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INTERACTIONS BETWEEN HERPESVIRUSES AND THE HUMAN IMMUNE SYSTEM IN OLD AGE

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1 ABSTRACT

English version

Advanced age is accompanied by a decline of immune functions, which may play a role in increased vulnerability to emerging pathogens and low efficacy of vaccinations in elderly people. The capacity to mount immune responses against new antigens is particularly affected in this population. However, its precise determinants are not fully understood. In particular, it is yet unclear at which level age-intrinsic mechanisms and external factors (namely the co-infections with persistent viruses) cooperate, contributing to immunosenescence.

The aim of this work was to unravel the age-dependent interplay between the host immune system and three main herpesviruses which are widespread among the general population: HSV-1, CMV ad EBV. To reach this scope, I focused first on the effect of persistent infections on immunosenescence and, later, on the effect of immunosenescence on the immune responses to infections. Three main questions were addressed:

1) May persistent viral infections influence the T-cell compartment and immune responsiveness in older adults?
2) How age affects the memory responses to herpesviruses?
3) How age affects the naïve responses to primary infections (SARS-CoV-2)?

To this aim, I first assessed immunological parameters, related to $CD8^+$ and $CD4^+$ T-cell responsiveness, according to the serological status for common latent herpesviruses in two independent cohorts: 1. healthy individuals aged 19y to 95y (n= 150) and 2. individuals above 70y old enrolled in a primo-vaccination clinical trial (n= 137). Results show a prevalent effect of age and CMV infection on $CD8^+$ and $CD4^+$ naive T cells respectively. CMV seropositivity was associated with blunted $CD4^+$ T-cell and antibody responses to primary vaccination against tick-borne encephalitis virus, chosen as model of *de novo* vaccination. The concomitant presence of different persistent infections is also responsible of the accumulation (inflation) of late-differentiated memory T cells in older subjects.

Then, I measured HSV-, CMV- and EBV-specific cellular responses in middle-aged and elderly adults from Cohort 1 dissecting the secretory capacity of distinct memory subsets. Notably,

an age-related increase of late-differentiated, HSV-specific memory $CD8^+$ T cells is observed, suggesting that HSV-1 participate to the memory inflation of the CD8 compartment. In addition, a concomitant decrease of secretory capacity upon general TCR stimulation is noticed in early/intermediate differentiated memory $CD8^+$ T cells.

Finally, to confirm whether primary $CD8^+$ T-cell responses are defective in advanced age and assess potential consequences, an *in vitro* approach to prime SARS-CoV-2-specific naive $CD8^+$ T cells from healthy, unexposed donors of different age groups was exploited. Being an emerging infection, SARS-CoV-2 is the perfect model to study primary responses in unexposed subjects. Compared to younger adults, older individuals display a poor SARS-CoV-2-specific T-cell priming capacity in terms of both magnitude and quality of the response. In addition, older subjects recognize a lower number of epitopes.

Collectively, these data implicate that immune ageing is associated with altered primary responses. This implies that elderly subjects present with a low SARS-CoV-2-specific cellular immunity. As virus-specific $CD8^+$ T-cells have shown to be protective toward critical COVID-19 manifestations, an age-related suboptimal cellular immunity may contribute to the age-pattern of the disease. Moreover, the concomitant infection with CMV negatively affects, in elderly individuals, CD4-mediated response and thus both cellular and humoral immunity. This may further explain the reduced responses to emerging infections and de-novo vaccination with advanced age.

Overall, recall T-cell responses are less affected by age. Nonetheless, herpesviruses cause an "inflation" of late-differentiated memory cells that could negatively impact on immunological memory towards other antigen specificities (e.g. previously administered vaccines) and is a sign of frequent viral reactivation in life.

Altogether, these data provide insights on the changes in adaptive immunity over time and the associated decline in vaccine efficacy with ageing. This knowledge is important for the management of infectious diseases in elderly populations.

Versione Italiana

Il processo di invecchiamento è accompagnato da un declino delle funzioni immunitarie che può svolgere un ruolo importante nell'aumento della vulnerabilità ai patogeni emergenti e nella bassa efficacia delle vaccinazioni nelle persone anziane, la cui capacità di attivare risposte immunitarie contro nuovi antigeni è particolarmente compromessa. Tuttavia, le cause alla base di questo fenomeno non sono state ancora completamente comprese. In particolare, non è chiaro se esista una cooperazione fra meccanismi intrinseci legati all'età e fattori esterni (ed, in particolare, le infezioni con virus persistenti) nella progressione dell'immunosenescenza.

Lo scopo di questo lavoro è di studiare l'interazione tra il sistema immunitario dell'ospite e tre principali herpesvirus che sono diffusi tra la popolazione generale (HSV-1, CMV ed EBV), nonché di valutare come questa interazione vari con l'età. Per raggiungere questo scopo, mi sono concentrato prima sull'effetto delle infezioni persistenti sull'immunosenescenza e, successivamente, sull'effetto dell'immunosenescenza sulle risposte immunitarie alle infezioni. Sono state quindi affrontate tre domande principali:

1) Le infezioni virali persistenti possono influenzare il compartimento delle cellule T e la risposta immunitaria negli anziani?

2) In che modo l'età influenza le risposte memoria agli herpesvirus?

3) In che modo l'età influenza le risposte alle infezioni primarie (prendendo, a modello, SARS-CoV-2)?

A questo scopo, ho inizialmente valutato parametri immunologici relativi ai linfociti T $CD8^+$ e $CD4^+$ in base allo stato sierologico per i comuni herpesvirus latenti in due coorti indipendenti: 1. individui sani di età compresa tra 19 e 95 anni (n = 150) e 2. individui di età superiore a 70 anni arruolati in uno studio clinico vaccinale (n = 137). I risultati mostrano, rispettivamente, un effetto prevalente dell'età e dell'infezione da CMV sulle cellule T naive $CD8^+$ e $CD4^+$. Infatti, la sieropositività al CMV è stata associata a una ridotta risposta CD4 e anticorpale in seguito ad una vaccinazione primaria contro encefalite da zecca (TBE, scelta come antigene modello). La concomitante presenza di diverse infezioni persistenti è inoltre responsabile dell'accumulo ("inflazione") di cellule T di memoria altamente differenziate nei soggetti più anziani.

Quindi, ho misurato le risposte cellulari specifiche per HSV-1, CMV ed EBV in adulti e anziani della Coorte 1, analizzando la capacità secretoria delle diverse sottopopolazioni memoria. In

particolare, si è osservato un aumento correlato all'età di cellule T $CD8^+$ di memoria altamente differenziate specifiche per HSV-1, suggerendo che questo virus partecipi all'inflazione del compartimento memoria CD8. Inoltre, si è notato una concomitante diminuzione della capacità secretoria in seguito a stimolazione generale del TCR nelle cellule T $CD8^+$ di memoria poco differenziate.

Infine, per confermare se le risposte primarie delle cellule T $CD8^+$ siano difettose in età avanzata ed investigarne le potenziali conseguenze, è stato sfruttato un approccio *in vitro* per indurre il *priming* di cellule T $CD8^+$ naive specifiche per SARS-CoV-2 di donatori sani (non esposti al virus) appartenenti a diversi gruppi di età. Essendo un'infezione emergente, SARS-CoV-2 rappresenta il modello ideale per studiare le risposte primarie in soggetti non esposti al virus. Rispetto agli adulti più giovani, gli individui anziani mostrano una scarsa capacità di innesco delle cellule T specifiche per SARS-CoV-2 in termini sia di intensità che di qualità della risposta. Inoltre, i soggetti più anziani riconoscono un numero inferiore di epitopi.

Nel loro insieme, questi dati suggeriscono fortemente che l'invecchiamento immunitario è associato a risposte primarie alterate. Ciò implica che i soggetti anziani presentino una bassa immunità cellulare specifica per SARS-CoV-2. Poiché le cellule T $CD8^+$ virus-specifiche hanno dimostrato di essere protettive nei confronti delle manifestazioni critiche di COVID-19, un'immunità cellulare subottimale correlata all'età può contribuire alla suscettibilità dei soggetti anziani a forme gravi di COVID-19. Inoltre, l'infezione concomitante con CMV influenza negativamente, negli individui anziani, la risposta mediata da CD4 e quindi l'immunità sia cellulare che umorale. Ciò potrebbe ulteriormente spiegare le risposte ridotte alle infezioni emergenti e alla vaccinazione riscontrate in età avanzata.

Nel complesso, le risposte delle cellule T memoria sono meno influenzate dall'età. Tuttavia, gli herpesvirus causano un aumento numerico ("infazione") delle cellule di memoria differenziate che potrebbero avere un impatto negativo sulla memoria immunologica verso altre specificità antigeniche (ad es. vaccini precedentemente somministrati). Questo fenomeno ed è anche un indice di frequenti riattivazioni virali avvenute nel corso della vita.

Questi dati gettano nuova luce sui cambiamenti dell'immunità adattativa nel tempo e sul declino dell'efficacia dei vaccini associato all'invecchiamento, aprendo nuove opportunità preventive e terapeutiche per la gestione delle malattie infettive nella popolazione anziana.

2 GENERAL INTRODUCTION

2.1 The immune system

The human immune system is composed by two major branches, the innate immunity and the adaptive immunity. The innate immunity is the first and nonspecific reaction initiated after every infection, with the aim to block the spread of the pathogen. Moreover, innate immune cells favor the onset of the adaptive immunity, mostly through two mechanisms:

1. activation of pro-inflammatory signaling pathways, resulting in the production of antimicrobial molecules, of pro-inflammatory cytokines and chemokines and of co-stimulatory molecules that, soluble or expressed on the cell surface, activate adaptive immune cells;
2. phagocytosis of the pathogen by macrophages and dendritic cells (DCs). This is followed by the processing of the antigen and their presentation, through MHC molecules, to T lymphocytes (see below).

The adaptive (or acquired) immunity is composed by cell-mediated and humoral responses, mediated respectively by the T and the B lymphocytes. Adaptive immunity is antigen-specific, slower in its development if compared to innate responses and retains an immunological memory against previously encountered antigens.

2.1.1 Humoral response

The humoral immunity is mediated by immunoglobulins (Ig), secreted by B lymphocytes. Ig molecules are constituted a variable and a constant region. While the variable region of Igs determines the antigen specificity, the constant region contributes to the creation of the antigen binding domain and determines the effector functions of antibodies (Abs) and their fate of being secreted or remaining on the cell surface. Abs anchored to the cell membrane constitute the B cell receptor (BCR) that induces the antigen recognition by B cells, while secreted Abs traffic into the tissues and across mucosal surface. Moreover, the constant regions determine the subdivision of Abs into five different types: IgA, IgD, IgE, IgG, IgM, each with different properties and functions.

BCR allows also the endocytosis of the antigen and its presentation, through class II MHC molecules, to $CD4^+$ T cells, thus inducing T-dependent antibody responses. Indeed, B cells can secrete Igs with and without T helper cells. In the latter case, these responses are usually fast and dominated by IgM, while T-dependent antibody responses promote isotype switching

(from IgM to IgG) and the generation of B memory cells and long lived plasmacells (PC). T-cell help to antibody responses is provided by specific T helper lymphocytes previously primed by DCs; these $CD4^+$ T cells, according to the kind of cytokines produced, control the isotype of IgG secreted; in particular, IL-4 induces IgG1 production while IFN-γ (secreted by both $CD4^+$ and $CD8^+$ T cells) and IL-6 induce IgG2a production. Class switching to IgG2a and survival of memory B cells secreting IgG2a require T-bet expression [1].

Antibody may "neutralize" the antigen blocking its interaction with host receptors, or they may eliminate microbes and toxins through different mechanisms that include the involvement of some innate mechanisms as complement activation, phagocytosis or antibody-dependent cellular cytotoxicity by NK cells. The involvement of phagocytes or NK cells requires the interaction of the heavy constant chain of Ig with the leukocyte Fc receptor that, after having been triggered, will deliver signals to activate the cell and stimulate its microbicidal or phagocytic activity (opsonization).

2.1.2 Cellular response

The pathogen-specific cellular immunity is mediated by $CD4^+$ and $CD8^+$ T lymphocytes; T lymphocytes mount specific responses against antigens (Ags) that are presented through major histocompatibility complex (MHC) molecules; indeed, intracellular or internalized antigens are processed by the cellular proteolytic systems ("antigen processing") that generates small peptides (epitopes); these small peptides, associated to the MHC molecules, migrate to the cell surface to be recognized by the specific T cell receptor. Intracellular Ags are presented through MHC class I molecules, present on all nucleate cells, and activate $CD8^+$ T cells, while internalized antigens are presented through MHC class II molecules, present on "professional" APCs like DCs, macrophages and B cells, and activate $CD4^+$ T cells.

T lymphocytes recognize the presented antigen through the TCR, a complex of proteins that includes a dimer (usually formed by α and β subunits, but in some cases γ and δ subunits can be found), responsible for the binding with the epitope, and the CD3 chains (consisting in other three protein dimers), responsible for the signal transduction, which includes the activation of several kinases (such as the mammalian target of rapamycin, mTOR) and ends with the entering into the nucleus of some transcription factors (TFs), like NFAT, AP-1 and NFκB, that stimulate the expression of key T-cell associated genes. Further to TCR stimulation, also defined as "signal 1", T-cell activation requires costimulatory signals ("signal 2") and

other additional signals from the environment, like the presence of pro-inflammatory cytokines ("signal 3") [2].

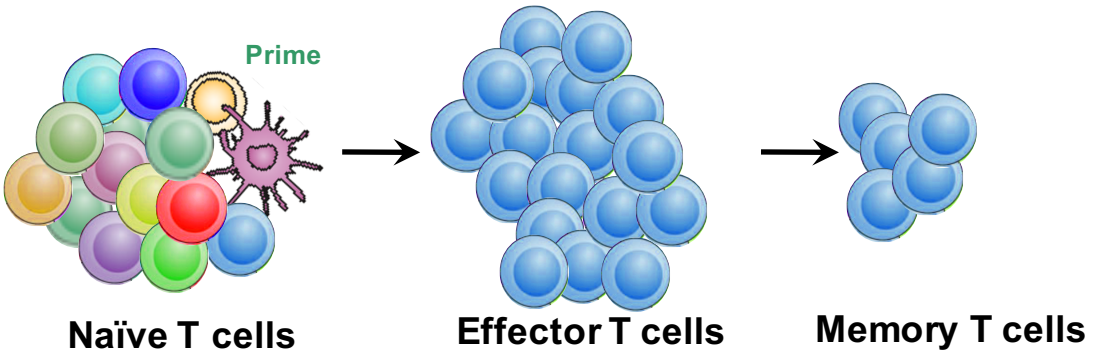

Figure 2.1. *Development of the cellular response*

The modulation of these 3 signals during the priming of naïve T lymphocytes is of great importance to generate a productive response leading to strong effector functions, cell survival and memory generation. Following antigen stimulation, antigen-specific T cells undergo a remarkable phase of "expansion": naïve precursors massively proliferate and differentiate into "effectors" (Figure 2.1), in charge of the clearance of the pathogen [3].

2.1.2.1 Effector T cells

Effector $CD4^+$ T cells may accomplish their duty of "orchestrating" the immune response both remaining in lymph nodes to help B cells or migrating into the site of infection, thus supporting the activities of innate immune cells or cytotoxic T lymphocytes (CTLs), directly involved in the clearance of the pathogen through the secretion cytotoxic proteins. T helper lymphocytes that recognize the epitope bound to MHC class II molecules on the surface of the same DC presenting the antigen to the $CD8^+$ T cell can help the activation of the $CD8^+$ T cell itself through the secretion of IL-2 (beneficial for CTLs expansion) or enhancing, on DCs, the expression of co-stimulatory molecules and the production of cytokines in favour of the $CD8^+$ T cell. The ability of T helper cells to sustain (or depress) a certain kind of immunity depends by the lineage that they acquire (e.g. Th1, Th2, Th17 or Treg).

Effector $CD8^+$ T cells (CTLs) are responsible of the elimination of virus-infected or tumor cells through the secretion of cytotoxic molecules such as perforin or granzymes. They can also secrete pro-inflammatory molecules such as IFNγ. The effector functions of $CD8^+$ T cells is under the control of the transcription factor T-bet expression [4, 5]. Remarkably, expression

of T-bet in $CD4^+$ and $CD8^+$ T cells is induced downstream of TCR, IFNγ and IL-12 signaling, and promotes the transcription of effector genes [6].

2.1.2.2 Memory T cells

After the antigen is cleared (or the pathogens go into latency), the vast majority of effector T cells die during the so called "contraction phase", and the survivors differentiate into memory cells that, in case of secondary infection, will be ready to give birth to a new immune response [7]. The death of effector cells once the antigenic challenge has been met, is of essential importance to avoid tissue damages and enlargement of secondary lymphoid organs, and usually involves the 90% of the clonal population [8].

Both $CD4^+$ and $CD8^+$ memory T cells do not require antigen stimulation for their maintenance, but are dependent on IL-7 and IL-15 signalling that mediates homeostasis and survival, up-regulating anti-apoptotic molecules such as Bcl-2 [9]. Memory cells can be categorized into two broad subpopulations: "central memory" (CM) and "effector memory" (EM), although the development of multiparametric flow cytometry allowed the characterization of other T cell subsets (Figure 2.2). CM reside mostly in lymph nodes and are responsible for the clonal expansion after re-exposure to the antigen, while EM are disseminated within peripheral tissues where they display immediate effector functions. Their different role and anatomical distribution is associated with their phenotype; for example, lymph node homing receptors like CD62L and CCR7 are expressed on CM but not on EM. However, this differentiation seems more a simplistic way of describing the T-cell memory organization than a clear picture of the reality, because several experiments have demonstrated that CM can display secretory capacity and EM may proliferate [10]. The use of additional surface marker to phenotypically define T-cell subsets may, in part, help in the understanding the plethora of different memory subpopulations. For instance, T cells that have lost CCR7 expression (as EM) but keep CD27 (as CM) are usually transitioning toward a more differentiated phenotype and are, for this reason, called "transitional memory" (TM), harbouring intermediate properties between CM and EM (Figure 2.2). Instead, highly differentiated EM tend to re-express CD45RA, usually present only in naïve (N) T cells, although do not present with N-like properties; indeed, this cell subsets represents the more differentiated one ("terminally differentiated EM" or "EM that re-express CD45RA" – EMRA), uncapable of proliferating but characterized by high

activation levels and strong secretory capacity, especially related to pro-inflammatory molecules, and are more prone to exhaustion.

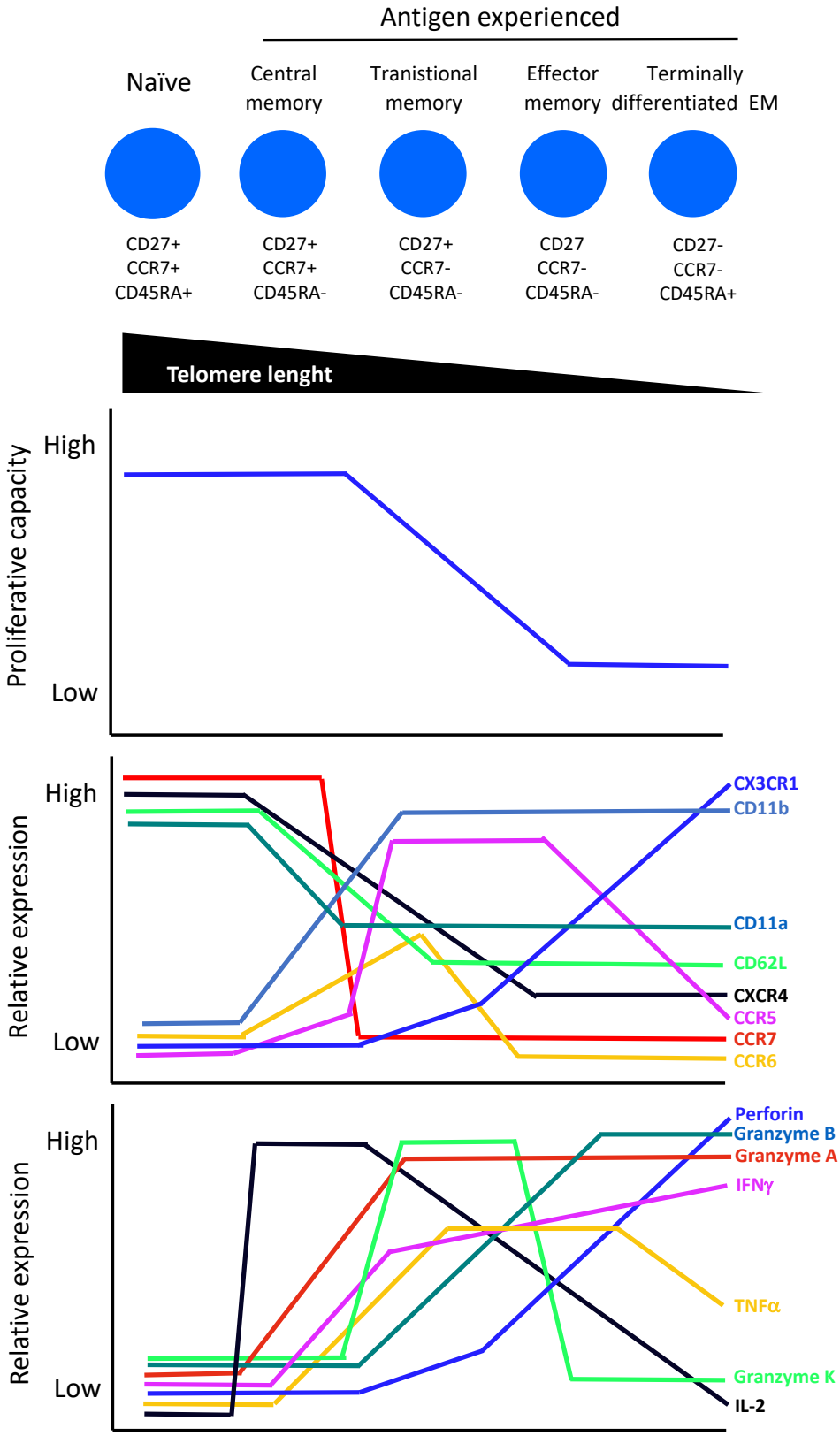

Figure 2.2. *Memory T-cell subsets*

In general, it is well established that the secondary response, mediated by memory cells, is faster and more intense than the primary response [11, 12].

In the case of chronic or latent infections, and particularly during the HIV infection, the persistence of the pathogen misleads the physiological development of the cellular response, affecting the functionality of memory cells, that can exhibit poor recall proliferation, exhausted phenotype, loss of effector functions and a skewed composition of T cell memory subpopulations [13].

2.1.3 $CD8^+$ T cell programming

Long lived memory $CD8^+$ T cells seem to originate from a subset of effector cells called MPEC, memory precursor effector cells, in contrast to SLEC, short lived effector cells. The balance of MPEC/SLEC among a population of effectors depend by the overall amount and duration of the three above mentioned signals, thus the first 48-96 hours of stimulation determine the fate of the future memory population. In particular, the development of CM cells is dictated by a short and reduced antigenic stimulation [14, 15]. The composition of the effector T-cell subsets and, as a consequence, the generation of long lived memory T cells, is related to the balance of the two TFs T-bet and Eomes; indeed, T-bet is important for the generation of SLECs, and its expression has to decrease for the development of a functional memory population, while Eomes is crucial for the ability of memory T cells to respond to IL-7 signaling, important for memory homeostasis [5, 16]. The expression of T-bet and Eomes is regulated by the mTOR kinase, whose activity promote T-bet at the expense of Eomes; thus, mTOR inhibition promote the accumulation of memory precursors $CD8^+$ T cells, the increased and sustained expression of prosurvival genes (Bcl-2 and Bcl-3) and the development of fully competent memory $CD8^+$ T cells [5], as well as the maintenance and the antigen-recall responses of memory $CD8^+$ T cells [17].

Other TFs are involved in the development of memory T cells like the Foxo family, Bcl-6 and Blimp-1. The forkhead box O1 (FOXO1) is downstream to mTORc2 and under the stringent control of AKT-mediated phosphorylation and nuclear exclusion, induced by IL-12 and IFNα signalling [18]. FOXO1 targets IL-7 receptor subunit-α, CD62L, Bcl-2 and Eomes, thus promoting memory differentiation [18]. FOXO3 too needs phosphorylation, induced by IL-7 and IL-15 [19], for nuclear exclusion, and its inhibition leads to an enhanced expansion protecting effector cells from apoptosis; this results in an increased accumulation of memory $CD8^+$ T cells, without effects on the phenotype [20].

Bcl-6, the transcriptional repressor of Granzyme B, is important for $CD4^+$ and $CD8^+$ memory T-cell formation [21] and interacts with T-bet for Th1 development [22]. Bcl-6 is negatively regulated by Blimp-1, a TF required for T cell homeostasis, cytokine secretion [23] and more expressed in SLEC than MEPC and CM [24]; of note, Blimp-1/Bcl-6 balance is involved also in the formation and maintenance of memory B cells.

The interaction with $CD4^+$ T cells and DCs play a fundamental role in determining the $CD8^+$ T cells programming. Indeed an increased interaction between DCs and T cells due to a higher epitope density does not affect the expansion but enhances the survival of $CD8^+$ T cells during the contraction phase through an increased expression of both Bcl-6 and Eomes [25], the TFs required for long lived memory cells development. Moreover, DCs interact with $CD4^+$ T cells to promote the development of $CD8^+$ EM cells [26]. Together, DCs and $CD4^+$ T cells may influence $CD8^+$ T cells programming through the cytokines they secrete. For instance, IL-2 promotes the expansion of SLECs and the accumulation of EM [26], IL-15 favors the formations of MPECs [27] while pro-inflammatory cytokines such as IL-12 and IFNγ enhance the contraction phase [28] and the formation of SLECs through the induction of T-bet [29].

2.1.4 Immunosenescence

Life expectancy has increased considerably over the last century as a consequence of advances in medicine and improved public health systems. However, old age is associated with a high prevalence of chronic diseases and an increased susceptibility to cancer and emerging pathogens, such as SARS-CoV-2 [30]. Age-related deficits in the immune system are thought to play a key role in the development of many pathological conditions [31-33]. Immune ageing is characterized by a progressive erosion of the naive T cell compartment, which impairs *de novo* immune responses against newly encountered antigens [34-36]. Alongside this decline in absolute numbers [37], naive T cells in elderly individuals also exhibit various functional limitations [38], including suboptimal differentiation in response to T cell receptor (TCR)-mediated activation [34]. Conversely, older individuals show an accumulation of terminally differentiated, exhausted effector memory T cells, that contribute to the hyperinflammatory status and to the reduced control of chronic infections [39]. Indeed, ageing is characterized an increased concentration of pro-inflammatory molecules ("inflammageing"), associated with several metabolic abnormalities and oxidative stress [40], that likely contributes to the rise on non-communicable morbidities in elderly subjects.

2.2 The Herpesviruses

More than 100 herpesviruses have been at least partially characterized so far, and 8 of them have been routinely found in humans: herpes simplex virus type 1 (HSV-1), herpes simplex virus type 2 (HSV-2), varicella-zoster virus (VZV), cytomegalovirus (CMV), Epstein-Barr virus (EBV), human herpesvirus 6 (HHV6), human herpesvirus 7 (HHV7) and human herpesvirus 8 also named Kaposi's Sarcoma herpesvirus (HHV8).

Their structure is similar among all herpevisures (Figure 2.3), with a large, double-stranded DNA genome (120 to 250 kilobase pairs) enclosed by an icosapentahedral capsid (100-110 nm), surrounded by an amorphous protein coat (tegument) encased in a glycoprotein-bearing lipid bilayer envelope. The envelope consists of polyamines, lipids, and glycoproteins. The lipid bilayer is derived from host cell membranes, and the glycoproteins confer distinctive properties to each virus and provide unique antigens to which the host is capable of responding.

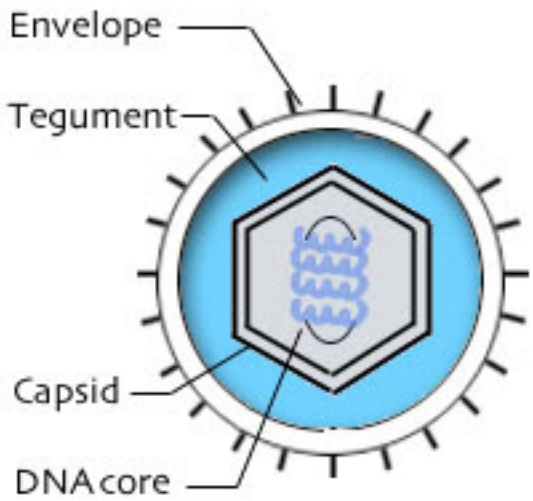

Figure 2.3. *Herpesvirus virion structure*

Herpesviruses are divided into three groups:

- α herpesviruses: HSV-1, HSV-2 and VZV; they are characterized by an extremely short reproductive cycle (hours), prompt destruction of the host cell and the ability to replicate in a wide variety of host tissues. They characteristically establish latent infection in sensory nerve ganglia.

- β herpesviruses: CMV, HHV6 and HHV7; they have a restricted host range. Their reproductive life cycle is long (days) and they may form enlarged cells. These viruses can establish latent infection in secretory glands, cells of the reticuloendothelial system, and the kidneys.
- γ herpesviruses: EBV and HHV8; they present the most limited host range. Latent virus has been demonstrated in lymphoid tissue.

Their viral cycle is very similar (Figure 2.4): transcription, genome replication, and capsid assembly occur in the host cell nucleus. Genes are replicated in a specific order: (1) immediate-early genes, which encode regulatory proteins; (2) early genes, which encode enzymes for replicating viral DNA; and (3) late genes, which encode structural proteins. The tegument and envelope are acquired as the virion buds out through the nuclear membrane or endoplasmic reticulum.

