 1:800 | 1:400 |
| CD45RA | PerCP-Cy5.5 | HI100 | Mouse IgG2b, kappa | Biolegend | 304122 | 1:50 | - |
| CD56 | PE-Cy7 | HCD56 | Mouse IgG1, kappa | Biolegend | 318318 | 1:100 | - |
| CD69 | BV785 | FN50 | Mouse IgG1, kappa | Biolegend | 310932 | 1:100 | 1:100 |
| CD122 | PE-Cy7 | TU27 | Mouse IgG1, kappa | Biolegend | 339014 | 1:50 | - |
| CD226 | BV786 | DX11 | Mouse IgG1, kappa | BD | 742497 | 1:100 | - |
| KIR2D | PE | NKVFS1 | Mouse IgG1, kappa | Miltenyi Biotec | 130-092-688 | 1:400 | 1:400 |
| KIR3DL1 | PE | 5.133 | Mouse IgG1 | Miltenyi Biotec | 130-095-205 | 1:25 | 1:200 |
| NKG2A | APC | REA110 | Recombinant human IgG1 | Miltenyi Biotec | 130-113-563 | 1:400 | 1:800 |
| TIGIT | PE-eFluor610 | MBSA43 | Mouse IgG1, kappa | eBioscience | 61-9500-42 | 1:50 | - |

**Supplementary Table 4.** Continued.

| | Fluorochrome | Clone | Isotype | Manufacturer | Catalogue nr. | Dilution | Whole blood Assay Dilution |
|---|---|---|---|---|---|---|---|
| Fixable Viability Stain | FVS780 | N/A | N/A | BD Horizon | 565388 | 1:2000 | - |
| **Intracellular markers** | | | | | | | |
| CD3 | FITC | UCHT1 | Mouse IgG1, kappa | Biolegend | 300440 | 1:200 | - |
| CD107a | PE-Cy7 | H4A3 | Mouse IgG1, kappa | Biolegend | 328618 | 1:50 | - |
| Helios | PE-Cy7 | 22F6 | Armenian hamster IgG | Biolegend | 137236 | 1:200 | - |
| IFN-gamma | BUV737 | 4S.B3 | Mouse IgG1, kappa | BD | 564620 | 1:50 | - |
| IL-2 | BV785 | MQ1-17H12 | Rat IgG2a, kappa | Biolegend | 500348 | 1:50 | - |
| TNF-alpha | PerCP-Cy5.5 | Mab11 | Mouse IgG1, kappa | Biolegend | 502926 | 1:50 | - |


6

# GENERAL DISCUSSION AND FUTURE PERSPECTIVES

Identification of changes that T cells undergo during aging may provide new insights and starting points for fundamental T-cell research and for future therapeutic strategies in older patients which may improve health in the elderly. This thesis focusses on the fundamentals of changes that occur to T cells during aging in both mice and humans. We provide new leads to explain decreased immune protection in the elderly. In mice and humans we found that the kinetics of T-cell responses slow down at older age. Moreover, we report an upsurge of expression of regulatory T-cell features with age and we discovered a novel $CD8^+$ T-cell subset with immunosuppressive capacities, which we named $KIR^+RA^+$ T cells.

## AGING RESULTS IN DISTINCT CHANGES OF T-CELL RESPONSES

### T-cell Response Kinetics are a Hallmark of Immune Aging

Changes that T cells undergo during aging are often addressed by assessing the *ex vivo* phenotype of T cells and/or measuring the *in vitro* T-cell response at a single point in time. However, insight into the impact of aging on the kinetics of the T-cell response over several days is limited [1,2]. An example that emphasizes the importance of timely and robust T-cell responses is the ongoing SARS-CoV-2 pandemic. Symptomatic and asymptomatic individuals that suffered from COVID-19 produced IgG antibodies in response to SARS-CoV-2, but these antibody levels declined rapidly within the following two to three months [3,4]. Disappearance of IgG antibodies removes a protective immunological barrier and increases the chance of secondary SARS-CoV-2 infection. In the absence of this line of defense, the need for a rapid T-cell response is vital to prevent viral dissemination throughout the lung. Importantly, the need for a rapid T-cell response does not only apply to SARS-CoV-2 infection, but also infection with respiratory viruses such as influenza virus and RSV. Expansion and activation of naive T cells to form a memory T-cell subset that is directed against respiratory viruses, ready to act upon secondary exposure is a highly important process to provide immediate protection, as well as future protection. Delay in T-cell activation and proliferation as a consequence of aging may therefore lead to impaired generation and maintenance of T-cell memory and removal of infected lung cells, allowing viral dissemination and resulting in more severe disease.

In **chapter 2** we investigated the impact of aging on the kinetics of T-cell responses in young and aged mice and conclude that these response kinetics are an important factor to address in T-cell aging research. We measured expression of the classical activation markers CD25 and CD69 [5,6] on T-cell

subsets *in vitro* stimulated with anti-CD3 and/or anti-CD28 over time and found that upregulation of CD25 and CD69 expression by T cells of aged mice is reduced and delayed compared to young mice. Moreover, proliferation of T cells of aged mice over time after anti-CD3 and/or anti-CD28 stimulation was significantly reduced. Additionally, we found that production of IL-2, a major driver of T-cell proliferation was impaired in aged mice. Supplementation of T-cell cultures with IL-2 did not enhance proliferation of aged T cells, indicating that this pathway of T-cell stimulation is impaired in aged mice.

In **chapter 3**, we addressed whether reduced and delayed T-cell activation and proliferation could be attributed to compromised DNA repair. In contrast to our findings in **chapter 2**, T cells of *Ercc1*$^{-/\Delta 7}$ mice showed minimal impaired induction of CD25 expression nor T-cell proliferation in response to anti-CD3 and/or anti-CD28 stimulation. This at least indicates that compromised DNA repair does not explain reduced T-cell activation we found in aged wild type mice. Interestingly however, the T-cell response of *Ercc1*$^{-/\Delta 7}$ mice to anti-CD3 and supplemented IL-2 showed a trend towards impaired induction of CD25 and impaired proliferation, which was similar to findings in wild type aged mice. These findings led us to conclude that impairment of IL-2-mediated signaling at old age may at least partially be caused by compromised DNA repair, whereas impaired CD28-mediated signaling may not.

In **chapter 4** we described a new aging-related subset of human CD8$^+$ T cells. These TIGIT$^+$ Helios$^+$ CD8$^+$ T cells were increasingly present in the blood of older adults and showed reduced induction of CD25 expression, delayed expression of CD69, and diminished proliferation. These findings are similar to the altered response kinetics of CD8$^+$ T cells from aged mice (**chapter 2**). Altered *in vitro* response kinetics of CD8$^+$ T cells from aged mice can thus be found in an aging-related subset of human CD8$^+$ T cells and therefore highlights that altered response kinetics is a common aging-related phenomenon. Whether human CD4$^+$ T cells also show comparable response kinetics as found in mice remains to be established. Thus, we conclude that aging slows down the capacity of T cells to express activation markers and to proliferate in response to stimulation.

### *Do slower kinetics of T-cell responses lead to a loss of protective immunity against infectious diseases?*

An important question that now remains is the *in vivo* relevance of these findings. During respiratory infectious diseases, expression of CD69 is required for the egress of lymphocytes to the lymph system [7] and the infected lung [8] and therefore plays a role in the continuation of the immune response.

Moreover, CD69 has been shown to be critical for the generation of memory helper T cells in bone marrow [9]. Slower induction of CD69 expression by T cells may therefore indicate that these T cells egress less efficiently or slower from the blood, which may lead to a delay in the protective immune response. Additionally, induction of CD25 expression is required for formation of the high-affinity IL-2 receptor together with CD122 and CD132, which is a driving factor of T-cell proliferation once in contact with IL-2 [10]. Reduced expression of CD25 may therefore explain impaired T-cell proliferation. Lastly, proliferation of T cells as a result of stimulation (e.g. primary response to foreign antigen) is not only vital for the formation of protective T-cell memory, but also to maintain protection during secondary pathogen encounter [11]. A decline of the capacity of T cells to proliferate may therefore influence both the expansion in the presence of a novel antigen, as well as the memory T-cell response, which may lead to susceptibility to infectious diseases and reduced responses to vaccination at older age. Thus, in the light of viral infections, slower T-cell responses may facilitate rapid viral replication and infection, ultimately leading to decreased protection and more prolonged and/or severe disease.

**Novel Markers for Human CD8$^+$ T-cell Aging: TIGIT and Helios**

The gradual loss of co-stimulatory receptors CD28 and CD27 has previously been linked to differentiation of CD8$^+$ T cells as a consequence of chronic or latent viral infections [12] and aging [13-15]. The current view is that early-differentiated CD27$^+$CD28$^+$ CD8$^+$ T cells convert to intermediate-differentiated CD27$^+$CD28$^-$ cells, before reaching the CD27$^-$CD28$^-$ late-differentiation stage due to repeated activation. Late-differentiated cells are currently regarded senescent T cells due to their impaired proliferation, expression of senescence-associated markers CD57 and killer cell lectin-like receptor G1 (KLRG1), and presence of shortened telomeres [13,15]. Due to these findings, research on T-cell senescence has therefore mainly focused on late-differentiated cells. In **chapter 4**, we identify that co-expression of TIGIT and Helios, two recently described immunosuppression-markers, can be used as a marker for immunosenescent CD8$^+$ T cells, as TIGIT$^+$Helios$^+$ cells showed a trend towards higher expression of CD57 and KLRG1, and functionally showed impaired capacity to proliferate and activate. Interestingly, we found these cells not only accumulating in the late-differentiated T-cell subset at old age, but also to accumulate within the intermediate-differentiated T-cell subset of older adults. The importance of the intermediate-differentiated CD8$^+$ T-cell subset is only recently becoming more apparent, as it has been shown that elevated numbers of the intermediate subset can be used as a biomarker that predicts frailty [16], although the phenotype of

these cells remained to be explored. Our findings now indicate that aging drives immunosenescence of the CD8⁺ T-cell population already in the intermediate-differentiated state and therefore challenge the current view that primarily late-differentiated CD8⁺ T cells are immunosenescent.

## ACCUMULATION OF REGULATORY T CELLS WITH AGE

Accumulation of regulatory T cells (Tregs) has been hypothesized to be one of the causes for reduced T-cell mediated protection observed during aging, as Tregs may dampen protective T-cell responses and their (relative) numbers increase with age. It has indeed been shown in mice that accumulated Tregs impair protective immune responses [17,18], and this is thought to be due to the sheer increase in Treg numbers, as the suppressive capacity remains comparable between Tregs of young and aged mice [17,19] and humans [20,21]. However, the underlying cause of Treg accumulation remained unclear. In **chapter 2** we report accumulation of Tregs in the spleen of aged mice and we show that these cells were particularly enriched within their naive CD4⁺ T-cell subset. Moreover, we found similar accumulation of Tregs within the naive CD4⁺ T-cell pool of mice with a deficiency in the *Ercc1* gene (*Ercc1*$^{-/\Delta 7}$ mice) (**Chapter 3**). *Ercc1*-deficiency compromises several DNA damage repair mechanisms and leads to accumulation of DNA damage [22-24], which is an important hallmark of aging [25,26]. As a consequence, *Ercc1*$^{-/\Delta 7}$ mice age very rapidly and provide a model for cell intrinsic aging that minimizes the effects of aging on a chronological scale. From our findings in **chapter 2 and 3**, we conclude that the accumulation of Tregs within the naive CD4⁺ T-cell subset is at least partly promoted by compromised DNA-repair.

Placing our findings from **chapter 2 and 3** within the current literature, we postulate a new potential mechanism that accounts for the accumulation of Tregs with age (**Figure 1**). Senescent tissue cells are known to accumulate in several tissues and organs of wildtype aged and *Ercc1*-deficient mice [27-29]. These senescent cells do not proliferate anymore, but secrete the senescence-associated secretory phenotype (SASP), which consists of pro-inflammatory factors, including IL-6 [30]. Increased levels of IL-6 have been observed in *Ercc1*-deficient mice [27,31,32] and have also been reported in older adults [33]. Importantly, IL-6 increases in serum of wildtype aged mice and promotes accumulation of Tregs in these mice [34], indicating that elevated IL-6 at old age may be the driver of Treg accumulation. Based on these findings, we propose a potential mechanism in which the rise of senescent cells during aging is accompanied by increased levels of IL-6, that in turn drives the accumulation

of Tregs. Possibly, the accumulation of Tregs is a mechanism to counter the pro-inflammatory microenvironment created by senescent cells secreting the SASP. Local and tissue-specific secretion of pro-inflammatory proteins may therefore also explain why accumulation of Tregs has mostly been reported to occur outside of the peripheral blood [17]. Whether IL-6 directly interacts with Tregs and promotes Treg expansion or whether this occurs indirectly remains to be established. Thus, the findings presented in **chapter 2 and 3** of this thesis provide the link between aging, cellular senescence, inflammaging, and the accumulation of Tregs as shown in **Figure 1**.

