Human Cytomegalovirus as a driving force towards Immunosenescence
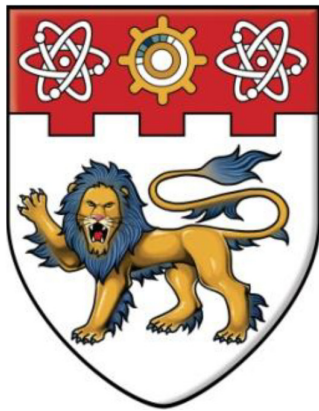
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Human Cytomegalovirus as a driving force towards

Immunosenescence

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Abstract

As the number of elderly in the human population is increasing, aging is becoming a severe problem especially for public health. The importance of understanding this development is highlighted by the vulnerability of the elderly for injuries and their increased need for medical care. With regard to pathogen defence, aging is associated with a significant decrease of immune system functions. This phenomenon, known as 'immunosenescence', is correlated with alteration in T cell populations, namely the expansion of highly differentiated memory T cells and the decrease of naive cells. However, immunosenescence may not be driven solely by chronological aging. Also chronic antigenic exposure caused, for instance, by latent viral infections may contribute to the deteriorations. Our study aims to dissect the effect of persistent infection on the aging of the immune system. We specifically focussed on the trajectory of memory T cell differentiation caused by chronic CMV infection. For this, we analysed the cytokine profiles and T cell phenotypes of blood samples from two cohorts of 267 young (age: 21 yrs) and 250 elderly Singaporeans (age: 74 yrs). While aging is known to be associated with an increased level of pro-inflammatory molecules (low grade inflammation), we could not detect such an increase in the young CMV+ individuals. Notably however, in this group we observed CMV-associated alterations T cell populations that closely resembled the senescence-related profile of the elderly. This was evident for instance in a significant increase in the T_{EM} and T_{TE} populations of both CD4+ and CD8+ T cells, a key-marker for defining immunosenecence. This senescence-specific shift was apparently specific for CMV infection, as other latent infections in the young by Helicobacter pylori and herpes simplex virus-2 did not induce detectable alterations in T cell populations. Our work thus demonstrates that primary CMV infection has a strong impact on the shaping of the T cell senescence profile. Preliminary data from a NSG humanized mouse model we established

may further help to evaluate the effects of persistent CMV infection on T cells early in life and their contribution to the immunological trajectories of aging.

1 Introduction

1.1 Chronological aging

The average human life span has doubled over the past century, which has resulted in a general aging of the world's population. This trend is continuing as it has been estimated that by 2050 the number of people over 65 years will double, reaching one billion elderly worldwide [1]. The required medical care for an elderly person is extensively higher than for a young individual, which is mostly due to the numerous effects of aging on the body. From a purely economic and logistical point of view the increased attendance of medical institutions by elderly individuals is becoming a burden, therefore studies of aging are crucial to better understand the phenomenon and the associated diseases.

Until now, the mechanisms involved in the aging process remain highly elusive. From the evolutionary point of view it is usually defined as the progressive loss of function resulting in decreased fertility and increased mortality. However many physiological functions are decreasing with age, and many organs are negatively affected by aging as will be described alter on. This raises questions to how and why this has evolved since it impairs an individual and therefore decreases fitness, the main attribute of evolutionary development. Interestingly, some species show no age-associated decreased fertility or increased mortality, such as freshwater medusa *Hydra magnipapillata*, or desert tortoise *Gopherusagassizii,* for which mortality even decreases with age. Consequently, aging cannot be explained simply as an inevitable biological wear-and-tear[2]. It has been suggested that programmed aging could have originated and is maintained via altruistic group selection. The accelerated turnover of generations would maintain high genetic variability and the plasticity of species to adapt to

environment changes. However, many evolutionary biologists argue that species selection is very weak and is surpassed by individual selection [3-5].

Several other explanations of the existence of aging have been proposed. The mutation-accumulation theory implies that late-acting mutations exerting deleterious effect in old individuals may be accumulated due to the selection shadow later in life (where natural selection exerts strong selection at reproductive age only). It is also possible that beneficial mutations at young ages would be selected even though they would exert a negative effect in older ages, a phenomenon known as antagonistic pleiotropy[6]. Lastly it has been argued that maintenance of a putative repair mechanism, which would slow down or prevent aging, would cost too much energy. Individuals without this mechanism could therefore invest more resources into their progeny, which would overpower the progeny of the "self-repair" organisms (disposable-soma theory).