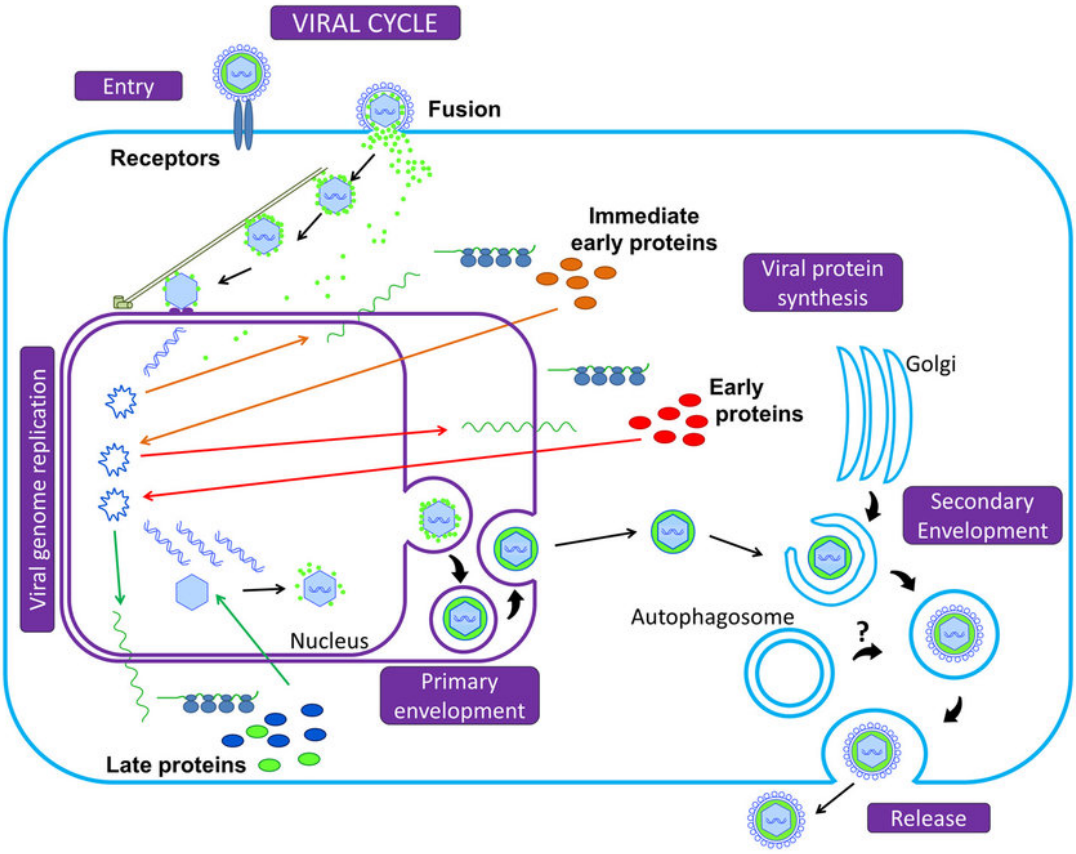

Figure 2.4. *Herpesvirus viral cycle*

Virions are transported to the cell membrane via the Golgi complex, and the host cell dies as mature virions are released. Alternatively, in selected cell types, the virus may be maintained in a latent state. The latent viral genome may reactivate at any time.

In this thesis, I have mainly focused on 3 herpesviruses: HSV-1, CMV and EBV. In particular, a larger space has been given to CMV, as results indicate its preponderant role in the immunosenescent process, and HSV-1, which is the less studied in terms of virus-specific responses.

2.2.1 HERPES SIMPLEX VIRUS TYPE 1 (HSV-1)

HSV-1 is one of the most ubiquitary viruses affecting about 90% of the people worldwide.

HSV-1 virions possess a round shape with a diameter of 120-200 nm. The envelope consists of a lipid bilayer in which are embedded 12 glycoproteins, responsible for the entry in host cell. The tegument is an unstructured layer composed by approximately 20 proteins including viral protein (VP) 16 responsible for the initial viral transcription, VP22 involved in virus transport along microtubules and the virion host shutoff (vhs) that blocks host cell protein synthesis. The capsid is composed by 162 capsomers in an icosahedral structure. The core is the central structure containing the viral genome [41].

The HSV-1 genome is a linear double stranded DNA long 152 kbp and can be divided into two unique sequences, designated as unique long (UL) and unique short (US), flanked by repeated sequences internal (IRL and IRS) and terminal (TRL and TRS) [42]. The HSV-1 genome encodes approximately 90 unique transcriptional units, of these at least 84 encode proteins, of which about 40 are necessary for viral replication.

After primary infection, occurring usually in the mucosal epithelium of the mouth, the virus enters nerve endings of local sensory neurons and it is transported retrograde to the nucleus where it establishes latency. In response to physical, hormonal or emotional stress, the virus can periodically reactivate and it is transported anterograde to or near the site of primary infection. Depending on several factors, including the host immune status, the reactivation may be asymptomatic or lead to a recurrent lesions [43].

Thus, when HSV-1 is inside a cell has two alternatives: its replication that ends with the lysis of host cell and the release of new viral progeny or the establishment of latency.

Both of these pathways anyway are preceded by the common event of the entry of the virus inside the cell. The access of HSV-1 into cells requires the concerted activity of at least 4 envelope glycoproteins: gB, gD and heterooligomers of gH and gL [44].

Once inside the cell, some tegument proteins remain in the cytoplasm while other are transported to the nucleus (VP16) or remain associated with the capsid that travel to the nucleus via microtubule network. As soon as the viral DNA enters the nucleus it is rapidly circularized and viral genes are expressed in a tightly regulated, interdependent temporal sequence [45]. α or immediate early (IE) genes, are first expressed, about 2-4 hours post-infection, by the combined action of the tegument protein VP16 with host cell elements, and stimulate the expression of β genes. Expression of β or early (E) genes reaches a peak at 4-8 hours post-infection. Several E genes are involved in viral DNA synthesis, event that starts after their expression. γ or late (L) genes encode structural proteins, and they reach a peak in expression during late stages of infection after viral DNA synthesis has started.

DNA synthesis occurs with the rolling-circle mechanism: DNA concatamers are cleaved and single monomers are incorporated in the nucleocapsids [46]. Viral particles mature by budding through the nuclear membrane. Enveloped viral particles travel along endoplasmic reticulum and Golgi apparatus, where probably are further processed, to finally exit the host cell by exocytosis, that lead to lytic cell death [47].

In fully permissive tissue culture cells, the entire process of viral replication takes about 18 hours.

However, HSV-1 persists lifelong in a latent state in sensory neurons innervating the site of primary infection. In particular HSV-1 has been observed to establish latency in trigeminal ganglia (TG). During latency viral genome is tightly associated with cellular histones at episomal level, thus no gene expression is detected except for some mRNA, named latency associated transcripts (LATs) whose coding sequence are located in flanking regions of UL [48].

2.2.1.1 Immune response to HSV-1/2

HSV may frequently reactivate without clinical symptoms but, when symptomatic, its manifestations vary from cold scores to encephalitis. The individual immune responses, both innate and adaptive, play a major role in determining the susceptibly of subjects to severe manifestations [49-51]. Keratinocytes are the first front barrier against HSV entry and,

triggered by the huge variety of pattern recognition receptors (PRRs) they express, they respond secreting type-I interferon (IFN) [52] in addition to several different chemokines and cytokines, thus to initiate the immune response [53]. In the epidermis, HSV can also infect Langerhans cells (LCs)[53]. HSV-infected LCs then migrate to the dermis, where they undergo apoptosis and are taken up by dermal macrophages and DCs [54] for subsequent antigen presentation to T cells [55]. The role of macrophages in HSV-associated disease is however controversial [56, 57]. Macrophages are indeed involved in HSV control [58]; in particular, M1 are refractory to HSV infection and respond to it with massive pro-inflammatory cytokine secretion which however, if excessive, contribute to disease exacerbation [56, 59]. Further studies aimed at better characterizing the myeloid compartment, including the presence of Myeloid-Derived-Suppressor-Cells, that have been shown in other viral infections to dampen T-cell responses [60], are required to dissect the role of the different myeloid cell subsets at the infection and latency sites [61].

Herpetic lesions are also highly infiltrated by plasmacytoid dendritic cells (pDC), producers of type-I IFN and responsible for the HSV-specific T-cell activation [62], and Natural Killer (NK) cells [62]. NK depletion is associated with increased susceptibility to HSV infection and systemic spread [63], and impaired NK cell cytotoxic capacity and IFN-γ secretion are associated with loss of viral control [64]. NK cells may be sensed through TLR2 [65], which is directly stimulated by HSV glycoproteins [66], but require co-stimulation from HSV-specific $CD4^+$ T-cells to be activated and start releasing IFN-γ [67]. Notably, and in contrast to classical immunological paradigms, it has been shown NK cells can present HSV-derived epitopes to $CD4^+$ T-cells [65, 68, 69].

Further to mucosal responses, immunity in the nervous system, and in particular in trigeminal ganglia where HSV establishes a latency, is important for protection [70]. Microglia, resident macrophages and monocytes are found in TG and brain of HSV-infected animals, and recognition through TLR2 and TLR9 is important for their activation [71-74] and NO production (required for viral clearance) [70, 75]. A further PRR crucial for HSV control is STING [76, 77]. Notably, STING stimulation on APCs has been shown, in several infection and tumor models, to ameliorate the induction of T-cell responses [78, 79]. Also in the case of HSV, it is likely that the stimulation of STING increases protection not only through boosting innate responses but, mostly, improving the engagement of adaptive responses.

Indeed, up to date, adaptive immunity is considered essential for long-lasting HSV control, although several controversies exist on the identification of the exact correlates (if humoral or cellular responses) [80-82]. After administration of HSV-2 glycoprotein D (gD-2) during a clinical trial, a higher protection level was observed in subjects with high gD-2-specific humoral responses, while the same was not true for subjects with high cellular responses [80]. However, subunit vaccines are better suited to induce humoral, rather than cellular, responses [83], and data from this clinical trial should be taken more as an indication of the contribution given from HSV-specific antibodies rather than a proof of concept of their role as immune correlate of protection. Passive transfer of HSV-specific antibodies has not shown substantial effects on viral replication [51, 84], and B-cell-depleted mice suffered less severe consequences, compared T-cell-depleted animals, after HSV infection [85, 86]. In addition, in a pre-clinical vaccination study where an attenuated, replication competent, HSV vector was administered to mice, we observed that mice without IgG survived lethal challenge while some animals with detectable antibodies died [87, 88]. In accordance with other reports [84, 89-95], these observations, together, indicate that humoral responses may contribute but are insufficient to mediate protection. Of note, both neutralization functions as well as the capacity to mediate antibody-dependent cell-mediated cytotoxicity (ADCC) seem important [96, 97].

Conversely, several works indicate that protective HSV immunity is mainly mediated by $CD4^+$ and $CD8^+$ T cells at their late stages of differentiation and expressing transcription factors controlling effector functions, such as T-bet and Blimp-1 [64, 82]. IFN-γ production from $CD4^+$ T cells has been shown as an important correlate of protection [64], and $CD4^+$ T-cell deficient mice rapidly dye after HSV infection [86, 98]. $CD4^+$ T cells are rapidly migrating into herpetic lesions [99], where they can persist also after healing [100], and secrete chemokines for $CD8^+$ T-cell recruitment [98, 101]. $CD8^+$ T cells found at both mucosal sites [93, 94, 102] and TGs [72, 74], and in particular those with an effector memory phenotype, are indeed directly responsible for the control of ocular and vaginal herpes [81, 82, 87, 103]. The importance of this T-cell subset has been demonstrated in several other contexts including vaccinia virus [104], HIV and SIV [105-109] and Lysteria monocytogenes [110]. Conversely, T cells from subjects with frequent recurrences of ocular herpes or from mice succumbing to intravaginal infection display a predominant central memory phenotype [81, 82, 103].

EM $CD8^+$ T-cells present with low proliferative but high cytolytic potential and, mostly, with the capacity to localize into tissues and develop into resident T memory (TRM) cells [101, 102, 110-112]. Mucosal responses are known to be crucial to control HSV [93, 94, 102] and, in case of genital herpes, mucosal rather than systemic immunity protects mice from lethal HSV challenges [102, 113]. However, for this function, prior $CD4^+$ T-cell presence in herpetic lesions seems required [101]. HSV-specific $CD4^+$ and $CD8^+$ TRM cells will then persist in *loco* also after the lesion resolution, adjacent to peripheral nerve endings, ready to control future reactivations in peripheral sites [87, 114, 115]. Their in-depth transcriptional analysis has revealed a high expression of genes usually associated with ongoing effector responses, suggesting that they are frequently stimulated by antigens but that, at the same time, they can effectively inhibit HSV reactivation and thus viral spread [116].

Intriguingly, HSV-specific, late differentiated, T cells in asymptomatic subjects seem to overcome the several immune suppression mechanisms usually put in place during viral infections. For instance, they are directed toward immunodominant epitopes [81, 82, 87, 102, 117] and are characterized by low PD-1 and high Tim-3 expression [117, 118]. Instead, those specific for subdominant epitopes are usually less differentiated but express higher levels of inhibitory checkpoints molecules [87, 117]. Moreover, their metabolism is unaffected by HSV replication. Indeed, the functionality of late-differentiated T cells is usually supported, energetically, by an increased glycolysis [119]. As also HSV-1 infected-cells display an increased glycolysis [120], a competition for glucose may exist, and T cells may be forced to adapt their metabolism exploiting other pathways [121]. However, HSV-specific TRM cells keep high glycolytic levels [116], suggesting that their metabolic pattern is not affected by HSV-infected cells. Therefore, this particular T cell subset seems they key player in HSV control and should be targeted in future therapeutic approaches.

2.2.2 CYTOMEGALOVIRUS

CMV infection can lead to three distinct clinical syndromes:

- congenital CMV infection, which may results in severe symptomatic congenital cytomegalic inclusion disease (hepatosplenomegaly, retinitis, a petechial/purpuric skin rash, and involvement of the central nervous system);

- mononucleosis syndrome, characterized by fever, malaise, atypical lymphocytosis, pharyngitis and, rarely, cervical adenopathy or hepatitis. Cytomegalovirus mononucleosis can be distinguished from EBV mononucleosis by serologic assays.
- CMV infection in severely immunocompromised individuals, that may experience life-threatening disease from either primary or reactivated cytomegalovirus infection. In these patients, infection can involve the lungs, gastrointestinal tract, liver, retina, and central nervous system

Replication of cytomegalovirus is most prominent in cells of glandular origin (salivary glands and the kidneys), and large quantities of virus can be shed in saliva and urine. Cytomegalovirus can cause persistent infection in various tissues, including those of the salivary glands, breasts, kidneys, endocervix, seminal vesicles and peripheral blood leukocytes. This persistent infection leads to chronic viral excretion by the involved organ.

Cytomegalovirus infections are among the most prevalent virus infections worldwide. Transmission of virus from mother to child can occur by one of several routes, including infected breast milk, cervical secretions, and saliva. Conversely, a child can transmit infection to the mother through infected secretions or urine.

2.2.2.1 CMV AND AGEING

Despite a strong immune response, CMV is not eliminated and remains as a latent infection. This virus is known to leave a major imprint on our immune system, in particular reflected by the accumulation of $CD27^{-}CD28^{-}CCR7^{-}CD45RA^{+}CD57^{+}$ T cells, identified as late differentiated/exhausted EM T cells which produce pro-inflammatory cytokines [122]. CMV infection has also been associated with changes of naive T-cell frequencies, though not in all studies [123], and with consistently increased levels of systemic inflammatory cytokines [122]. Its impact on vaccine responsiveness has been controversial. It has been mostly studied in the context of vaccination against influenza [124-127], with some studies reporting suboptimal influenza vaccine responses in CMV-infected patients, and other studies showing no effect or even an enhancement of vaccine humoral and cellular immune responses or heterologous immunity by CMV [128-130]. Lack of consensus may be related to the variability of the parameters investigated in different studies, including: $CD8^{+}$ *versus* $CD4^{+}$ T cells, T-cell frequencies *versus* absolute counts, young *versus* old subjects, primary *versus* recall vaccine

responses. Therefore the influence on adaptive immunity by latent infection with CMV and other persistent viruses remains an open question with several unresolved issues.

2.2.3 EPSTEIN-BARR VIRUS

EBV is trophic for B-lymphocytes. Replication has been documented in the parotid gland, as well as other lymphatic tissues. Evidence of lytic disease, as evidenced by the formation of multinucleated giant cells, is not apparent with infection caused by Epstein-Barr virus. EBV infection is associated with classic mononucleosis, characterized by are malaise, myalgia, pharyngitis, cervical adenopathy, splenomegaly, and atypical lymphocytosis. EBV has also been incriminated as a cause of lymphoproliferative disease in highly immunocompromised individuals. The development of lymphoproliferative malignancy in heart and bone marrow transplant recipients has been documented, and is felt to be associated with the presence of virus. The virus has been linked to a wide range of malignancies: sucposttransplant lymphoproliferative diseases (PTLDs), nasopharyngeal carcinoma (NPC), Hodgkin's lymphoma, and gastric carcinoma (MS).

In healthy individuals, EBV is highly prevalent, as it affects more than 90% of individuals worldwide. The age of primary infection was found to vary according to socioeconomic factors that are reflected by crowdedness and low sanitation.

3 OBJECTIVE

It is common nowadays to live beyond 80 years old, which has an important impact on human demographics and represents a growing challenge in terms of health care. Advanced age is accompanied by the increase in acute and chronic diseases, resulting in an important decline in quality of life. In turn, this is associated with the need to care for elderly individuals with poor health. In particular, elderly people suffer more often and more severely from infections than young individuals [131-133]. The current SARS-CoV-2 pandemic represents a particularly relevant example of the issues that can be encountered in this context [134, 135]. It highlights the importance of understanding better the evolution of the immune system with ageing, which is key in the vulnerability of old people to many diseases including COVID-19. Moreover the response of older people to vaccination is considered generally suboptimal [136]. A recent study shows that the immunogenicity to the SARS-CoV-2 vaccine BNT162b2 is indeed reduced in the elderly [137].

The need for in-depth knowledge on immune responsiveness to infections and vaccinations in the elderly has never been greater, and this work aims at unravelling the interplay between the human immune system and the herpesviruses with ageing. Herpesviruses are highly prevalent and establish a persistent infection which may give rise to period reactivation. This can affect the whole immune system, and the first part of thesis was aimed at assessing at which extent T-cell responses are affected in adults and elderly subjects carrying one or more herpesvirus infection. Particular attention was given to numerical and functional alterations of the naïve T-cell compartment. Moreover, the eventual interference of herpesvirus infections on the development of primary responses against a *de novo* vaccine was studied using the vaccine against tick-borne encephalitis virus (TBEv) as model antigen.

In the second part of the thesis, I focused on the opposite direction, studying HSV-1-, CMV- and EBV-specific T-cell responses and their eventual fluctuations with advancing age. I undertake a comprehensive characterization of CD8 responses mediated by different memory subsets, to analyze potential age-related alterations of specific memory T-cell subpopulations.

While recall responses are important for the control of vaccine-preventable diseases or persistent and recurrent infections, primary immunity is crucial in the context of emerging

pathogens or *de-novo* vaccination. Therefore the third part of the thesis was aimed at studying age-associated alteration to epitope-specific naïve $CD8^+$ T cells. I undertook the study in the context of the COVID-19 pandemic, analyzing the capacity of elderly individuals to mount primary responses to SARS-CoV-2, which represents a perfect model to study primary responses in unexposed subjects.

The overall objective of the thesis is thus to evaluate the age-associated changes in T-cell immunity toward both primary (SARS-CoV-2) and recall antigens (herpesviruses) and to ascertain the influence of persistent herpetic infections on these changes.

Thesis structure	Main questions
Section 1	Do persistent herpesvirus infections affect T-cell immunity during ageing?
Section 2	Does ageing affect memory T-cell responses to herpesviruses?
Section 3	Does ageing affect naive T-cell responses?

4 MATERIALS AND METHODS

4.1 Study subjects

Two cohorts of healthy volunteers were enrolled in this study. The first cohort (observational study, Resulta sections 1 and 2) consisted of 150 healthy adults (aged between 19 and 95 years old) with serology data for four common herpesviruses. Individuals <65y were recruited among blood donors (Etablissement francais du sang) in Paris (France) while those aged >75y were recruited at the geriatric department of the Pitié-Salpêtrière Hospital (Paris, France). Individuals with malignancies, acute diseases, or severe chronic diseases, such as atherosclerosis, congestive heart failure, poorly controlled diabetes mellitus, renal or hepatic disease, various inflammatory conditions, or chronic obstructive pulmonary disease, as well as individuals on immunosuppressive therapy, were excluded from the study. The second cohort (Results section 1) consisted of 137 individuals aged more than 70 years old, who received a full TBE vaccination course of three injections at week 0, 4 and 24 with a licensed inactivated whole virus vaccine (FSME Immun® CC, Baxter) as part of a clinical trial conducted at the Epidemiology, Biostatistics and Prevention Institute (EBPI) at the University of Zürich Switzerland. Here, we report the core data of this study including immunogenicity of TBE vaccination in healthy elderly stratified by CMV serostatus. Included individuals were healthy according to a health questionnaire and a physical evaluation at study entry; 57% reported no co-morbidities and no long-term medical treatment, 43% reported one co-morbidity requiring drug treatment (mostly hypertension). All individuals were immunocompetent and TBEv-naive and -seronegative. Immune response assays were conducted prior to vaccination, and at weeks 4, 8, 24 and 28 post-vaccination for humoral and week 26 for cellular immune responses.

The studies were approved by the local institutional ethics committees (i.e. Comité de Protection des Personnes of the Pitié Salpétrière Hospital, Paris and cantonal ethics committee, Zürich, Switzerland - number of the ethics approval is EK1309) and all participants provided written informed consent.

For priming experiments of SARS-CoV-2 specific naïve $CD8^+$ T cells (Results section 3), peripheral blood samples (n=19) were obtained in an anonymous fashion from blood donors through the Blood Bank of the Ferrara Hospital. The protocol was approved by the Regional health authority (AUSL), which supervises blood donatios. Donors were either sampled before

December 2019 or during 2020. In the latter case, donors were serologically negative for SARS-CoV-2 antibodies, as determined in plasma samples by ELISA (Mabtech) according to manufacturer's instructions.

PBMCs were isolated from venous blood samples via Ficoll-Paque (GE-Healthcare) density gradient centrifugation according to standard protocols and cryopreserved in complete medium supplemented with dimethyl sulfoxide (DMSO; 10% v/v; Sigma-Aldrich) and fetal calf serum (FCS; 90% v/v; Sigma-Aldrich) or as dry pellet for DNA extraction. Complete medium (R+) consisted of RPMI 1640 supplemented with non-essential amino acids (1% v/v), penicillin/streptomycin (100 U/mL), L-glutamine (2 mM), and sodium pyruvate (1 mM) (all from Thermo Fisher Scientific).

4.2 Herpesvirus serological assays

Herpesvirus serological tests were performed using clinical-grade assays measuring IgG levels, according to the manufacturer's instructions. Anti-CMV IgGs were measured using the BioPlex 2200 ToRC IgG kit on the BioPlex 2200 analyzer (Bio-Rad). Anti-EBV, anti-HSV1 and anti-HSV2 IgGs were measured using the BioPlex 2200 EBV, HSV-1 and HSV-2 IgG kit on the BioPlex 2200 analyzer (Bio-Rad).

4.3 Measurement of inflammation associated cytokines

Plasma cytokines were measured by Simoa digital ELISA using commercial assays for IL-6, TNF, IL-10 and IFN-γ (Quanterix) according to the manufacturer's instructions [138].

4.4 Phenotypic analysis on fresh blood and *ex vivo* phenotye

PBMCs of donors from Cohort 1 were stained for surface markers using combinations of the following directly conjugated monoclonal antibodies: anti-CCR7–PE-Cy7 (clone 3D12; BD Biosciences), anti-CD3–BV605 (clone SK7; BD Biosciences), anti-CD8–APC-Cy7 (clone SK1; BD Biosciences), anti-CD4 BUV395 (clone SK3; BD Biosciences) anti-CD27–AF700 (clone O323; BioLegend), anti-CD45RA–ECD (clone 2H4LDH11LDB9; Beckman Coulter) and anti-CD95–FITC (clone DX2; BD Biosciences). Non-viable cells were eliminated from the analysis using LIVE/DEAD Fixable Aqua (Thermo Fisher Scientific). Samples were acquired using an LSR Fortessa (BD Biosciences).

Ex vivo immunophenotype from samples used in Results section 3 was performed on thawed PBMCs upon exclusion of dead cells with LIVE/DEAD Fixable Aqua (Life Technologies) and the

following directly-conjugated monoclonal antibodies were used: anti-CD4 eFluor450, HLA-DR-APC and anti-CD45RA PerCP-Cy5.5 (Life Technologies); anti-CD8 APC-Cy7 and anti-CCR7 PE-Cy7 (BD); anti CD27 FITC anti-PD1 PE (Miltenyi Biotec). Naive T cells were defined as $CCR7^{+}CD427^{+}CD45RA^{+}$. Samples were acquired using an FACS Canto II (BD Biosciences).
Data were analyzed using FACSDiva software version 7 (BD Biosciences) and/or FlowJo software version 10 (FlowJo LLC).

4.5 Analysis of TBEv-specific humoral and cellular immune responses

TBEv-specific antibody titers were measured before (week 0) during (week 4, 8 and 24) and after (week 28) the tick-borne encephalitis virus (TBE) vaccination course by ELISA according to the manufacturer instruction. The TBEv-specific cellular immune response was assessed at week 0 and 26 by IFNγ enzyme-linked immunosorbent spot assay (ELISpot) using pools of overlapping peptides for all structural proteins of TBEv. Briefly, $2x10^5$ thawed PBMCs / well from week 0 and week 26 of the same donor were stimulated in anti-IFNγ (clone 1-D1K, Mabtech) coated 96-well ELISpot-plates (MAIP S45, Millipore) for 18h with $2x10^4$ freshly generated autologous monocyte-derived DCs. For antigen-specific stimulation, five pools of overlapping peptides encompassing all structural proteins of TBEv were used at 1mg/ml final peptide concentration (15-mers overlapping by 5; BMC Microcollections, Germany). Washed plates were incubated with anti-IFNγ-Biotin (7-B6-1, Mabtech) followed by Streptavidin-alkaline Phosphatase (Mabtech), developed with color reagents (170-6432, Biorad) and analyzed in an automated ELISpot reader (AID). The number of total spot forming units (SFU) was calculated after background subtraction of the unstimulated control. For intracellular cytokine staining (ICS) $1x10^6$ PBMCs from week 26 were stimulated with pools of TBEv overlapping peptides (1mg/ml final peptide concentration) in the presence of Brefeldin A overnight at 37°C. Stimulation with Staphylococcus enterotoxin B (SEB) or VZV lysate were also performed as comparison. Medium alone was used as a negative control. On the next day, cells were surface stained with anti-CD3-PerCP (clone SK7; BD Biosciences), anti-CD4-AmCyan (clone SK3; BD Biosciences), and anti-CD8-APC-Cy7 (clone SK1; BD Biosciences). Samples were then fixed and permeabilized, before staining with anti-IFNγ-APC (clone B27; BD Biosciences). Samples were acquired using an LSR Fortessa (BD Biosciences) and data were analyzed using FACSDiva software version 7 (BD Biosciences) and/or FlowJo software version 10 (FlowJo LLC). Percentages of antigen specific T cells were calculated after background

subtraction of the unstimulated control.

4.6 DNA extraction and TREC analysis on PBMCs

DNA was extracted from dry pellet using RSC Blood DNA according to manufacturer instructions (Promega).

sjTRECs are episomal circular DNAs generated during TCR chain recombination and were quantified by multiplex quantitative PCR together with the Albumin for cell normalization on a ViiA7 (Thermofisher Scientific). The protocol is based on a quantitative PCR of genomic DNA extracted from whole blood, using the Biomark HD system (Fluidigm France, Paris, France). 1 to 2 µg of genomic DNA was preamplified for 3 minutes at 95°C and then 18 cycles of 95°C for 15 seconds, 60°C for 30 seconds and 68°C for 30 seconds, in a 50 µl reaction that contained the primers, 200 µM of each dNTP, 2.5 mM MgSO4 and 1.25 unit of Platinum Taq DNA pol High Fidelity (ThermoFisher Scientific, Courtaboeuf, France) in 1x buffer. Columns of 48.48 Dynamic array IFCs were loaded with 5 µl containing 2.25 µl of a 1/400th dilution of preamplified DNA, 2.5 µl of 2x Takyon Low Rox Probe MM (Eurogentec) and 0.25 µl of sample Loading Reagent and raws with an equal mixture of 2x Assay loading Reagent and 2x Assay Biomark that contains only the 2 primers and the probe specific for each assay.

sjTRECs were normalized to 150 000 cells (around 1 µg of DNA) using the Albumin gene quantification.

4.7 DNA and RNA extraction on sorted cell populations

After thawing, PBMCs were stained with CCR7 BV650 (Clone 3D12; BD Biosciences), CD95 FITC (Clone DX2; BD Biosciences), CD27 PE (Clone M-T271; BD Biosciences), CD8 APC (Clone RPA-T8; BD Biosciences), CD4 APC-Cy7 (Clone RPA-T4; BD Biosciences), CD45RA ECD (Clone IM271111; Beckman Coulter), CD57 PB (Clone HCD57; Biolegend), CD49d PeCY7 (Clone 9F10; Biolegend), and eFluor 506 for viability (Biolegend). Naive CD4 and CD8 cells, defined as $CD454A^{+}$, $CD27^{+}$ $CCR7^{+}$, $CD95^{-}$, $CD49d^{-}$, were sorted on an ARIAIII (Becton Dickinson). DNA and RNA from sorted cells were extracted using AllPrep DNA/RNA Micro kit (Qiagen) according to manufacturer instruction.

4.8 sjTREC digital droplet PCR

Ten µl of genomic DNA (50-200 ng) were mixed in 1x Master Mix Takyon (Eurogentec) 200 nM of sjTREC FAM-DarkQuencher Probe, 400 nM of sjTREC Forward and Reverse Primers, 200

nM of BCKDHA YakimaYellow-TAMRA probe, 900 nM of BCKDHA Forward and Reverse primers [140] and 1,2 µL of surfactant. Droplets were generated with a RainDance Source system. PCR amplification (95°C 5 min then 45 cycles of 95°C 15 sec and 60°C, 1 min) was performed and read using the RainDance Sense apparatus. sjTREC were normalized for the number of cell using BCKDHA quantification.

4.9 Peptides

The following peptides were synthesized by solid phase method and purified by HPLC to >97% purity: 37 SARS-CoV-2-derived peptides, the EV10 peptide (ELAGIGILTV) and the peptides used to stimulate memory responses (NLVPMVATV from CMV; CLGGLLTMV and GLCTLVAML from EBV; ALMLRLLRI, NLLTTPKFT, RMLGDVMAV, FLGAGALAV, ALLGLTLGV and GIFEDRAPV from HSV-1). The SARS-CoV-2-derived peptides were selected from wider lists of predicted and/or confirmed epitopes, as indicated in Table 5.3.1. Peptides, either as single or in pool/matrixes, were suspended in DMSO and used at the concentration of 1 µM. Matrixes were composed of 6 peptides each (Table 5.3.1).

4.10 In vitro priming of human antigen-specific $CD8^+$ T cells

Thawed PBMCs were resuspended at 10^7 cells/ml in 24-well tissue culture plates (5 x 10^6 cells/well) or in a 75-cm^2 tissue culture flask (7 x 10^7 cells/flask) containing AIM medium (Life Technologies) supplemented with FLT3L (50 ng/ml; R&D Systems) to mobilize resident DCs. After 24 hours (day 1), the peptides were added to the cultures, and DC maturation was induced with TNFα (1000 U/ml, Miltenyi Biotech), IL-1β (10 ng/ml, Miltenyi Biotec), IL-7 (0.5 ng/ml, R&D Systems), and prostaglandin E2 (1 µM, Calbiochem). On day 2, FBS (Euroclone) was added at a final v/v ratio of 10%. Medium was then replaced at day 4 and 7 with fresh RPMI 1640 (Euroclone) enriched with 10% FBS, nonessential amino acids (Euroclone) and sodium pyruvate (Sigma-Aldrich). Antigen-specific $CD8^+$ T cells were characterized on day 10.

4.11 ELISpot assay for SARS-CoV-2-specific T-cell responses

Human IFNγ ELISpot PLUS (HRP) kits with precoated plates were obtained from Mabtech. PBMCs (1 x 10^5 upon priming or 2.5 x 10^5 for ex vivo stimulation) were seeded in duplicate and stimulated with peptide pools or matrixes. Cells incubated with medium alone were used as negative control, whereas those stimulated with an anti-CD3 monoclonal antibody (Mabtech) represented the positive control. Plates were incubated for 24 hours, processed

according to the manufacturer's instruction and read by an automated reading system (AELVIS). The number of specific IFNγ-secreting T cells, expressed as spot-forming units (SFU) per 10^6 cells, was calculated by subtracting the negative control values. Responses were considered after background subtraction, when more than 50 SFU/million cells were present. This threshold was taken after protocol optimization [142] and maintained throughout all assays. Results were excluded if the positive control was negative.