Lastly, our hypothesis that Tregs accumulate as a consequence of senescence-induced inflammation may also provide a potential link to the accumulation of regulatory $KIR^+RA^+$ T cells at older age as we report on in **chapter 5**. We found that $KIR^+RA^+$ T cells highly express the transcription factor Helios. Interestingly, a recent study shows that the proportion of $Helios^+$ $CD8^+$ T cells positively correlates with plasma levels of pro-inflammatory factors, such as IP-10, TNF, and IL-6 in HIV-infected individuals [35]. Part of these $Helios^+$ $CD8^+$ T cells described in this report likely comprise $KIR^+RA^+$ T cells. The association between $Helios^+$ $CD8^+$ T cells and pro-inflammatory factors described in this report may indicate that a similar event occurs during the course of aging: increased pro-inflammatory plasma levels increase the abundance of T cells with a regulatory profile to counterbalance the pro-inflammatory environment. It would therefore be highly interesting to assess whether pro-inflammatory cytokine levels are also increased in individuals with higher $KIR^+RA^+$ T-cell proportions. Moreover, promoting or blocking pro-inflammatory factors in future *in vivo* mouse studies should provide evidence whether the proportion of the murine equivalent of $KIR^+RA^+$ T cells alters within a pro-inflammatory microenvironment. Potentially, $KIR^+RA^+$ T cells could be used as a biological marker for an increased inflammatory state.

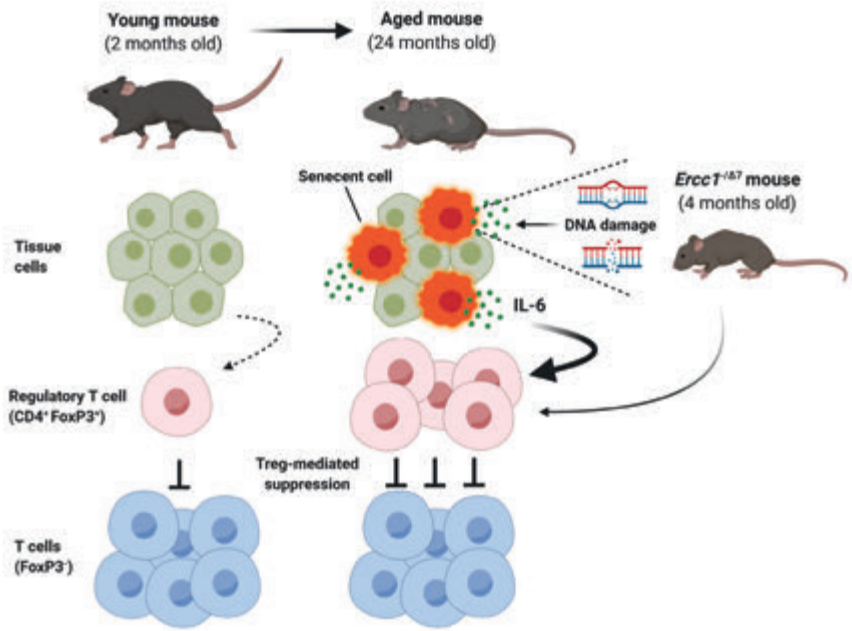



**Figure 1. Potential mechanism of regulatory T-cell accumulation with age.**
Schematic representation of the hypothesized causes for the accumulation of Tregs with age. At young age, tissue cells are fully functional and homogeneous. The balance between the proportions of Treg and helper T cells (Th cells) is shifted to Th cells. During aging, tissue cells increasingly develop into senescent cells that secrete the pro-inflammatory senescence-associated phenotype (SASP), including high levels of IL-6. Presence of pro-inflammatory IL-6 promotes the accumulation of Tregs, leading to a shift in balance to Tregs. Insight into this process is highlighted by the *Ercc1*$^{-/\Delta 7}$ mouse model, which is a model for compromised DNA-repair induced senescent cells, increased levels of IL-6, and accumulation of Tregs.



## VIRTUAL MEMORY T CELLS: A SUBSET COMPRISING TWO DIFFERENT CELL TYPES

### Virtual Memory T cells

In addition to ongoing research on more established T-cell subsets, novel T-cell subsets are still being discovered. A recently described CD8$^+$ T-cell subset are 'virtual memory T cells'. Virtual memory cells were initially identified in wildtype and germ-free mice, and were described as CD8$^+$ T cells that express memory phenotype and functionality without having encountered cognate antigen [36]. This led to the hypothesis that virtual memory cells arise via homeostatic proliferation in response to cytokines derived from other cells in their microenvironment. Virtual memory cells have been shown to control *Listeria monocytogenes* infection in mice [37] in an IL-15-dependent manner acting

as bystander cells [38]. Moreover, virtual memory cells with characteristics of cellular senescence accumulate with age in unprimed aged mice [39]. Together, these observations imply that virtual memory cells are capable of contributing to protective immunity, which is subject to age-related changes that may diminish their effectivity despite their accumulation with age.

Virtual memory cells have also been identified in humans, characterized by expression of CD45RA$^+$ CD8$^+$ T cells that express several Killer-cell Immunoglobulin like Receptors (KIR) *and/or* the inhibitory natural-killer cell (NK cell) receptor NKG2A [40]. Similar to findings in mice, human virtual memory cells accumulate with age and show reduced capacity to proliferate [41]. However, further phenotypical and functional characterization of human virtual memory cells and their clinical relevance in the context of infectious disease in older adults remained unexplored.

**Identification of KIR$^+$RA$^+$ and NKG2A$^+$RA$^+$ CD8$^+$ T cells within the Human Virtual Memory Subset**

In **chapter 5** we discovered that the accumulation of virtual memory cells with age was caused by an increase in the proportion of KIR$^+$ NKG2A$^-$ cells, whereas we found a decline in KIR$^-$ NKG2A$^+$ cells with age (**Figure 2A**). These findings indicated that the human virtual memory subset may actually consist of two different cell subsets. We show that these cells indeed express differing transcriptional phenotypes and that the transcriptional phenotype of CD45RA$^+$ KIR$^+$ NKG2A$^-$ cells (which we named KIR$^+$RA$^+$ T cells based on their distinctive co-expression of KIR and CD45RA) was enriched for regulatory markers (**Figure 2B**). These findings encouraged us to further explore the phenotype and functionality of KIR$^+$RA$^+$ T cells, as these cells accumulated with age and appeared to be a relatively large subset amongst the CD8$^+$ T-cell population. At the protein level, we found that KIR$^+$RA$^+$ T cells express a phenotype that could be linked to regulatory T cells, as they expressed TIGIT, Helios, and CD122 [42-46]. Furthermore, we found that KIR$^+$RA$^+$ T cells have a low proliferative capacity and low IL-2 production.

Interestingly, we found that KIR$^+$RA$^+$ T cells suppressed proliferation of other CD8$^+$ T cells and therefore can be considered to be a regulatory cell subset. Moreover, we found KIR$^+$RA$^+$ T cells to be highly activated in the blood of older adults in response to acute respiratory viral infection. Importantly, activation of KIR$^+$RA$^+$ T cells was associated with prolonged presence of symptoms in older adults infected with Influenza A virus, indicating that these regulatory KIR$^+$RA$^+$ T cells may aggravate respiratory disease following viral infection, possibly by suppressing anti-viral CD8$^+$ T cells.

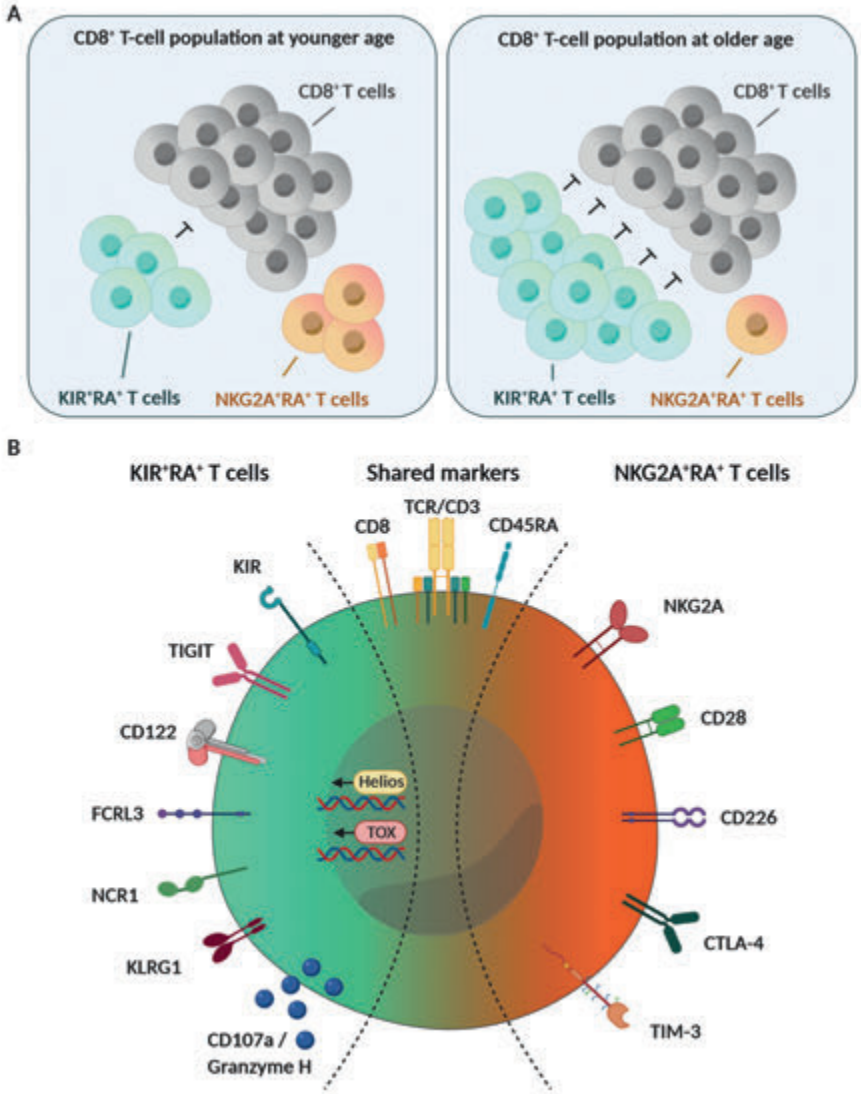



**Figure 2. The redefined human virtual memory cell subset: KIR$^+$RA$^+$ T cells and NKG2A$^+$RA$^+$ T cells.**

(**A**) Schematic representation of the CD8$^+$ T-cell population in young individuals and aged individuals, indicating accumulation of suppressive KIR$^+$RA$^+$ T cells (CD45RA$^+$ KIR$^+$ NKG2A$^-$) and the decline of NKG2A$^+$RA$^+$ T cells (CD45RA$^+$ KIR$^-$ NKG2A$^+$) with age. (**B**) Based on our combined RNA sequencing, flow cytometric analyses, and stimulation assays, we present KIR$^+$RA$^+$ T cells and NKG2A$^+$RA$^+$ T cells as two phenotypically distinct subsets. We found KIR$^+$RA$^+$ T cells to express markers related to regulation (TIGIT, Helios, CD122, FCRL3), killing (CD107a, Granzyme H, NCR1), and exhaustion (TOX, KLRG1), whereas NKG2A$^+$RA$^+$ T cells express markers related to co-stimulation (CD28, CD226), and co-inhibition (CTLA-4, TIM-3).

Finally, we could extend these findings to COVID-19, as we had the unique opportunity at the start of the COVID-19 pandemic to assess KIR$^+$RA$^+$ T cells in individuals infected with SARS-CoV-2, in whom KIR$^+$RA$^+$ T cells became a highly activated cell subset during acute SARS-CoV-2 infection. Based on our efforts, we know how to characterize both KIR$^+$RA$^+$ and NKG2A$^+$RA$^+$ CD8$^+$ T cells (**Figure 2B**) and we defined a new regulatory CD8$^+$ T-cell subset that adds to the current classification of T-cell subsets and may contribute to aggravation of viral respiratory disease in older adults.

**Potential Suppressive Mechanisms of KIR$^+$RA$^+$ T cells**

An important question that remains unanswered is by what mechanism KIR$^+$RA$^+$ T cells suppress proliferation of other CD8$^+$ T cells and thereby potentially impair anti-viral CD8$^+$ T-cell responses. It is important to identify how KIR$^+$RA$^+$ T cells exert suppression. This to eventually develop strategies to enhance protection and/or treat severe disease in older adults if KIR$^+$RA$^+$ indeed suppress protective anti-viral responses. Here, we speculate on potential ways that KIR$^+$RA$^+$ T cells exert immunosuppressive action based on the characteristic markers we found in **chapter 5**. These hypotheses are also summarized in **figure 3**.