Interestingly, it has been proven experimentally that some genes have a significant impact on the rate of aging. For *Caenorhabditis elegans,* longevity of certain strains[7, 8] was associated with superoxide dismutases (SODs)[9], responsible for scavenging free radicals, and genes involved in the central-nutrient response, such as Insulin receptor 1 (IGF-1)[10] and serine-threonine kinase Sch9[11]. A similar association for some of these genes was also observed in *Saccharomyces cerevisiae*[12].While a prevailing theory suggests that free radicals being the primary cause of aging, it is noteworthy that over-expression of SODs in *Saccharomyces cerevisiae* led to increased survival for only 5-30% of the population[9]. Mutations in central nutrient-response pathways, in turn, resulted in twofold to threefold lifespan expansion[10, 11]. Similar observations have also been made in fat-specific insulin receptor knock-out mice[13, 14] suggesting that aging is primarily the result of physiological processes, mainly metabolism[15, 16]. However, regardless of its origin and mechanism,

chronological aging results in general decline of functionality on multi-system level (**Figure 1-1**).
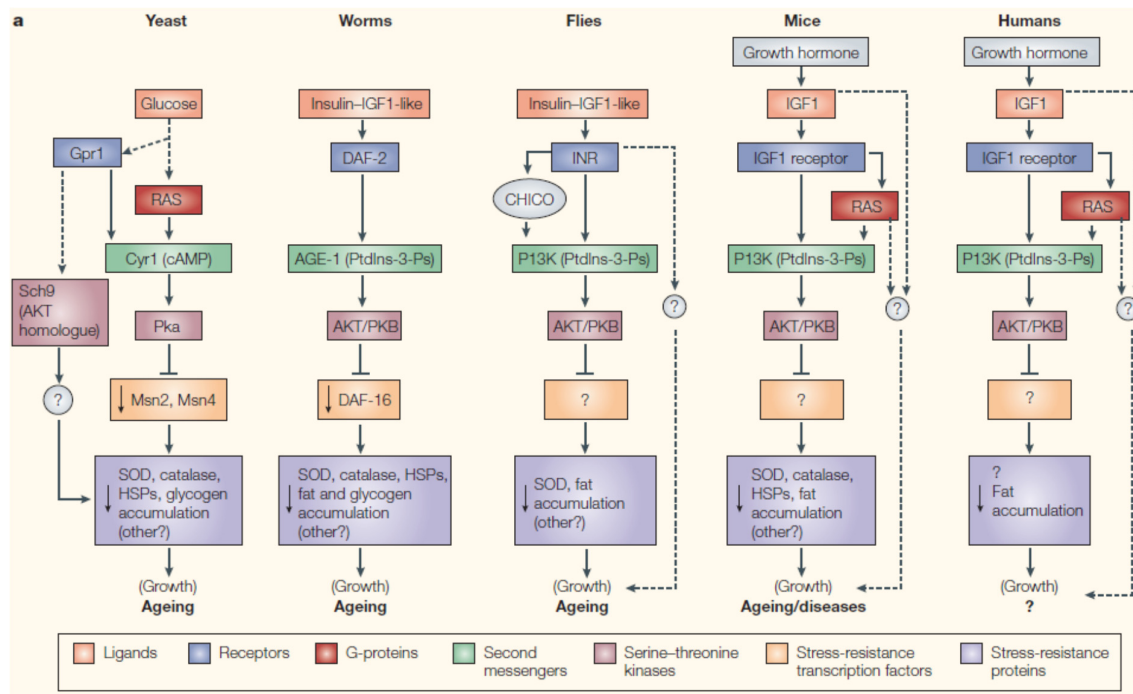
Figure 1-1 **Longevity pathways in yeast, worms, flies and mice**

Common pathways shared by yeast, worms, flies, mice and humans related to the alteration of lifespan. These pathways include genes involved in free radical scavenging (SODs), heatshock proteins (HSPs) and genes involved in metabolic pathways such as insulin receptor or serine-threonine kinase Sch9. Figure adapted from [15]

1.2 Biological aging

As the degree of functional decline varies among individuals of the same chronological age, chronological aging is not necessarily a reliable indicator of the degree of physiological deterioration. The 'biological age', defined by a set of objective biomarkers, could be a better measure. While it could be a possible predictor of mortality in aged populations, it was originally established that in young populations [17, 18]. *Belsky et al.*[19] showed, in a 12-