4.12 Phenotypic analysis and intracellular cytokine staining on cryopreserved PBMCs

Intracellular cytokine staining was performed to detect T-cell responses specific for SARS-CoV-2, HSV-1, CMV and EBV. Two x 10^6 $PBMC_S$ were incubated with media alone or with the pool of 37 peptides for 6 hours. Anti-CD107 Vioblue (Miltenyi Biotec) was added at the time of stimulation, whereas brefeldin and monensin were added after 1 hour of stimulation. After incubation for 5 hours at 37°, cells were stained with LIVE/DEAD Fixable Aqua and anti-CD8 APC-Cy7. After fixation and permeabilization (Cytofix/Cyoperm, BD), cells were incubated with anti-IFNγ FITC (Life Technologies) and anti-TNF PE (BD) antibodies. Results obtained from cells incubated with media alone (NT) were subtracted from those obtained from cells incubated with the peptide pool (background subtraction). Boolean gates were performed on IFNγ, TNF and CD107. Cytokine responses were background subtracted individually before further analysis. All acquisitions were performed on a FACS CANTO II (BD) upon compensation conducted with antibody capture beads (BD). Flow cytometry data was analyzed using FlowJo (version 10.1, Tree Star Inc.).

4.13 Protein sequences and alignment

Complete protein sequences for the envelope protein (E), membrane glycoprotein (M), nucleocapsid phosphoprotein (N), RNA dependent RNA polymerase (RdRp), spike protein (S), ORF3a and ORF6 of SARS-CoV-2 and, when applicable, the HCoVs NL63, 229E, OC43 and HKU1, were downloaded from NCBI (accession numbers reported in Table 4.1). MUSCLE algorithm was used to align multiple sequences.

Protein	Virus				
	SARS-CoV-2	**NL63**	**229E**	**OC43**	**HKU1**
E	YP_009724392.1	YP_003769.1	NP_073554.1	YP_009555243.1	YP_173240.1
M	YP_009724393.1	YP_003770.1	NP_073555.1	YP_009555244.1	YP_173241.1
N	YP_009724397.2	YP_003771.1	NP_073556.1	YP_009555245.1	YP_173242.1
RdRp	YP_009724389.1	AIW52828.1	QNT54753.1	YP_009555260.1	YP_459941.1
S	YP_009724390.1	YP_003767.1	NP_073551.1	YP_009555241.1	YP_173238.1
ORF3a	YP_009724391.1	YP_003768.1	-	-	-
ORF6	YP_009724394.1	-	-	-	-

Table 4.1 *Protein identities used*

4.14 Statistical analysis

Univariate statistical analyses were performed using GraphPad Prism, R [144] and Rstudio [145] softwares with the packages Tidyverse [146] Scales [147]. Groups were compared using the non-parametric Mann-Whitney or Kruskal-Wallis tests, as indicated in Figure legends. Bonferroni correction was applied when performing multiple group comparisons. Spearman's rank test was used to determine correlations. *P* values below 0.05 were considered statistically significant.

5 RESULTS[1]

5.1 Section 1: The effect of herpesviruses on the ageing immune system

Studying primary immune responses, in particular to vaccination, in older humans poses several challenges. Most vaccinations are given to boost preexisting immunity in this population. The responses to several recall vaccines, such as those against influenza virus, varicella zoster virus [148, 149], *Streptococcus pneumoniae* [150, 151] or diphtheria and tetanus antigens [152], have been studied in older individuals. These studies have revealed differential responses upon vaccination in elderly populations. Some vaccines provided an adequate boosting of immunological memory while others presented a waning capacity to maintain strong and protective responses [153]. In contrast, available data sets from studies on Yellow Fever [154, 155], hepatitis B [156], or Japanese encephalitis virus [157] vaccines, indicate that primary responses in older individuals are mostly compromised. Vaccinated older people presented delayed and reduced primary antibody responses compared with young adults, and both their $CD8^+$ and $CD4^+$ T-cell responses were impaired. Overall, these data support a general impairment of *de novo* immune responses, while recall immunity seems more preserved in some cases.

Immunosurveillance against emerging viruses and *de novo* vaccine responsiveness are crucially dependent on the efficacy to mount primary responses [30], and thus the activation of naive T cells [158]. Several independent studies have confirmed that ageing results in general defects of the quality (e.g. activation, proliferation, telomere length, and differentiation) and the quantity of naive $CD8^+$ and $CD4^+$ T cells, reducing their ability to respond to previously unencountered antigens [34, 159, 160]. The quantitative loss of naive T cells is a key feature of immune ageing and can by itself be a cause of impaired vaccine-specific immune response. Several factors play a role in the age-related contraction of the naive T-cell compartment: (i) the naive T-cell pool is used and consumed throughout life to generate effector and memory T cells; (ii) old age-associated impaired lymphopoiesis and thymic involution result in diminished naive T-cell production and replenishment; (iii) naive

[1] I purposely decided, especially in the Results section, to use the plural “we” instead of the singular “I”. Research is a team work. In addition, this study has been done in collaboration with several institutions. Therefore, to acknowledge the support of the different colleagues and collaborators, I believe that the pronoun “we” is the most appropriate.

T-cell maintenance is affected by an altered homeostasis due to an unbalanced naive vs memory cell ratio.

Whether common chronic and latent viral infections impact immune responsiveness, e.g against emerging viruses or vaccines, is an important issue in this context. Persistent viruses, such as those of the herpesvirus family, are highly prevalent in the human population and, although classically benign in immunocompetent individuals, exert a long-term stress on host's immune cells. From a biological point of view, the herpesvirus family represents the most classic model of viruses that establish a latent infection, a strategy that allows them to remain in the host for the duration of its life. After primary acute infection, herpesviruses induce a persistent infection characterized by successive cycles of reactivation and authentic virological latency, established only in particular cell types and anatomical sites, different for each herpesvirus. Latent infection constitutes a reduced immunogenic stimulus for the infected host, while the reactivation phase is characterized by the initiation of the lytic viral cycle, the rapid destruction of target cells and the induction of inflammatory and virus-specific immune responses. The prevalence of these infections increases proportionally with age, and their role in age-related immune decline remains a matter of debate.

In this section, we aimed to identify cumulative and differential effects on $CD8^+$ and $CD4^+$ T-cell compartments by persistent long term viral infections in an elderly population. We focused on the influence of herpesvirus infection including CMV, EBV, HSV-1 and HSV-2 on the counts, maintenance and responsiveness of naive T cells. To this end, two cohorts were studied: a cohort of 150 healthy adults aged between 19 and 95 years old, and a cohort of 137 older healthy adults (aged more than 70 years old) who received a primary vaccination against tick-borne encephalitis (TBE). We found that long term infections with herpesviruses, in particular CMV, impacted the naive $CD4^+$, but not $CD8^+$, T-cell compartment, with consequences for vaccine responsiveness.

5.1.1 Different influence of age and persistent infections on systemic inflammation and the T-cell compartment

We studied a cohort of healthy adults (n=150, named "Cohort 1") aged from 19 to 95 years old, grouped according to the number of persistent viruses they were infected with on the basis of the serology data for four common herpesviruses: CMV, EBV, HSV-1 and HSV-2 (see Table 5.1.1 for a distribution of donors according to their serology).

		Cohort 1 N= 150				Cohort 2 N= 137	
		Mid		Old		Old	
		CMV-neg N= 36	CMV-pos N= 37	CMV-neg N= 23	CMV-pos N= 54	CMV-neg N= 68	CMV-pos N= 69
Age (year)	(median; range)	32 (19-65)	44 (20-64)	82 (76-94)	83 (75-95)	73 (70-86)	74 (70-87)
Gender	(female: male)	16:20	20:17	16:7	31:23	30:38	38:31
Body Mass Index (BMI)	(kg/m^2, mean, range)	N/A	N/A	21 (17-29)	24 (16-32)	24 (18-30)	24 (18-30)
Co-morbidities	with/ without	N/A	N/A	13/10	37/17	26/42	33/36
Medication	with/ without	N/A	N/A	16/7	42/12	26/42	32/37
Number of drugs	0	N/A	N/A	7	12	42	37
	1	N/A	N/A	4	13	19	26
	2+	N/A	N/A	12	29	7	6

Table 5.1.1. *Baseline characteristics of study participants*

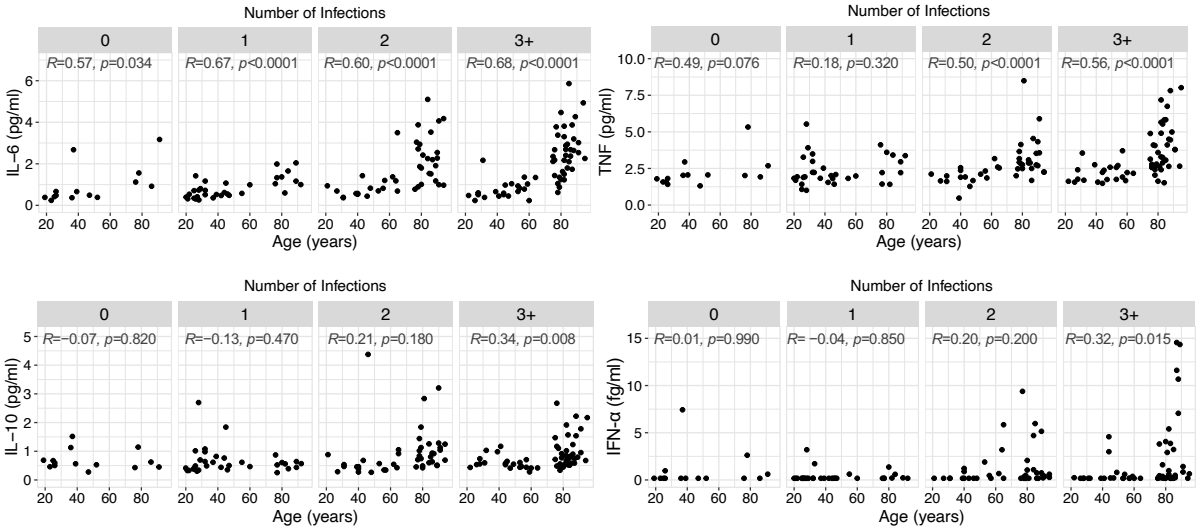

Figure 5.1.1*. Impact of age and herpesvirus infections on inflammation levels. Correlation between inflammatory cytokine levels and age according to the number of infections with herpesviruses (n=14, 33, 43 and 59 for 0, 1, 2, and 3+ infections). Statistical significance was determined by Spearman's rank correlation. Spearman's R and p values are shown for each panel.*

Persistent infections with these viruses may induce sustained inflammatory responses in their host and, to verify this hypothesis, we measured by digital ELISA circulating cytokines (IFN- γ, IL-6, TNF and IL-10) commonly associated with inflammatory manifestations. We observed an age-dependent increase in all four inflammatory markers, also associated with increasing numbers of herpesvirus infections (Figure 5.1.1).

However, the cumulative effect of latent infections on inflammatory cytokines could only be found in the long term, i.e. in older adults (above 75 years old), while younger subjects stratified by number of infections displayed similar cytokine levels (Figure 5.1.2A). This rise in inflammatory cytokine levels in older people was strongly associated with CMV infection, except for IL-6 levels, which presented a modest increase in CMV-negative older donors (Figure 5.1.2B). Of note, apart from IL-6, the increase in inflammatory cytokines with ageing was barely noticeable in the absence of infections (Figure 5.1.2B).

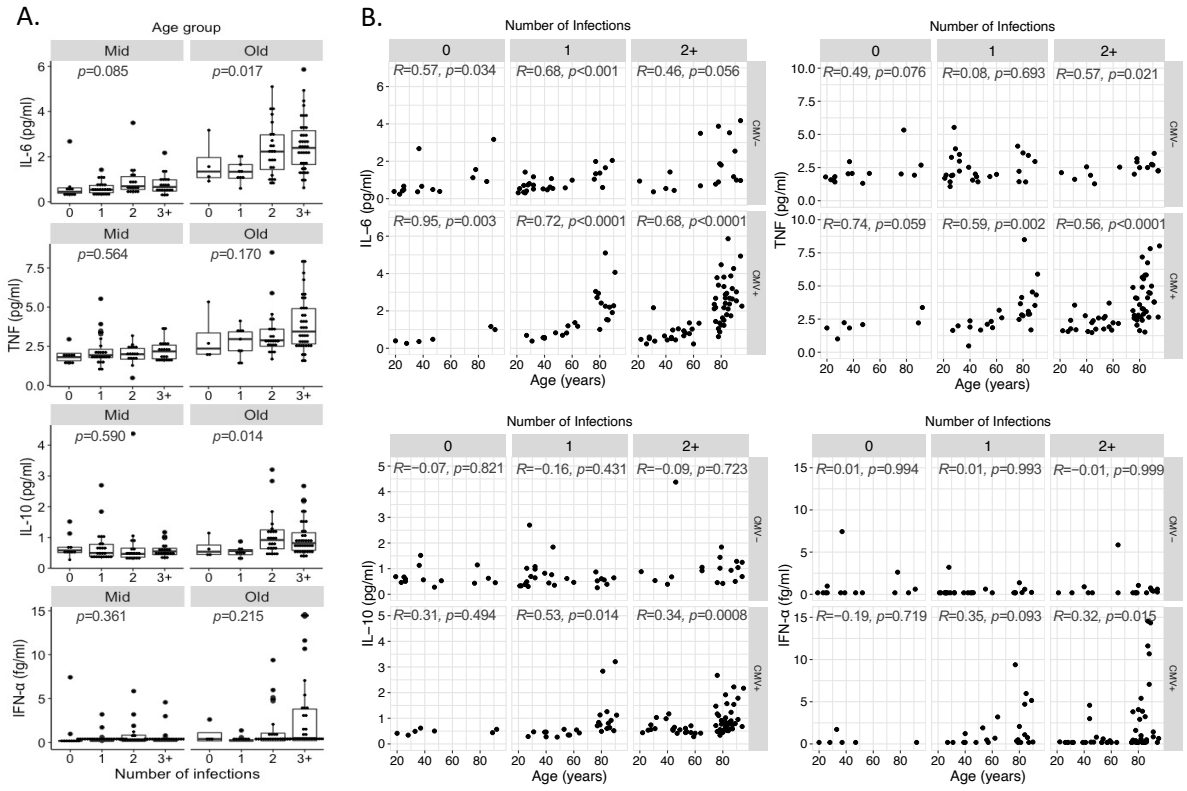

Figure 5.1.2. *Impact of age and herpesvirus infections on inflammation levels. (A) Inflammatory cytokine levels according to the number of infections with herpesviruses in subjects grouped according to age and shown as box and whiskers plot (n=73 for Mid, n=76 for Old). (B) Correlation between inflammatory cytokine levels and age according to number of infections with herpesviruses (excluding CMV) and CMV seropositivity (n= 149). Spearman's R and p values are shown for each panel. Statistical significance was determined by Kruskal-Wallis test (A) or Spearman's rank correlation (B).*

These data indicate that herpesvirus infections, especially CMV, exacerbate the inflammatory profile of adults only in the long term, i.e. in older people.

These common herpesviruses, in particular CMV, are known to induce strong T-cell responses through the activation of naive T cells and differentiation into effector memory (EM) T cells. Total $CD4^+$ and $CD8^+$ T-cell counts were unaffected by age while only the number of $CD8^+$ T cells was increased by the presence of multiple infections (Figure 5.1.3).

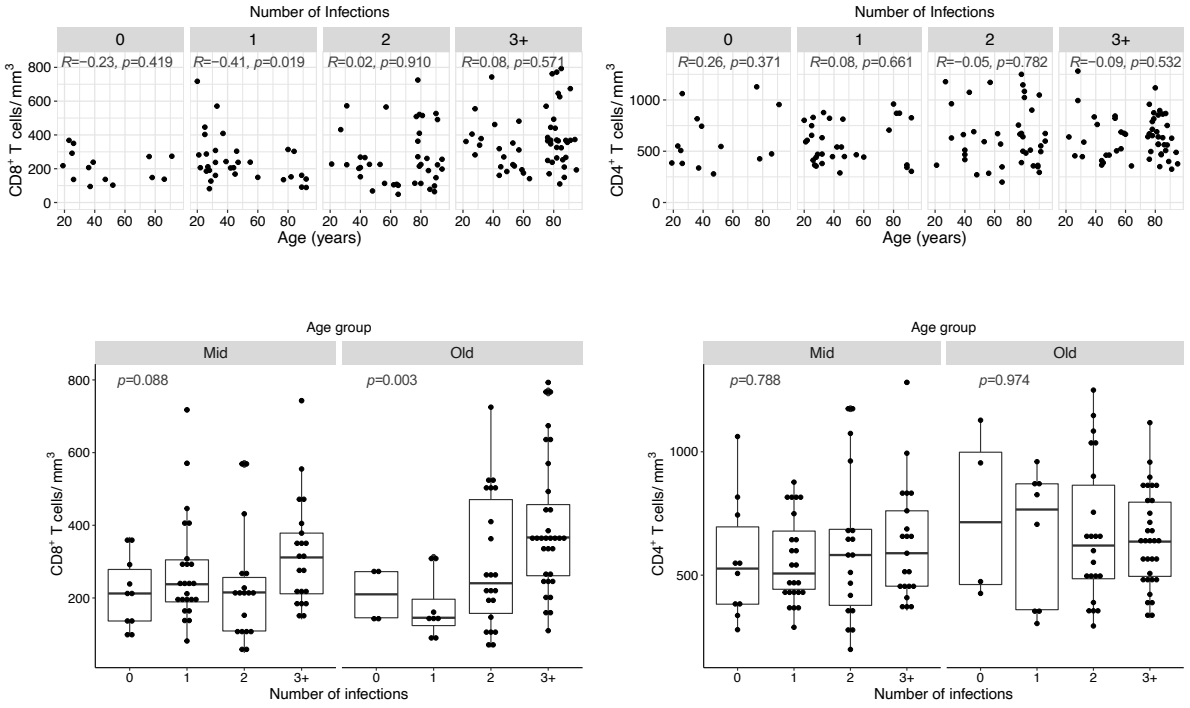

Figure 5.1.3. *Impact of age and herpesvirus infections on T-cell counts. (A) Correlation between total $CD8^+$ and $CD4^+$ T-cell absolute counts and age according to the number of infections with herpesviruses (n=13, 32, 39 and 49 for 0, 1, 2, and 3+ infections). Statistical significance was determined by Spearman's rank correlation. Spearman's R and p values are shown for each panel. (B) Absolute counts of total $CD8^+$ and $CD4^+$ T-cells according to the number of infections with herpesviruses in subjects grouped according to age and shown as box and whiskers plot (n=73 for Mid, n=60 for Old). Statistical significance was determined by Kruskal-Wallis test.*

In line with the inflammatory cytokine profile, an increase in both $CD4^+$ and $CD8^+$ EM T cells (defined as $CD3^+$ $CD27^-$ $CD45RA^{+/-}$ $CCR7^-$) was observed overtime with herpesvirus infections (Figure 5.1.4).

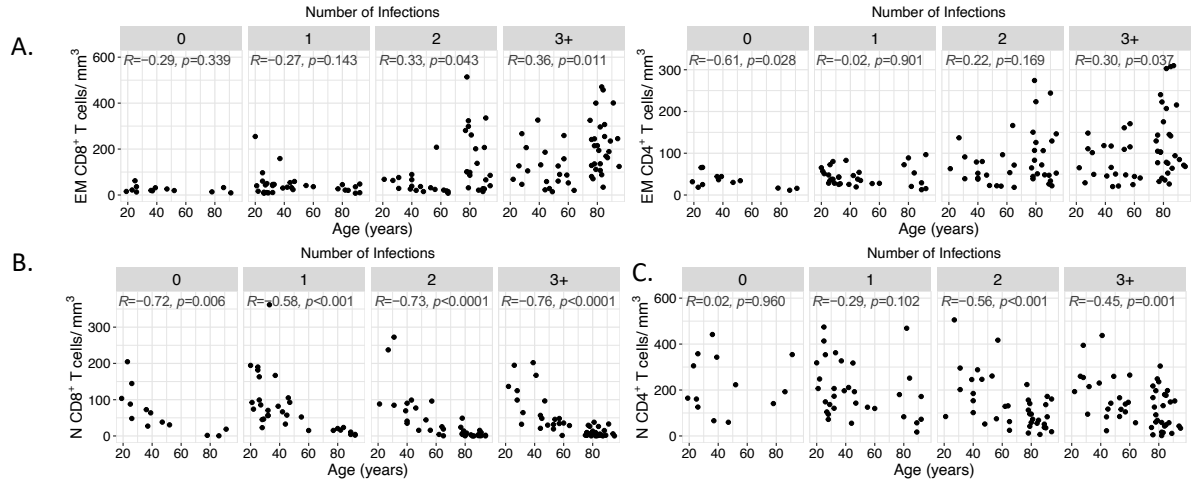

Figure 5.1.4. *Impact of age and herpesvirus infections on effector memory and naive T-cell counts.*
Correlation between effector memory CD8⁺ and CD4⁺ (A), and naive CD8⁺ (B) and CD4⁺ (C) T-cell absolute counts and age according to the number of infections with herpesviruses (n=13, 32, 39 and 49 for 0, 1, 2, and 3+ infections). Statistical significance was determined by Spearman's rank correlation. Spearman's R and p values are shown for each panel.

This rise, which was mainly driven by CD45RA⁺ EM among CD8⁺ T cells and CD45RA⁻ EM among CD4⁺ T cells (Figure 5.1.5), could be attributed again almost-exclusively to CMV, especially in the CD8 compartment, as it was not observed in CMV uninfected donors (Figure 5.1.6A).

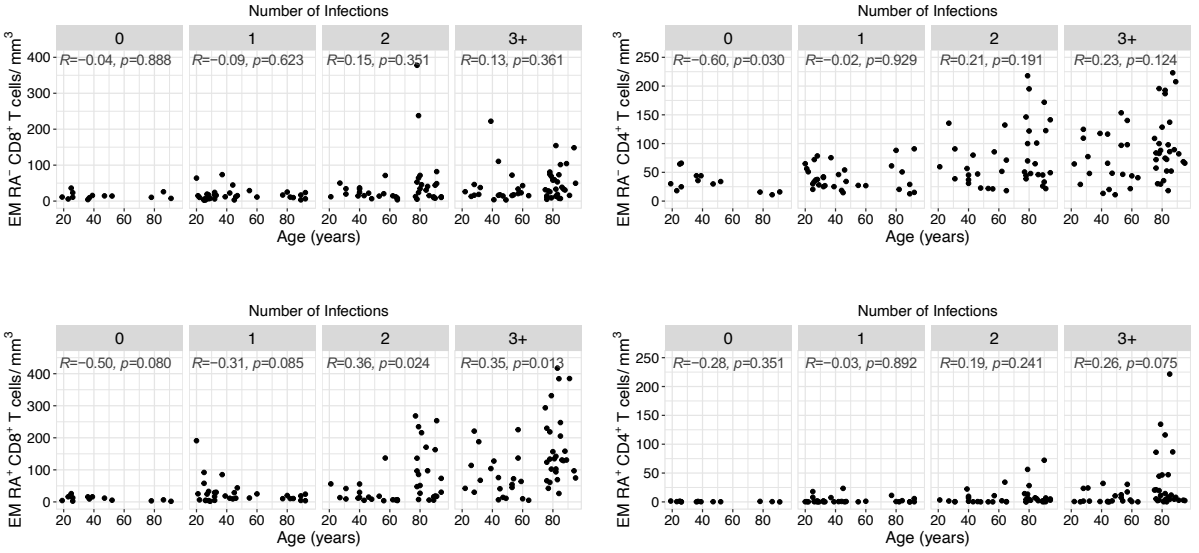

Figure 5.1.5*. Impact of age and herpesvirus infections on effector memory T-cell subsets. Correlation between effector memory CD45RA⁻ and CD45RA⁺ CD8⁺ and CD4⁺ T-cell absolute counts and age according to the number of infections with herpesviruses (n=13, 32, 39 and 49 for 0, 1, 2, and 3+ infections). Statistical significance was determined by Spearman's rank correlation. Spearman's R and p values are shown for each panel.*

Of note, this infection-driven accumulation of EM T cells was statistically significant in both old and young age (Figure 5.1.6.B). These data add to and confirm the multiple lines of evidence that CMV is the main cause of a disturbed EM T-cell compartment and inflation of this subset, which is observable at both younger and older ages.

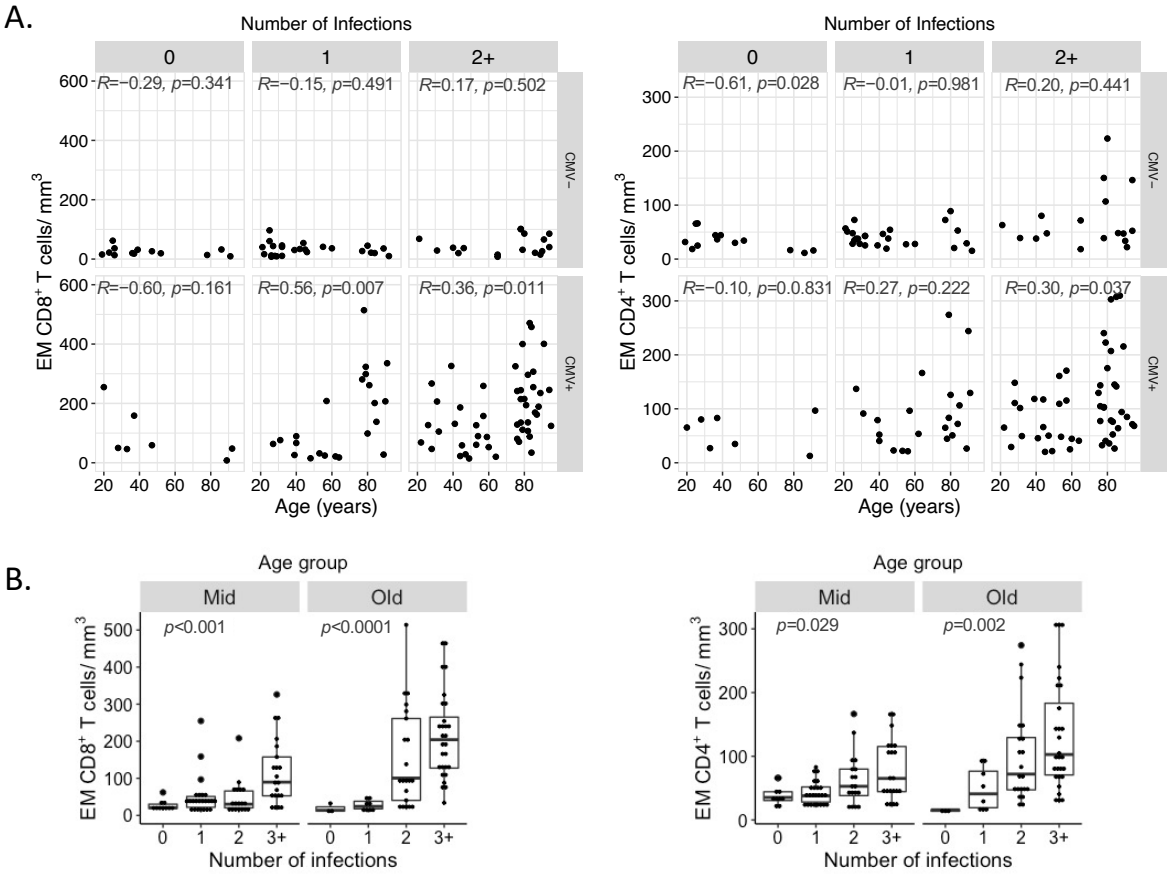

Figure 5.1.6*. Impact of age and herpesvirus infections on effector memory T-cell levels. (A) Correlation between absolute counts of effector memory CD8⁺ and CD4⁺ T cells and age according to number of infections with herpesviruses (excluding CMV) and CMV seropositivity (n=133). (B) Absolute counts of effector memory CD8⁺ and CD4⁺ T cells according to the number of infections with herpesviruses in subjects grouped according to age and shown as box and whiskers plot (n=73 for Mid, n=60 for Old Statistical significance was determined by Spearman's rank correlation (A) or Kruskal-Wallis test (B).*

The influence of persistent viral infections on $CD8^+$ and $CD4^+$ naive T-cell pools has certainly more relevance in the context of *de novo* responsiveness, as naive T cells are required for mounting adaptive immune responses to newly encountered antigens. Naive T cells were defined here as $CD3^+$ $CD27^+$ $CD45RA^+$ $CCR7^+$ $CD95^-$, thus excluding so-called $CD95^+$ "stem cell memory T cells". Interestingly, the evolution of the naive T-cell pool did not mirror that of the

EM T-cell pool, and there was a clear difference between the evolution of $CD8^+$ and $CD4^+$ naive T-cell counts with ageing and herpesvirus infections. The loss of the naive $CD8^+$ T-cell compartment was profound in older subjects (reaching numbers below 10 cells/mm^3 of blood in oldest individuals), irrespective of infection number or CMV serostatus (Figures 5.1.4B and 5.1.7A).

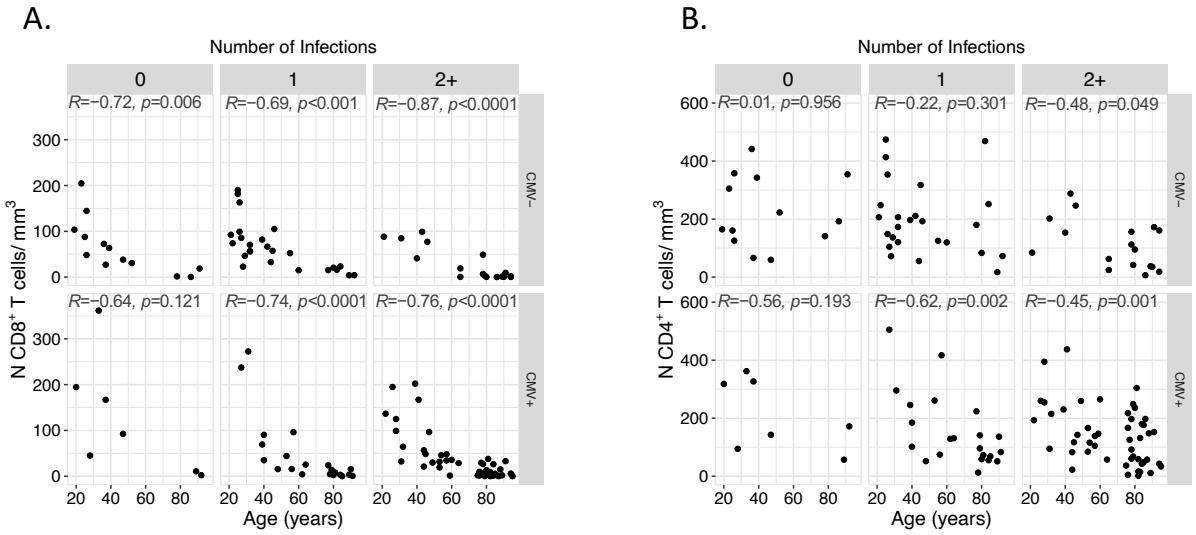

***Figure 5.1.7**. Impact of age and herpesvirus infections on $CD8^+$ and $CD4^+$ naive T-cell levels. Correlation between absolute counts of naive $CD8^+$ (A) and $CD4^+$ (B) T cells and age according to number of infections with herpesviruses (excluding CMV) and CMV seropositivity (n=133). Statistical significance was determined by Spearman's rank correlation, Spearman's R and p values are shown for each panel.*

In contrast, the reduction in naive $CD4^+$ T-cell counts with ageing was more moderate and associated with herpesvirus infections (Figures 5.1.4C and 5.1.7B). While CMV had the strongest association with reduced naive $CD4^+$ T cells, CMV-negative donors presented nonetheless a modest but statistically significant (R=-0.48, p=0.049) reduction of these cells if infected with two other herpesviruses (Figure 5.1.7B). Altogether, these data highlight a differential influence of ageing and persistent viral infections on the naive $CD8^+$ and $CD4^+$ T-cell compartments, and raise two questions: the first one related to the basis of these differences, and the second one related to the eventual impact of common herpesvirus infections on the capacity of older people to mount T-cell responses against previously unencountered antigens.