*The co-inhibitory receptor TIGIT*

To co-inhibitory receptor TIGIT is primarily expressed by CD4$^+$ Tregs [47] as well as by part of the CD8$^+$ T-cell population [48,49] and competes with the co-stimulatory receptor CD266 for binding to the same two ligands CD155 and CD112 [50,51]. We found that KIR$^+$RA$^+$ T cells highly express TIGIT, while expression of CD226 was low, which indicates that binding of KIR$^+$RA$^+$ T cells to CD155 and/or CD112 is primarily through TIGIT. The result of interaction of TIGIT with its ligand depends on the cell type that expresses TIGIT. For effector CD4$^+$ and CD8$^+$ T cells TIGIT-ligand interactions results in cell intrinsic impaired proliferation and effector function [48,50,52], whereas for CD4$^+$ Tregs this interaction promotes suppression of the immune response [43,47,48]. As KIR$^+$RA$^+$ T cells are TIGIT$^+$, suppressive mechanisms mediated by TIGIT in CD4$^+$ Tregs may point towards potential mechanisms of KIR$^+$RA$^+$ T cell-mediated suppression. TIGIT-mediated suppression by CD4$^+$ Tregs is currently thought to be an indirect suppressive mechanism. First, binding of TIGIT to CD155 on dendritic cells resulted in an increase of IL-10 production and a decrease of IL-12 production by dendritic cells and conversion of the dendritic cell into a tolerogenic dendritic cell [51]. Second, interaction of TIGIT$^+$ CD4$^+$ Tregs with effector CD4$^+$ T cells indirectly suppressed effector-cell proliferation, as CD4$^+$ Tregs secreted immunosuppressive IL-10 and fibrinogen-like protein 2 (Fgl2)

[47]. Thus, these reports suggest that KIR$^+$RA$^+$ T cells may interact with dendritic cells and other T cells to indirectly suppress the immune response (**Figure 3A**).

*The IL-15 receptor*

We show that KIR$^+$RA$^+$ T cells express high levels of CD122, the β-chain of the IL-2 and IL-15 receptors. In mice, it has been shown that virtual memory T cells are highly responsive against IL-15 and require IL-15 for their development [38,53], which indicates that KIR$^+$RA$^+$ T cells may interact with IL-15 more efficiently compared to other T cells. The response of virtual memory cells to IL-2 is still unclear and still remains to be addressed. Similar to virtual memory cells, CD8$^+$ Tregs in mice were reported to be dependent on IL-15 for their development and suppressive function [54], indicating that the capacity to suppress may depend on IL-15 signaling. CD4$^+$ Tregs can suppress immune responses by acting as a 'sink' for IL-2 [55]. Hypothetically, high expression of CD122 may indicate that KIR$^+$RA$^+$ T cells act as an IL-15 'sink': extracting IL-15 that is present in the cellular microenvironment, thereby scavenging the IL-15 out of the system so that it can't be used by other T cells (**Figure 3B**). Absence of IL-15 during influenza A virus infection in IL-15 knockout mice reduced deadly lung pathology inflicted by effector CD8$^+$ T-cell responses [56], which may indicate that KIR$^+$RA$^+$ T cells act as regulators that may limit lung pathology by indirectly suppressing T effector cells through scavenging of IL-15. Whereas we observed that a higher activation status of KIR$^+$RA$^+$ T cells positively correlated with prolonged disease, it may be that the IL-15 levels exceeded the 'sink capacity' of KIR$^+$RA$^+$ T cells allowing effector T cells to kill infected lung cells leading to prolonged presence of symptoms. Measuring the impact of IL-15 *in vitro* on KIR$^+$RA$^+$ T-cell responses as well as measuring the level of IL-15 present in older adults suffering from viral respiratory infection may provide further insight into this process.

*Killing of other CD8$^+$ T cells*

Suppression of T-cell responses through killing of T cells is one of the mechanisms that has been reported for CD4$^+$ Tregs [57,58]. In these reports, CD4$^+$ Tregs targeted activated CD4$^+$ T cells, CD8$^+$ T cells, monocytes, and dendritic cells, causing cell death of these target cells mediated by Granzyme B or perforin in a contact-dependent manner. Although we did not find Granzyme B or perforin to be enriched in KIR$^+$RA$^+$ T cells in an unstimulated state, we found increased transcripts for Granzyme H. Moreover, we found highly increased levels of the degranulation marker CD107a in KIR$^+$RA$^+$ T cells after *in vitro* stimulation. Granzyme H is closely related to Granzyme B and can cause target-

cell killing similarly [59], indicating a potential killing mechanism of KIR$^+$RA$^+$ T cells. Increased levels of CD107a is indicative of release of granules containing cytotoxic factors, such as granzymes and perforins [60,61], indicating that KIR$^+$RA$^+$ T cells may suppress through secretion of these factors (**Figure 3C**).

Additionally, KIR$^+$RA$^+$ T cells showed enrichment for the natural cytotoxicity triggering receptor 1 (NCR1/NKp46). NCR1 has been shown to promote expression of the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) [62], which can induce target-cell apoptosis upon binding to death receptor-5 (DR5) [63] (**Figure 3D**). Interestingly, activated CD4$^+$ Tregs have been reported to express TRAIL and induce cell death through TRAIL-DR5 interaction [64]. Together, these findings indicate that the suppressive capacity of KIR$^+$RA$^+$ T cells we describe in **chapter 5** may possess several mechanisms that point towards target cell killing. It would be highly relevant for future research to address the potential target cell killing mechanisms that may result in suppression.

**Similarities between CD8$^+$ Tregs , KIR$^+$RA$^+$ T cells, and NKG2A$^+$RA$^+$ T cells**

The existence and role of CD8$^+$ Tregs in humans is controversial, as CD8$^+$ T cells are mostly regarded a population of cells that are highly effective killer cells that target infected cells or tumor cells. In **chapter 5**, we provide evidence for a subset of CD8$^+$ Tregs in humans which we named KIR$^+$RA$^+$ T cells. But how are KIR$^+$RA$^+$ T cells triggered to exert suppression? And what is their relationship to NKG2A$^+$RA$^+$ T cells? Interestingly, we found remarkable similarities between murine CD8$^+$ Tregs and human KIR$^+$RA$^+$ T cells which may provide answers to this question (**Figure 4**).

*Phenotypical and functional links between KIR$^+$RA$^+$ T cells and CD8$^+$ Tregs*

A subset of CD8$^+$ Tregs identified in mice express receptors belonging to the Ly49 receptor family [65]. Ly49 receptors are the murine analogue of human KIRs, and both families are characterized by activating and inhibitory receptors [65,66]. In mice, 90% of the Ly49$^+$ CD8$^+$ Tregs express inhibitory Ly49F, which is thought to bind it's ligand (possibly MHC I [67]) and prevent overactivation of these cells to ensure their survival [68]. These findings in mice may indicate that survival of KIR$^+$RA$^+$ T cells humans may also be mediated by binding of KIR to MHC-I, more specifically by binding of KIR to HLA-B or HLA-C [69]. In **chapter 5**, we assessed bulk KIR expression and we could therefore not further dissect differences in the expression of either activating or inhibitory KIRs, although the human variant of Ly49F, KIR3DL1, was present in our bulk analysis of KIRs. Possibly, isolating KIR$^+$RA$^+$ T cells that express KIR3DL1 may specifically select

for regulatory KIR$^+$RA$^+$ T cells. Additionally, strikingly similar to our findings on KIR$^+$RA$^+$ T cells, murine CD8$^+$ Ly49$^+$ Tregs express CD122 and the transcription factor Helios [42,65]. Expression of Helios was shown to be vital for the survival and suppressive function of CD8$^+$ Ly49$^+$ Tregs, as deletion of Helios in these cells results in loss of suppression and in autoimmune disease [42]. Together, these findings indicate that Ly49$^+$ CD8$^+$ Tregs are the murine equivalent of human KIR$^+$RA$^+$ T cells and that expression of KIR and Helios may promote survival of KIR$^+$RA$^+$ T cells (**Figure 4**).

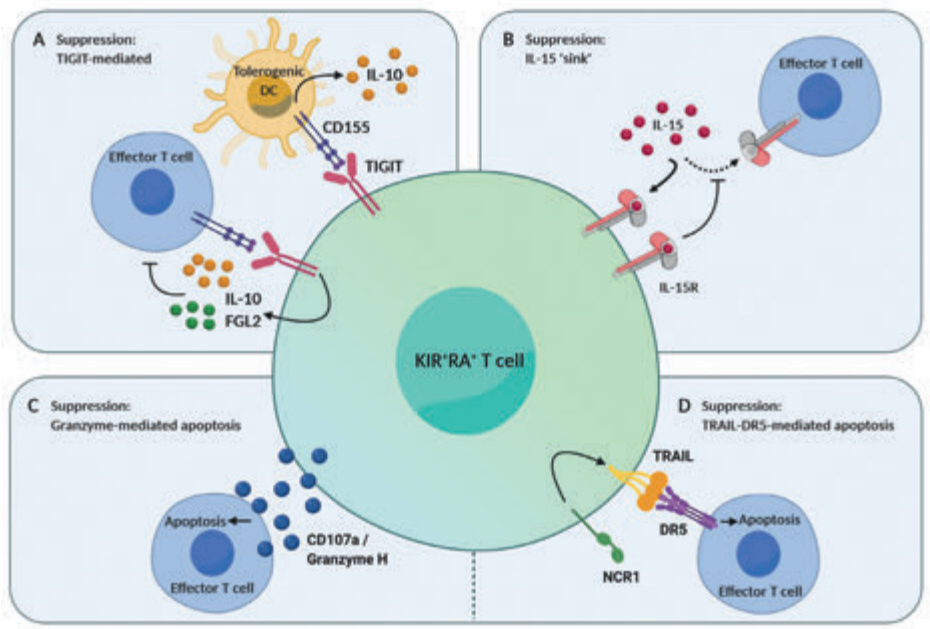



**Figure 3. Hypothetical suppression mechanisms of KIR$^+$RA$^+$ T cells.**
Schematic representation of potential mechanisms for suppression of effector T cells by KIR$^+$RA$^+$ T cells. (**A**) High expression of the co-inhibitory receptor TIGIT enables binding to its ligand CD155, resulting in two indirect suppressive mechanisms: first, enticing CD155-expressing dendritic cells (DCs) to convert to tolerogenic DCs that produce suppressive IL-10, and second, suppressing CD155-expressing effector T cells by secretion of IL-10 and FGL2. (**B**) High expression of CD122 indicates that KIR$^+$RA$^+$ T cells are capable of scavenging IL-15 from the cellular microenvironment, thereby acting as an IL-15 sink and preventing activation and proliferation of effector T cells. (**C**) KIR$^+$RA$^+$ T cells highly express degranulation marker CD107a after activation and the transcriptome of KIR$^+$RA$^+$ T cells is enriched for Granzyme H mRNA, indicating Granzyme-mediated killing of effector T cells. (**D**) Expression of NCR1 enhances expression of TRAIL, which enables binding of KIR$^+$RA$^+$ T cells to DR5 present on effector T cells, resulting in killing of the effector T cell.

*NKG2A-HLA-E interaction counteracts suppressive responses in CD8⁺ Tregs*
Murine CD8⁺ Ly49⁺ Tregs can prevent autoantibody-induced autoimmune disease by binding Qa-1b (HLA-E in humans) present on dysregulated follicular helper T cells [54,65]. Interestingly, a subset of CD8⁺ Tregs recognizing HLA-E has also been shown in humans and failure of these cells to recognize HLA-E may lead to type-1 diabetes [70]. Moreover, senescent human fibroblasts have recently been shown to express HLA-E, which interacted with NKG2A on CD8⁺ T cells and thereby prevented fibroblast elimination [71]. Thus, HLA-E can be bound by either the T-cell receptor (TCR) or the NKG2A/CD94 receptor of CD8⁺ Tregs, with different outcomes: binding of the TCR to HLA-E on target T cells leads to suppression of target T cells by killing of these T cells in mice [72], whereas this suppressive mechanism can be inhibited when NKG2A binds HLA-E, as NKG2A-signalling abrogates TCR signaling [73] (**Figure 4**). The peptide presented in HLA-E that is recognized by human CD8⁺ Tregs is still unknown, but heat shock protein 60 (HSP60) as well as self-antigens are thought to be presented by HLA-E and recognized by CD8⁺ Tregs [68,70,74]. T cells can upregulate expression of HSP60 presented in MHC class I and II after activation and can in turn be suppressed by Tregs that recognize MHC/HSP60 complexes [75]. Thus, these findings suggest that the interaction of NKG2A with HLA-E limits the TCR-mediated capacity of CD8⁺ Tregs to suppress, whereas interaction of KIR with its ligand results in survival by preventing overactivation (**Figure 4**).