years longitudinal study of 948 individuals (aged 26-38 years), that biological age already varies in the 3rd and 4th decade of human life. Biological aging was established here via the measurement of systemic changes in 18 aging related morbidity associated markers (**Figure 1-2**).Therefore, health trajectories could be determined at already at early ages.
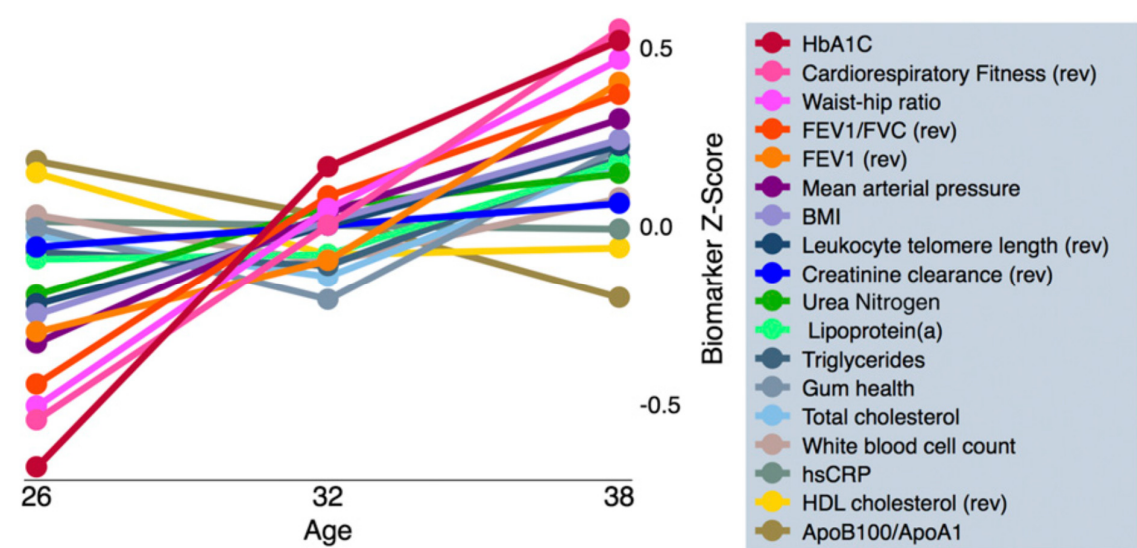
Figure 1-2 **Biological Aging**

This figure depicts the shifts in age-related biomarkers over the course of 12 years. 18 different parameters from 1037 individuals (95% detention) related to aging, morbidity and mortality, metabolism, immune system or fitness were measured to establish the rate and extent of age-related deteriorations. Biomarker values were standardized to have mean = 0 and SD = 1 across the 12 y of follow-up (Z scores) and parameters declining with age were given a reversed value to give increasing Z score with age. All markers show here that already at the 3rd and 4th decade of live there is a considerable difference in the level of biological aging. Figure was taken from.[19].

1.3 Multi-system alterations associated with age

The Geriatric syndrome of multiple coexisting conditions: weakness, immobility or poor tolerance to physiologic or psychological stressors, is called 'frailty'. Frailty has been defined as the loss of complexity in resting dynamics triggering a vicious cycle towards functional decline and other adverse clinical outcomes[20].The most widely accepted definition of a frail individual, the Fried's definition, states that an individual is frail if he/she exhibits 3 or more of the following: weight loss, weakness, low physical activity, slowed motor performance and exhaustion[20, 21]. Frailty syndrome defined as 3 or more of the above mentioned conditions is independently predictive of numerous serious adverse health conditions, such as illness, mortality, hospitalization, disability. Frailty is estimated to affect 7-10% of the individuals above 65 years and around 30% of those above 80 years old[20, 21]. Even though Frailty has been recognized as an independent clinical entity (showing only partial overlap with disability and co-morbidity) the biological causes of frailty remain poorly understood. Recently it has been shown that certain biological parameters are related to Frailty, such as anemia, high white blood cells count or low levels of eGFR in plasma[22].

Frailty is also related to lifestyle, physical exercise and cognitive interventions[23]. Furthermore frailty is strongly related to sarcopenia, the age-related deterioration of muscles[24] and some chronic infections[25]. One such infection that is strongly associated with higher morbidity and mortality is Cytomegalovirus (CMV), which is described later in more detail. It is often not clear whether a disease is a consequence of tissue deterioration or whether the worsening of tissue functionality and accumulation of errors lead to the diseases. Moreover, the clinical manifestations of aging also differ between individuals. This natural heterogeneity makes it difficult to determine which alteration is a cause and which is a

consequence of aging. Despite the poor understanding of aging at the organism level, aging of the cells is much more defined thanks to numerous *in vitro* models of cellular aging[26], referred to as cellular senescence.