5.1.2 Distinct evolution of the naive CD8⁺ and CD4⁺ T-cell compartments with increasing age[2]

Our data highlight a stronger contraction of naive CD8⁺ T cells (i.e. 10-time reduction) over naive CD4⁺ T cells (i.e. less than 2-time reduction) with ageing, comparing adults *versus* elderly subjects (Figure 5.1.8A). This is in line with the preservation of the CD4⁺ naive T-cell pool in healthy individuals of 20-69 years of age comparatively to naive CD8⁺ T cells [123]. This is also consistent with a higher thymic output of CD4⁺ T cells in the adult age range [161] and in elderly individuals [162], although in these previous studies the impact of infections was not considered. Here, production of new naive T cells or thymic output, as measured by T-cell receptor excision circle (TREC) levels in total PBMCs, was strongly associated with ageing and was profoundly reduced in older individuals, independently of the number of herpesvirus infections (Figure 5.1.8B). This pattern was similar to the decline of naive CD8⁺ T-cell counts (Figure 5.1.4B), which suggests a strong dependency of naive CD8⁺ T-cell maintenance on thymic production. The naive CD8⁺ T-cell compartment seems to contract as the thymus involutes and thymopoiesis wanes with ageing. The lack of influence of CMV or EBV seropositivity on the thymic production of new T cells *per se* was documented in a large-scale population study of healthy adults [139].

As the maintenance of naive CD4⁺ T cells showed a different dynamic to that of naive CD8⁺ T cells, we hypothesized that they are less dependent on thymic output, but more linked to homeostatic proliferation. To examine this possibility, we measured TREC levels directly in naive T cells, using sensitive digital PCR assays on FACS-sorted cells.

We observed reduced TREC levels in naive T cells from old donors. This reveals a dilution of TRECs in the naive cells through cell division, likely reflecting higher homeostatic proliferation in older compared to younger subjects (Figure 5.1.8C). Interestingly, the reduction in TREC levels was more pronounced in the CD4⁺ than the CD8⁺ pool. Moreover, higher levels of the proliferation marker Ki67 were found in naive CD4⁺ T cells compared to naive CD8⁺ T cells, in particular from older individuals (Figure 5.1.4D).

[2] Experiments performed in collaboration with the research group of prof. Antoine Toubert, Université de Paris, Institut de Recherche Saint Louis, EMiLy, Inserm U1160, Paris, France.

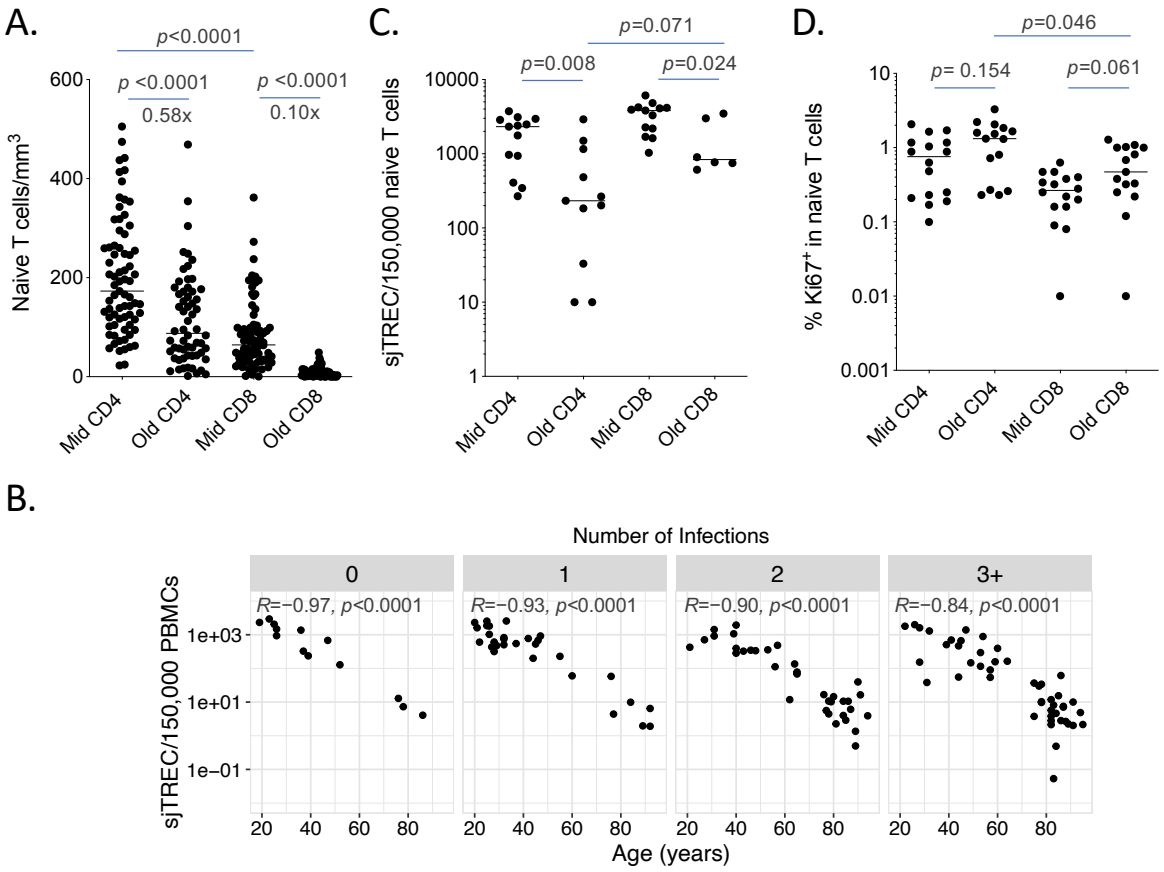

Figure 5.1.8. *Thymic output and naive T-cell homeostatic proliferation. (A) CD4⁺ and CD8⁺ naive T-cell absolute counts in middle-aged (<65y) and old (>75y) subjects (n= 73 for Mid CD4 and CD8, n=60 for Old CD4 and CD8). The magnitude of the reduction in the number of naive CD4⁺ and CD8⁺ T cells between Mid and Old is indicated. (B) Correlation between sjTRECs levels measured in total PBMCs and age according to the number of infections with herpesviruses (n=13, 29, 35 and 48 for 0, 1, 2, and 3+ infections). (C) TRECs levels determined by digital PCR in FACS sorted CD8⁺ and CD4⁺ naive T-cell subsets from middle-aged (<65y) and old (>75y) subjects (n=13 for Mid CD4 and CD8, n=11 for Old CD4, n=6 for Old CD8). (D) Ki67 expression levels determined by flow cytometry in CD4⁺ and CD8⁺ naive T-cell subsets from middle-aged (<65y) and old (>75y) subjects (n=16 for Mid CD4 and CD8, n=15 for Old CD4 and CD8). Each dot represents one donor and line median values (A, C, D). Statistical significance was determined by Mann-Whitney test and Bonferroni adjustment (A, C, D) or Spearman's rank correlation (B). Spearman's R and p values are shown for each panel (B).*

Together, these data indicate that the pool of naive CD8⁺ T cells contracts with ageing due to reduced thymic production, while the pool of naive CD4⁺ T cells is maintained to some extent through robust homeostatic proliferation. The naive CD4⁺ T-cell pool is nonetheless affected by infections with persistent viruses, in particular CMV, but also other herpesviruses.

5.1.3 Reduced responsiveness to *de novo* immunization in CMV-infected older adults[3]

To understand the impact of persistent viral infections on the ability to mount T-cell responses against neoantigens in older people, we concentrated on CMV infection, being the virus with the strongest impact on the T-cell compartment in older people. We therefore studied the induction of *de novo* immune responses upon vaccination in a second cohort of older people. Healthy elderly individuals (n=137, named "Cohort 2") were recruited in a clinical trial to receive for the first time a vaccination against tick-borne encephalitis (TBE) using the recommended prime-boost regimen with three injections (at week 0, 4 and 24). The individuals selected for this study had no serum anti-TBE virus (TBEv) antibodies prior to vaccination, indicating that they had never been exposed to nor vaccinated against TBEv. In line with the Cohort 1 studied above, CMV-seropositive subjects presented lower $CD4^+$ (but not $CD8^+$) naive T-cell counts than CMV-seropositive subjects, confirming the association between CMV infection and lower naive $CD4^+$ T-cell counts with old age (Figure 5.1.9A).

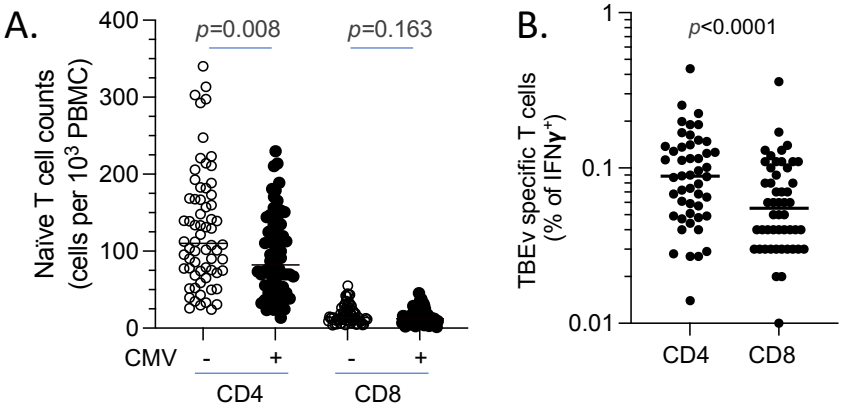

***Figure 5.1.9**. Impact of CMV on $CD8^+$ T-cell priming efficacy in older subjects. (A) Absolute counts of naive $CD4^+$ or $CD8^+$ T cells according to the CMV serostatus of subjects (n=66 for CMV- and n=66 CMV+). (B) Frequency of TBEv-specific $CD4^+$ or $CD8^+$ T cells determined by intracellular IFNγ staining upon stimulation with TBEv overlapping peptides at week 26 after vaccination in older subjects (>70y) (n=49). Statistical significance was determined by Mann-Whitney test. Each dot represents one donor and line median values.*

De novo cellular immune responses to TBE vaccination were monitored at week 26 post-first immunization (2 weeks after the last boost), and compared to baseline values, using IFN-γ

[3] Experiments performed in collaboration with the research group of prof. Urs Karrer, Division of Infectious Diseases and Hospital Epidemiology, University Hospital of Zurich, Zurich, Switzerland.

ELISpot with pools of overlapping peptides against all structural proteins of TBEv. Total TBEv-specific T-cell responses were statistically significantly lower in CMV^+ compared to CMV^- elderly individuals (Figure 5.1.10A). The frequency of TBEv-specific T cells induced upon vaccination correlated with the counts in naive $CD4^+$ T cells prior to vaccination (Figure 5.1.10B), but not naive $CD8^+$ T cells (data not shown). This indicates a reliance of the capacity to mount *de novo* vaccine T-cell responses on the pool of naive $CD4^+$ T cells, and suggests that CMV infection may therefore impact this capacity due to its effect on the naive $CD4^+$ T-cell pool.

To get further insights into these observations, intracellular IFN-γ staining and assessment by flow cytometry was performed on Elispot-positive donors in order to distinguish TBEv-specific $CD8^+$ and $CD4^+$ T cells. This revealed that the TBEv-specific cellular response was mostly dominated by $CD4^+$ rather than $CD8^+$ T cells in vaccinated individuals (Figure 5.1.9B), consistent with its reliance on the naive $CD4^+$ T-cell pool. CMV seropositivity had no impact on TBEv-specific $CD8^+$ T-cell levels. However, it was associated with lower TBEv-specific $CD4^+$ T-cell responses (Figure 5.1.10C), consistent with the impact of CMV on the naive $CD4^+$, but not $CD8^+$, T-cell compartment in older donors. For comparison, we assessed the influence of CMV on memory T-cell reactivity. In contrast to TBEv-specific *de novo* responses, VZV-specific memory $CD8^+$ and $CD4^+$ T-cell levels were equivalent regardless of the CMV serostatus (Figure 5.1.10D).

Of note, we observed a higher number of IFN-γ producing $CD8^+$ and $CD4^+$ T cells upon stimulation with Staphylococcus enterotoxin B (SEB) in CMV^+ donors (Figure 5.1.10E), reflecting higher levels of effector memory (thus IFN-γ producing) cells in CMV^+ donors, that can be activated with this superantigen. These results support a reduced $CD4^+$ T-cell responsiveness to neoantigens in CMV-infected older adults.

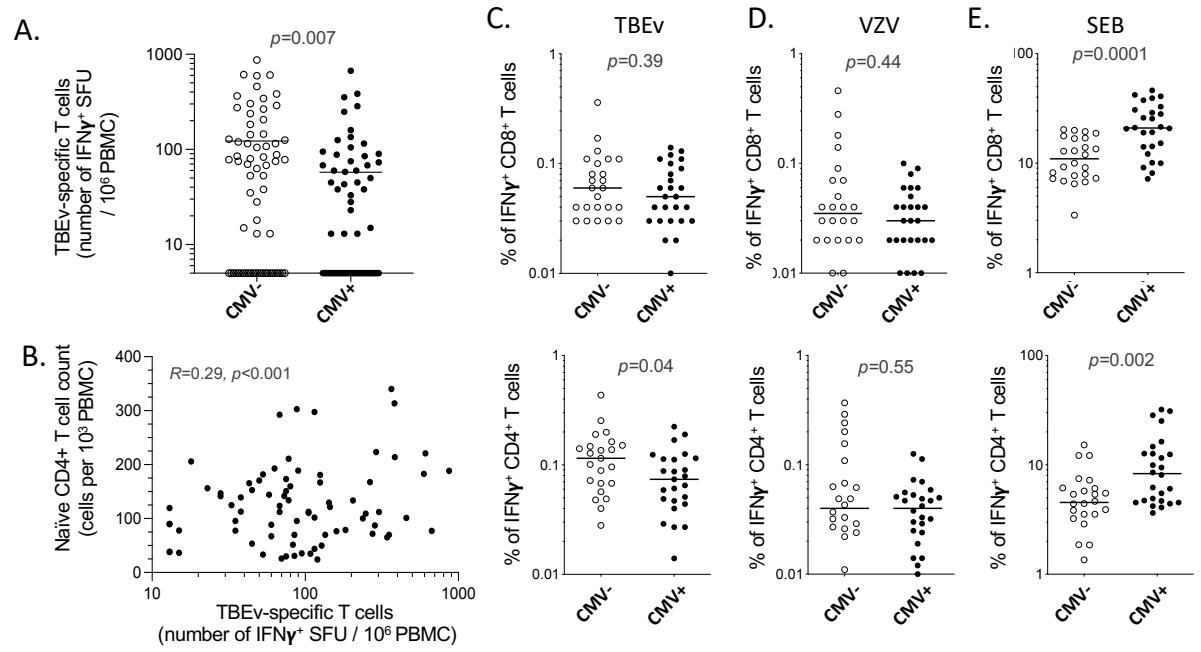

Figure 5.1.10. *Impact of CMV on TBEv vaccine T-cell responsiveness in older subjects. (A) Frequency of TBEv specific T cells determined by IFNγ Elispot upon stimulation with TBEv overlapping peptides at week 26 after the first vaccination in CMV-seronegative and CMV-seropositive subjects (>70y) (n=68 for CMV- and n=69 CMV+). Data shown are subtracted of no stimulation background values. (B) Correlation between naive CD4$^+$ T-cell counts prior to vaccination and the frequency of TBEv-specific T cells at week 26 after the first vaccination (n=132). (C, D, E) Frequencies of IFNγ producing CD4$^+$ or CD8$^+$ T cells upon stimulation with TBEv antigens (C), VZV antigens (D) or SEB (E), determined by intracellular cytokine staining at week 26 after the first vaccination in TBEv Elispot positive CMV-seronegative or CMV-seropositive subjects (>70y) (n=68 for CMV- and n=69 CMV+). Each dot represents one donor and line median values (A, C-E). Representative flow cytometry plots are shown for unstimulated control and TBEv stimulation conditions (percentages of IFNγ producing CD4$^+$ or CD8$^+$ T cells are indicated). Data shown are subtracted of no stimulation background values. Statistical significance was determined by Mann-Whitney test (A, C-E) or Spearman's rank correlation (B).*

We next assessed vaccine-induced humoral immunity measuring TBEv-specific binding levels at different time points after vaccination. We observed that both TBEv-specific antibody responses were delayed and decreased in elderly vaccinees with latent CMV infection (Figure 5.1.11A). Of note, there were clear associations between the induction of TBEv-specific T-cell responses and the levels of both TBEv-specific binding antibody levels upon vaccination (Figure 5.1.11.B). Altogether, these data indicate that CMV infection has a negative impact on the mounting of *de novo* cellular and humoral immune responses in the elderly.

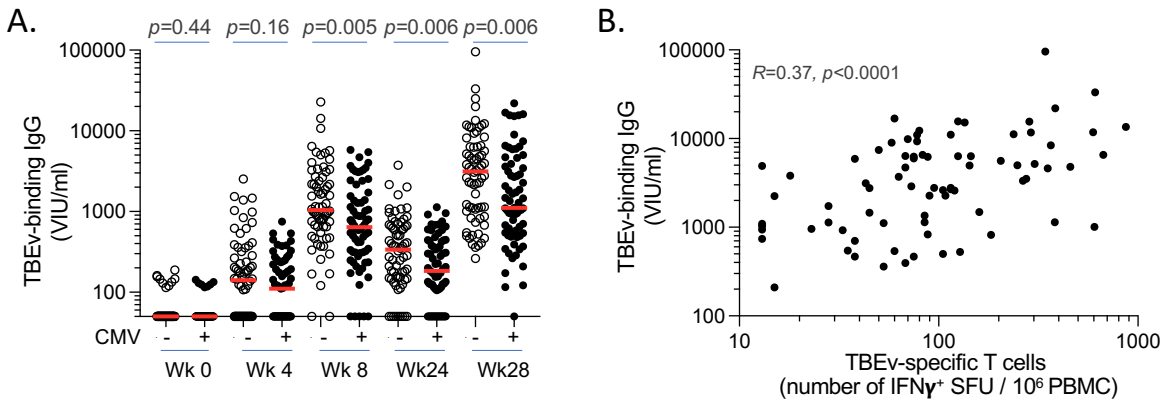

Figure 5.1.11. *Impact of CMV on TBEv vaccine humoral responsiveness in older subjects (A) Levels of TBEv specific binding antibodies (IgG) determined at week 0, 4, 8, 24 and 28 after the first vaccination in CMV-seronegative or CMV-seropositive subjects (>70y). All individuals received three vaccine doses at week 0, 4 and 24 (n=68 for CMV- and n=69 CMV+). Each dot represents one donor and line median values. (B) Correlation between the frequency of TBEv-specific T cells at week 26 after the first vaccination and the levels of TBEv-specific antibodies (IgG) determined at week 28 (n=137). Statistical significance was determined by Mann-Whitney test and Bonferroni adjustment (A) or Spearman's rank correlation (B). Spearman's R and p values are shown for each panel.*

5.1.4 Discussion

Through chronic activation and mobilization of immune resources, infections with herpesviruses, in particular CMV, result in continuous naive T-cell activation and inflation of EM T cells [122]. However, we observed here that the associations of naive and EM T-cell numbers with ageing and chronic infections did not mirror each other and that the contraction or maintenance of the naive T-cell pool depends on multiple factors. In a context of limited or even absent T-cell renewal capacity, the naive T-cell compartment can be particularly affected, as shown by studies in young adults thymectomized during early childhood. In this model of T-cell ageing independent of chronological age, the combination of reduced thymic output (i.e. due to thymectomy) and chronic immune activation (i.e. due to infection with a persistent virus like CMV) resulted in the alteration of naive T-cell frequencies and homeostasis [163, 164]. With ageing, thymic involution and long-term immunity against persistent viruses may combine to exhaust immune resources.

In the present work, we observed that the contraction of the naive $CD8^+$ T-cell compartment with old age was independent of infections by herpesviruses, including CMV, in line with a previous study [37]. We propose that naive $CD8^+$ T-cell pool maintenance with increasing age relies strongly on cell replenishing capacity due to thymic output, so much that the influence

of persistent virus infections, although expected, was not observed. In contrast, the naive $CD4^+$ T-cell pool is maintained more by homeostatic proliferation, allowing for the long-term effects of persistent viral infections to be observed.

Nonetheless we observed that herpesvirus infections, in particular CMV but also EBV/HSV, were associated with decreased absolute counts of naive $CD4^+$ T cells with ageing. Considering that the frequency of naive cell precursors specific for a given antigen are typically very low, ranging approximately between one cell in 10^5 to 10^6 T lymphocytes in adults [165, 166], this quantitative reduction in naive cells, including potentially unique antigen-reactive ones, may affect the mounting of neoantigen-specific immune responses. Indeed, we found that CMV seropositivity was associated with a reduction in $CD4^+$ T-cell responsiveness to neoantigens (i.e. TBEv antigens), but not to recall antigens (VZV antigens), in older people. With advanced age, *de novo* $CD4^+$ T-cell responses appear to be affected by a persistent virus like CMV, in line with the impact on naive $CD4^+$ T-cell counts.

Interestingly, distinct patterns were observed regarding T-cell subsets and inflammatory markers that were associated with ageing and persistent infections. For example, the naive $CD8^+$ T-cell decline and IL-6 level increase are inversely related ($p<0.0001$, not shown), mostly dependent on ageing but not latent infections. Ageing-related increased levels of IL-6, which is known to contribute to thymic atrophy [167, 168], may thus play an important role in the contraction of the naive $CD8^+$ T cells by inhibiting their production in the thymus. On the other hand, the inflation of EM $CD8^+$ T cells and increased levels of inflammation associated cytokines such as TNF, IFNα and IL-10 in older individuals followed a similar pattern, and both were clearly associated with CMV infection. Strikingly, in healthy older subjects, the increases in systemic inflammatory cytokines were strongly associated with CMV serostatus. CMV by itself has therefore a central part in the increased inflammatory status characteristic of the elderly subjects.

Previous studies have found conflicting evidence concerning the influence of latent CMV-infection on influenza vaccine immunogenicity in different populations. Some showed decreased vaccine immunogenicity in CMV-seropositive elderly [124, 126], while others did not show any substantial influence [128, 169]. A beneficial effect of CMV infection on influenza vaccine immunogenicity was even reported in younger individuals [129]. By using influenza vaccine immunogenicity as a readout these studies mainly analyzed the influence of latent CMV on recall or memory responses. Using TBE vaccine in TBEv-naive elderly we

were able to assess the influence of latent CMV infection on a true neoantigen, and we found a delay and reduction in TBE-vaccine induced humoral immunity in CMV-infected older subjects. As previously proposed [170], CMV-related increased inflammation, possibly mediated by the action of TNF on B-cells, might be a mechanism through which CMV impacts the humoral response and antibody production upon vaccination. Moreover, CMV infection might indirectly affect germinal center and antibody formation through defective generation of follicular T helper (Tfh) cells due to lower naive T-cell numbers in CMV-seropositive subjects. Although it would have been interesting to evaluate antigen specific Tfh $CD4^+$ T cells directly, our study was limited to TBEv-specific IFNγ producing, i.e. Th1, $CD4^+$ T cells. Previous studies suggest nonetheless that Tfh cells are even more dampened than Th1 cells in older individuals, affecting the related humoral response [171-173]. In elderly adults, the reduced quality of Tfh cells and ability to provide help to B cells were reported to be partly responsible for declined influenza specific antibody titers upon vaccination [174-178]. From these studies, we may infer the induction of TBEv-specific Tfh cells should be lower upon vaccination in older people, in particular if they are CMV seropositive, resulting in impaired humoral immune responses. It will be important to decipher the exact role of CMV in the impaired humoral responsiveness with ageing.

5.2 Section 2: memory T-cell responses to herpesviruses during ageing

Memory T-cell responses are usually considered unaltered in elderly individuals [39, 179]. However, as described above, the ageing process implies the accumulation of highly and terminally differentiated memory cells (i.e. EM and EMRA), which have a good secretory capacity. Therefore, assays aimed at measuring epitope-specific cellular responses in the CD8 compartment could be biased by the high proportion of EM and EMRA in the elderly, and mask eventual differences at the level of early differentiated memory cells, such as CM and TM. To solve this issue, we measured epitope-specific CD8 responses in phenotypically defined memory subsets of middle-aged adults (Mid) and elderly subjects (Old) and compared the responses mediated by each memory subset.

We focused our attention on herpesvirus-specific responses. Immunosenescence has been considered a key process in the reactivation of herpesviruses which could easily exit from latency in the presence of a weak immune control. Nonetheless, this theory, as well as the fact the herpesvirus-specific responses are defective in the elderly, has never been formally proven.

5.2.1 Subjects

To analyze age-specific memory responses to herpesvirus infections, we used PBMCs from subjects of the previously described Cohort 1, focusing only on those with a positive serology for HSV-1 and for CMV or EBV and that were positive for HLA-A2. We tested 21 middle-aged subjects (Mid) against CMV/EBV and 19 against HSV-1, while we tested 15 elderly subjects (Old) against CMV/EBV and 20 against HSV-1 (in some cases, a low cell number did not allowed the testing against all antigens). The same volunteers and some HLA-A2 negative subjects were also tested after stimulation with coated anti-CD3 and soluble anti-CD28 (21 Mid and 30 Old).

5.2.2 Total CD8 responses do not differ between middle-aged and old adults

To determine the secretory capacity of the memory CD8 compartment, we first determined the expression of CD107 (a marker of degranulation) and the release of IFN-γ and TNF after a 6 hours stimulation with coated anti-CD3 and soluble anti-CD28. This short-term activation excludes the induction of responses mediated by naïve T cells which requires longer time.

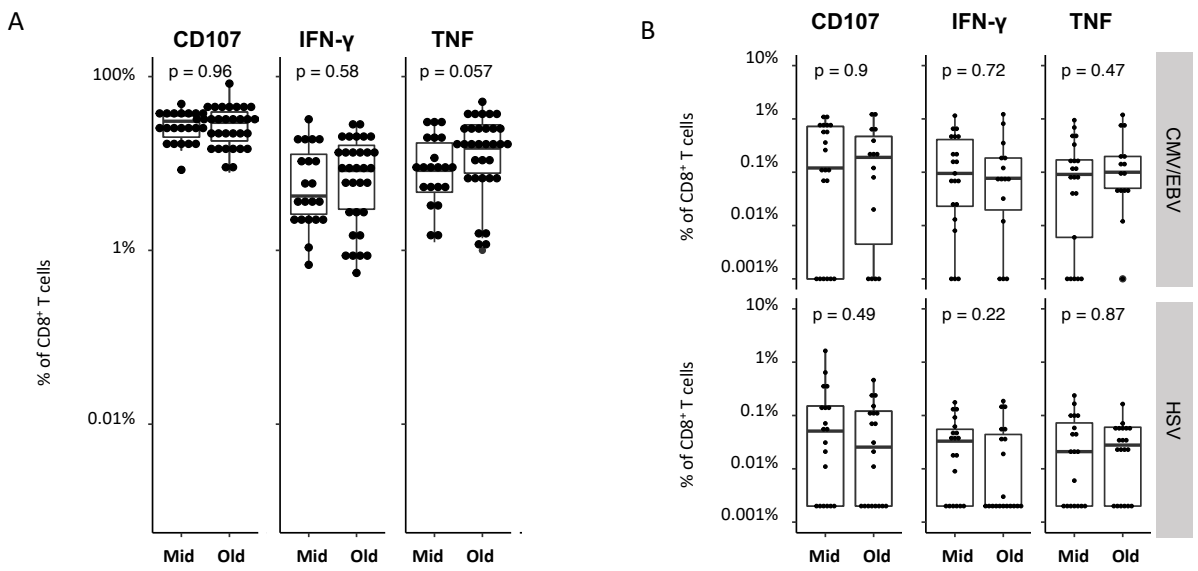

Figure 5.2.1. *Total CD8 responses in middle-aged and elderly adults. PBMCs from Mid and Old subjects were stimulated for 6 hours with anti-CD3/CD28 (A) or with a pool of peptides derived from CMV/EBV (B, top) or HSV-1 (B, bottom). The expression of CD107 or the release of IFN-γ and TNF were measured by intracellular cytokine staining in total CD8⁺ T cells. N as in paragraph 5.2.1 ("Subjects"). Statistical significance was determined using the Mann-Whitney test. All p values are shown.*

As shown in Figure 5.2.1A, the secretory capacity of CD8+ T cells after general TCR ligation was comparable between subjects for the two age groups, although a tendency towards higher TNF release was observed in elderly subjects. This could reflect the accumulation of highly differentiated memory cells which dominate the CD8 compartment in old age.

To then assess if this phenomenon was true also when measuring antigen-specific responses, PBMCs of HLA-A2⁺ donors were stimulated with a pool of HLA-A2 presented epitopes (Table 5.2.1) from CMV and EBV (pool termed "CMV/EBV") or HSV-1 (pool termed "HSV-1). However, also after antigen-specific stimulation we did not observe differences in terms of secretory capacity between CD8⁺ T cells from adults and elderly subjects (Figure 5.2.1B). Together, these data suggest that the elderly present with an intact recall response upon general TCR ligation and antigen-specific stimulation of the whole CD8 compartment.

Virus	Peptide code	Peptide sequence	Antigen
CMV	**NLV**	NLVPMVATV	pp65
EBV	**CLG**	CLGGLLTMV	$LMP2A_{356-364}$
	GLC	GLCTLVAML	BMLF1
HSV-1	**ALM**	ALMLRLLRI	$UL9_{196-204}$
	NLL	NLLTTPKFT	$GB_{342-350}$
	RML	RMLGDVMAV	$GB_{561-569}$
	FLG	FLGAGALAV	$UL43_{272-280}$
	GIF	GIFEDRAPV	GB_{17-25}

Table 5.2.1. *CD8 restricted, HLA-A2 presented epitopes*

5.2.3 Intermediate differentiated memory cells from elderly subjects show a defective secretory capacity after TCR ligation.

To capture eventual alterations at the levels of phenotypically defined memory T-cell subsets, the expression of CD107 and the release of IFN-γ and TNF were measured in central (CM), transitional (TM), effector (EM) and terminally differentiated effector (EMRA) memory $CD8^+$ T cells upon activation with anti-CD3/CD28, the CMV/EBV peptide pool or the HSV peptide pool.