*Do NKG2A⁺RA⁺ T cells convert to KIR⁺RA⁺ T cells during aging?*
In **chapter 5**, we show that the proportion of NKG2A⁺RA⁺ T cells declines at older age, whereas the proportion of KIR⁺RA⁺ T cells increases. We describe NKG2A⁺RA⁺ and KIR⁺RA⁺ T cells as two independent cell subsets that were previously regarded to be one. However, we cannot exclude the possibility that KIR⁺RA⁺ T cells present at older age are derived from NKG2A⁺RA⁺ T cells that lost NKG2A and gained KIR expression during aging. An argument for this hypothesis can be derived from natural killer cells (NK cells). KIR and NKG2A are classical NK-cell markers and it has been shown that NKG2A⁺ NK cells may lose NKG2A during differentiation and start expressing KIRs [76,77]. KIR⁺ NK cells, but not NKG2A⁺ NK cells, degranulate when in contact with HLA-E expressing cells, indicating killing of these HLA-E⁺ cells by KIR⁺ NK cells. Based on our findings on KIR⁺RA⁺ T cells in the context of the studies mentioned above, we established a hypothesis (**Figure 4**). We propose that due to a yet to be identified process that is accelerated by aging, NKG2A⁺ CD8⁺ T cells may lose expression of NKG2A and gain expression of KIR. Gain of KIR expression ensures

survival of KIR$^+$RA$^+$ T cells by preventing overactivation. Remaining KIR$^+$RA$^+$ T cells can actively suppress other T cells as the negative feedback through NKG2A has disappeared. Moreover, we speculate that the transcription factor Helios contributes to survival of KIR$^+$RA$^+$ T cells as observed in murine CD8$^+$ Tregs.

As a side note, in contrast to our finding that the proportion of NKG2A$^+$RA$^+$ T cells declines with age, a recent study by Pereira *et al.* reported an increase of NKG2A$^+$ cells within the highly differentiated CD27$^-$CD28$^-$ CD8$^+$ T-cell subset at older age [71]. Re-analysis of our data to match the analysis of Pereira *et al.* still indicated a decline of NKG2A$^+$ CD8$^+$ T cells within the CD27$^-$CD28$^-$ cell subset with age (data not shown). One possible explanation for these contrasting results may be the CMV status of the study participants. All donors we included in our study were seronegative for CMV to exclude bias towards CMV-induced changes to CD8$^+$ T cells, such as induction of NKG2A expression by CD8$^+$ T cells [78]. CMV status was not reported in the study by Pereira *et al.*

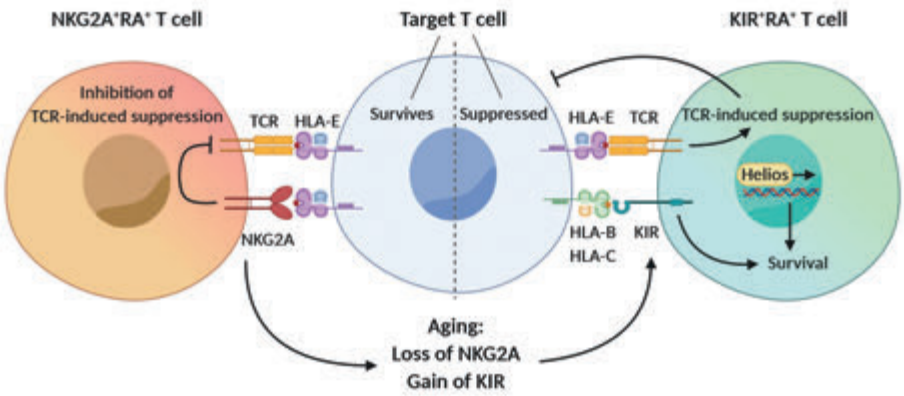



**Figure 4. Molecular interactions of NKG2A$^+$RA$^+$ and KIR$^+$RA$^+$ T cells with their target T cell.** Schematic representation of the hypothesized activation of NKG2A$^+$RA$^+$ T cells and KIR$^+$RA$^+$ T cells, proposed molecular interactions with their target T cell, and their hypothesized differentiation from NKG2A$^+$RA$^+$ into KIR$^+$RA$^+$ T cells during aging. In young individuals, the T-cell receptor (TCR) interacts with HLA-E on the target T cell, which leads to initiation of a suppressive response, potentially through killing. However, the NKG2A receptor may also interact with HLA-E, resulting in the inhibition of TCR activation and neutralization of the suppressive response. As a result, the target T cell survives. With age, expression of NKG2A is lost, whereas expression of KIR is gained. The absence of NKG2A allows full TCR signaling, resulting in suppression of the target cell, possibly by killing. Expression of inhibitory KIRs may interact with HLA-B or HLA-C on the target T cell, preventing overactivation of KIR$^+$RA$^+$ T cells and thereby contributes to survival of this cell subset. Similarly, KIR$^+$RA$^+$ T cells gain expression of the transcription factor Helios promoting survival.

### KIR$^+$RA$^+$ T cells in Infectious Disease: a Double-Edged Sword

In **chapter 5** we show that KIR$^+$RA$^+$ T cells can exert suppression and that the presence of activated KIR$^+$RA$^+$ T cells in peripheral blood positively correlates with the total duration of symptoms in influenza A virus-infected older adults. We therefore speculate that suppression of anti-viral CD8$^+$ T cells by KIR$^+$RA$^+$ T cells may lead to prolonged illness, thereby concluding that the presence of activated KIR$^+$RA$^+$ T cells during infection is unfavorable. This hypothesis is fueled by studies showing that CD4$^+$ Tregs are capable of suppressing protective CD8$^+$ T cell responses in influenza A-infected mice [79], and genetically disrupting CD8$^+$ Tregs enhances the T-cell response against acute viral infection [80]. However, it may still be that presence of highly activated KIR$^+$RA$^+$ T cells prevented the occurrence of even longer duration of symptoms: what would have happened if these KIR$^+$RA$^+$ T cells were not present? Hypothetically, absence of highly activated KIR$^+$RA$^+$ T cells as regulatory cells may as well have resulted in uncontrolled killing by effector CD8$^+$ T cells that fight virus-infected lung cells, potentially resulting in lung pathology and more severe disease. Arguing for this alternative hypothesis is a recent study showing that the co-inhibitory marker TIGIT limits pathological tissue damage after viral infection in mice [81]. This suggests that presence of TIGIT$^{Hi}$ KIR$^+$RA$^+$ T cells may prevent excessive lung damage. Lastly, it remains to be established whether KIR$^+$RA$^+$ T cells only suppress other activated effector T cells, or whether they can kill virally infected cells themselves as well. Recent evidence from research on HIV suggests that the human virtual memory cell subset may limit the viral reservoir by eliminating HIV-infected CD4$^+$ T cells [82], indicating that KIR$^+$RA$^+$ T cells may be a highly important cell subset in maintaining balance within the immune system by killing of infected cells as well as suppressing other T cells. Thus, future studies in humans and *in vivo* studies in mice should address whether the presence or absence of KIR$^+$RA$^+$ T cells in the lung is favorable or unfavorable during viral infection and whether there is a tipping point between the number of KIR$^+$RA$^+$ T cells being activated determining health or disease.

### KIR$^+$RA$^+$ T cells: Bystander or Antigen-Specific T cells?

Whether KIR$^+$RA$^+$ T cells are bystander cells during viral infection or are activated due to recognition of viral antigens remains to be elucidated. Three arguments can be put forward why it is likely that KIR$^+$RA$^+$ T cells are bystander cells during viral infection. First, we found that a large proportion of KIR$^+$RA$^+$ T cells are activated as a consequence of influenza A virus infection (26% on average), as well as during infection with other respiratory viruses (averages ranging from 6% to 23%). These proportions are more than expected for an

antigen-specific response. Second, we found that activated KIR$^+$RA$^+$ T cells are not specific for the highly conserved and immunodominant influenza A epitope GILGFVFTL, which may indicate a bystander role for these cells. However, whereas the response of CD8$^+$ T cells is immunodominant when GILGFVFTL is restricted by HLA-A*02, we cannot exclude that KIR$^+$RA$^+$ T cells recognize the GILGFVFTL epitope restricted by HLA-C*08 [83] via interaction with their KIR [66]. Third, to further provide evidence for a bystander role of KIR$^+$RA$^+$ T cells, we also assessed their presence and activation status in SARS-CoV-2 infected adults. Since humans were considered naive to this new virus at the time of our study, we would not expect a large pool of specific memory T cells responding during the event of COVID-19 in the patients included in our study. Although it has been shown that some individuals already have SARS-CoV-2 specific CD8$^+$ T cells prior to SARS-CoV-2 infection, this is still a minority of individuals (20%) [84]. In all SARS-CoV-2 infected individuals included in our study we found activated KIR$^+$RA$^+$ T cells, indicating bystander activation. Still, caution should be considered with regard to the specificity of these cells and our findings warrant new immune studies to investigate potential antigen-specificity of KIR$^+$RA$^+$ T cells.



## INTERVENING WITH AGE-RELATED CHANGES TO T CELLS VIA MTOR

Currently, therapeutic and preventive targeting of age-related events to reduce inflammation is widely being explored, with the ultimate goal to improve the response to vaccination, infection, and a healthy life span. The mammalian target of rapamycin (mTOR) pathway is an important driver of cellular aging [85] and is a well-known target in anti-aging research. For example, blocking of mTOR with rapamycin can expand life- and health span in mice [86-88], can improve the response to influenza vaccination in elderly individuals [89], and can reduce secretion of the SASP, including IL-6 *in vitro* [90]. In **chapter 3** of this thesis, we assessed whether blocking of mTOR by rapamycin could slow down the effect of aging by compromised DNA damage repair on the phenotype and response of T cells. The main conclusion of our study is that the majority of changes in T cells we observed due to compromised DNA damage repair cannot be reversed by inhibition of mTOR activation. For example, we show that the accumulation of Tregs in *Ercc1*$^{-/\Delta 7}$ mice cannot be reversed by rapamycin, but rather promoted these Tregs to express CD25 and PD-1 which may indicate improved immunosuppressive capacity of these cells [91]. However, the decline in proportion of naive T cells and the increase in memory T cells observed in WT

aged and *Ercc1*$^{-/\Delta 7}$ mice was partially dependent of mTOR activation. Treating *Ercc1*$^{-/\Delta 7}$ mice with rapamycin increased and/or preserved the proportion of naive T cells. Higher frequencies of naive T cells may lead to better memory T-cell mediated protection, as observed previously in rapamycin intervention studies [92,93]. The relatively minor effect of rapamycin treatment we observed on age-related T-cell changes does not necessarily imply that treatment with rapamycin is ineffective. One of the limitations of our study is that we used one dose of rapamycin, whereas it has been shown in lifespan studies that different doses of rapamycin lead to differing lifespan in WT mice [87,94]. This may indicate that different doses of rapamycin may affect T cells differently. Moreover, the lifespan of *Ercc1*$^{-/\Delta 7}$ mice is short compared to the lifespan of wild type mice. Therefore, the duration of rapamycin treatment in *Ercc1*$^{-/\Delta 7}$ mice was relatively short compared to other studies in wild type mice, which may have a different impact on T cells. The dose and duration of rapamycin that is administered and its effect on T cells therefore warrants future immune studies. Preventing intracellular DNA damage or results thereof over the course of life may help to improve immune protection at old age.

## THE SENSE IN BECOMING SENESCENT OR EXHAUSTED

The general term 'immunosenescence' comprises all age-related changes that occur to the immune system, including those that occur in the T-cell population. Two hallmarks of immunosenescence that occur to T cells during aging are T-cell senescence and T-cell exhaustion. These terms are partly overlapping, as they both mark T cells with impaired functionality, such as reduced proliferative capacity. However, senescent T cells are mostly viewed as cells that express senescence-associated markers CD57, KLRG1, and γH2AX (indicating DNA damage), whereas exhausted T cells have been characterized as T cells that constitutively express co-inhibitory receptors. The increase in senescent and exhausted T cells is a major topic in the field of immune aging. It is generally accepted that increased presence of senescent and exhausted T cells promotes viral persistence, susceptibility to infectious disease, and more severe disease following infection at older age. However, a final important factor to address is whether sustained expression of co-inhibitory markers by T cells could be beneficial to the host, within and outside the field of infectious diseases. What is the purpose of expression of these markers? And is it wise to interfere with these co-inhibitory markers to elicit stronger responses against vaccines and infectious diseases?

First, increased and sustained expression of co-inhibitory markers by T cells is a widely reported observation to occur during aging and is often approached as disadvantageous. However, this appears to be more subtle. A study that strikingly illustrates the importance of the co-inhibitory marker PD-1 has shown that genetically disrupting expression of PD-1 leads to overstimulation and severe exhaustion of anti-viral T cells [95]. This study led to the hypothesis that the PD-1 inhibitory pathway, as well as those of other co-inhibitory receptors may have evolved to prevent excessive damage induced by persistent infections by limiting T-cell function. These findings may therefore also highlight the importance of TIGIT$^+$ CD8$^+$ T cells we observe at older age (**chapters 4 and 5**). Besides that TIGIT$^+$ CD8$^+$ T cells mark a subset of regulatory T cells (**chapter 5**), we also observe sustained expression of TIGIT on other CD8$^+$ T cells, that are considered to be exhausted [49]. Possibly, sustained expression of TIGIT on these cells may promote their survival by preventing over-stimulation of these cells, and may also limit pathology following viral infection [81]. By this way, these cells survive, can still act as cells that protect against infectious disease, as well as reducing the collateral immune pathology done to the host.

Second, in this thesis we focus on aging of T cells in an infectious disease setting. Broadening interpretation of hallmarks of immunosenescence from the fields of tumor immunology and autoimmunity however, can further improve our insight and even shows potential benefits of cellular exhaustion. A recent study has strikingly shown that CD8$^+$ T cells with features of exhaustion indeed associate with poor clearance of viral infection, but on the other hand these cells predicted a better prognosis in several autoimmune diseases [96]. Additionally, the field of tumor immunology taught many lessons on exhaustion and senescence, and co-inhibitory receptors are often blocked to elicit anti-tumor responses. However, these therapies may also induce a break of self-tolerance, resulting in autoimmune disease [97]. Thus, taking these views into account, targeting of exhaustion and senescence related markers on T cells in the context of aging and infectious disease should aim for achieving a delicate balance between the T-cell response against infectious diseases and tumors, whilst not breaking tolerance to prevent autoimmune disease (**Figure 5**).

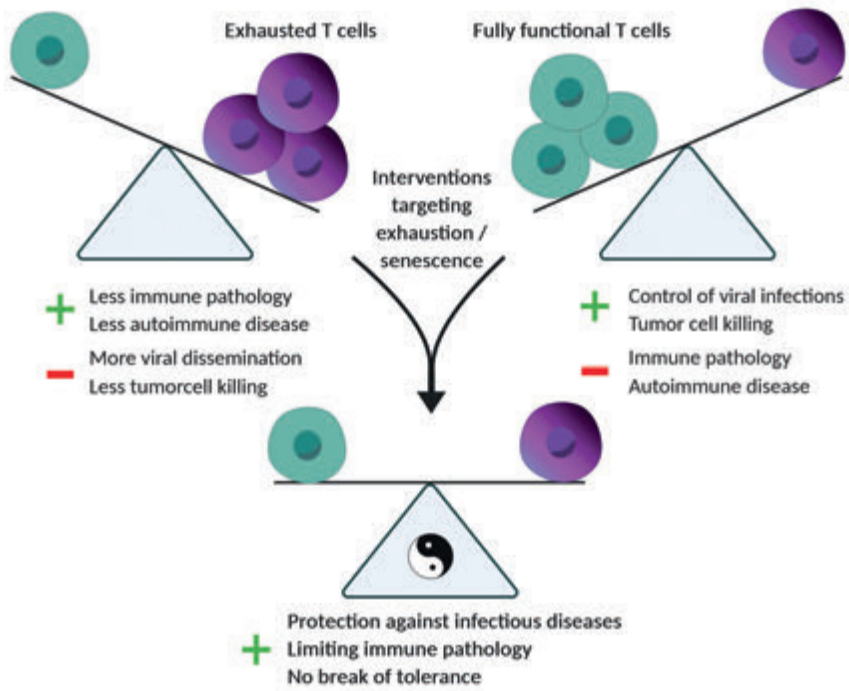



**Figure 5. Targeting age-related T-cell exhaustion and senescence should aim to achieve balance.**
Schematic representation of the balance between fully competent T cells and exhausted or senescent T cells, which may be regarded as immunoregulatory cells. As indicated, a high amount of exhausted T cells commonly observed with aging dampens the host's response to infectious diseases and limits killing of tumor cells, although pathogen-induced immune pathology and autoimmune responses caused by fully functional T cells is limited. In contrast, a low number of exhausted T cells may provide superior killing of virally infected cells and tumor cells, but at the risk of increased immune pathology and breaking tolerance. Interventions targeting exhausted or senescent T cells should aim to stabilize this balance, providing optimal protection against infectious diseases and tumors, whilst limiting immune pathology and autoimmune disease.

## FUTURE PERSPECTIVES

In this thesis, we provide new insights into the impact of aging on T cells in mice and humans. As we show the importance of studying the change of T-cell response kinetics with age, it would be of great significance to address the *in vivo* impact of delayed T-cell activation and impaired T-cell proliferation on the response to vaccination or infection. It has indeed been speculated that minor changes in these tightly regulated processes may have a great negative impact on T-cell mediated protection [98]. Thus, assessing the role of slower and lower

induction of activation markers on T cells may provide an answer to whether decreased protection against infectious diseases in older adults is caused by impaired activation and proliferative T-cell responses.

Throughout this thesis, we report an accumulation of regulatory-cell features in the T-cell population at older age and identify a new aging-related regulatory $CD8^+$ T-cell subset. One prominent question that remains unanswered is whether immunosenescent T cells expressing regulatory properties such as the $KIR^+RA^+$ T cells we define only act as suppressive cells during viral infection, or whether they also contribute to clearance of virally infected cells. Additionally, it would be of high interest to assess how $KIR^+RA^+$ T cells as well as other characteristics of immune suppression develop with progressing age and if so, whether this process could be invoked or halted. One of the possibilities to investigate is whether the accumulation of $KIR^+RA^+$ T cells can be accelerated by pro-inflammatory factors that have been reported to increasingly circulate at older age. Moreover, our research provides new links to potential intervention strategies to target $KIR^+RA^+$ T cells, such as Helios and TIGIT. Targeting these molecules may provide insight into survival and suppressive mechanisms involved in this $CD8^+$ T-cell subset. Together, these insights may provide new targets for preventive or therapeutic strategies to either block or promote $KIR^+RA^+$ T cells, not only in the context of aging, but also in the fields of cancer, transplantation, and autoimmunity.



## CONCLUDING REMARK

The search for novel hallmarks of T-cell aging is one of the most important fundamental challenges in the immune aging field. In this thesis, we provide new insights in murine and human T-cell aging which enhances understanding of the impact of aging on T cells. Our findings highlight the importance of studying the altered response kinetics of T cells at older age and the rise of regulatory-cell features in the T-cell population, which both may contribute to impaired protective immunity against infectious diseases in older individuals. Ultimately, these fundamental findings may provide new starting points for other fundamental, translational, and clinical studies that focus on vaccination of older adults, infectious disease resistance, and healthy aging.

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A

# APPENDIX

# NEDERLANDSE SAMENVATTING

Het *immuunsysteem*, ook wel het afweersysteem genoemd, vormt een fascinerend geheel waar je een leven aan wetenschappelijk onderzoek aan zou kunnen wijden. Het immuunsysteem is te vergelijken met een puzzel van duizenden stukjes, waarvan je meestal niet eens weet hoe de stukjes eruitzien. De taak van een onderzoeker in de immunologie is te achterhalen hoe de stukjes eruitzien en hoe deze vervolgens in het grote geheel passen. Naast de grote rol van het immuunsysteem in kanker en auto-immuunziekten zoals reuma, speelt het immuunsysteem een onmisbare rol in de strijd tegen veroorzakers van *infectieziekten*, veroorzaakt door bijvoorbeeld virussen en bacteriën. De afgelopen decennia heeft wetenschappelijk onderzoek naar het immuunsysteem (immunologie) grote stappen gemaakt waardoor bijvoorbeeld vaccinaties tegen infectieziekten en nieuwe medicatie hebben bijgedragen aan verbeteringen van de kwaliteit van leven. Er wordt bijvoorbeeld geschat dat vaccinatie per jaar twee tot drie miljoen doden voorkómt. Dit geeft aan hoe belangrijk immunologisch onderzoek is om uiteindelijk bij te dragen aan een gezond leven. Maar er valt ook nog veel te ontdekken over het immuunsysteem. De complexiteit van het immuunsysteem vraagt om creativiteit van onderzoekers en dat maakt waarom immunologisch wetenschappelijk onderzoek ook zo interessant is en blijft.

De complexiteit van het immuunsysteem komt doordat de samenstelling en de werking van het systeem constant verandert. Een grote factor die bijdraagt aan de continue veranderingen is *veroudering*: hoe ouder een persoon wordt, hoe meer veranderingen er plaatsvinden binnen de verschillende cellen van het immuunsysteem. Er wordt gedacht dat deze veranderingen de vatbaarheid voor infectieziekten toe laat nemen naarmate mensen ouder worden. Daarnaast blijkt ook dat ouderen langer en vaak ernstiger ziek zijn ten gevolge van infectieziekten in vergelijking met jongeren. Voorbeelden van infectieziekten zijn virussen die luchtweginfecties en longontstekingen kunnen veroorzaken, zoals het influenzavirus (de veroorzaker van de griep) en het nieuwe coronavirus (SARS-CoV-2) wat de veroorzaker is van COVID-19. Vooral onder ouderen zorgen deze virussen voor ernstige luchtweginfecties met opnames in het ziekenhuis en soms sterfte als gevolg. Wanneer een ernstige luchtweginfectie overwonnen wordt door het immuunsysteem zorgt de nasleep van de ziekte vaak voor problemen in het dagelijks leven van ouderen. Daarnaast is het ook bekend dat vaccinaties een verminderd beschermend effect hebben in groepen oudere mensen. Kortom, naarmate iemand ouder wordt neemt de vatbaarheid voor infectieziekten en de ernst van ziekte toe, terwijl de effectiviteit van beschermende vaccins afneemt.

Doordat de gemiddelde levensverwachting van de wereldbevolking sterk is gestegen gedurende de afgelopen decennia wordt onderzoek naar veroudering van het immuunsysteem steeds belangrijker. De stijging van het aandeel mensen met hoge leeftijd is onder andere toe te schrijven aan de ontwikkeling van vaccinaties, antibiotica en sterke verbeteringen van de medische zorg. Het aandeel mensen van zestig jaar en ouder van de totale wereldbevolking is op dit moment een stuk hoger dan de afgelopen jaren en er wordt verwacht dat deze stijging de komende jaren alleen nog maar meer toeneemt. De World Health Organization (WHO) stelt zelfs dat het punt waarop de wereldbevolking uit meer ouderen dan jongeren bestaat er al snel aankomt. Dit benadrukt hoe belangrijk immunologisch verouderingsonderzoek is.

**In de Nederlandse samenvatting van dit proefschrift hoop ik jullie mee te nemen in deze fascinerende immunologische wereld en hoe dit proefschrift bijdraagt aan de huidige immunologische kennis met betrekking tot veroudering. Er zullen hier en daar onbekende woorden aan bod komen. Aan het eind van deze samenvatting staat een verklarende woordenlijst, waarbij de onderstreepte woorden verder uitgelegd worden.**

**Het Immuunsysteem**

Het immuunsysteem bestaat uit veel verschillende soorten onderdelen die grofweg opgedeeld kunnen worden in twee takken: het *aangeboren* deel en het *aangeleerde* deel. Deze twee delen werken samen, vullen elkaar aan en hebben elkaar nodig om optimale bescherming te bieden tegen infectieziekten. Onder deze twee delen vallen allerlei soorten cellen die allemaal een specifieke functie uitoefenen om zo goed mogelijk bescherming te bieden tegen ziekteverwekkers.