As shown in Figure 5.2.2 A and B, CD107 expression was generally very low in CM upon both anti-CD3/CD28 or peptide-mediated activation, in line with the low secretory capacity of this T-cell subset. Elderly subjects presented with statistically significant lower proportion of $CD107^+$ TM $CD8^+$ T cells compared to middle-aged adults upon anti-CD3/CD28 activation. However, this phenomenon did not involve more differentiated $CD8^+$ T-cell subsets (i.e. EM and EMRA) (Figure 5.2.2A). Moreover, the levels of EBV/CMV- and HSV-specific TM cells secreting CD107 were comparable between age groups (Figure 5.2.2B).

Interestingly, and in line with the age-dependent inflation of high differentiated memory T-cell subsets with age, a non-statistical significant trend toward higher HSV-specific EM responses was noticed in elderly subjects (Figure 5.2.2B). Surprisingly, the same was not true for CMV/EBV-specific EM or EMRA $CD8^+$ T cells.

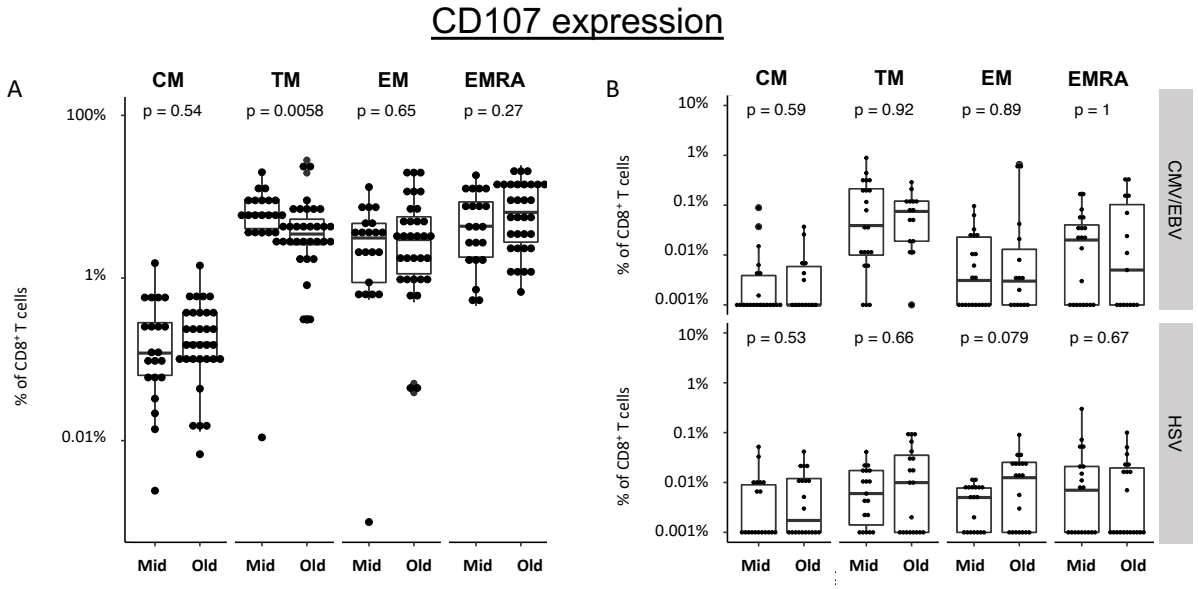

Figure 5.2.2. *CD107 expression in memory CD8⁺ T cells in middle-aged and elderly adults. PBMCs from Mid and Old subjects were stimulated as indicated in Figure 5.2.1 . The expression of CD107 was measured by intracellular cytokine staining in central memory (CM), transitional memory (TM), effector memory (EM) or terminally differentiated EM (EMRA) CD8⁺ T cells. N as in paragraph 5.2.1 ("Subjects"). Statistical significance was determined using the Mann-Whitney test. All p values are shown.*

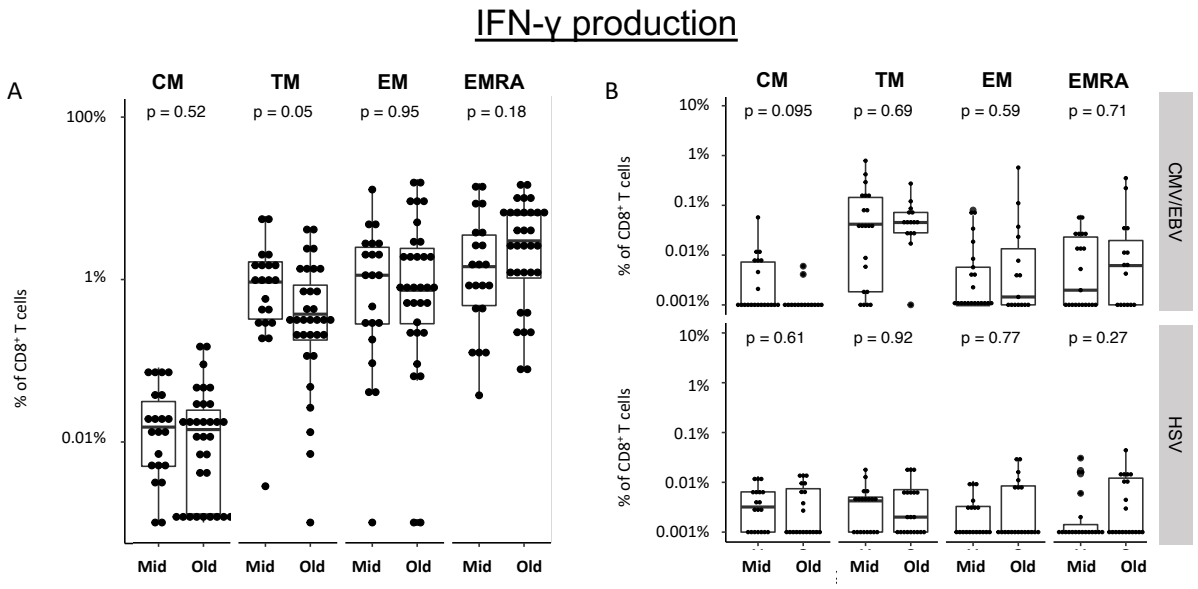

Figure 5.2.3. *IFN-γ production in memory CD8⁺ T cells in middle-aged and elderly adults. PBMCs from Mid and Old subjects were stimulated as indicated in Figure 5.2.1 . The expression of IFN-γ was measured by intracellular cytokine staining in central memory (CM), transitional memory (TM), effector memory (EM) or terminally differentiated EM (EMRA) CD8⁺ T cells. N as in paragraph 5.2.1 ("Subjects"). Statistical significance was determined using the Mann-Whitney test. All p values are shown.*

A similar pattern was observed when measuring IFN-γ production, as TM from elderly subjects showed lower levels of responses compared to those of middle-aged individuals but only after anti-CD3/CD28 activation (Figure 5.2.3A) and not upon antigen-specific stimulation (Figure 5.2.3B). Instead, and irrespectively of the stimulus used, recall responses mediated by EM and EMRA were comparable among the two age-groups.

We next measured TNF production in different memory $CD8^+$ T-cell subsets. As shown in Figure 5.2.4A, Mid and Old subjects showed a comparable pattern of response upon general TCR ligation via anti-CD3, although elderly individuals presented with a non-significant increase in the EMRA subset. Responses toward the CMV/EBV peptide pool were similar in all $CD8^+$ T-cell memory subsets in both age groups (Figure 5.2.4B). However, in analogy with results collected measuring CD107 expression, elderly subjects showed higher HSV-specific EM $CD8^+$ T cells.

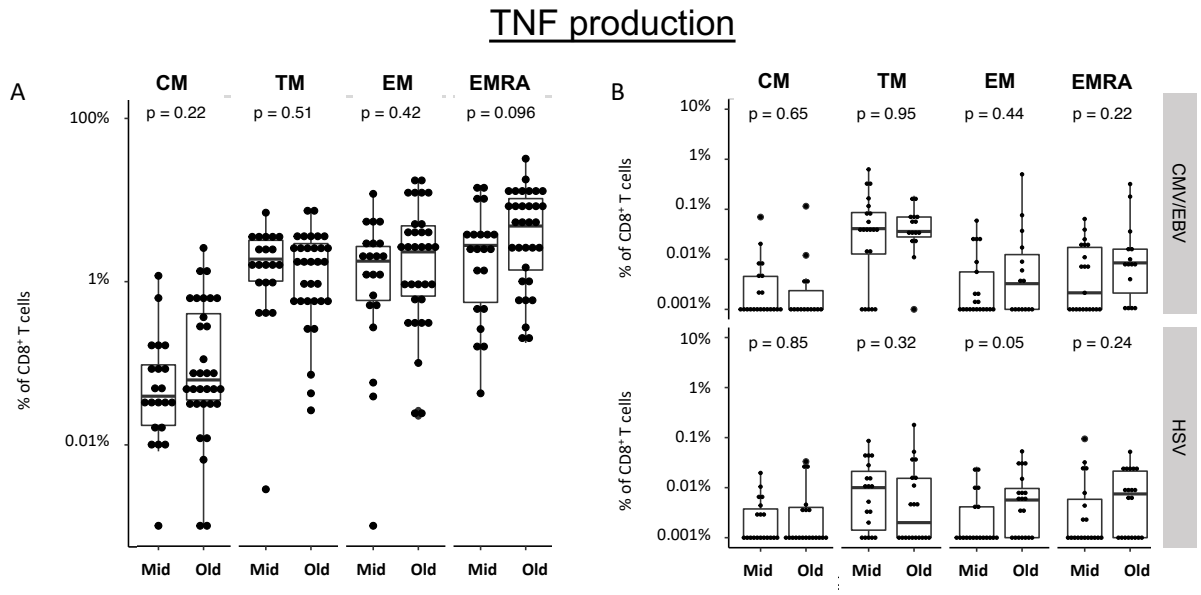

Figure 5.2.4. *TNF production in memory $CD8^+$ T cells in middle-aged and elderly adults. PBMCs from Mid and Old subjects were stimulated as indicated in Figure 5.2.1 . The expression of IFN-γ was measured by intracellular cytokine staining in central memory (CM), transitional memory (TM), effector memory (EM) or terminally differentiated EM (EMRA) $CD8^+$ T cells. N as in paragraph 5.2.1 ("Subjects"). Statistical significance was determined using the Mann-Whitney test. All p values are shown.*

Collectively, these findings suggest a partially altered memory CD8 pool with advance age, characterized by the poor responses mediated by early differentiated memory cells (TM) and the accumulation of epitope-specific late differentiated $CD8^+$ T cells.

5.2.4 Discussion

As described in the Results section 1, age has a strong negative impact on the naïve T-cell compartment, while it is associated with the accumulation of late/terminally differentiated memory cells. It has been shown that this is, at least in part, due to memory T cells specific for CMV and EBV, that tend to inflate contributing to the age-associated increase of EM and EMRA $CD8^+$ T cells [122]. However, we did not observed increased CMV/EBV-specific EM/EMRA $CD8^+$ T cells in elderly subjects of our cohort. This could be due to the fact that we enumerated just secreting, and not all epitope-specific, memory T cells. Previous studies yielded conflicting results regarding whether the CMV-specific T-cell expanded during the ageing process maintain their function [180]. Nonetheless, it has been reported that CMV-specific T cells in the elderly carry a dysfunctional phenotype and a bias towards a more anti-inflammatory response [181]. Therefore, the comparable frequency of $CD107^+$, IFN-γ^+ or TNF^+ EM and EMRA $CD8^+$ cells that we observed between middle-aged and old adults could hide numerical differences of total CMV- or EBV-specific T cells with blunted functionality. Moreover, as the main focus of the analysis were HSV-specific responses, we activated CMV and EBV-specific memory cells using just 1 and 2 peptides, respectively, combined in a single pool. We cannot exclude that stimulation with a higher number of peptide epitopes could highlight eventual differences.

Interestingly, our results suggest that the inflation of HSV-specific EM, as already shown in murine models [182]. The increased proportion of terminally differentiated effector memory T-cell populations in the elderly is accompanied by oligoclonality of the TCR diversity [39] and has been shown to be driven by immune activation resulting from the recurrent reactivation (even subclinical) of persistent infections. As terminally differentiated memory T cells are characterized by a marked inflammatory profile, their accumulation along time likely participates to the so-called “inflammageing”. However, as EM and EMRA are constrained by limited proliferative capabilities and their activation can be regulated through the action of various immune check points (e.g., PD-1) which can lower the degree of expansion and differentiation, this regulation may potentially limit the efficacy of the cellular immune response in the setting of infectious or malignant diseases. Moreover, increasing age may be related to a narrowing of the TCR repertoire, resulting in the loss of effective T cells, as shown in the context of influenza [183], or in an increased proportion of low-avidity virus-specific $CD8^+$ T cells, as shown in the case of CMV [184].

Therefore, we can envisage a paradigm where recurrent (even when asymptomatic) reactivation of persistent infections such as HSV-1 prompt memory inflation and thus the accumulation of highly differentiated memory $CD8^+$ T cells, possibly oligoclonal, poorly proliferating and, at some extents, dysfunctional. This, in turn, may eventually lead to the poor control of further reactivation, in a vicious cycle that leads to more inflammation, with a plethora of downstream consequences, linked to both the increase of pro-inflammatory cytokines and the severe consequences of viral reactivation itself. For instance, seropositivity to HSV is associated with increased frailty index [185], cardiovascular disease risk [186] and neurodegeneration [187].

Up to current knowledge, it is unclear at which level memory inflation may corrode the pool of memory cells toward unrelated antigens (e.g. toward previously administered vaccines). Although we did not specifically addressed this question, our results show lower TM-mediated recall responses upon general TCR ligation via coated anti-CD3 and soluble anti-CD28. As this stimulation is supposed to activate all T cells, irrespectively to antigen-specificity, these results suggest an overall dysfunctional phenotype in the TM subset, arguing in favor of an age-dependent loss of recall responses mediated by early/medium differentiated memory cells. This subsets usually displays high proliferative capacity; further studies are therefore warranted to analyze the expansion capacity upon secondary antigenic challenge of early differentiated memory cells in the elderly.

5.3 Section 3: naive T-cell responses to SARS-CoV-2 during ageing

SARS-CoV-2 is the causative agent of the coronavirus disease 2019 (COVID-19), whose mortality rate progressively increases with age [188]. Despite clear correlates of protection are still unknown, studies suggest that an uncoordinated adaptive immunity, and in particular the lack of virus-specific cellular responses, is associated with severe forms of disease [189, 190]. Lower frequencies of SARS-CoV-2 specific $CD8^+$ T cells have been found in critically ill patients [191, 192], while the onset of wide cellular responses precedes infection resolution [193]. Furthermore, SARS-CoV-2-specific memory $CD8^+$ T cells seem important for protection against reinfections [194].

The functional capacity of SARS-CoV-2-specific T cells is lower in elderly patients [189, 195], which harbor poor virus-specific $CD8^+$ T-cell responses [191]. As a new threat emerged in 2019, the induction of SARS-CoV-2-specific cellular immunity relies mainly on the activation of naive T cells. This subset progressively decreases with age [34, 158, 196], exposing elderly people to higher risks of severe consequences upon infection with emerging pathogens [30]. To evaluate whether the age impacts on primary SARS-CoV-2-specific responses, we exploited an *in vitro* T-cell priming approach to stimulate naive precursors from unexposed subjects of different age groups. Owing to this disease-free system, which permits to focus solely on immune cell features without other confounders (e.g. physiological stressors induced by the disease), we could observe that the induction of *de novo* SARS-CoV-2-specific T-cell immunity is altered with ageing, resulting in narrower and less functional responses.

5.3.1 *In vitro* priming of SARS-CoV-2-specific naive $CD8^+$ T cells in healthy donors

To assess the induction of primary SARS-CoV-2-specific $CD8^+$ responses in unexposed healthy individuals, we exploited an *in vitro* system initially developed to prime naive T cells specific for the HLA-A2-restricted melanoma epitope EV10 in melanoma-naive donors (Figure 5.3.1A). This priming approach mirrors what happens *in vivo* in both mice and humans [34, 78].

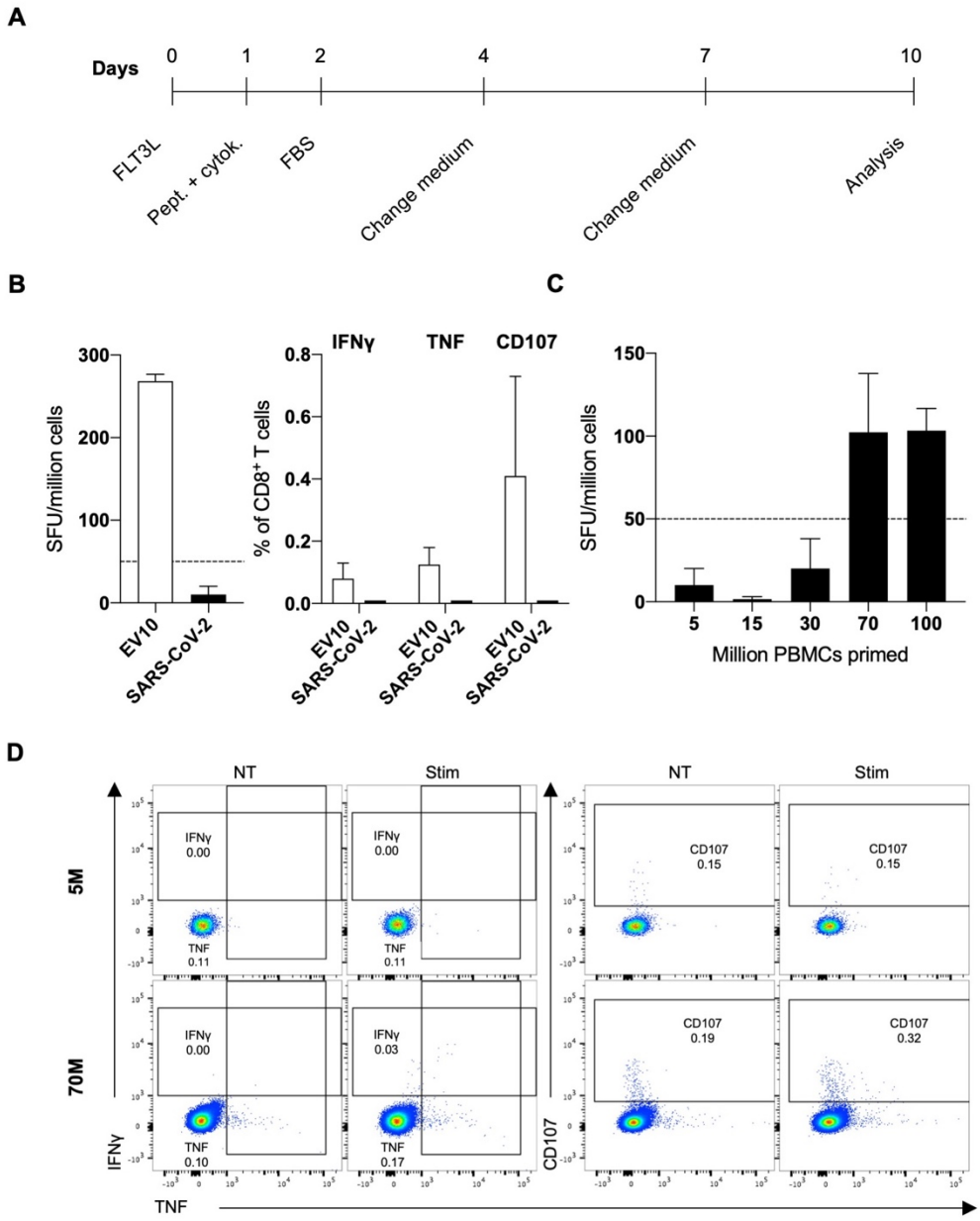

Figure 5.3.1. *In vitro priming of SARS-CoV-2-specific naive CD8⁺ T cells in healthy donors. (A) Scheme of the in vitro priming protocol. (B) PBMCs (5 x 10^6) were primed with EV10 or a pool of 37 SARS-CoV-2-derived peptides. After ten days, the frequency of peptide-specific primed naive CD8⁺ T cells was measured by IFNγ ELISpot (left) or assessing, by ICS, the expression of IFNγ, TNF and CD107 (right). Data are shown, after background (NT) subtraction, as the mean + S.E.M. of 2 donors. (C-D) PBMCs (5-100 x 10^6) were primed with a pool of 37 SARS-CoV-2-derived peptides. After ten days, the frequency of epitope-specific primed naive CD8⁺ T cells was measured by IFNγ ELISpot or assessing, by ICS, the expression of IFNγ, TNF and CD107. Data are shown, after background (NT) subtraction, as the mean + S.E.M. of 3 donors (C). One representative dot plot for stimulation of 5 and 70 x 10^6 PBMCs is shown (D).*

In the present study, peripheral blood mononuclear cells (PBMCs) from HLA-A2 positive blood donors were stimulated with a pool of 37 predicted and/or described HLA-A2-restricted peptide epitopes derived from different SARS-CoV-2 proteins (Table 5.3.1) [193, 197-199].
In parallel experiments, melanoma and SARS-CoV-2 peptides were used to prime naive $CD8^+$ T cells. CD8-mediated responses were evaluated 10 days after stimulation by IFNγ-ELISpot and measuring the expression of IFNγ, TNF and CD107 by intracellular cytokine staining (ICS). As shown in Figure 5.3.1B, EV10- but not SARS-CoV-2-specific $CD8^+$ T cells were expanded. This *in vitro* priming protocol was developed to work with as few as 2.5 million PBMCs, exploiting the peculiar high frequency of naive $CD8^+$ T cells specific for the EV10 epitope [200]. Since the frequency of SARS-CoV-2-specific naive T cells is lower [201], ranging from 1 out $5x10^5$ to less than 1 out 10^7, we escalated up to 100 million the number of PBMCs to be primed, without changing the conditions of readout assays. Starting from 70 million PBMCs, SARS-CoV-2-specific primary T-cell responses could be induced in unexposed donors (Figures 5.3.1C and D).

Protein	Code	Sequence	Start-end	Length	Matrix	Predicted in
E	**SLV**	SLVKPSFYV	50-58	9	1, 7	[193, 198, 199]
M	**KLL**	KLLEQWNLV	15-23	9	1, 8	[193, 198, 199]
M	**TLA**	TLACFVLAAV	61-70	10	1, 9	[193, 197]
M	**GLM**	GLMWLSYFI	89-97	9	1, 10	[193]
N	**ALN**	ALNTPKDHI	138-146	9	2, 8	[193]
N	**LQL**	LQLPQGTTL	159-167	9	2, 9	[193]
N	**LAL**	LALLLLDRL	219-227	9	2, 10	[193]
N	**LLL**	LLLDRLNQL	222-230	9	1, 11	[193, 197-199]
N	**RLN**	RLNQLESKM	226-234	9	2, 11	[193]
N	**GMS**	GMSRIGMEV	316-324	9	1, 12	[193, 197]
N	**ILL**	ILLNKHIDA	351-359	9	2, 7	[193, 197]
RdRp	**NLI**	NLIDSYFVV	4456-4464	9	2, 12	[193, 198]
RdRp	**YTM**	YTMADLVYAL	4514-4523	10	3, 7	[193, 198]
RdRp	**SLL**	SLLMPILTL	4631-4639	9	3, 8	[193, 198]
RdRp	**KIF**	KIFVDGVPFV	4724-4733	10	3, 9	[193, 198]
RdRp	**RLA**	RLANECAQV	5046-5054	9	3, 10	[193, 198]
RdRp	**YLP**	YLPYPDPSRIL	5220-5230	11	3, 11	[193, 198]

RdRp	**LMI**	LMIERFVSL	5246-5254	9	3, 12	[193, 198]
RdRp	**MLD**	MLDMYSVML	5291-5299	9	4, 7	[193, 198]
S	**TLD**	TLDSKTQSL	109-117	9	6, 12	[199]
S	**YLQ**	YLQPRTFLL	269-277	9	13	[193, 197-199]
S	**KIA**	KIADYNYKL	417-425	9	4, 8	[193, 198]
S	**KLP**	KLPDDFTGCV	424-433	10	6, 7	[193]
S	**SII**	SIIAYTMSL	691-699	9	4, 9	[193, 198]
S	**LLF**	LLFNKVTLA	821-829	9	4, 10	[193, 198]
S	**ALN**	ALNTLVKQL	958-966	9	6, 8	[193]
S	**VLN**	VLNDILSRL	976-984	9	4, 11	[193, 197-199]
S	**RLD**	RLDKVEAEV	983-991	9	4, 12	[193, 198]
S	**RLQ**	RLQSLQTYV	1000-1008	9	5, 7	[193, 198]
S	**HLM**	HLMSFPQSA	1048-1056	9	5, 8	[193, 198]
S	**VVF**	VVFLHVTYV	1060-1068	9	5,11	[193]
S	**RLN**	RLNEVAKNL	1185-1193	9	5, 10	[193, 197]
S	**NLN**	NLNESLIDL	1192-1200	9	5, 12	[193, 197]
S	**FIA**	FIAGLIAIV	1220-1228	9	5,9	[193, 197, 198]
ORF3a	**ALS**	ALSKGVHFV	72-80	9	6, 10	[193, 199]
ORF3a	**LLY**	LLYDANYFL	139-147	9	6, 9	[193, 199]
ORF6	**HLV**	HLVDFQVTI	3-11	9	6, 11	[193, 199]

Table 5.3.1. *HLA-A2-restricted, SARS-CoV-2-derived peptides used in the study.*

5.3.2 Reduced antigenic repertoire of SARS-CoV-2 specific primary CD8$^+$ T cell responses in older adults

We then exploited this *in vitro* priming system to test samples from HLA-A2 positive, SARS-CoV-2 unexposed healthy donors belonging to two age groups (Table 5.3.2): middle-aged adults (Mid, median age 30-years-old, range 19-49, n=10) and older adults (Old, median age 67-years-old, range 65-69y, n=9).

To characterize the magnitude and breadth of the *in vitro*-induced responses, PBMCs from Mid and Old subjects were stimulated with the pool of the 37 peptides to prime SARS-CoV-2-specific naive CD8$^+$ T cells. The cultures were tested 10 days after stimulation against a bidimensional peptide matrix system (Table 5.3.1) by IFNγ-ELISpot. Data were first analyzed for the intensity of matrix-specific responses in the two age-groups.

Subject	Sex	Age
Mid 01	F	19
Mid 02	M	21
Mid 03	M	23
Mid 04	M	23
Mid 05	M	26
Mid 06	M	33
Mid 07	M	35
Mid 08	M	36
Mid 09	F	39
Mid 10	M	49
Old 01	M	65
Old 02	F	65
Old 03	F	65
Old 04	M	65
Old 05	M	66
Old 06	F	66
Old 07	M	69
Old 08	M	69
Old 09	M	69

Table 5.3.2. *Study subjects.*

As shown in Figure 5.3.2A, IFNγ release upon stimulation with single matrixes was generally higher in middle-aged adults. In particular, statistically significant differences were observed against M1 and M7 matrixes (Figure 5.3.2B). However, responses against some matrixes (i.e. M6 and M12) were similar between the age-groups in terms of both magnitude and frequency (Figures 5.3.2A-B). To assess the presence of cross-reactive responses due to memory T cells specific for common cold coronaviruses (HCoV), we stimulated PBMCs directly *ex-vivo* with the same matrixes or with a pool of HLA-A2-presented epitopes derived from CMV, EBV and

HSV-1, as a control of recall responses. IFNγ secretion was measured 24 hours after stimulation. No positive responses toward the SARS-CoV-2 epitope matrixes were observed while donors reacted to the pool of CMV, EBV and HSV-1 peptides (Figure 5.3.3).