*T-cellen: een belangrijke cel voor bescherming tegen ziekteverwekkers*

Wanneer een virus, bijvoorbeeld het nieuwe coronavirus, voor het eerst in iemands leven in de luchtwegen terechtkomt en daar longcellen infecteert komt het aangeboren immuunsysteem in actie. Immuuncellen die bij het aangeboren deel van het immuunsysteem horen kunnen in de longen stukjes van het virus opnemen. Deze immuuncellen laten de stukjes virus vervolgens zien aan immuuncellen van het aangeleerde immuunsysteem. *T-cellen* zijn één van de celtypen die behoren tot het aangeleerde immuunsysteem. T-cellen in een neutrale ofwel *naïeve* staat (*naïeve T-cellen* genoemd) kunnen de stukjes virus die gepresenteerd worden door het aangeboren immuunsysteem herkennen. Wanneer naïeve T-cellen een stukje virus herkennen kunnen zij reageren. Deze

reactie wordt ook wel een *T-cel respons* genoemd. Een T-cel respons is van veel factoren afhankelijk en bestaat voornamelijk uit *activatie* en *celdeling* van naïeve T-cellen. Allereerst heeft deze T-cel respons als doel om het virus op te ruimen. Dit doen ze door bijvoorbeeld de longcellen die door het virus geïnfecteerd zijn te doden, waardoor het virus niet meer verder kan groeien in deze longcellen. Op die manier kan een virus uiteindelijk minder goed verspreiden naar andere longcellen en daardoor minder schade aanrichten. Daarnaast leren de naïeve T-cellen ook iets. Celdeling van naïeve T-cellen zorgt ervoor dat uit deze naïeve T-cellen een hele groep T-cellen ontstaat die klaarstaat voor wanneer er in de toekomst hetzelfde of een soortgelijk virus weer longcellen infecteert. Deze T-cellen worden dan niet meer naïeve T-cellen genoemd, maar *geheugen T-cellen*. Het voordeel van deze geheugen T-cellen is dat ze veel sneller tot actie kunnen overgaan dan naïeve T-cellen, omdat ze de ziekteverwekker al herkennen. Hierdoor wordt de kans op ziekte veroorzaakt door hetzelfde virus voorkómen of beperkt.

*Balans van een T-cel respons: regulatoire T-cellen*

Een belangrijk aspect van een T-cel respons is om balans te houden. Ten eerste moeten T-cellen de virus-geïnfecteerde longcellen zo snel en sterk mogelijk aanvallen om verspreiding van het virus te voorkomen en daarmee ziekte zoveel mogelijk te beperken. De manier waarop T-cellen dit doen is door de geïnfecteerde longcel te doden. Aan het doden van cellen zit alleen ook een keerzijde. Het doden van geïnfecteerde longcellen door T-cellen kan veel schade aanrichten wanneer dit op een ongecontroleerde manier gebeurt, omdat longcellen onder andere verantwoordelijk zijn voor de zuurstofuitwisseling. T-cellen moeten daarom de juiste balans weten te vinden tussen het wel of niet doden van geïnfecteerde longcellen, zonder dat dit al te veel schade en ernstigere ziekte oplevert dan het virus zelf.

Om de balans zo goed mogelijk te bewaren zijn er allerlei mechanismen aanwezig om T-cellen onder controle te houden. Allereerst bestaat de buitenkant van een T-cel uit een soort wand: het celmembraan. De buitenkant van dit celmembraan wordt ook wel het oppervlak van de T-cel genoemd. Op het oppervlak van T-cellen zijn verschillende kenmerken aanwezig om de cel af te remmen. Deze kenmerken worden *receptoren* genoemd. Op een enkele T-cel zitten al snel duizenden van deze verschillende receptoren waarmee T-cellen met andere cellen in hun omgeving contact maken. Elke receptor heeft een andere uitwerking, maar over het algemeen kan een receptor een T-cel activeren of juist afremmen. Wanneer een afremmende receptor op een T-cel een signaal ontvangt van omliggende cellen, stuurt de receptor het signaal door naar de

kern van de T-cel. Hierdoor worden verschillende processen die zorgen voor het doden van longcellen op dat moment gelijk stilgelegd.

Ten tweede bestaat er ook nog een kleine groep van gespecialiseerde T-cellen die andere T-cellen kunnen afremmen of *reguleren*. Deze afremmende T-cellen worden ook wel *regulatoire T-cellen* of '*Tregs*' genoemd. Tregs kunnen op verschillende manieren de activatie en celdeling van andere T-cellen afremmen, bijvoorbeeld door aan de receptoren te binden of zelfs door andere T-cellen te doden. Ook op deze manier wordt het doden van longcellen stilgelegd. Ondanks dat de groep Tregs redelijk klein is ten opzichte van het aandeel andere T-cellen is het belang van Tregs gigantisch: zonder deze cellen zouden er door het hele lichaam spontaan allerlei ontstekingen ontstaan waardoor leven praktisch onmogelijk is.

Kortom, T-cellen zijn een van de belangrijkste barrières tegen infectieziekten en zijn daarom ook de hoofdrolspeler van dit proefschrift. Zonder T-cellen zou een persoon niet kunnen leven. T-cellen voorkomen niet zozeer dat een persoon geïnfecteerd raakt, maar ze komen juist in actie wanneer de infectie al heeft plaatsgevonden. Ze voorkomen dat het virus verder kan verspreiden terwijl ze zichzelf onder controle houden en beschermen daardoor tegen ernstige ziekte.

*Tot zover is het belang van T-cellen dus duidelijk.*
*Maar wat gebeurt er met T-cellen tijdens het verouderingsproces?*

**Veroudering & T-cellen**

Het is bekend dat naarmate een persoon ouder wordt, het uiterlijk en de functie van T-cellen ook anders worden. Aangezien T-cellen een van de belangrijkste verdedigingslinies zijn tegen infectieziekten, waaronder de bovengenoemde luchtwegvirussen, zijn belangrijke vragen waarop dit proefschrift ingaat: wat verandert er aan T-cellen tijdens het ouder worden en waardoor ontstaan die veranderingen? En verklaren deze veranderingen de vatbaarheid voor infectieziekten en verhoogde kans op een ernstiger ziekteverloop op oudere leeftijd?

Het is bekend dat tijdens het verouderingsproces het uiterlijk van T-cellen verandert en daarnaast ook de functie van T-cellen veranderd en/of afneemt. Deze veranderingen dragen deels bij aan de vatbaarheid voor ziekte en verminderde reactie op vaccinaties. Een voorbeeld van veranderingen aan T-cellen met veroudering is de afname van het aantal naïeve T-cellen en de toename van geheugen T-cellen. Zoals hierboven uitgelegd ontstaan geheugen T-cellen wanneer naïeve T-cellen reageren tegen een nieuwe ziekteverwekker.

De geheugen T-cellen die hierdoor ontstaan zijn goed in hun reactie tegen deze specifieke ziekteverwekker, maar kunnen weinig doen tegen nieuwe ziekteverwekkers. Een recent voorbeeld hiervan is het nieuwe coronavirus. Doordat dit virus nieuw is voor het immuunsysteem moeten er voldoende naïeve T-cellen aanwezig zijn om te reageren. De T-cellen van een net geboren kind bestaan voor het overgrote deel uit deze naïeve T-cellen, maar naarmate een persoon ouder wordt neemt het aantal van deze naïeve T-cellen af en blijven er dus steeds minder van deze cellen over om te kunnen reageren op een nieuwe ziekteverwekker. Deze afname van naïeve T-cellen zou dus een van de oorzaken kunnen zijn waarom ouderen ernstiger ziek worden door het nieuwe coronavirus.

Ten tweede wordt er gedacht dat tijdens het verouderingsproces het aantal Tregs toeneemt. Zoals hierboven beschreven kunnen Tregs andere T-cellen afremmen om zo ernstige schade aan de longen te voorkómen. Een toename van het aantal Tregs in oudere personen zou er dus toe kunnen leiden dat T-cellen die moeten reageren tegen een ziekteverwekker eerder of te snel afgeremd worden, waardoor het virus langer in het lichaam/de longen verblijft.

Dit proefschrift gaat in op de vraag óf en hoe T-cellen veranderen tijdens het ouder worden. In deze Nederlandse samenvatting worden de bevindingen van elk van de hoofdstukken van dit proefschrift samengevat. De ondertitel van dit proefschrift benadrukt al wat onze bevindingen zijn over T-cellen tijdens het ouder worden: de balans verschuift richting de regulatoire kenmerken en regulatoire T-cellen. Dit proefschrift draagt bij aan bestaande inzichten over ouder worden met T-cellen op een basaal niveau, ofwel *fundamenteel immunologisch onderzoek*. Dit houdt in dat de bevindingen in dit proefschrift niet *direct* te verplaatsen zijn naar de kliniek in het ziekenhuis, maar wel aan het begin kunnen staan van nieuwe mogelijkheden om infectieziekten onder ouderen te begrijpen en te bestrijden. Uiteindelijk zouden de bevindingen in dit proefschrift dus nieuwe beginpunten aan kunnen duiden voor ander onderzoek waarin vaccinatie van oudere volwassenen, weerbaarheid tegen infectieziekten en gezonde veroudering centraal staan. Dit heeft niet als uiteindelijke doel om mensen zo oud mogelijk te laten worden, maar om het aantal levensjaren in goede gezondheid te laten toenemen en zo ook de druk op de zorg te verminderen.



**T-cel responsen veranderen tijdens het verouderingsproces**

Inzicht in de invloed van veroudering op T-cel responsen is van belang, omdat veranderingen in dit proces direct kunnen leiden tot vatbaarheid voor infectieziekten en ernstige ziekte als gevolg van een infectie. Hoewel de invloed

van veroudering op T-cellen vaak bekeken wordt op één bepaald punt in de tijd na het activeren van T-cellen, is de T-cel respons over meerdere punten in de tijd minder onderzocht. Hoe veroudering de T-cel respons over de tijd beïnvloedt is belangrijk om te achterhalen, omdat bescherming tegen infectieziekten bepaald kan worden door de snelheid waarin T-cellen vermenigvuldigen door celdeling en hoe snel en hoe lang ze geactiveerd zijn. In het geval van een virusinfectie, zoals infectie met SARS-CoV-2 of een griepvirus telt namelijk iedere minuut doordat virussen in het algemeen snel verspreiden en cellen infecteren. Om dit proces van verspreiding een halt toe te roepen is een snelle en adequate T-cel respons van groot belang.

*T-cel responsen in muizen*

In **hoofstukken 2** en **3** hebben wij gebruik gemaakt van muizen in onze experimenten. Het gebruik van dieren voor wetenschappelijke experimenten wordt (terecht) nooit lichtzinnig genomen en wordt altijd getoetst door een ethische commissie of experimenten weloverwogen worden uitgevoerd. Vooral in immunologisch onderzoek is het heel lastig, al dan niet onmogelijk, om vervangende systemen te gebruiken waardoor er geen proefdieren nodig zouden zijn. Het immuunsysteem is zó complex dat dit (met de huidige kennis) niet na te bootsen is.

Er zijn twee redenen waarom in **hoofdstuk 2** en **hoofdstuk 3** gebruik gemaakt is van muizen in plaats van menselijk materiaal. Ten eerste omdat muizen in een laboratoriumomgeving relatief weinig ziekteverwekkers tegenkomen die de T-cellen kunnen hebben beïnvloed. Daarnaast zijn, in tegenstelling tot bij mensen, van alle muizen de leefomstandigheden hetzelfde waardoor er minder variaties aanwezig zijn door omgevingsfactoren. Hierdoor kunnen wij specifiek kijken naar het effect van veroudering op T-cellen, zonder factoren die dit beeld kunnen vertroebelen. Ten tweede kunnen onze studies met muizen extra inzichten geven in dit veelgebruikte diermodel voor andere lopende immunologische verouderingsstudies in muizen. Tot slot neemt dit niet weg dat dierexperimenten waar mogelijk vermeden moeten worden.

In **hoofdstuk 2** van dit proefschrift is de invloed van veroudering op de reactie van T-cellen over de tijd beschreven, ook wel de kinetiek van een T-cel respons genoemd. Het onderzoek in dit hoofdstuk is gedaan met T-cellen van jonge en oude muizen. In **hoofdstuk 2** concluderen wij dat de kinetiek van T-cel responsen een belangrijke factor is om te bestuderen. Zo hebben wij ontdekt dat de activatie van T-cellen van oude muizen trager op gang komt en ook langer aanhoudt, terwijl de piek van activatie van T-cellen van jonge muizen hoog en kort is. Het gevolg van het bekijken van T-cel activatie op één punt in

de tijd kan dus leiden tot een andere en te beperkte conclusie. Naast dit verschil zagen we dat de deling van T-cellen van oude muizen ook sterk verminderd was vergeleken met de deling van T-cellen van jonge muizen. Kortom, de algehele T-cel respons lijkt met veroudering langzamer op gang te komen, wat een verklaring kan zijn voor vatbaarheid voor infectieziekten op latere leeftijd.

Tijdens het verouderingsproces vinden er allerlei veranderingen plaats in veel verschillende soorten cellen van het lichaam, binnen en buiten het immuunsysteem. Een van de meest belangrijke kenmerken van veroudering die te vinden is in allerlei organismen, waaronder de mens, is de opstapeling van schade aan het DNA. DNA is aanwezig in bijna alle cellen van het lichaam en is verpakt in de celkern. Gedurende het hele leven delen er continu cellen, bijvoorbeeld om organen te vernieuwen. Bij een celdeling vind er DNA replicatie plaats. Dat houdt in dat het DNA uit elkaar gehaald wordt, verdubbeld wordt door er stukken aan te bouwen en vervolgens wordt het DNA gelijk verdeeld over de twee nieuwe cellen. Een bijeffect van DNA replicatie is dat elke celdeling een kleine mogelijkheid van schade aan DNA met zich meebrengt. Deze schade kan gerepareerd worden, maar op den duur bouwt er toch wat blijvende DNA schade op. Dit beschadigde DNA blijft dan in de cel aanwezig totdat de cel doodgaat. Wat de invloed van DNA schade is op T-cellen is nog niet veel onderzocht, terwijl het wel bekend is dat cellen in een ouder persoon meer DNA schade bij zich dragen dan cellen van een jong persoon. In **hoofdstuk 3** gaan wij in op wat de mogelijke effecten kunnen zijn van de opstapeling van DNA schade op T-cellen. Hiervoor maakten wij gebruik van muizen met een bepaalde verandering in het erfelijke materiaal waardoor zij sneller DNA schade opbouwen. Door deze schade worden de muizen sneller oud. Het doel van dit onderzoek was om in kaart te brengen in hoeverre het uiterlijk van T-cellen en de T-cel respons beïnvloed worden door deze DNA schade. In **hoofdstuk 3** zagen wij dat T-cellen van muizen met DNA schade in sommige omstandigheden verschillen vertoonden in de activatie en celdeling van T-cellen vergeleken met muizen die geen DNA schade hadden. Daarom concluderen wij dat DNA schade in kleine mate bijdraagt aan de vertraagde T-cel kinetiek die we wel zien bij de oude muizen in **hoofdstuk 2**.

*T-cel responsen in mensen*

In **hoofdstuk 4** onderzoeken wij ook de invloed van veroudering op de kinetiek van T-cel responsen, maar dan bij T-cellen uit het bloed van mensen. Allereerst beschrijven wij een tot nog toe onontdekt uiterlijk van een bepaalde groep T-cellen: deze cellen brengen namelijk bepaalde kenmerken tot uiting die ‘CD8’, ‘TIGIT’ en ‘Helios’ heten. Wij ontdekten dat deze ‘TIGIT$^{+}$Helios$^{+}$ CD8$^{+}$ T-cellen’

in grotere hoeveelheden aanwezig zijn in het bloed van oudere volwassenen vergeleken met jongere volwassenen. Net zoals wij bij T-cellen van muizen in **hoofdstuk 2** ontdekten laten deze menselijke TIGIT$^{+}$Helios$^{+}$ CD8$^{+}$ T-cellen ook tragere activatie en verminderde celdeling zien over de tijd. Onze bevindingen in muizen is op deze manier dus uiteindelijk vertaald naar de mens en dit laat dus zien dat tragere kinetiek van T-cellen dus een algemeen kenmerk is van het effect van veroudering op T-cellen en niet alleen in muizen te vinden is. Onze bevindingen leiden naar de volgende belangrijke vraag:

*Zijn de veranderingen in de T-cel respons verantwoordelijk voor de verminderde bescherming door T-cellen tegen infectieziekten?*

Of onze bevindingen van vertraagde kinetiek van T-cellen ook plaatsvinden in het bloed gedurende een infectie is nog onbekend. Wel kan er over dit antwoord gespeculeerd worden. Tijdens een infectie is het bekend dat T-cellen geactiveerd raken en daardoor naar de lymfeklieren kunnen verplaatsen. De verplaatsing van T-cellen naar de lymfeklieren zorgt ervoor dat er nog meer cellen van het immuunsysteem kunnen vechten tegen de infectie. Deze stap is dan ook cruciaal om van de infectie af te komen. Wanneer de activatie en celdeling van T-cellen in het lichaam langzamer zou verlopen zoals wij hebben gezien in onze experimenten zou dit dus kunnen leiden tot een vertraging van een beschermende immuunrespons, met als mogelijk gevolg dat een virus zich sneller kan verspreiden.

**Op latere leeftijd nemen T-cellen met regulatoire kenmerken toe**

In de huidige literatuur wordt er verondersteld dat er een toename van afremmende Tregs plaatsvindt op latere leeftijd. Deze toename wordt vaak gezien als een van de oorzaken voor vatbaarheid voor infectieziekten, omdat deze Tregs de activatie en celdeling van andere T-cellen kunnen onderdrukken die juist tegen de ziekteverwekker moeten beschermen.

Naast het bestuderen van T-cel responsen hebben wij ook naar deze Tregs gekeken. In **hoofdstuk 2** zagen wij bijvoorbeeld dat oude muizen meer Tregs in hun milt hebben. Aangezien het bekend is dat Tregs de activatie en celdeling van andere T-cellen kunnen afremmen is onze observatie een teken dat er in oude muizen meer van dit soort afremming plaats zou kunnen vinden. Het gevolg van deze afremming zou kunnen zijn dat de reactie op een virus minder goed tot stand komt, omdat de Tregs deze reactie afremmen. In **hoofdstuk 3** vonden wij interessant genoeg een zelfde toename van Tregs in muizen die DNA schade opbouwen. Van deze bevindingen uit beide hoofdstukken kunnen wij

dus nu concluderen dat de toename van Tregs in oude muizen in ieder geval gedeeltelijk toe te schrijven is aan de toename van DNA schade.

Op basis van onze bevindingen in deze hoofdstukken stellen wij ook een nieuwe hypothese op voor toekomstig onderzoek. Wij stellen nu dat de toename van Tregs komt door de omgeving waarin T-cellen zich bevinden. Tijdens het verouderingsproces ontstaan er langzaam aan cellen door het hele lichaam heen die onstekingsstofjes uitscheiden. Op deze ontstekingsstofjes kunnen T-cellen vervolgens reageren. Doordat Tregs de T-cellen zijn die balans moeten houden tussen ontsteking en geen ontsteking reageren Tregs op de onstekingsstofjes in het bloed door te gaan delen en vermeerderen. Hierdoor ontstaan er op den duur steeds meer T-cellen met een regulatoir uiterlijk en regulatoire functie. Vervolgonderzoek zal moeten aantonen of onze hypothese inderdaad klopt.

**Ontdekking van nieuwe uiterlijke kenmerken voor CD8⁺ T-cellen in oudere volwassenen**

De bevindingen in **hoofdstuk 2 en 3** zijn interessant genoeg ook terug te vinden in mensen. In **hoofdstuk 5** kijken wij naar T-cellen van jongere en oudere volwassenen tussen de 21 en 82 jaar oud. In deze groep mensen hebben wij een nieuw soort T-cel ontdekt die kenmerken vertoont van Tregs: het afremmen van andere T-cellen door het onderdrukken van celdeling. Deze cellen hebben wij *KIR$^{\pm}$RA$^{\pm}$ T-cellen* genoemd op basis van hun uiterlijke kenmerken. In **hoofdstuk 5** zien wij dat het aandeel van deze KIR⁺RA⁺ T-cellen onder T-cellen toeneemt naarmate mensen ouder worden en uiterlijke kenmerken vertonen die ook voorkomen op regulatoire T-cellen. Samen wijzen deze bevindingen in de richting dat een toename van KIR⁺RA⁺ T-cellen op oudere leeftijd een van de factoren zou kunnen zijn die bijdragen aan verhoogde vatbaarheid voor infectieziekten en ernstigere ziekte.

Om te kijken of de aanwezigheid van KIR⁺RA⁺ T-cellen in oudere volwassenen ook verband houdt met toegenomen ziekte hebben wij een groep oudere volwassenen tussen de 61 en 83 jaar onderzocht. Deze mensen werd gevraagd aan te geven wanneer zij griepachtige verschijnselen vertoonden, zoals koorts en hoesten. Vervolgens werd er eerst vastgesteld door welk virus deze mensen ziek waren geworden. Daarnaast werd er, vlak nadat iemand aangaf deze griepverschijnselen te hebben, bloed afgenomen en vervolgens ook nog na twee en acht weken. In het bloed van de mensen die meededen aan onze studie vonden wij de KIR⁺RA⁺ T-cellen terug. Daarnaast zagen wij ook dat de KIR⁺RA⁺ T-cellen geactiveerd waren op het tijdstip van de eerste bloedafname, terwijl deelnemers nog griepachtige verschijnselen hadden. Op de latere tijdstippen zagen wij dat het aantal geactiveerde KIR⁺RA⁺ T-cellen weer afnam. Door

de recente pandemie van het coronavirus SARS-CoV-2 hadden wij ook de mogelijkheid om naar KIR$^+$RA$^+$ T-cellen te kijken in jongere volwassenen met een coronavirus infectie. Ook in deze mensen zagen wij dat KIR$^+$RA$^+$ T-cellen geactiveerd waren tijdens de infectie.

Op zich was de bevinding van KIR$^+$RA$^+$ T-cel activatie tijdens virale luchtwegziekte al een interessant gegeven, omdat dit aangeeft dat deze cellen mogelijk een rol spelen tijdens virale luchtweginfecties. Inderdaad zagen wij dat de duur van griepklachten verband hield met het aantal geactiveerde KIR$^+$RA$^+$ T-cellen: hoe meer van de KIR$^+$RA$^+$ T-cellen geactiveerd waren, hoe langer de griepklachten aanhielden. Dit zou kunnen betekenen dat de activatie van KIR$^+$RA$^+$ T-cellen ervoor zorgt dat de virusinfectie erger wordt, door bijvoorbeeld de T-cellen af te remmen die juist tegen het virus moeten reageren. Het zou dus een verklaring kunnen zijn waarom ouderen over het algemeen ernstigere ziekteverschijnselen vertonen dan jongeren.

Daarentegen zou het ook andersom benaderd kunnen worden: wat zou er gebeurd zijn als de geactiveerde KIR$^+$RA$^+$ T-cellen er niet zouden zijn? Mogelijk onderdrukten de KIR$^+$RA$^+$ T-cellen inderdaad de T-cellen die het virus op zouden moeten ruimen, maar voorkwamen ze hierdoor juist méér schade en ernstigere ziekte. De T-cellen die het virus opruimen doen dit namelijk voornamelijk door het doden van virus-geïnfecteerde longcellen. Dit is een goed mechanisme om de virusinfectie op te ruimen, maar als deze opruimactie te lang doorgaat treedt er schade op aan de longen door het afweersysteem zelf. Dit zou vervolgens weer kunnen leiden tot luchtwegproblemen en verhoogde kans op andere infecties, zoals bacteriële infecties.

Kortom, onze ontdekking van deze nieuwe soort T-cellen, die wij KIR$^+$RA$^+$ T-cellen hebben genoemd, zou een mogelijk startpunt kunnen zijn voor nieuw onderzoek in de richting van deze KIR$^+$RA$^+$ T-cellen. Belangrijke vragen die in de toekomst beantwoord zouden moeten worden zijn: wat is de rol van KIR$^+$RA$^+$ T-cellen tijdens luchtweginfecties? Hoe onderdrukken KIR$^+$RA$^+$ T-cellen de celdeling van andere T-cellen precies? En zijn KIR$^+$RA$^+$ T-cellen een van de factoren die bijdragen aan de verhoogde vatbaarheid voor luchtweginfecties in ouderen met een verhoogde kans op een ernstiger verloop? En wat is de bijdrage van KIR$^+$RA$^+$ T-cellen tijdens vaccinatie: reageren ouderen minder goed op vaccinaties doordat er meer KIR$^+$RA$^+$ T-cellen aanwezig zijn?

**Het grotere geheel: Wat zijn de voor- en nadelen van de stijging van regulatoire kenmerken van T-cellen met veroudering?**

Het onderzoek in dit proefschrift is geschreven vanuit het perspectief van de invloed van veroudering met T-cellen op de immuunrespons tegen infectieziekten

en vaccinaties. *Waardoor zijn ouderen vatbaarder voor infectieziekten, worden ze ernstiger ziek en reageren ze minder goed op vaccinaties?* Dit proefschrift draagt een van de vele mogelijke factoren voor deze vatbaarheid aan, namelijk de stijging van regulatoire kenmerken van T-cellen tijdens het ouder worden. De stijging van regulatoire kenmerken zien wij dus over het algemeen als iets negatiefs. Maar zijn er ook voordelen te vinden aan deze kenmerken? Waarom evolueert het immuunsysteem tot een systeem met meer regulatoire kenmerken?

Binnen het infectieziekten-onderzoek zijn er ook aanwijzingen dat bepaalde regulatoire uiterlijke kenmerken van T-cellen juist bijdragen aan het voorkómen van buitensporige schade, bijvoorbeeld aan de longen tijdens virale infecties. Kenmerken waar wij in dit proefschrift over rapporteren zouden daar ook aan kunnen bijdragen, zoals de aanwezigheid van TIGIT en de aanwezigheid van geactiveerde $KIR^{+}RA^{+}$ T-cellen.