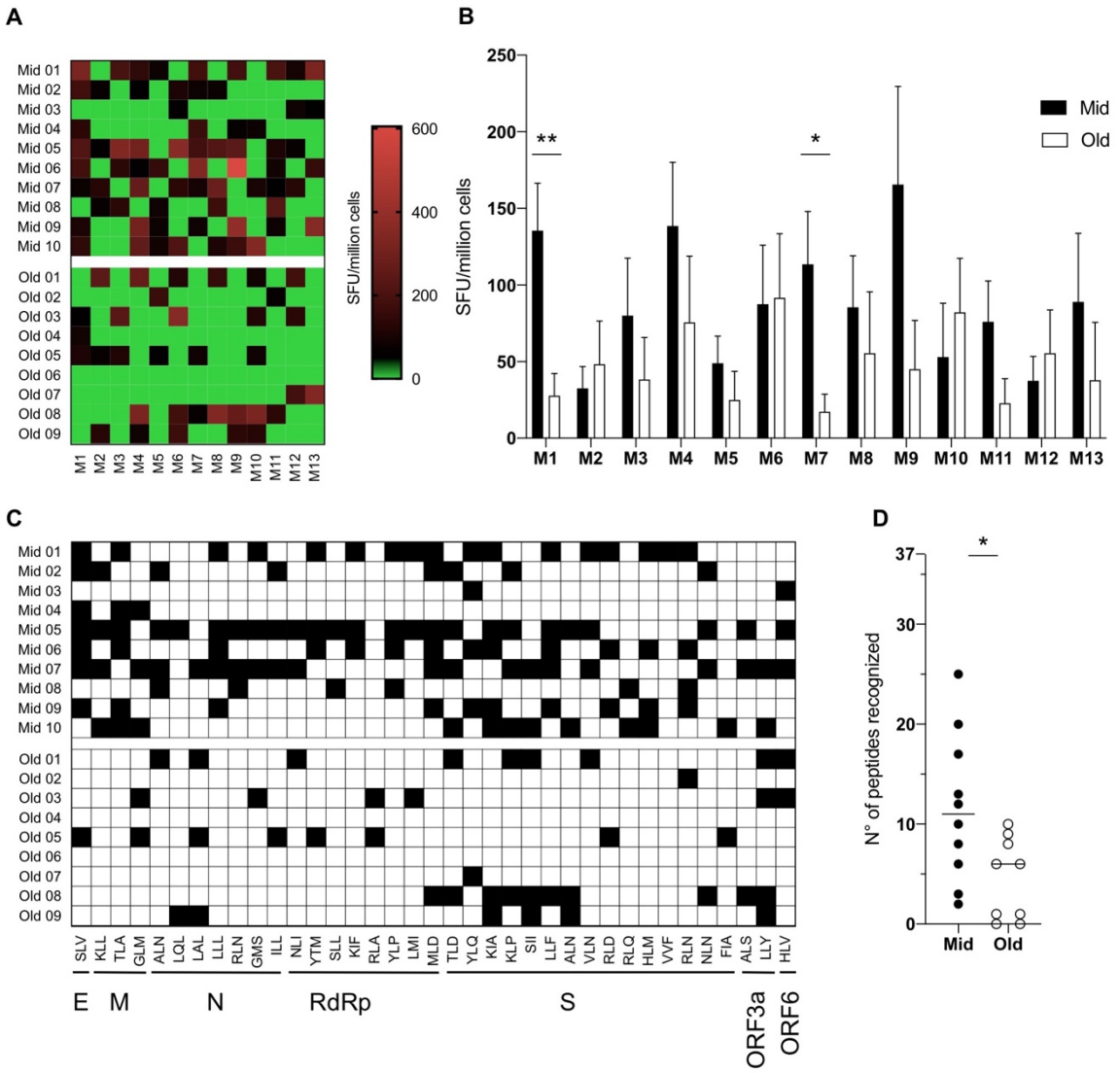

Figure 5.3.2. *Reduced antigenic repertoire of SARS-CoV-2 specific primary CD8⁺ T cell responses in older adults.*
*(A-D) PBMCs (7 x 10^7) were primed in vitro with a pool of 37 SARS-CoV-2-derived peptides. After ten days, the frequency of epitope-specific primed naive CD8⁺ T cells was measured, upon restimulation with 12 different matrixes composed by 6 peptides each or with the YLQ peptide (matrix 13), by IFNγ ELISpot. Responses above the threshold of 50 SFU/million cells were considered positive. Individual (A) and median values + S.E.M. (B) of responses to single matrixes are shown. Since each peptide is contained in two different matrixes, when responses to both matrixes were above 50 SFU/million cells, the response toward that single peptide was counted as positive and marked in black (C). The total number of recognized peptides by each donor was calculated and shown (D), and the lines represent the median values. Statistical significance was determined by Mann Whitney test, *p<0.05 and ** p<0.01.*

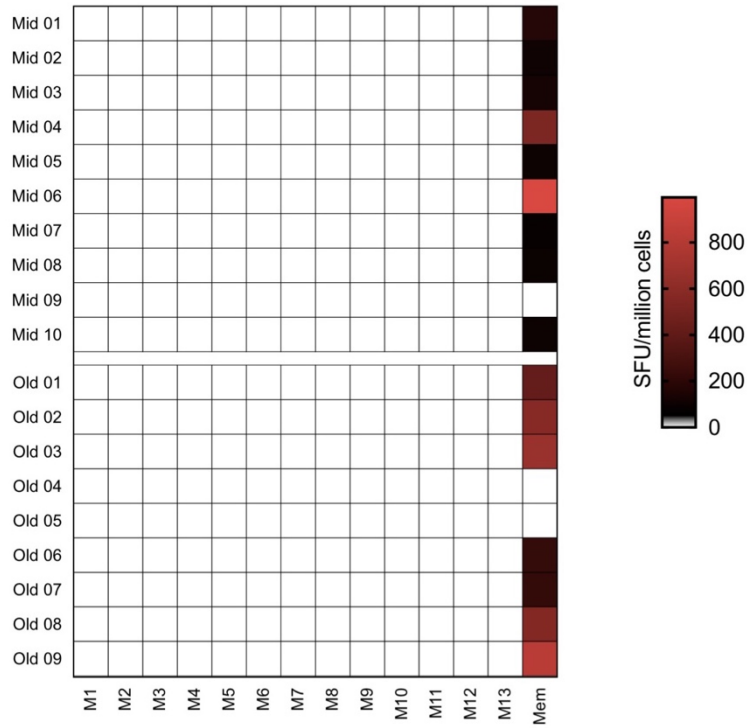

Figure 5.3.3. *Absence of memory CD8⁺ T cells cross-reacting to SARS-CoV-2-derived peptides. PBMCs (2.5 x 10^5/well) were stimulated for 24 hours with 12 different matrixes composed by 6 peptides each, with the YLQ peptide (M13) or with a pool of peptides from CMV, EBV and HSV-1 (Mem). IFNγ release was measured by ELISpot. Responses above the threshold of 50 SFU/million cells were considered positive. The magnitude of matrix responses are indicated in red scale.*

We then used bidimensional matrixes to deconvolve the recognition of individual peptide epitopes by primed naive CD8⁺ T cells. This analysis showed that all peptides were recognized by at least one donor (Figure 5.3.2C and Table 5.3.3). The SLV E-derived peptide, the MLD RdRp-derived peptide and the KIA S-derived peptide were the most frequently recognized (7-8 donors out of 19). Among these, responses against the SLV and MLD peptides were mostly detected in middle-aged adults, which overall recognized a statistically significant higher number of peptides compared to older adults (Figure 5.3.3D).

Antigen		Responders			Literature	% of homology with HCoV			
Prot.	**Code**	**Tot, n=19**	**Mid, n=10**	**Old, n=9**	**Reported Frequency[a]**	**NL63**	**229E**	**OC43**	**HKU1**
E	**SLV**	**8**	7	1	Not tested	22.2	22.2	44.4	44.4
M	**KLL**	**4**	4	0	N [202] L [201] L [203]	33.3	33.3	44.4	33.3
M	**TLA**	**6**	6	0	N [204] N [205]	20	20	20	10
M	**GLM**	**5**	3	2	N [203] L [206]	33.3	44.4	44.4	33.3
N	**ALN**	**5**	4	1	N [207] N [204]	11.1	22.2	66.6	44.4
N	**LQL**	**2**	1	1	N [207] N [204] N [202]	22.2	44.4	44.4	44.4
N	**LAL**	**4**	1	3	N [207] L [204] H [208]	22.2	22.2	11.1	11.1
N	**LLL**	**5**	5	0	N [207] N [205] N [208] L [204] L [191] L [193] L [203] H [202]	22.2	22.2	11.1	11.1
N	**RLN**	**3**	3	0	Not tested	0	11.1	11.1	11.1
N	**GMS**	**4**	3	1	L [207] L [204] L [201] L [203]	11.1	11.1	22.2	11.1
N	**ILL**	**4**	3	1	L [207]	22.2	11.1	33.3	22.2
RdRp	**NLI**	**3**	2	1	N [193]	44.4	22.2	44.4	44.4
RdRp	**YTM**	**4**	3	1	L [201]	80	70	80	80
RdRp	**SLL**	**2**	2	0	Not tested	55.5	33.3	55.5	55.5
RdRp	**KIF**	**3**	3	0	Not tested	70	70	90	90
RdRp	**RLA**	**2**	0	2	Not tested	77.7	77.7	100	100
RdRp	**YLP**	**4**	4	0	Not tested	100	81.8	81.8	100
RdRp	**LMI**	**3**	2	1	N [204]	55.5	55.5	88.8	88.8

RdRp	**MLD**	**7**	6	1	Not tested	44.4	44.4	66.6	66.6
S	**TLD**	**6**	4	2	N [202] L [203]	11.1	0	11.1	0
S	**YLQ**	**5**	4	1	L [203] H [209] H [191] H [201] H [193] H [205]	0	0	44.4	44.4
S	**KIA**	**7**	5	2	N [203] L [209]	22.2	11.1	22.2	33.3
S	**KLP**	**6**	4	2	N [203] L [209]	22.2	33.3	22.2	22.2
S	**SII**	**5**	2	3	N [209] L [201] L [206] L [203]	0	0	11.1	22.2
S	**LLF**	**6**	5	1	N [203] L [209]	55.5	33.3	66.6	66.6
S	**ALN**	**4**	2	2	N [207] N [204] N [205] L [209]	66.6	55.5	66.6	66.6
S	**VLN**	**4**	3	1	N [204] N [203] L [209] H [201] H [206]	33.3	33.3	66.6	66.6
S	**RLD**	**4**	3	1	N [203]	44.4	44.4	66.6	66.6
S	**RLQ**	**2**	2	0	N [205] L [203] H [209]	44.4	44.4	55.5	55.5
S	**HLM**	**4**	4	0	L [203]	44.4	33.3	44.4	44.4
S	**VVF**	**1**	1	0	N [193], L [203]	33.3	44.4	44.4	33.3
S	**RLN**	**5**	4	1	N [204] N [203] N [205] L [209]	11.1	22.2	55.5	33.3
S	**NLN**	**4**	3	1	N [209] N [204]	33.3	55.5	55.5	55.5

					N [203]				
S	**FIA**	**2**	1	1	N [203] N [202] N [207] N [204] L [209] L [205] H [206]	0	33.3	11.1	11.1
ORF3a	**ALS**	**3**	2	1	N [203] L [193] H [202]	33.3	NA[b]	NA	NA
ORF3a	**LLY**	**6**	2	4	N [203] H [204] H [191] H [193]	11.1	NA	NA	NA
ORF6	**HLV**	**5**	3	2	L [206]	NA	NA	NA	NA

Table 5.3.3. *Responses to the HLA-A2-restricted, SARS-CoV-2-derived peptides used and homology with HCoVs*

[a]Reported frequencies were calculated as follows:
None, N (Tested but no responses detected in SARS-CoV-2-positive individuals);
Low, L (>0%, <33% of responses detected in SARS-CoV-2-positive individuals);
High, H (>33% of responses detected in SARS-CoV-2-positive individuals).
[b]NA= not applicable

Of note, naive $CD8^+$ T cell frequencies were statistically significant lower in older adults and showed a positive correlation with the number of recognized peptides (although not statistically significant, Figure 5.3.4). Other phenotypic baseline measurements, including activation levels assessed by HLA-DR and PD1 expression, were comparable among the age-groups (Figure 5.3.4A). Thus, although the readout of our experimental setting could also potentially capture alterations at the level of antigen presenting cells, these results suggest that the narrower responses observed in older subjects may be due to holes in the naive repertoire.

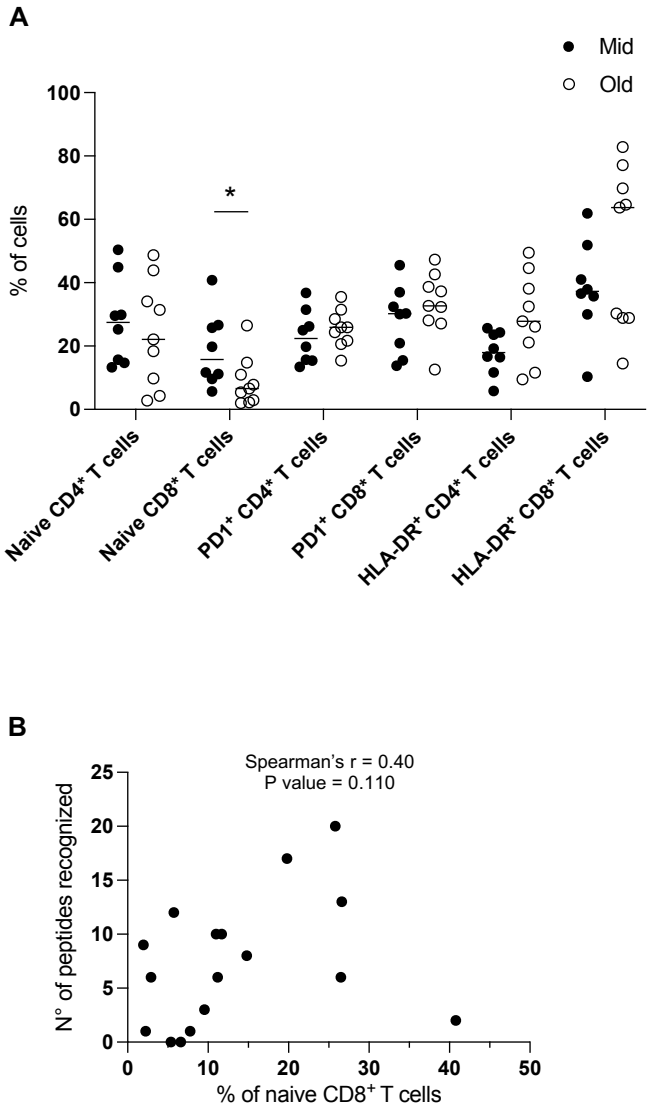

***Figure 5.3.4**. Differentiation and activation phenotypes of study subjects. (A) PBMCs of study subjects stratified by age were analyzed for the expression of differentiation markers (CD45RA, CD27 and CCR7) to identify naive T cells and of activation markers (PD1 and HLA-DR). (B) The correlation between the number of peptides recognized determined by IFNγ ELISpot and the percentage of naïve CD8⁺ T cells is shown. Statistical significance was determined by Mann Whitney test (A) and Spearman's rank correlation (B), *p<0.05.*

The percent of homology with the 4 HCoVs (NL63, 229E, OC43 and HKU1) was then computed. The majority of epitopes shared a similarity less than 50% (Table 5.3.3), while identical sequences were observed in some RdRp-derived peptides (RLA and YLP). However, we did not find any positive correlation between the percentage of homology and the numbers of donors responding to the peptides (Figure 5.3.5), suggesting that cross-reactive immunity does not impact the CD8-mediated peptide-specific responses identified. Altogether, these

data suggest that primary CD8⁺ T-cell responses to SARS-CoV-2 are quantitatively altered and narrower in older people.

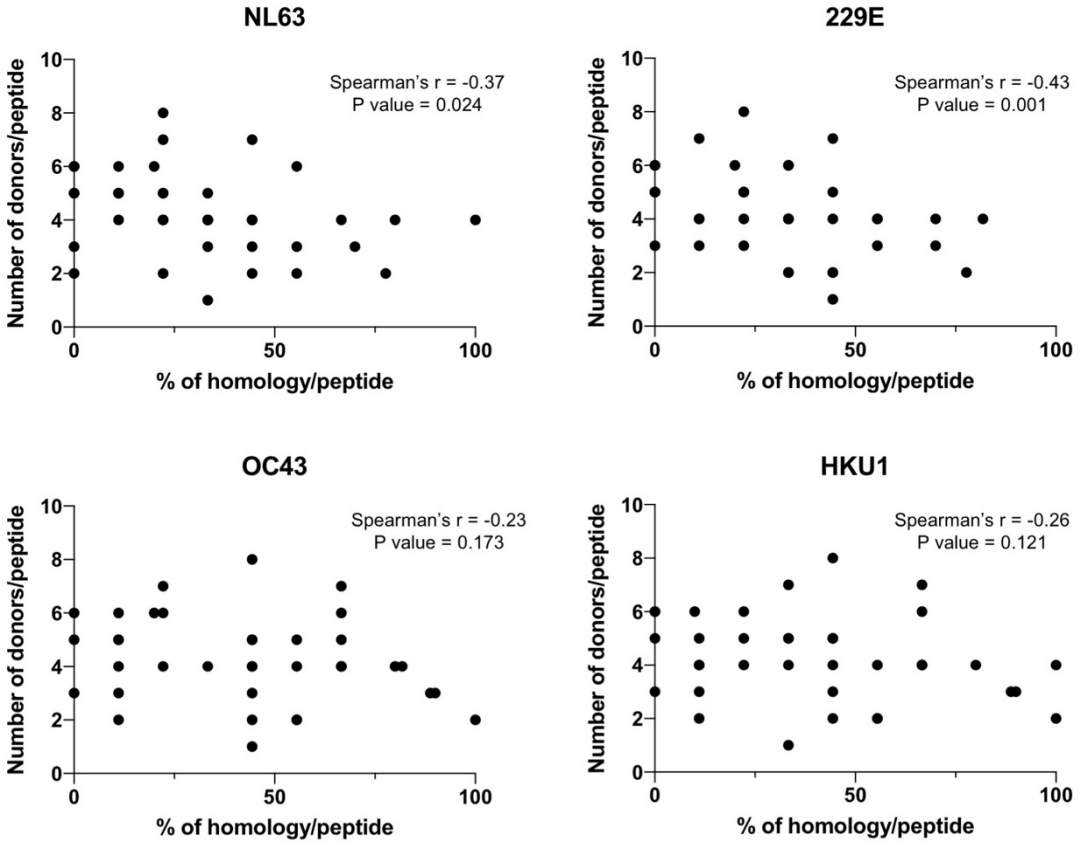

Figure 5.3.5. *Correlation between peptide homology and number of responders to the priming.*
The percentage of homology between SARS-CoV-2-derived peptides and the 4 HCoVs NL63, 229E, OC43 and HKU1 was calculated and correlated with the number of donors showing positive responses toward each peptide upon priming. Statistical significance was determined by the Spearman's rank correlation.

5.3.3 Altered functions of primed SARS-CoV-2-specific CD8⁺ T cells in older subjects

Finally, we sought to determine whether the age-associated alterations that we observed in naive CD8⁺ T cells primed with SARS-CoV-2 peptides could be appreciated evaluating the expression of IFNγ, TNF and CD107 by ICS after stimulation with the pool of the 37 peptides. IFNγ production was generally very low, with a trend towards higher responses in middle-aged adults (Figures 5.3.6 and 5.3.7A). The same pattern was observed for TNF production, although it reached higher levels compared to IFNγ in both age groups. Notably, the marker

of degranulation CD107 was expressed at statistically significant higher levels in Mid compared to Old donors (Figures 5.3.6 and 5.3.7A).

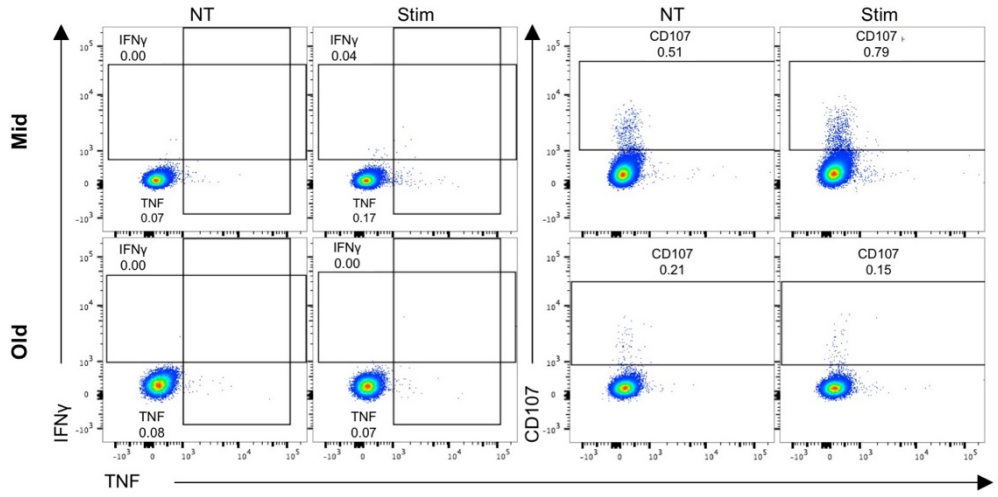

Figure 5.3.6. *Age impacts on the functions of primed SARS-CoV-2-specific CD8⁺ T cells. PBMCs (7 x 10^7) were primed in vitro with a pool of 37 SARS-CoV-2-derived peptides. After ten days, the frequency of epitope-specific primed naïve CD8⁺ T cells was measured, upon restimulation with the same peptide pool, assessing by ICS the expression of IFNγ, TNF and CD107. Representative dot plots for Mid and Old are shown.*

Consistently with data from convalescent individuals [199, 202, 210, 211], we observed a high proportion of SARS-CoV-2-specific monofunctional CD8⁺ T cells, especially in older subjects (Figure 5.3.7B). Of note, the frequency of TNF⁺ and CD107⁺ CD8⁺ T cells directly correlated with the number of recognized peptides (Figure 5.3.7C). The same was observed in respect to IFNγ production, although results did not reach statistical significance. Together, these data confirm that middle-aged individuals have the capacity to mount superior *de novo* T-cell responses against SARS-CoV-2 compared to older subjects.

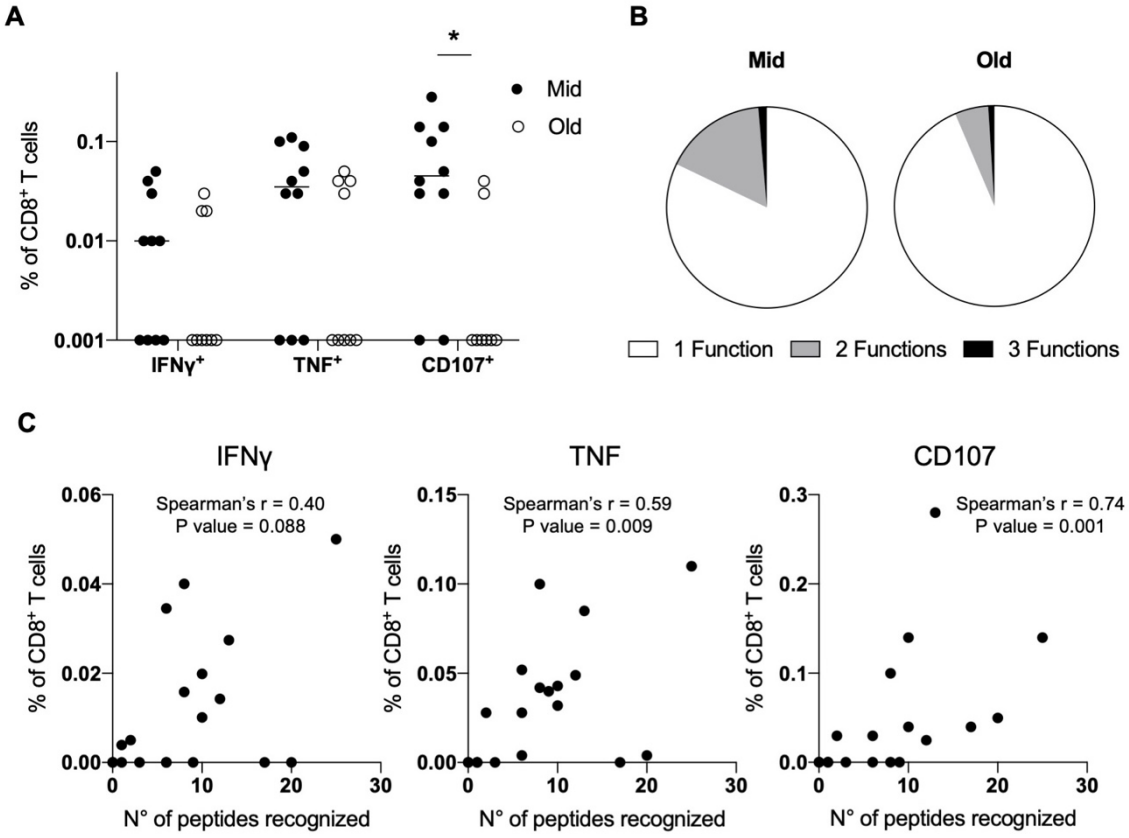

Figure 5.3.7. *Altered functions of primed SARS-CoV-2-specific CD8⁺ T cells in older subjects. (A-C) PBMCs (7 x 10^7) were primed in vitro with a pool of 37 SARS-CoV-2-derived peptides. After ten days, the frequency of epitope-specific primed naive CD8⁺ T cells was measured, upon restimulation with the same peptide pool, assessing by ICS the expression of IFNγ, TNF and CD107. Data are shown as percentage of CD8⁺ T cells after background (NT) subtraction, and lines represent median values (A). Polyfunctional capacity was determined and shown as a mean for Mid and Old donors (B). The correlation between the number of peptides recognized determined by IFNγ ELISpot and the production of IFNγ or TNF and the expression of CD107 determined by ICS is shown (C). Statistical significance was determined by Mann Whitney test (A) and Spearman's rank correlation (C), *p<0.05.*

5.3.4 Discussion

Immune ageing is characterized by the presence of few and dysfunctional naive T cells [34, 159], and their loss is associated with severe COVID-19 clinical manifestations [189]. This suggests that elderly individuals mount poor *de novo* responses toward SARS-CoV-2. To address this issue, we developed an *in vitro* approach to study the priming of SARS-CoV-2-specific CD8⁺ T-cell responses from naive cells in healthy unexposed individuals of different age groups, without the influence of the infection or comorbidities in infected patients. To

our knowledge, SARS-CoV-2-specific $CD8^+$ T-cell responses have been rarely detected in healthy donors, as studied so far stimulating low cell numbers with the aim of identifying cross-reactive memory responses [201, 204, 209]. In our study, we used 8-10 amino acid long peptides with a rather low homology with HCoVs. Of note, the majority of SARS-CoV-2-specific responses were against peptides with a minimal degree of homology. Furthermore, in a 24h stimulation assay which should activate cross-reactive HCoVs-specific memory $CD8^+$ T cells [212], we did not observe any detectable recall response. Although the expansion of low-differentiated memory cells in our *in vitro* priming system cannot be totally ruled out, we would exclude its major contribution, consistently with recent reports of a limited impact of HCoV exposure in shaping $CD8^+$ T-cell responses [191, 207, 213, 214]. Nonetheless, the issue of cross-reactive immunity deserves further investigations since memory $CD4^+$ T cells specific for common cold coronaviruses and cross-reacting to SARS-CoV-2 have been frequently reported in uninfected individuals [207, 213, 215-218].

Among the 37 selected peptides, some were recognized by >30% of donors. However, the immunodominance pattern observed upon *in vitro* priming does not mirror that found in infected patients (Table 5.3.3). For example, the YLQ epitope, although immunodominant in infected patients, is not frequently recognized in unexposed controls. Similarly, the RLQ epitope, also found as immunodominant in convalescent patients, is recognized very rarely upon priming. Notably, in infected individuals, the response towards the YLQ epitope is more diverse than that toward the RLQ epitope [209]. Thus, the inability to prime RLQ-specific naive T cells may derive by the low frequency of their clones. Considering the narrowing T-cell repertoire in old people, it is not surprising that we could only observe once the priming of YLQ-specific primary responses, and never RLQ-specific ones, in subjects >65-years-old. Conversely, and in agreement with Shomuradova's studies, we observed the induction of primary responses toward the SII and NLN epitopes, which are subdominant in infected individuals [209]. Distinct immunodominant patterns between infected and healthy individuals after T-cell expansion using overlapping 15mers covering the N, NSP17 and NSP13 proteins were also reported by Le Bert et al [215]. These data suggest that immune responses towards certain epitopes are preferentially selected during the infection. Considering that several factors, including viral load and infection duration, determine the magnitude and the breadth of $CD8^+$ T-cell responses [197], the precise causes behind this shift of the repertoire require more in-depth analysis.

The T-cell response against SARS-CoV-2 seems to be mainly dominated by $CD4^+$ T lymphocytes [219, 220]. Nevertheless, cytotoxic T cells have been shown to be important for protection [191, 192, 194]. Low disease severity is strongly associated with higher number of SARS-CoV-2-specific $CD8^+$ T cells and of naïve $CD8^+$ T cells [189]. Although few studies investigated age-specific patterns of antiviral immunity, it has been shown that elderly infected patients harbor low frequencies of virus-specific $CD8^+$ T cells [191], and age correlates with few IFNγ-producing T cells [189, 195]. Our data indicate a poor priming capacity of SARS-CoV-2 specific $CD8^+$ T-cell responses in healthy older subjects. Furthermore, we demonstrate that primary cellular responses are directed toward a low number of peptide epitopes in older subjects, consistently with the holes in the T-cell repertoire occurring with ageing [158]. Although we did not use overlapping peptides spanning the whole sequence of the 7 antigens targeted in our study, we could appreciate that some peptides were recognized more by younger subjects, such as the SLV E-derived peptide, 2 M-derived peptides (KLL and TLA) and the MLD RdRp-derived peptide. Some S-derived peptides were nonetheless recognized at similar frequencies in the two age groups (e.g. SII and ALN). This suggests that older individuals may still mount *de novo* responses against this antigen, even if narrower and potentially suboptimal, not arguing against immunization campaigns targeting the elderly. Further studies are however necessary to investigate the magnitude, duration and protection levels of long-term responses induced by the natural infection or by the vaccine in the most-at-risk populations. In the meantime, it is crucial to pay special attention to older subjects, even when vaccinated.

6 CONCLUSIONS

Age-related decline of the immune system, or immunosenescence, is thought to play an important role in the increased rate of severe infection or cancer, and reduced responsiveness to vaccination observed in the elderly. Improving our understanding of immune ageing has broad implications in disease prevention and optimization of vaccine immunogenicity with advanced age. There is an established paradigm that the ability to mount primary cellular immune responses from naive T-cells is waning in older people. For instance, reduced immunogenicity to SARS-CoV-2 vaccination has recently been demonstrated in elderly people. However, the factors that can impact the naive T-cell compartment and primary immune responsiveness in older adults are not fully understood. The potential influence of persistent viral infections, known to continuously stimulate and consume immune resources, remains controversial due to conflicting evidence.

In this study, the cumulative and differential impact of infections with common persistent herpesviruses (i.e. CMV, EBV, HSV-1 and HSV-2) on the T-cell compartments and T-cell responsiveness was investigated. Results show that infection with these viruses, in particular CMV, results in a reduction of the naive $CD4^+$ T-cell compartment with advanced age. Consistently, CMV infection is associated with reduced cellular as well as humoral responsiveness to a primary vaccination in old people. Conversely, primary responses mediated by naïve $CD8^+$ T cells strongly decline with age, irrespectively of the co-infection with herpesviruses. The effect seems due only to advancing age and the consequent numerical reduction of the naïve $CD8^+$ T-cell compartment which is, on turn, caused by thymic involution. This translates, in elderly subjects, in low and dysfunctional CD8-mediated responses toward neoantigens such as emerging infections like SARS-CoV-2, providing further explanations to the susceptibility of old individuals to its severe consequences.

Whether recall responses to pathogens encountered earlier in life are compromised with advanced ageing is still a matter of debate. The results shown in this thesis show that ageing by itself does not alter the numerical proportion of memory $CD4^+$ and $CD8^+$ T cells. However, the presence of persistent herpesvirus infections is responsible of increased levels of highly differentiated memory T cells. In respect to their functions, and consistently with a lack of impact of age only on recall responses, results show minimal age-related alterations to herpesvirus-specific memory cellular responses. However, we could nonetheless observe, in

elderly individuals, a lower frequency of early-differentiated memory $CD8^+$ T-cells with secretory capacity and the accumulation of late differentiated HSV-specific late differentiated memory $CD8^+$ T cells.

Our results suggest that a strong contraction of the naive $CD8^+$ T-cell repertoire associated with age (from Results section 1) likely results in an important reduction of naive $CD8^+$ T-cell responsiveness to new antigens in elderly human populations, explaining findings from the Results section 3. On the same line, diminished YF vaccine-specific $CD8^+$ T-cell responses were found to be correlated with baseline naive T-cell frequencies [155]. Older individuals may thus become more vulnerable to diseases for which the induction of $CD8^+$ T-cell immunity plays a protective role, such as against emerging viruses and tumors.

The present study provides potential insights underlying the vulnerability of elderly people to pathogens. Related to the contraction of the naive T-cell compartment, older CMV positive individuals may indeed present blunted adaptive immune responses to new antigens, such as those against SARS-CoV-2, possibly affecting their capacity to control viral replication. This is in line with a recent report of a favorable impact of thymic rebound during COVID lymphopenia [221] and with the observed reduced priming capacity of SARS-CoV-2 naïve $CD8^+$ T cells with ageing. Future studies will be necessary to establish if serology to herpesviruses, in particular CMV, may inform on the capacity to mount a good immune response against SARS-CoV-2 and other neoantigens, as well as if it may affect the recall responses mediated by early differentiated memory T cells.

In this respect, this study shows for the first time the inflation of effector-memory HSV-specific $CD8^+$ T-cells in older subjects, suggesting that all herpeviruses (and not only CMV) contribute to expansion of late-differentiated memory $CD8^+$ T cells with ageing. This is in line with results from section 1 showing that multiple infections are associated with higher numbers of EM $CD8^+$ and $CD4^+$ T cells. Whether this may alter the recall responses to other antigens is yet unknown. However, as a lower responses to general TCR ligation via anti-CD3 stimulation were observed in TM cells of older subjects, it is likely that the phenomenon could regard also antigen-specific early/intermediate differentiated memory T-cells. Further studies and appropriate methodologies (e.g. studying the proliferative capacity, which is typical of CM and TM) are required to better dissect this phenomenon.