Buiten het veld van infectieziekten is het beeld soms minder negatief. Hoe ouder een persoon wordt, des te groter de kans wordt op het ontwikkelen van auto-immuunziekten en tumoren. Zo is er uit een studie gebleken dat T-cellen met regulatoire kenmerken niet goed zijn voor de bescherming tegen infectieziekten, maar wel bijdragen aan een betere prognose voor ernstige auto-immuunziekten zoals reuma. Daarnaast is er uit onderzoek naar tumoren gebleken dat het uitschakelen van regulatoire T-cellen wel kan leiden tot verbeterde prognoses, maar dat het nadeel hiervan weer is dat de kans op auto-immuunziekten toeneemt.

Op basis van dit overkoepelende overzicht van de verschillende gebieden binnen de immunologie valt er dus het volgende te stellen: tijdens het verouderingsproces moet het immuunsysteem zich aanpassen aan allerlei invloeden van binnen en buitenaf. Het immuunsysteem groeit als het ware mee met de persoon. De toename van regulatoire kenmerken die in dit proefschrift naar voren komen zou een manier van het immuunsysteem kunnen zijn om om te gaan met de veranderende omstandigheden tijdens het hele leven. De toename van regulatoire T-cel kenmerken voorkómt enerzijds de toename van auto-immuunziekten op latere leeftijd en daarnaast zorgt het mogelijk ook voor verminderde schade aan organen ten gevolge van infectieziekten doordat krachtige T-cel responsen sterker onderdrukt worden. Het nadeel van deze verandering is dat wanneer de regulatoire kenmerken teveel doorslaan richting het onderdrukkende effect, de kans op kanker en hogere vatbaarheid voor infectieziekten toeneemt en de effectiviteit van vaccinatie minder kan worden. Samen benadrukt dit het grote belang van balans: voldoende regulatoire T-celkenmerken om de kans op auto-immuunziekten te verminderen, maar niet

zóveel regulatoire kenmerken om vatbaarheid voor infectieziekten ook zo laag mogelijk te houden.

## CONCLUSIE

De zoektocht naar nieuwe kenmerken van T-cellen op latere leeftijd is een van de meest belangrijke uitdagingen van fundamenteel immunologisch verouderingsonderzoek. Dit proefschrift laat nieuwe kenmerken zien van veroudering met T-cellen in muizen en mensen. Deze nieuwe kenmerken benadrukken dat een T-cel respons op verschillende punten lager en trager is op hogere leeftijd, wat een verklaring zou kunnen zijn voor de vatbaarheid voor infectieziekten op hogere leeftijd en daarnaast een hogere kans op een ernstiger verloop van de ziekte. Daarnaast is de stijging van regulatoire kenmerken van T-cellen op hogere leeftijd een mogelijke verklaring voor de afgenomen bescherming tegen infectieziekten in ouderen. Uiteindelijk kunnen deze fundamentele inzichten nieuwe startpunten zijn voor verder fundamenteel en/of klinisch onderzoek die als doel hebben om de effectiviteit van vaccinaties in ouderen te verbeteren, en weerbaarheid tegen infectieziekten en gezonde veroudering te bereiken.

## VERKLARENDE WOORDENLIJST

**Immuunsysteem**: ook wel afweersysteem genoemd, bestaat uit veel verschillende cellen en stofjes (eiwitten) die door het gehele lichaam aanwezig zijn om samen bescherming te bieden tegen infectieziekten en een rol spelen bij auto-immuunziekten en tumoren.

**Infectieziekten**: ziekten die veroorzaakt wordt door bacteriën, virussen, schimmels en parasieten die normaal niet in het lichaam voorkomen.

**Immuuncel**: een cel van het immuunsysteem. Elk soort immuuncel heeft een heel eigen functie om ziekteverwekkers op te ruimen of andere immuuncellen te ondersteunen bij het opruimen. Deze cellen worden door onderzoekers en artsen ook wel witte bloedcellen of leukocyten genoemd.

**Aangeboren immuunsysteem**: deel van het immuunsysteem dat reageert op ziekteverwekkers en dit niet (perse) doet op basis van geheugen. Dit deel van het immuunsysteem kan (stukjes van) veel ziekteverwekkers opnemen en vervolgens

doden en/of presenteren aan cellen van het aangeleerde immuunsysteem om zo nog meer cellen in te zetten tegen de ziekteverwekker.

**Aangeleerd immuunsysteem**: deel van het immuunsysteem dat zich ontwikkelt gedurende het leven. Wanneer het aangeboren immuunsysteem een ziekteverwekker presenteert aan cellen van het aangeleerde immuunsysteem, zoals T-cellen, dan kunnen deze T-cellen zich verder ontwikkelen en specialiseren tot T-cellen die heel goed zijn om één bepaalde ziekteverwekker heel goed te herkennen, vroegtijdig op te sporen en te doden, zodat een tweede infectie met dezelfde ziekteverwekker weinig tot geen ziekte veroorzaakt (je bouwt dan geheugen op/bent dan immuun voor deze specifieke ziekte).

**Virussen**: zijn kleine pakketjes genetisch materiaal die levende cellen, zoals longcellen van mensen, nodig hebben om zich te vermenigvuldigen en daarna weer verder te verspreiden naar omliggende cellen en andere personen. Voorbeelden van virussen zijn het influenzavirus (veroorzaker van de griep), het nieuwe coronavirus (SARS-CoV-2, veroorzaker van COVID-19) en het mazelenvirus.

**T-cellen**: immuuncellen die behoren tot het aangeleerde deel van het immuunsysteem. Er bestaan veel verschillende soorten T-cellen die allemaal een andere functie hebben. Er zijn T-cellen die virus-geïnfecteerde cellen doden, T-cellen die andere immuuncellen ondersteunen of juist afremmen.

**Naïeve T-cellen**: zijn T-cellen die nog nooit een ziekteverwekker zijn tegengekomen. Op jonge leeftijd zijn er veel van deze naïeve T-cellen aanwezig. Naarmate iemand ouder wordt zijn steeds meer naïeve T-cellen een ziekteverwekker tegengekomen, waardoor ze zijn veranderd in geheugen T-cellen. Daarnaast neemt ook de aanmaak van naïeve T-cellen af naarmate mensen ouder worden; de cellen worden dus als het ware 'opgebruikt'.

**Geheugen T-cellen**: zijn T-cellen die ooit gedurende het leven een ziekteverwekker zijn tegengekomen. Ze blijven altijd in het lichaam aanwezig om in actie te komen wanneer dezelfde ziekteverwekker voor een tweede keer terugkomt. Wanneer geheugen T-cellen een ziekteverwekker voor een tweede keer tegenkomen kunnen deze cellen snel delen en de ziekteverwekker verwijderen zodat ziekte beperkt of zelfs afwezig blijft. Het voordeel van geheugen T-cellen is dat ze dus veel sneller kunnen reageren, omdat de eerste stappen van een immuunrespons overgeslagen kunnen worden.

**T-cel respons**: reactie die begint wanneer T-cellen een ziekteverwekker herkennen en/of door ontstekingsstofjes geactiveerd worden. Een goede en beschermende T-cel respons bestaat voornamelijk eerst uit T-cel activatie en daarna T-cel celdeling. Een beschermende T-cel respons zorgt voor het opruimen van ziekteverwekkers, aansturen en helpen van andere cellen en het aanmaken van geheugencellen om voor bescherming te zorgen in de toekomst.

**T-cel activatie**: is onderdeel van een T-cel respons. T-cel activatie houdt in dat een T-cel iets in zijn omgeving herkent als zodanig 'bedreigend' dat er actie nodig is. T-cel activatie is te herkennen aan receptoren die op het celoppervlak tevoorschijn komen die er eerder in een ruststaat niet waren. Met deze receptoren kunnen er verschillende functies uitgevoerd worden, zoals het verplaatsen van de T-cel door het lichaam of het opvangen van stimulerende stofjes (cytokines).

**T-cel celdeling**: is onderdeel van een T-cel respons. Een naïeve T-cel die een ziekteverwekker herkent wil zich vermeerderen om een zo goed mogelijke kans te maken om tegen de ziekteverwekker te vechten. Dit gebeurt door opsplitsing van één T-cel naar twee T-cellen en deze kunnen vervolgens weer opsplitsen naar vier T-cellen enzovoorts. Het kan bijvoorbeeld zo zijn dat één enkele naïeve T-cel in vijf tot acht dagen tijd vermeerdert tot tienduizend T-cellen die vervolgens tegen de ziekteverwekker kunnen vechten.

**Receptoren**: zijn een soort 'voelsprieten' van cellen, waaronder T-cellen, die de omgeving aftasten op zoek naar stofjes of andere cellen in de buurt waar de receptoren in verbinding mee kunnen komen. Elke receptor heeft een specifieke en unieke functie. Wanneer deze receptoren in contact komen met hetgeen wat ze herkennen sturen ze een signaal door naar de kern van de T-cel, waarna de T-cel bijvoorbeeld afgeremd of juist extra aangezet wordt.

**Regulatoire receptoren**: een bepaalde groep receptoren die ervoor zorgen dat de T-cel waarop zij zich bevinden afgeremd wordt. In dit proefschrift zien wij een toename van deze regulatoire receptoren op T-cellen van oudere muizen en volwassenen.

**Regulatoire T-cellen (Tregs)**: zijn een kleine maar speciale groep onder de T-cellen die andere T-cellen kunnen afremmen. Tregs zijn van groot belang voor het behouden van de balans tussen het opruimen van ziekteverwekkers

door andere T-cellen en het voorkomen van grote schade aan organen, zoals de longen. In dit proefschrift zien wij een toename van T-cellen met een regulatoire functie.

**KIR$^{+}$RA$^{+}$ T-cellen**: zijn T-cellen die wij in dit promotieonderzoek hebben ontdekt. Deze cellen hebben regulatoire kenmerken. KIR$^{+}$RA$^{+}$ T-cellen hebben wij zo genoemd op basis van de karakteristieke receptoren die op het celoppervlak aanwezig zijn (de KIR en CD45RA receptoren). Van dit celtype hebben wij ontdekt dat hun aandeel in het bloed toeneemt met hogere leeftijd, andere T-cellen kunnen afremmen en dat ze sterk actief zijn in ouderen met luchtweginfecties die veroorzaakt zijn door verschillende virussen, waaronder het influenzavirus en het nieuwe coronavirus.

# CURRICULUM VITAE

Daan Pieren was born on November 28th, 1989 in Heemstede, The Netherlands. He finished his secondary school at the Coornhert Lyceum in Haarlem in 2008. After working in elderly healthcare and voluntary work at the Food bank he started his bachelor Health and Lifesciences at the Vrije Universiteit (VU) in Amsterdam in 2009. As part of his bachelor's degree, he performed an internship at the Center for Tropical Medicine and Travel Medicine at the Academic Medical Center under supervision of dr. Rosanne Wieten, studying Yellow Fever vaccination in travelers using immunosuppressive medication.

After obtaining his Bachelor of Science in 2012, he continued his studies at the VU with the master Biomedical Sciences, specializing in immunology and infectious diseases. As part of the master's degree, he performed an internship at the Department of Medical Microbiology and Infection Control of the VU Medical Center under supervision of dr. Saara Vainio. There, he studied a new diagnostic test to rapidly detect bacterial resistance to antibiotics. He performed another internship at the Department for Immune Mechanisms (IMM) of the RIVM under supervision of dr. Teun Guichelaar, studying innate immune responses against Respiratory Syncytial Virus in the experimental cotton rat model. To finalize his master's degree, he wrote his literature thesis at the Leiden University Medical Center at the Department of Parasitology under supervision of dr. Meta Roestenberg and dr. Milly van Dijk (VU), which describes immune protection in the skin after vaccination with live attenuated malaria parasites. After obtaining his Master of Science in 2015, the research activities during his studies encouraged him to pursue a PhD position.

In 2015 he started as a PhD candidate at the IMM department of the RIVM in collaboration with the division of Infectious Diseases and Immunology at the Utrecht University. His supervisors were dr. Teun Guichelaar, prof. dr. Willem van Eden, and prof. dr. Debbie van Baarle. The research of his PhD project focused on the impact of aging on T cells, which resulted in this thesis.

Daan currently works at the RIVM as a short-term postdoctoral researcher under supervision of dr. Jelle de Wit and prof. dr. Cécile van Els. In summer 2021, Daan will continue his scientific career by moving and working abroad together with one of the most important persons in his life: Julien.

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# DANKWOORD

**Here are we, one magical moment**
**Such is the stuff, from where dreams are woven**
**-** David Bowie, Station to Station (1976) **-**

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