The inflation of HSV-specific memory T cells observed in elderly subjects is a probable sign of frequent viral reactivation (which could also be subclinical). As the reactivation of

herpesviruses is thought to be a cause of an increased inflammatory status and of the onset of several noncommunicable diseases, these results argue in favor of the role of HSV in the establishment of both cardiovascular disease and dementia in elderly subjects.

Collectively, these findings provide clear insights into the alterations of adaptive immunity over time and the decline in vaccine efficacy with ageing (Figure 6.1). In particular, they show that the infection with persistent viruses, such as the herpesviruses which are common in the general population, can alter host immune resources, affecting both vaccine efficacy and the development of co-morbidities. Therefore, strategies aimed at limiting CMV, HSV-1 and EBV infections in the general population are required to increase the quality of life especially of elderly individuals.

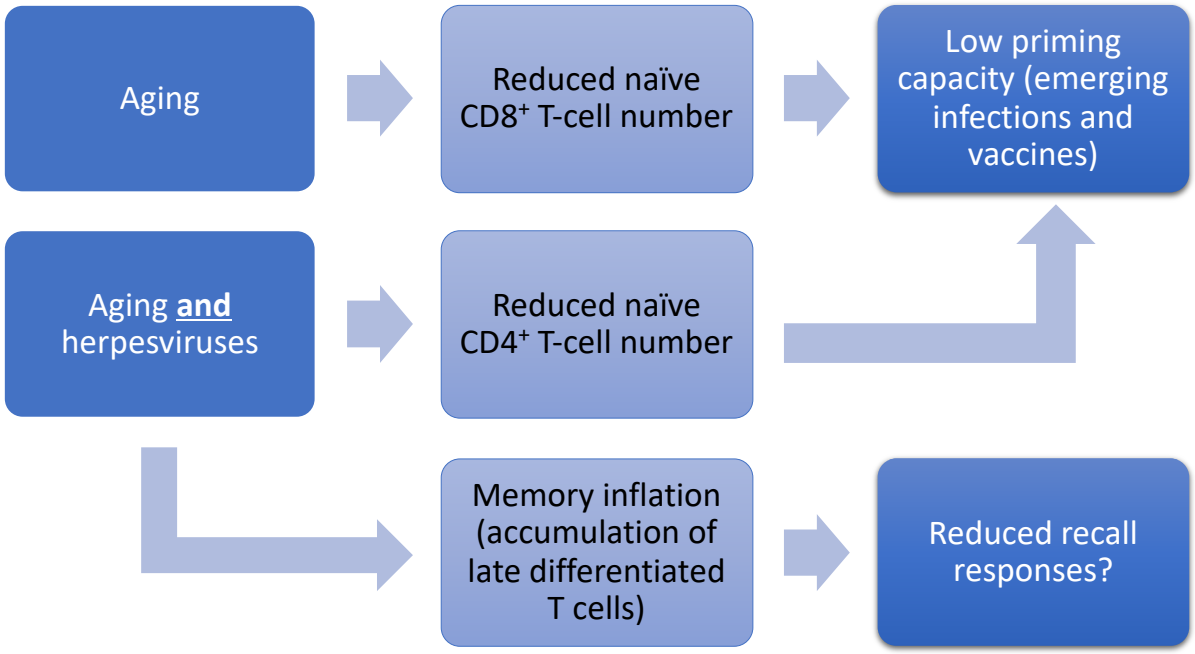

Figure 6.1. *Interplay between herpesvirus infections and adaptive T-cell immunity.*

7 REFERENCES

1. Wang NS, McHeyzer-Williams LJ, Okitsu SL, Burris TP, Reiner SL, McHeyzer-Williams MG. **Divergent transcriptional programming of class-specific B cell memory by T-bet and RORalpha**. *Nat Immunol* 2012; 13(6):604-611.

2. Curtsinger JM, Mescher MF. **Inflammatory cytokines as a third signal for T cell activation**. *Curr Opin Immunol* 2010; 22(3):333-340.

3. Blattman JN, Antia R, Sourdive DJ, Wang X, Kaech SM, Murali-Krishna K, et al. **Estimating the precursor frequency of naive antigen-specific CD8 T cells**. *J Exp Med* 2002; 195(5):657-664.

4. Takemoto N, Intlekofer AM, Northrup JT, Wherry EJ, Reiner SL. **Cutting Edge: IL-12 inversely regulates T-bet and eomesodermin expression during pathogen-induced CD8+ T cell differentiation**. *J Immunol* 2006; 177(11):7515-7519.

5. Rao RR, Li Q, Odunsi K, Shrikant PA. **The mTOR kinase determines effector versus memory CD8+ T cell fate by regulating the expression of transcription factors T-bet and Eomesodermin**. *Immunity* 2010; 32(1):67-78.

6. Rutishauser RL, Kaech SM. **Generating diversity: transcriptional regulation of effector and memory CD8 T-cell differentiation**. *Immunol Rev* 2010; 235(1):219-233.

7. Nolz JC, Harty JT. **Protective capacity of memory CD8+ T cells is dictated by antigen exposure history and nature of the infection**. *Immunity* 2011; 34(5):781-793.

8. Badovinac VP, Porter BB, Harty JT. **Programmed contraction of CD8(+) T cells after infection**. *Nat Immunol* 2002; 3(7):619-626.

9. Kurtulus S, Tripathi P, Moreno-Fernandez ME, Sholl A, Katz JD, Grimes HL, et al. **Bcl-2 allows effector and memory CD8+ T cells to tolerate higher expression of Bim**. *J Immunol* 2011; 186(10):5729-5737.

10. Unsoeld H, Krautwald S, Voehringer D, Kunzendorf U, Pircher H. **Cutting edge: CCR7+ and CCR7- memory T cells do not differ in immediate effector cell function**. *J Immunol* 2002; 169(2):638-641.

11. Green S, Ennis FA, Mathew A. **Long term recall of memory CD8 T cells in mice to first and third generation smallpox vaccines**. *Vaccine* 2011; 29(8):1666-1676.

12. Jabbari A, Harty JT. **Secondary memory CD8+ T cells are more protective but slower to acquire a central-memory phenotype**. *J Exp Med* 2006; 203(4):919-932.

13. West EE, Youngblood B, Tan WG, Jin HT, Araki K, Alexe G, et al. **Tight regulation of memory CD8(+) T cells limits their effectiveness during sustained high viral load**. *Immunity* 2011; 35(2):285-298.

14. Sarkar S, Teichgraber V, Kalia V, Polley A, Masopust D, Harrington LE, et al. **Strength of stimulus and clonal competition impact the rate of memory CD8 T cell differentiation**. *J Immunol* 2007; 179(10):6704-6714.

15. Williams MA, Bevan MJ. **Shortening the infectious period does not alter expansion of CD8 T cells but diminishes their capacity to differentiate into memory cells**. *J Immunol* 2004; 173(11):6694-6702.

16. Intlekofer AM, Takemoto N, Wherry EJ, Longworth SA, Northrup JT, Palanivel VR, et al. **Effector and memory CD8+ T cell fate coupled by T-bet and eomesodermin**. *Nat Immunol* 2005; 6(12):1236-1244.

17. Li Q, Rao RR, Araki K, Pollizzi K, Odunsi K, Powell JD, et al. **A central role for mTOR kinase in homeostatic proliferation induced CD8+ T cell memory and tumor immunity**. *Immunity* 2011; 34(4):541-553.

18. Rao RR, Li Q, Gubbels Bupp MR, Shrikant PA. **Transcription factor Foxo1 represses T-bet-mediated effector functions and promotes memory CD8(+) T cell differentiation**. *Immunity* 2012; 36(3):374-387.

19. Dejean AS, Beisner DR, Ch'en IL, Kerdiles YM, Babour A, Arden KC, et al. **Transcription factor Foxo3 controls the magnitude of T cell immune responses by modulating the function of dendritic cells**. *Nat Immunol* 2009; 10(5):504-513.

20. Sullivan JA, Kim EH, Plisch EH, Peng SL, Suresh M. **FOXO3 regulates CD8 T cell memory by T cell-intrinsic mechanisms**. *PLoS Pathog* 2012; 8(2):e1002533.

21. Ichii H, Sakamoto A, Hatano M, Okada S, Toyama H, Taki S, et al. **Role for Bcl-6 in the generation and maintenance of memory CD8+ T cells**. *Nat Immunol* 2002; 3(6):558-563.

22. Oestreich KJ, Huang AC, Weinmann AS. **The lineage-defining factors T-bet and Bcl-6 collaborate to regulate Th1 gene expression patterns**. *J Exp Med* 2011; 208(5):1001-1013.

23. Kallies A, Hawkins ED, Belz GT, Metcalf D, Hommel M, Corcoran LM, et al. **Transcriptional repressor Blimp-1 is essential for T cell homeostasis and self-tolerance**. *Nat Immunol* 2006; 7(5):466-474.

24. Rutishauser RL, Martins GA, Kalachikov S, Chandele A, Parish IA, Meffre E, et al. **Transcriptional repressor Blimp-1 promotes CD8(+) T cell terminal differentiation and**

represses the acquisition of central memory T cell properties. *Immunity* 2009; 31(2):296-308.

25. Leignadier J, Labrecque N. **Epitope density influences CD8+ memory T cell differentiation**. *PLoS One* 2010; 5(10):e13740.

26. Zaragoza B, Evaristo C, Kissenpfennig A, Libri V, Malissen B, Rocha B, et al. **Cell-to-cell interactions and signals involved in the reconstitution of peripheral CD8 T(CM) and T(EM) cell pools**. *PLoS One* 2011; 6(3):e17423.

27. Obar JJ, Lefrancois L. **Early signals during CD8 T cell priming regulate the generation of central memory cells**. *J Immunol* 2010; 185(1):263-272.

28. Badovinac VP, Porter BB, Harty JT. **CD8+ T cell contraction is controlled by early inflammation**. *Nat Immunol* 2004; 5(8):809-817.

29. Joshi NS, Cui W, Chandele A, Lee HK, Urso DR, Hagman J, et al. **Inflammation directs memory precursor and short-lived effector CD8(+) T cell fates via the graded expression of T-bet transcription factor**. *Immunity* 2007; 27(2):281-295.

30. Nicoli F, Solis-Soto MT, Paudel D, Marconi P, Gavioli R, Appay V, et al. **Age-related decline of de novo T cell responsiveness as a cause of COVID-19 severity**. *Geroscience* 2020; 42(4):1015-1019.

31. Dorshkind K, Swain S. **Age-associated declines in immune system development and function: causes, consequences, and reversal**. *Curr Opin Immunol* 2009; 21(4):404-407.

32. Ventura MT, Casciaro M, Gangemi S, Buquicchio R. **Immunosenescence in ageing: between immune cells depletion and cytokines up-regulation**. *Clin Mol Allergy* 2017; 15:21.

33. Fulop T, Larbi A, Dupuis G, Le Page A, Frost EH, Cohen AA, et al. **Immunosenescence and Inflamm-Ageing As Two Sides of the Same Coin: Friends or Foes?** *Front Immunol* 2017; 8:1960.

34. Briceno O, Lissina A, Wanke K, Afonso G, von Braun A, Ragon K, et al. **Reduced naive CD8(+) T-cell priming efficacy in elderly adults**. *Ageing Cell* 2016; 15(1):14-21.

35. Nikolich-Zugich J. **Ageing of the T cell compartment in mice and humans: from no naive expectations to foggy memories**. *J Immunol* 2014; 193(6):2622-2629.

36. Gallerani E, Proietto D, Dallan B, Campagnaro M, Pacifico S, Albanese V, et al. **Impaired Priming of SARS-CoV-2-Specific Naive CD8(+) T Cells in Older Subjects**. *Front Immunol* 2021; 12:693054.

37. Wertheimer AM, Bennett MS, Park B, Uhrlaub JL, Martinez C, Pulko V, et al. **Ageing and cytomegalovirus infection differentially and jointly affect distinct circulating T cell subsets in humans**. *J Immunol* 2014; 192(5):2143-2155.

38. Goronzy JJ, Fang F, Cavanagh MM, Qi Q, Weyand CM. **Naive T cell maintenance and function in human ageing**. *J Immunol* 2015; 194(9):4073-4080.

39. Pinti M, Appay V, Campisi J, Frasca D, Fulop T, Sauce D, et al. **Ageing of the immune system: Focus on inflammation and vaccination**. *Eur J Immunol* 2016; 46(10):2286-2301.

40. Franceschi C, Bonafe M, Valensin S, Olivieri F, De Luca M, Ottaviani E, et al. **Inflamm-ageing. An evolutionary perspective on immunosenescence**. *Ann N Y Acad Sci* 2000; 908:244-254.

41. Davison AJ. **Overview of classification**. 2007.

42. Wadsworth S, Jacob RJ, Roizman B. **Anatomy of herpes simplex virus DNA. II. Size, composition, and arrangement of inverted terminal repetitions**. *J Virol* 1975; 15(6):1487-1497.

43. Perng GC, Jones C. **Towards an understanding of the herpes simplex virus type 1 latency-reactivation cycle**. *Interdiscip Perspect Infect Dis*; 2010:262415.

44. Akhtar J, Shukla D. **Viral entry mechanisms: cellular and viral mediators of herpes simplex virus entry**. *Febs J* 2009; 276(24):7228-7236.

45. Jones PC, Roizman B. **Regulation of herpesvirus macromolecular synthesis. VIII. The transcription program consists of three phases during which both extent of transcription and accumulation of RNA in the cytoplasm are regulated**. *J Virol* 1979; 31(2):299-314.

46. Hay J, Ruyechan WT. **Alphaherpesvirus DNA replication**. 2007.

47. Baines JD. **Envelopment of herpes simplex virus nucleocapsids at the inner nuclear membrane**. 2007.

48. Stevens JG, Haarr L, Porter DD, Cook ML, Wagner EK. **Prominence of the herpes simplex virus latency-associated transcript in trigeminal ganglia from seropositive humans**. *J Infect Dis* 1988; 158(1):117-123.

49. Khanna KM, Lepisto AJ, Decman V, Hendricks RL. **Immune control of herpes simplex virus during latency**. *Current opinion in immunology* 2004; 16(4):463-469.

50. Khanna KM, Lepisto AJ, Hendricks RL. **Immunity to latent viral infection: many skirmishes but few fatalities**. *Trends in immunology* 2004; 25(5):230-234.

51. Chew T, Taylor KE, Mossman KL. **Innate and adaptive immune responses to herpes simplex virus**. *Viruses* 2009; 1(3):979-1002.

52. Zhang LJ, Sen GL, Ward NL, Johnston A, Chun K, Chen Y, et al. **Antimicrobial Peptide LL37 and MAVS Signaling Drive Interferon-beta Production by Epidermal Keratinocytes during Skin Injury**. *Immunity* 2016; 45(1):119-130.

53. Truong NR, Smith JB, Sandgren KJ, Cunningham AL. **Mechanisms of Immune Control of Mucosal HSV Infection: A Guide to Rational Vaccine Design**. *Front Immunol* 2019; 10:373.

54. Kim M, Truong NR, James V, Bosnjak L, Sandgren KJ, Harman AN, et al. **Relay of herpes simplex virus between Langerhans cells and dermal dendritic cells in human skin**. *PLoS Pathog* 2015; 11(4):e1004812.

55. Nopora K, Bernhard CA, Ried C, Castello AA, Murphy KM, Marconi P, et al. **MHC class I cross-presentation by dendritic cells counteracts viral immune evasion**. *Frontiers in immunology* 2012; 3:348.

56. Lee DH, Ghiasi H. **Roles of M1 and M2 Macrophages in Herpes Simplex Virus 1 Infectivity**. *J Virol* 2017; 91(15).

57. Carr DJJ. **Herpes Simplex Virus 1, Macrophages, and the Cornea**. *J Virol* 2017; 91(21).

58. Mott K, Brick DJ, van Rooijen N, Ghiasi H. **Macrophages are important determinants of acute ocular HSV-1 infection in immunized mice**. *Invest Ophthalmol Vis Sci* 2007; 48(12):5605-5615.

59. Brun P, Qesari M, Marconi PC, Kotsafti A, Porzionato A, Macchi V, et al. **Herpes Simplex Virus Type 1 Infects Enteric Neurons and Triggers Gut Dysfunction via Macrophage Recruitment**. *Front Cell Infect Microbiol* 2018; 8:74.

60. Telatin V, Nicoli F, Frasson C, Menegotto N, Barbaro F, Castelli E, et al. **In Chronic Hepatitis C Infection, Myeloid-Derived Suppressor Cell Accumulation and T Cell Dysfunctions Revert Partially and Late After Successful Direct-Acting Antiviral Treatment**. *Front Cell Infect Microbiol* 2019; 9:190.

61. O'Connor MA, Rastad JL, Green WR. **The Role of Myeloid-Derived Suppressor Cells in Viral Infection**. *Viral Immunol* 2017; 30(2):82-97.

62. Donaghy H, Bosnjak L, Harman AN, Marsden V, Tyring SK, Meng TC, et al. **Role for plasmacytoid dendritic cells in the immune control of recurrent human herpes simplex virus infection**. *J Virol* 2009; 83(4):1952-1961.

63. Ashkar AA, Rosenthal KL. **Interleukin-15 and natural killer and NKT cells play a critical role in innate protection against genital herpes simplex virus type 2 infection**. *J Virol* 2003; 77(18):10168-10171.

64. Svensson A, Nordstrom I, Sun JB, Eriksson K. **Protective immunity to genital herpes simplex [correction of simpex] virus type 2 infection is mediated by T-bet**. *J Immunol* 2005; 174(10):6266-6273.

65. Kim M, Osborne NR, Zeng W, Donaghy H, McKinnon K, Jackson DC, et al. **Herpes simplex virus antigens directly activate NK cells via TLR2, thus facilitating their presentation to CD4 T lymphocytes**. *J Immunol* 2012; 188(9):4158-4170.

66. Marino A, Pergolizzi S, Cimino F, Lauriano ER, Speciale A, D'Angelo V, et al. **Role of Herpes Simplex Envelope Glycoprotein B and Toll-Like Receptor 2 in Ocular Inflammation: An ex vivo Organotypic Rabbit Corneal Model**. *Viruses* 2019; 11(9).

67. Chen B, Lee AJ, Chew MV, Ashkar AA. **NK cells require antigen-specific memory CD4(+) T cells to mediate superior effector functions during HSV-2 recall responses in vitro**. *J Leukoc Biol* 2017; 101(4):1045-1052.

68. Costa-Garcia M, Ataya M, Moraru M, Vilches C, Lopez-Botet M, Muntasell A. **Human Cytomegalovirus Antigen Presentation by HLA-DR+ NKG2C+ Adaptive NK Cells Specifically Activates Polyfunctional Effector Memory CD4+ T Lymphocytes**. *Front Immunol* 2019; 10:687.

69. Pillarisetty VG, Katz SC, Bleier JI, Shah AB, Dematteo RP. **Natural killer dendritic cells have both antigen presenting and lytic function and in response to CpG produce IFN-gamma via autocrine IL-12**. *J Immunol* 2005; 174(5):2612-2618.

70. Zolini GP, Lima GK, Lucinda N, Silva MA, Dias MF, Pessoa NL, et al. **Defense against HSV-1 in a murine model is mediated by iNOS and orchestrated by the activation of TLR2 and TLR9 in trigeminal ganglia**. *J Neuroinflammation* 2014; 11:20.

71. Marques CP, Hu S, Sheng W, Lokensgard JR. **Microglial cells initiate vigorous yet non-protective immune responses during HSV-1 brain infection**. *Virus Res* 2006; 121(1):1-10.

72. Lima GK, Zolini GP, Mansur DS, Freire Lima BH, Wischhoff U, Astigarraga RG, et al. **Toll-like receptor (TLR) 2 and TLR9 expressed in trigeminal ganglia are critical to viral control during herpes simplex virus 1 infection**. *Am J Pathol* 2010; 177(5):2433-2445.

73. Kodukula P, Liu T, Rooijen NV, Jager MJ, Hendricks RL. **Macrophage control of herpes simplex virus type 1 replication in the peripheral nervous system**. *J Immunol* 1999; 162(5):2895-2905.

74. Liu T, Tang Q, Hendricks RL. **Inflammatory infiltration of the trigeminal ganglion after herpes simplex virus type 1 corneal infection**. *J Virol* 1996; 70(1):264-271.

75. Lucinda N, Figueiredo MM, Pessoa NL, Santos BS, Lima GK, Freitas AM, et al. **Dendritic cells, macrophages, NK and CD8(+) T lymphocytes play pivotal roles in controlling HSV-1 in the trigeminal ganglia by producing IL1-beta, iNOS and granzyme B**. *Virol J* 2017; 14(1):37.

76. Reinert LS, Lopusna K, Winther H, Sun C, Thomsen MK, Nandakumar R, et al. **Sensing of HSV-1 by the cGAS-STING pathway in microglia orchestrates antiviral defence in the CNS**. *Nat Commun* 2016; 7:13348.

77. Skouboe MK, Knudsen A, Reinert LS, Boularan C, Lioux T, Perouzel E, et al. **STING agonists enable antiviral cross-talk between human cells and confer protection against genital herpes in mice**. *PLoS Pathog* 2018; 14(4):e1006976.

78. Gutjahr A, Papagno L, Nicoli F, Kanuma T, Kuse N, Cabral-Piccin MP, et al. **The STING ligand cGAMP potentiates the efficacy of vaccine-induced CD8+ T cells**. *JCI Insight* 2019; 4(7).

79. Nicoli F, Paul S, Appay V. **Harnessing the Induction of CD8(+) T-Cell Responses Through Metabolic Regulation by Pathogen-Recognition-Receptor Triggering in Antigen Presenting Cells**. *Front Immunol* 2018; 9:2372.

80. Belshe RB, Heineman TC, Bernstein DI, Bellamy AR, Ewell M, van der Most R, et al. **Correlate of immune protection against HSV-1 genital disease in vaccinated women**. *J Infect Dis* 2014; 209(6):828-836.

81. Srivastava R, Khan AA, Spencer D, Vahed H, Lopes PP, Thai NT, et al. **HLA-A02:01-restricted epitopes identified from the Herpes Simplex Virus tegument protein VP11/12 preferentially recall polyfunctional effector memory CD8+ T cells from seropositive asymptomatic individuals and protect humanized HLA-A*02:01 transgenic mice against ocular Herpes**. *J Immunol* 2015; 194(5):2232-2248.

82. Khan AA, Srivastava R, Spencer D, Garg S, Fremgen D, Vahed H, et al. **Phenotypic and functional characterization of Herpes Simplex Virus glycoprotein B epitope-specific effector and memory CD8+ T cells from symptomatic and asymptomatic individuals with ocular Herpes**. *J Virol* 2015; 89(7):3776-3792.

83. Finessi V, Nicoli F, Gallerani E, Sforza F, Sicurella M, Cafaro A, et al. **Effects of different routes of administration on the immunogenicity of the Tat protein and a Tat-derived peptide**. *Hum Vaccin Immunother* 2015; 11(6):1489-1493.
84. Morrison LA, Zhu L, Thebeau LG. **Vaccine-induced serum immunoglobin contributes to protection from herpes simplex virus type 2 genital infection in the presence of immune T cells**. *J Virol* 2001; 75(3):1195-1204.
85. Dudley KL, Bourne N, Milligan GN. **Immune protection against HSV-2 in B-cell-deficient mice**. *Virology* 2000; 270(2):454-463.
86. Harandi AM, Svennerholm B, Holmgren J, Eriksson K. **Differential roles of B cells and IFN-gamma-secreting CD4(+) T cells in innate and adaptive immune control of genital herpes simplex virus type 2 infection in mice**. *J Gen Virol* 2001; 82(Pt 4):845-853.
87. Nicoli F, Gallerani E, Skarlis C, Sicurella M, Cafaro A, Ensoli B, et al. **Systemic immunodominant CD8 responses with an effector-like phenotype are induced by intravaginal immunization with attenuated HSV vectors expressing HIV Tat and mediate protection against HSV infection**. *Vaccine* 2016; 34(19):2216-2224.
88. Sicurella M, Nicoli F, Gallerani E, Volpi I, Berto E, Finessi V, et al. **An attenuated herpes simplex virus type 1 (HSV1) encoding the HIV-1 Tat protein protects mice from a deadly mucosal HSV1 challenge**. *PLoS One* 2014; 9(7):e100844.
89. Nagafuchi S, Oda H, Mori R, Taniguchi T. **Mechanism of acquired resistance to herpes simplex virus infection as studied in nude mice**. *J Gen Virol* 1979; 44(3):715-723.
90. Halford WP, Maender JL, Gebhardt BM. **Re-evaluating the role of natural killer cells in innate resistance to herpes simplex virus type 1**. *Virol J* 2005; 2:56.
91. Liu T, Khanna KM, Chen X, Fink DJ, Hendricks RL. **CD8(+) T cells can block herpes simplex virus type 1 (HSV-1) reactivation from latency in sensory neurons**. *J Exp Med* 2000; 191(9):1459-1466.
92. Koelle DM, Posavad CM, Barnum GR, Johnson ML, Frank JM, Corey L. **Clearance of HSV-2 from recurrent genital lesions correlates with infiltration of HSV-specific cytotoxic T lymphocytes**. *J Clin Invest* 1998; 101(7):1500-1508.
93. Kuklin NA, Daheshia M, Chun S, Rouse BT. **Role of mucosal immunity in herpes simplex virus infection**. *J Immunol* 1998; 160(12):5998-6003.

94. McDermott MR, Goldsmith CH, Rosenthal KL, Brais LJ. **T lymphocytes in genital lymph nodes protect mice from intravaginal infection with herpes simplex virus type 2**. *J Infect Dis* 1989; 159(3):460-466.

95. Cuburu N, Wang K, Goodman KN, Pang YY, Thompson CD, Lowy DR, et al. **Topical herpes simplex virus 2 (HSV-2) vaccination with human papillomavirus vectors expressing gB/gD ectodomains induces genital-tissue-resident memory CD8+ T cells and reduces genital disease and viral shedding after HSV-2 challenge**. *J Virol* 2015; 89(1):83-96.

96. Awasthi S, Hook LM, Shaw CE, Pahar B, Stagray JA, Liu D, et al. **An HSV-2 Trivalent Vaccine Is Immunogenic in Rhesus Macaques and Highly Efficacious in Guinea Pigs**. *PLoS Pathog* 2017; 13(1):e1006141.

97. Jenks JA, Goodwin ML, Permar SR. **The Roles of Host and Viral Antibody Fc Receptors in Herpes Simplex Virus (HSV) and Human Cytomegalovirus (HCMV) Infections and Immunity**. *Front Immunol* 2019; 10:2110.

98. Bourne N, Perry CL, Banasik BN, Miller AL, White M, Pyles RB, et al. **Increased Frequency of Virus Shedding by Herpes Simplex Virus 2-Infected Guinea Pigs in the Absence of CD4(+) T Lymphocytes**. *J Virol* 2019; 93(4).

99. Cunningham AL, Turner RR, Miller AC, Para MF, Merigan TC. **Evolution of recurrent herpes simplex lesions. An immunohistologic study**. *J Clin Invest* 1985; 75(1):226-233.

100. Schiffer JT, Corey L. **Rapid host immune response and viral dynamics in herpes simplex virus-2 infection**. *Nat Med* 2013; 19(3):280-290.

101. Nakanishi Y, Lu B, Gerard C, Iwasaki A. **CD8(+) T lymphocyte mobilization to virus-infected tissue requires CD4(+) T-cell help**. *Nature* 2009; 462(7272):510-513.

102. Shin H, Iwasaki A. **A vaccine strategy that protects against genital herpes by establishing local memory T cells**. *Nature* 2012; 491(7424):463-467.

103. Khan AA, Srivastava R, Lopes PP, Wang C, Pham TT, Cochrane J, et al. **Asymptomatic memory CD8+ T cells: from development and regulation to consideration for human vaccines and immunotherapeutics**. *Hum Vaccin Immunother* 2014; 10(4):945-963.

104. Bachmann MF, Wolint P, Schwarz K, Oxenius A. **Recall proliferation potential of memory CD8+ T cells and antiviral protection**. *J Immunol* 2005; 175(7):4677-4685.

105. Addo MM, Draenert R, Rathod A, Verrill CL, Davis BT, Gandhi RT, et al. **Fully differentiated HIV-1 specific CD8+ T effector cells are more frequently detectable in controlled than in progressive HIV-1 infection**. *PLoS One* 2007; 2(3):e321.

106. Billingsley JM, Rajakumar PA, Connole MA, Salisch NC, Adnan S, Kuzmichev YV, et al. **Characterization of CD8+ T Cell Differentiation following SIVDeltanef Vaccination by Transcription Factor Expression Profiling**. *PLoS Pathog* 2015; 11(3):e1004740.

107. Yamamoto T, Johnson MJ, Price DA, Wolinsky DI, Almeida JR, Petrovas C, et al. **Virus inhibition activity of effector memory CD8(+) T cells determines simian immunodeficiency virus load in vaccinated monkeys after vaccine breakthrough infection**. *J Virol* 2012; 86(10):5877-5884.

108. Hansen SG, Vieville C, Whizin N, Coyne-Johnson L, Siess DC, Drummond DD, et al. **Effector memory T cell responses are associated with protection of rhesus monkeys from mucosal simian immunodeficiency virus challenge**. *Nat Med* 2009; 15(3):293-299.

109. Hess C, Altfeld M, Thomas SY, Addo MM, Rosenberg ES, Allen TM, et al. **HIV-1 specific CD8+ T cells with an effector phenotype and control of viral replication**. *Lancet* 2004; 363(9412):863-866.

110. Olson JA, McDonald-Hyman C, Jameson SC, Hamilton SE. **Effector-like CD8(+) T Cells in the Memory Population Mediate Potent Protective Immunity**. *Immunity* 2013; 38(6):1250-1260.

111. Hikono H, Kohlmeier JE, Takamura S, Wittmer ST, Roberts AD, Woodland DL. **Activation phenotype, rather than central- or effector-memory phenotype, predicts the recall efficacy of memory CD8+ T cells**. *J Exp Med* 2007; 204(7):1625-1636.

112. Jung YW, Rutishauser RL, Joshi NS, Haberman AM, Kaech SM. **Differential localization of effector and memory CD8 T cell subsets in lymphoid organs during acute viral infection**. *J Immunol* 2010; 185(9):5315-5325.

113. Zhu J, Peng T, Johnston C, Phasouk K, Kask AS, Klock A, et al. **Immune surveillance by CD8alphaalpha+ skin-resident T cells in human herpes virus infection**. *Nature* 2013; 497(7450):494-497.

114. Zhu J, Koelle DM, Cao J, Vazquez J, Huang ML, Hladik F, et al. **Virus-specific CD8+ T cells accumulate near sensory nerve endings in genital skin during subclinical HSV-2 reactivation**. *J Exp Med* 2007; 204(3):595-603.

115. Posavad CM, Zhao L, Dong L, Jin L, Stevens CE, Magaret AS, et al. **Enrichment of herpes simplex virus type 2 (HSV-2) reactive mucosal T cells in the human female genital tract**. *Mucosal Immunol* 2017; 10(5):1259-1269.

116. Peng T, Zhu J, Phasouk K, Koelle DM, Wald A, Corey L. **An effector phenotype of CD8+ T cells at the junction epithelium during clinical quiescence of herpes simplex virus 2 infection**. *J Virol* 2012; 86(19):10587-10596.

117. Carroll KL, Avery L, Treat BR, Kane LP, Kinchington PR, Hendricks RL, et al. **Differential Expression of Immune Checkpoint Molecules on CD8(+) T Cells Specific for Immunodominant and Subdominant Herpes Simplex Virus 1 Epitopes**. *J Virol* 2020; 94(2).

118. Srivastava R, Coulon PG, Roy S, Chilukuri S, Garg S, BenMohamed L. **Phenotypic and Functional Signatures of Herpes Simplex Virus-Specific Effector Memory CD73(+)CD45RA(high)CCR7(low)CD8(+) TEMRA and CD73(+)CD45RA(low)CCR7(low)CD8(+) TEM Cells Are Associated with Asymptomatic Ocular Herpes**. *J Immunol* 2018; 201(8):2315-2330.

119. Nicoli F, Papagno L, Frere JJ, Cabral-Piccin MP, Clave E, Gostick E, et al. **Naive CD8(+) T-Cells Engage a Versatile Metabolic Program Upon Activation in Humans and Differ Energetically From Memory CD8(+) T-Cells**. *Front Immunol* 2018; 9:2736.

120. Chattopadhyay D, Mukhopadhyay A, Ojha D, Sadhukhan P, Dutta S. **Immuno-metabolic changes in herpes virus infection**. *Cytokine* 2018; 112:52-62.

121. Nicoli F. **Angry, Hungry T-Cells: How Are T-Cell Responses Induced in Low Nutrient Conditions?** *Immunometabolism* 2020; 2(1):e200004.

122. Klenerman P, Oxenius A. **T cell responses to cytomegalovirus**. *Nat Rev Immunol* 2016; 16(6):367-377.

123. Patin E, Hasan M, Bergstedt J, Rouilly V, Libri V, Urrutia A, et al. **Natural variation in the parameters of innate immune cells is preferentially driven by genetic factors**. *Nat Immunol* 2018; 19(3):302-314.

124. Derhovanessian E, Theeten H, Hahnel K, Van Damme P, Cools N, Pawelec G. **Cytomegalovirus-associated accumulation of late-differentiated CD4 T-cells correlates with poor humoral response to influenza vaccination**. *Vaccine* 2013; 31(4):685-690.

125. Frasca D, Diaz A, Romero M, Landin AM, Blomberg BB. **Cytomegalovirus (CMV) seropositivity decreases B cell responses to the influenza vaccine**. *Vaccine* 2015; 33(12):1433-1439.

126. Trzonkowski P, Mysliwska J, Szmit E, Wieckiewicz J, Lukaszuk K, Brydak LB, et al. **Association between cytomegalovirus infection, enhanced proinflammatory response and**

low level of anti-hemagglutinins during the anti-influenza vaccination--an impact of immunosenescence. *Vaccine* 2003; 21(25-26):3826-3836.

127. Derhovanessian E, Maier AB, Hahnel K, McElhaney JE, Slagboom EP, Pawelec G. **Latent infection with cytomegalovirus is associated with poor memory CD4 responses to influenza A core proteins in the elderly**. *J Immunol* 2014; 193(7):3624-3631.

128. den Elzen WP, Vossen AC, Cools HJ, Westendorp RG, Kroes AC, Gussekloo J. **Cytomegalovirus infection and responsiveness to influenza vaccination in elderly residents of long-term care facilities**. *Vaccine* 2011; 29(29-30):4869-4874.

129. Furman D, Jojic V, Sharma S, Shen-Orr SS, Angel CJ, Onengut-Gumuscu S, et al. **Cytomegalovirus infection enhances the immune response to influenza**. *Sci Transl Med* 2015; 7(281):281ra243.

130. Smithey MJ, Venturi V, Davenport MP, Buntzman AS, Vincent BG, Frelinger JA, et al. **Lifelong CMV infection improves immune defense in old mice by broadening the mobilized TCR repertoire against third-party infection**. *Proc Natl Acad Sci U S A* 2018; 115(29):E6817-E6825.

131. Hayes EB, Komar N, Nasci RS, Montgomery SP, O'Leary DR, Campbell GL. **Epidemiology and transmission dynamics of West Nile virus disease**. *Emerg Infect Dis* 2005; 11(8):1167-1173.

132. Jia N, Feng D, Fang LQ, Richardus JH, Han XN, Cao WC, et al. **Case fatality of SARS in mainland China and associated risk factors**. *Trop Med Int Health* 2009; 14 Suppl 1:21-27.

133. Kline KA, Bowdish DM. **Infection in an ageing population**. *Curr Opin Microbiol* 2016; 29:63-67.

134. Guan WJ, Ni ZY, Hu Y, Liang WH, Ou CQ, He JX, et al. **Clinical Characteristics of Coronavirus Disease 2019 in China**. *N Engl J Med* 2020; 382(18):1708-1720.

135. Wu Z, McGoogan JM. **Characteristics of and Important Lessons From the Coronavirus Disease 2019 (COVID-19) Outbreak in China: Summary of a Report of 72314 Cases From the Chinese Center for Disease Control and Prevention**. *JAMA* 2020; 323(13):1239-1242.

136. Gustafson CE, Kim C, Weyand CM, Goronzy JJ. **Influence of immune ageing on vaccine responses**. *The Journal of allergy and clinical immunology* 2020; 145(5):1309-1321.

137. Collier DA, Ferreira I, Kotagiri P, Datir R, Lim E, Touizer E, et al. **Age-related immune response heterogeneity to SARS-CoV-2 vaccine BNT162b2**. *Nature* 2021.

138. Rodero MP, Decalf J, Bondet V, Hunt D, Rice GI, Werneke S, et al. **Detection of interferon alpha protein reveals differential levels and cellular sources in disease**. *J Exp Med* 2017; 214(5):1547-1555.

139. Clave E, Araujo IL, Alanio C, Patin E, Bergstedt J, Urrutia A, et al. **Human thymopoiesis is influenced by a common genetic variant within the TCRA-TCRD locus**. *Sci Transl Med* 2018; 10(457).

140. Zhong Q, Bhattacharya S, Kotsopoulos S, Olson J, Taly V, Griffiths AD, et al. **Multiplex digital PCR: breaking the one target per color barrier of quantitative PCR**. *Lab Chip* 2011; 11(13):2167-2174.

141. Papagno L, Kuse N, Lissina A, Gostick E, Price DA, Appay V, et al. **The TLR9 ligand CpG ODN 2006 is a poor adjuvant for the induction of de novo CD8(+) T-cell responses in vitro**. *Sci Rep* 2020; 10(1):11620.

142. Sforza F, Nicoli F, Gallerani E, Finessi V, Reali E, Cafaro A, et al. **HIV-1 Tat affects the programming and functionality of human CD8(+) T cells by modulating the expression of T-box transcription factors**. *AIDS* 2014; 28(12):1729-1738.

143. Nicoli F, Gallerani E, Sforza F, Finessi V, Chachage M, Geldmacher C, et al. **The HIV-1 Tat protein affects human CD4+ T-cell programing and activation, and favors the differentiation of naive CD4+ T cells**. *AIDS* 2018; 32(5):575-581.

144. **R: A Language and Environment for Statistical Computing**. In. Vienna, Austria: R Core Team, R Foundation for Statistical Computing; 2019.

145. **RStudio: Integrated Development Environment for R**. In. Boston, MA: RStudio Team, RStudio, Inc.; 2016.

146. Wickham H. **Tidyverse: Easily Install and Load the 'Tidyverse'**. In; 2017.

147. Wickham H. **Scales: Scale Functions for Visualization**. In; 2018.

148. Tseng HF, Harpaz R, Luo Y, Hales CM, Sy LS, Tartof SY, et al. **Declining Effectiveness of Herpes Zoster Vaccine in Adults Aged >/=60 Years**. *J Infect Dis* 2016; 213(12):1872-1875.

149. Weinberg A, Canniff J, Rouphael N, Mehta A, Mulligan M, Whitaker JA, et al. **Varicella-Zoster Virus-Specific Cellular Immune Responses to the Live Attenuated Zoster Vaccine in Young and Older Adults**. *J Immunol* 2017; 199(2):604-612.

150. Schenkein JG, Park S, Nahm MH. **Pneumococcal vaccination in older adults induces antibodies with low opsonic capacity and reduced antibody potency**. *Vaccine* 2008; 26(43):5521-5526.

151. van Werkhoven CH, Huijts SM, Bolkenbaas M, Grobbee DE, Bonten MJ. **The Impact of Age on the Efficacy of 13-valent Pneumococcal Conjugate Vaccine in Elderly**. *Clin Infect Dis* 2015; 61(12):1835-1838.

152. Grasse M, Meryk A, Schirmer M, Grubeck-Loebenstein B, Weinberger B. **Booster vaccination against tetanus and diphtheria: insufficient protection against diphtheria in young and elderly adults**. *Immun Ageing* 2016; 13(1):26.

153. Dugan HL, Henry C, Wilson PC. **Ageing and influenza vaccine-induced immunity**. *Cell Immunol* 2020; 348:103998.

154. Roukens AH, Soonawala D, Joosten SA, de Visser AW, Jiang X, Dirksen K, et al. **Elderly subjects have a delayed antibody response and prolonged viraemia following yellow fever vaccination: a prospective controlled cohort study**. *PLoS One* 2011; 6(12):e27753.

155. Schulz AR, Malzer JN, Domingo C, Jurchott K, Grutzkau A, Babel N, et al. **Low Thymic Activity and Dendritic Cell Numbers Are Associated with the Immune Response to Primary Viral Infection in Elderly Humans**. *J Immunol* 2015; 195(10):4699-4711.

156. Weinberger B, Haks MC, de Paus RA, Ottenhoff THM, Bauer T, Grubeck-Loebenstein B. **Impaired Immune Response to Primary but Not to Booster Vaccination Against Hepatitis B in Older Adults**. *Front Immunol* 2018; 9:1035.

157. Wagner A, Garner-Spitzer E, Jasinska J, Kollaritsch H, Stiasny K, Kundi M, et al. **Age-related differences in humoral and cellular immune responses after primary immunisation: indications for stratified vaccination schedules**. *Sci Rep* 2018; 8(1):9825.

158. Appay V, Sauce D. **Naive T cells: the crux of cellular immune ageing?** *Exp Gerontol* 2014; 54:90-93.

159. Li G, Yu M, Lee WW, Tsang M, Krishnan E, Weyand CM, et al. **Decline in miR-181a expression with age impairs T cell receptor sensitivity by increasing DUSP6 activity**. *Nat Med* 2012; 18(10):1518-1524.

160. Gustafson CE, Cavanagh MM, Jin J, Weyand CM, Goronzy JJ. **Functional pathways regulated by microRNA networks in CD8 T-cell ageing**. *Ageing Cell* 2019; 18(1):e12879.

161. Mold JE, Reu P, Olin A, Bernard S, Michaelsson J, Rane S, et al. **Cell generation dynamics underlying naive T-cell homeostasis in adult humans**. *PLoS Biol* 2019; 17(10):e3000383.

162. Ferrando-Martinez S, Ruiz-Mateos E, Hernandez A, Gutierrez E, Rodriguez-Mendez Mdel M, Ordonez A, et al. **Age-related deregulation of naive T cell homeostasis in elderly humans**. *Age (Dordrecht, Netherlands)* 2011; 33(2):197-207.

163. Sauce D, Larsen M, Fastenackels S, Duperrier A, Keller M, Grubeck-Loebenstein B, et al. **Evidence of premature immune ageing in patients thymectomized during early childhood**. *J Clin Invest* 2009; 119(10):3070-3078.

164. Sauce D, Larsen M, Fastenackels S, Roux A, Gorochov G, Katlama C, et al. **Lymphopenia-driven homeostatic regulation of naive T cells in elderly and thymectomized young adults**. *J Immunol* 2012; 189(12):5541-5548.

165. Alanio C, Lemaitre F, Law HK, Hasan M, Albert ML. **Enumeration of human antigen-specific naive CD8+ T cells reveals conserved precursor frequencies**. *Blood* 2010; 115(18):3718-3725.

166. Su LF, Kidd BA, Han A, Kotzin JJ, Davis MM. **Virus-specific CD4(+) memory-phenotype T cells are abundant in unexposed adults**. *Immunity* 2013; 38(2):373-383.

167. Sempowski GD, Hale LP, Sundy JS, Massey JM, Koup RA, Douek DC, et al. **Leukemia inhibitory factor, oncostatin M, IL-6, and stem cell factor mRNA expression in human thymus increases with age and is associated with thymic atrophy**. *J Immunol* 2000; 164(4):2180-2187.

168. Carbajosa S, Gea S, Chillon-Marinas C, Poveda C, Del Carmen Maza M, Fresno M, et al. **Altered bone marrow lymphopoiesis and interleukin-6-dependent inhibition of thymocyte differentiation contribute to thymic atrophy during Trypanosoma cruzi infection**. *Oncotarget* 2017; 8(11):17551-17561.

169. Wald A, Selke S, Magaret A, Boeckh M. **Impact of human cytomegalovirus (CMV) infection on immune response to pandemic 2009 H1N1 influenza vaccine in healthy adults**. *J Med Virol* 2013; 85(9):1557-1560.

170. Frasca D, Blomberg BB. **Ageing, cytomegalovirus (CMV) and influenza vaccine responses**. *Hum Vaccin Immunother* 2016; 12(3):682-690.

171. Lefebvre JS, Lorenzo EC, Masters AR, Hopkins JW, Eaton SM, Smiley ST, et al. **Vaccine efficacy and T helper cell differentiation change with ageing**. *Oncotarget* 2016; 7(23):33581-33594.

172. Lefebvre JS, Masters AR, Hopkins JW, Haynes L. **Age-related impairment of humoral response to influenza is associated with changes in antigen specific T follicular helper cell responses**. *Sci Rep* 2016; 6:25051.
173. Herati RS, Reuter MA, Dolfi DV, Mansfield KD, Aung H, Badwan OZ, et al. **Circulating CXCR5+PD-1+ response predicts influenza vaccine antibody responses in young adults but not elderly adults**. *J Immunol* 2014; 193(7):3528-3537.
174. Eaton SM, Burns EM, Kusser K, Randall TD, Haynes L. **Age-related defects in CD4 T cell cognate helper function lead to reductions in humoral responses**. *J Exp Med* 2004; 200(12):1613-1622.
175. Johnson SA, Cambier JC. **Ageing, autoimmunity and arthritis: senescence of the B cell compartment - implications for humoral immunity**. *Arthritis Res Ther* 2004; 6(4):131-139.
176. Song H, Price PW, Cerny J. **Age-related changes in antibody repertoire: contribution from T cells**. *Immunol Rev* 1997; 160:55-62.
177. Frasca D, Landin AM, Riley RL, Blomberg BB. **Mechanisms for decreased function of B cells in aged mice and humans**. *J Immunol* 2008; 180(5):2741-2746.
178. Hill DL, Whyte CE, Innocentin S, Lee JL, Dooley J, Wang J, et al. **Impaired HA-specific T follicular helper cell and antibody responses to influenza vaccination are linked to inflammation in humans**. *eLife* 2021; 10.
179. Asanuma H, Sharp M, Maecker HT, Maino VC, Arvin AM. **Frequencies of memory T cells specific for varicella-zoster virus, herpes simplex virus, and cytomegalovirus by intracellular detection of cytokine expression**. *J Infect Dis* 2000; 181(3):859-866.
180. Chiu YL, Lin CH, Sung BY, Chuang YF, Schneck JP, Kern F, et al. **Cytotoxic polyfunctionality maturation of cytomegalovirus-pp65-specific CD4 + and CD8 + T-cell responses in older adults positively correlates with response size**. *Sci Rep* 2016; 6:19227.
181. Ouyang Q, Wagner WM, Zheng W, Wikby A, Remarque EJ, Pawelec G. **Dysfunctional CMV-specific CD8(+) T cells accumulate in the elderly**. *Exp Gerontol* 2004; 39(4):607-613.
182. Lang A, Brien JD, Nikolich-Zugich J. **Inflation and long-term maintenance of CD8 T cells responding to a latent herpesvirus depend upon establishment of latency and presence of viral antigens**. *J Immunol* 2009; 183(12):8077-8087.
183. Gil A, Yassai MB, Naumov YN, Selin LK. **Narrowing of human influenza A virus-specific T cell receptor alpha and beta repertoires with increasing age**. *J Virol* 2015; 89(8):4102-4116.

184. Griffiths SJ, Riddell NE, Masters J, Libri V, Henson SM, Wertheimer A, et al. **Age-associated increase of low-avidity cytomegalovirus-specific CD8+ T cells that re-express CD45RA**. *J Immunol* 2013; 190(11):5363-5372.
185. Wang GC, Han C, Detrick B, Casolaro V, Levine DM, Fried LP, et al. **Herpesvirus Infections and Risk of Frailty and Mortality in Older Women: Women's Health and Ageing Studies**. *J Am Geriatr Soc* 2016; 64(5):998-1005.
186. Kotronias D, Kapranos N. **Herpes simplex virus as a determinant risk factor for coronary artery atherosclerosis and myocardial infarction**. *In Vivo* 2005; 19(2):351-357.
187. Mancuso R, Sicurella M, Agostini S, Marconi P, Clerici M. **Herpes simplex virus type 1 and Alzheimer's disease: link and potential impact on treatment**. *Expert Rev Anti Infect Ther* 2019; 17(9):715-731.
188. O'Driscoll M, Ribeiro Dos Santos G, Wang L, Cummings DAT, Azman AS, Paireau J, et al. **Age-specific mortality and immunity patterns of SARS-CoV-2**. *Nature* 2021; 590(7844):140-145.
189. Rydyznski Moderbacher C, Ramirez SI, Dan JM, Grifoni A, Hastie KM, Weiskopf D, et al. **Antigen-Specific Adaptive Immunity to SARS-CoV-2 in Acute COVID-19 and Associations with Age and Disease Severity**. *Cell* 2020; 183(4):996-1012 e1019.
190. Tan AT, Linster M, Tan CW, Le Bert N, Chia WN, Kunasegaran K, et al. **Early induction of functional SARS-CoV-2-specific T cells associates with rapid viral clearance and mild disease in COVID-19 patients**. *Cell Rep* 2021; 34(6):108728.
191. Ferretti AP, Kula T, Wang Y, Nguyen DMV, Weinheimer A, Dunlap GS, et al. **Unbiased Screens Show CD8(+) T Cells of COVID-19 Patients Recognize Shared Epitopes in SARS-CoV-2 that Largely Reside outside the Spike Protein**. *Immunity* 2020; 53(5):1095-1107 e1093.
192. Kroemer M, Spehner L, Vettoretti L, Bouard A, Eberst G, Pili Floury S, et al. **COVID-19 patients display distinct SARS-CoV-2 specific T-cell responses according to disease severity**. *J Infect* 2021; 82(2):282-327.
193. Kared H, Redd AD, Bloch EM, Bonny TS, Sumatoh HR, Kairi F, et al. **SARS-CoV-2-specific CD8+ T cell responses in convalescent COVID-19 individuals**. *J Clin Invest* 2021.
194. McMahan K, Yu J, Mercado NB, Loos C, Tostanoski LH, Chandrashekar A, et al. **Correlates of protection against SARS-CoV-2 in rhesus macaques**. *Nature* 2021; 590(7847):630-634.

195. Sattler A, Angermair S, Stockmann H, Heim KM, Khadzhynov D, Treskatsch S, et al. **SARS-CoV-2-specific T cell responses and correlations with COVID-19 patient predisposition**. *J Clin Invest* 2020; 130(12):6477-6489.

196. Nikolich-Zugich J, Li G, Uhrlaub JL, Renkema KR, Smithey MJ. **Age-related changes in CD8 T cell homeostasis and immunity to infection**. *Semin Immunol* 2012; 24(5):356-364.

197. Vibholm LK, Nielsen SS, Pahus MH, Frattari GS, Olesen R, Andersen R, et al. **SARS-CoV-2 persistence is associated with antigen-specific CD8 T-cell responses**. *EBioMedicine* 2021; 64:103230.

198. Grifoni A, Sidney J, Zhang Y, Scheuermann RH, Peters B, Sette A. **A Sequence Homology and Bioinformatic Approach Can Predict Candidate Targets for Immune Responses to SARS-CoV-2**. *Cell Host Microbe* 2020; 27(4):671-680 e672.

199. Sekine T, Perez-Potti A, Rivera-Ballesteros O, Stralin K, Gorin JB, Olsson A, et al. **Robust T Cell Immunity in Convalescent Individuals with Asymptomatic or Mild COVID-19**. *Cell* 2020; 183(1):158-168 e114.

200. Reiser JB, Legoux F, Gras S, Trudel E, Chouquet A, Leger A, et al. **Analysis of relationships between peptide/MHC structural features and naive T cell frequency in humans**. *J Immunol* 2014; 193(12):5816-5826.

201. Habel JR, Nguyen THO, van de Sandt CE, Juno JA, Chaurasia P, Wragg K, et al. **Suboptimal SARS-CoV-2-specific CD8(+) T cell response associated with the prominent HLA-A*02:01 phenotype**. *Proc Natl Acad Sci U S A* 2020; 117(39):24384-24391.

202. Nelde A, Bilich T, Heitmann JS, Maringer Y, Salih HR, Roerden M, et al. **SARS-CoV-2-derived peptides define heterologous and COVID-19-induced T cell recognition**. *Nat Immunol* 2021; 22(1):74-85.

203. Tarke A, Sidney J, Kidd CK, Dan JM, Ramirez SI, Yu ED, et al. **Comprehensive analysis of T cell immunodominance and immunoprevalence of SARS-CoV-2 epitopes in COVID-19 cases**. *Cell Rep Med* 2021; 2(2):100204.

204. Schulien I, Kemming J, Oberhardt V, Wild K, Seidel LM, Killmer S, et al. **Characterization of pre-existing and induced SARS-CoV-2-specific CD8(+) T cells**. *Nat Med* 2021; 27(1):78-85.

205. Rha MS, Jeong HW, Ko JH, Choi SJ, Seo IH, Lee JS, et al. **PD-1-Expressing SARS-CoV-2-Specific CD8(+) T Cells Are Not Exhausted, but Functional in Patients with COVID-19**. *Immunity* 2021; 54(1):44-52 e43.

206. Prakash S, Srivastava R, Coulon PG, Dhanushkodi NR, Chentoufi AA, Tifrea DF, et al. **Genome-Wide Asymptomatic B-Cell, CD4 (+) and CD8 (+) T-Cell Epitopes, that are Highly Conserved Between Human and Animal Coronaviruses, Identified from SARS-CoV-2 as Immune Targets for Pre-Emptive Pan-Coronavirus Vaccines**. *bioRxiv* 2020.

207. Peng Y, Mentzer AJ, Liu G, Yao X, Yin Z, Dong D, et al. **Broad and strong memory CD4(+) and CD8(+) T cells induced by SARS-CoV-2 in UK convalescent individuals following COVID-19**. *Nat Immunol* 2020; 21(11):1336-1345.

208. Joag V, Wijeyesinghe S, Stolley JM, Quarnstrom CF, Dileepan T, Soerens AG, et al. **Cutting Edge: Mouse SARS-CoV-2 Epitope Reveals Infection and Vaccine-Elicited CD8 T Cell Responses**. *J Immunol* 2021; 206(5):931-935.

209. Shomuradova AS, Vagida MS, Sheetikov SA, Zornikova KV, Kiryukhin D, Titov A, et al. **SARS-CoV-2 Epitopes Are Recognized by a Public and Diverse Repertoire of Human T Cell Receptors**. *Immunity* 2020; 53(6):1245-1257 e1245.

210. Kusnadi A, Ramirez-Suastegui C, Fajardo V, Chee SJ, Meckiff BJ, Simon H, et al. **Severely ill COVID-19 patients display impaired exhaustion features in SARS-CoV-2-reactive CD8(+) T cells**. *Sci Immunol* 2021; 6(55).

211. Zheng HY, Zhang M, Yang CX, Zhang N, Wang XC, Yang XP, et al. **Elevated exhaustion levels and reduced functional diversity of T cells in peripheral blood may predict severe progression in COVID-19 patients**. *Cell Mol Immunol* 2020; 17(5):541-543.

212. Zelba H, Worbs D, Harter J, Pieper N, Kyzirakos-Feger C, Kayser S, et al. **A Highly Specific Assay for the Detection of SARS-CoV-2-Reactive CD4(+) and CD8(+) T Cells in COVID-19 Patients**. *J Immunol* 2021; 206(3):580-587.

213. Woldemeskel BA, Kwaa AK, Garliss CC, Laeyendecker O, Ray SC, Blankson JN. **Healthy donor T cell responses to common cold coronaviruses and SARS-CoV-2**. *J Clin Invest* 2020; 130(12):6631-6638.

214. Nicoli F, Paudel D, Solis-Soto MT. **Old and new coronaviruses in the elderly**. *Ageing (Albany NY)* 2021; 13(9):12295-12296.

215. Le Bert N, Tan AT, Kunasegaran K, Tham CYL, Hafezi M, Chia A, et al. **SARS-CoV-2-specific T cell immunity in cases of COVID-19 and SARS, and uninfected controls**. *Nature* 2020; 584(7821):457-462.

216. Mateus J, Grifoni A, Tarke A, Sidney J, Ramirez SI, Dan JM, et al. **Selective and cross-reactive SARS-CoV-2 T cell epitopes in unexposed humans**. *Science* 2020; 370(6512):89-94.

217. Grifoni A, Weiskopf D, Ramirez SI, Mateus J, Dan JM, Moderbacher CR, et al. **Targets of T Cell Responses to SARS-CoV-2 Coronavirus in Humans with COVID-19 Disease and Unexposed Individuals**. *Cell* 2020; 181(7):1489-1501 e1415.

218. Braun J, Loyal L, Frentsch M, Wendisch D, Georg P, Kurth F, et al. **SARS-CoV-2-reactive T cells in healthy donors and patients with COVID-19**. *Nature* 2020; 587(7833):270-274.

219. Zuo J, Dowell AC, Pearce H, Verma K, Long HM, Begum J, et al. **Robust SARS-CoV-2-specific T cell immunity is maintained at 6 months following primary infection**. *Nat Immunol* 2021; 22(5):620-626.

220. Dan JM, Mateus J, Kato Y, Hastie KM, Yu ED, Faliti CE, et al. **Immunological memory to SARS-CoV-2 assessed for up to 8 months after infection**. *Science* 2021; 371(6529).

221. Cuvelier P, Roux H, Couedel-Courteille A, Dutrieux J, Naudin C, Charmeteau de Muylder B, et al. **Protective reactive thymus hyperplasia in COVID-19 acute respiratory distress syndrome**. *Crit Care* 2021; 25(1):4.

8 APPENDIX

The following papers constitute part of the work performed during the PhD, whose results were not included in this thesis:

1) Use of a Novel Peptide Welding Technology Platform for the Development of B- and T-Cell Epitope-Based Vaccines.
   Nicoli F, Pacifico S, Gallerani E, Marzola E, Albanese V, Finessi V, Llewellyn-Lacey S, Price DA, Appay V, Marconi P, Guerrini R, Caputo A, Gavioli R (2021)
   Vaccines, 19:526.

2) Old and new coronaviruses in the elderly.
   Nicoli F, Paudel D, Solis-Soto MT (2021) Corresponding author
   Aging (Albany NY), 13:12295-12296.

3) Age-related decline of de novo T-cell responsiveness as a cause of COVID-19 severity.
   Nicoli F, Soli-Soto MT, Paudel D, Marconi P, Gavioli R, Appay V, Caputo A (2020) Corresponding author
   GeroScience, 42:1015-1019

4) A new approach to UV protection by direct surface functionalization of TiO2 with the antioxidant polyphenol dihydroxyphenyl benzimidazole carboxylic acid.
   Battistin M, Dissette V, Bonetto A, Durini E, Manfredini S, Marcomini A, Casagrande E, Brunetta A, Ziosi P, Molesini S, Gavioli R, **Nicoli F**, Vertuani S, Baldisserotto A. (2020)
   Nanomaterials, 10:231

5) HPV-specific systemic antibody responses and memory B cells are independently maintained up to 6 years and in a vaccine-specific manner following immunization with Cervarix and Gardasil in adolescent and young adult women in vaccination programs in Italy.

Nicoli F, Mantelli B, Gallerani E, Telatin V, Bonazzi I, Marconi P, Gavioli R, Gabrielli L, Lazzarotto T, Barzon L, Palù G, Caputo A. (2020)
Vaccines, 8:26

6) Angry, hungry T-cells: how are T-cell responses induced in low nutrient conditions?
Nicoli F. (2020)
Corresponding author
Immunometabolism, 2:e200004

7) Synthesis and characterization of new multifunctional self-boosted filters for UV protection: ZnO complex with dihydroxyphenyl benzimidazole carboxylic acid.
Battistin M, Durini E, Dissette V, Bonetto A, Marcomini A, Casagrande E, Brunetta A, Ziosi P, Molesini A, Gavioli R, **Nicoli F**, Manfredini S, Vertuani S, Baldisserotto A. (2019)
Molecules, 24:4546

8) Synthesis and biological activity of peptide α-ketoamide derivatives as proteasome inhibitors.
Pacifico S, Ferretti V, Albanese V, Fantinati A, Gallerani E, **Nicoli F**, Gavioli R, Zamberlan F, Preti D, Marastoni M. (2019)
ACS Med Chem Lett, 10:1086-1092

The following papers were published during the PhD but refer to work performed previously:

1) The Tat protein of HIV-1 prevents the loss of HSV-specific memory adaptive responses and favors the control of viral reactivation.
   Nicoli F, Gallerani E, Sicurella M, Pacifico S, Cafaro A, Ensoli B, Marconi P, Caputo A, Gavioli R. (2020)
   Corresponding author
   Vaccines, 8:E274

2) Impact of IgA isoforms on their ability to activate dendritic cells and to prime T cells.
   Gayet R, Michaud E, **Nicoli F**, Chanut B, Paul M, Rochereau N, Guillon C, He Z, Papagno L, Bioley G, Corthesy B, Paul S. (2020)
   Eur J Immunol, 50:1295-1306

3) The TLR9 ligand CpG ODN 2006 is a poor adjuvant for the induction of de novo $CD8^+$ T-cell responses in vitro.
   Papagno L, Kuse N, Lissina A, Gostick E, Price DA, Appay V, **Nicoli F**. (2020) Corresponding author
   Sci Rep, 10:11620

4) Relationship between vaccination and nutritional status in children: analysis of recent Demographic and Health Surveys.
   Solis-Soto MT, Paudel D, **Nicoli F**. (2020)
   Corresponding author
   Demographic Research, 42:1-14

5) Guidelines for the use and interpretation of assays for monitoring autophagy (4th edition)
   Klionsky et al, (2021)
   Autophagy, 17:1-382

6) The STING ligand cGAMP potentiates the efficacy of vaccine-induced $CD8^+$ T cells.

Gutjahr A, Papagno L, **Nicoli F**, Kanuma T, Kuse N, Cabral-Piccin MP, Rochereau N, Gostick E, Lioux T, Perouzel E, Price DA, Takiguchi M, Verrier B, Yamamoto T, Paul S, Appay V. (2019)

JCI Insight, 4:e125107

7) In chronic hepatitis C infection, myeloid-derived suppressor cell accumulation and T cell dysfunctions revert partially and late after successful direct-acting antiviral treatment.

Telatin V*, **Nicoli F*,** Frasson C, Menegotto N, Barbaro F, Castelli E, Erne E, Palu G, Caputo A. (2019)

***Shared first autorship**

Front Cell Infect Microbiol, 9:190

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