1.4 Aging on the cellular level - cellular senescence

Generally, cells have only a restrained number of divisions they can undergo termed as Hayflick's limit[27] named after Leonard Hayflick who showed that cells undergo a checkpoint after several divisions [28] (on average this number ranges from 35 to 60). This checkpoint is caused by the attrition of telomeres, the repetitive DNA sequence on both chromosomal ends, thus exposing the genetic material to alterations [29]. The telomere shortening can be restored by up-regulating telomerase activity, a reverse transcriptase ribonucleoprotein, which synthesizes the last part of the lagging strand of DNA utilizing its RNA molecule template and preventing the telomere attrition upon each cell division [30, 31]. The expression of telomerase is sequestered in most cell types [32] .

The aging of cells is related to aging of an organism, as it has been shown that shortened telomeres in cells significantly correlated with an increased risk of mortality [33, 34]. In general, the functionality of senescent cells is impaired, principally at the proliferative level, however they can demonstrate potency in other functions. The proliferation arrest-replicative senescence was associated to telomere shortening. When the length of telomeres reaches a critical threshold, it is recognized by the DNA damage repair (DDR) pathway, which triggers replicative arrest until the DNA damage is repaired. However if the damage is too severe and irreparable, the replicative arrest becomes permanent [35].

The altered/multifunctional feature of senescent cells is manifested by so called senescence associated secretory phenotype (SASP) [36]. SASP manifests as a significant

increase in production of inflammatory cytokines (IL-1, IL-6, IL-12), growth factors (GM-SCF, G-SCF), chemokines (IL-8, MMP-2, MMP-3) and other molecules[36]. Some of the produced molecules contribute to local and possibly even systemic inflammation and the recruitment of immune cells can ultimately result in chronic inflammation. SASP cell accumulation does not only contribute to local tissue damage, but may contribute to or establish the chronic low-grade inflammation status observed in the elderly.

1.5 Immunosenescence

The deteriorated immune function in elderly is described as immunosenescence[37, 38]. While the decreased functionality impacts on both innate and adaptive arms of the immune system [39-42], the hallmarks of immunosenescence are the changes observed in T cells. Aging has often been associated with the accumulation of differentiated memory T cells and the decrease of naïve T cells [43]. Especially CD8$^+$memory T cells, which are accumulated with aging, show modified functionality during differentiation, characterized by a lower proliferation capacity and IL-2 production but higher cytotoxic capacity [44]. This functional switch presumably results from an induced T cell differentiation in response to the pathogen exposure and not from chronological aging *per se*. It is still unknown whether these T cells also acquire the SASP.T cells with high replicative history have shorter telomeres [45], lack expression of CD28 and/or CD27 and often express CD57, the best known marker of replicative senescence [46, 47]. The Swedish OCTO/NONA studies revealed that high frequency of CD8$^+$CD28$^-$ T cells and CMV seropositivity were part of a cluster of parameters predicting mortality in the older adults[48]. The NHANES follow-up studies also showed the association between CMV seropositivity and all-cause mortality, but independently of age [49].

The effect of persistent infections early in life and their potential impact later in life was demonstrated by the aged phenotype of T cells in CMV-seropositive infants and the maintenance of this profile long after primary infection [50]. Although those studies focused on a very vulnerable population of infants, whose immunity may have not completely matured at the time of primary infection, this clearly suggested that immunological history may play a significant role in the rate of aging and probably health.

1.5.1 Inflamm-aging

In the elderly, the cytokine network is distorted towards pro-inflammatory cytokines. It often results in a low-grade chronic systemic inflammation provoked by continuous antigen load and stress, a sub-clinical phenomenon is known as 'inflamm-aging'[51]. Many aging studies identified selected inflammatory molecules (IL-6, TNF-α and CRP) as predictors of mortality or being associated to morbidity in the elderly [52, 53]. The clinically important comorbidities associated with inflamm-aging are for instance metabolic and endocrine alterations [54], diabetes, obesity, insulin resistance and other age-specific diseases such as Alzheimer's disease, sarcopenia, cardiovascular disease, atherosclerosis or arthritis [55-58].

While the low-grade inflammation may directly contribute to aging associated with higher morbidity and mortality in elderly[57], some studies show that inflamm-aging could be just a compensatory mechanism for the immune system remodeling[59]. Other studies even suggest that chronic infections could actually be beneficial to its host. For instance, mice latently infected with either murine gammaherpesvirus 68 or murine cytomegalovirus, are resistant to bacterial infections of *Listeria monocytogenes* and *Yersinia pestis*. The protection is not antigen-specific but rather involved increased production of the cytokine IFN-γ and systemic activation of macrophages[60].

A beneficial role of latent CMV infection has also been shown in humans. CMV-seropositive young adults exhibited enhanced antibody responses to influenza vaccination, increased CD8^{+} T cell sensitivity, and elevated levels of circulating IFN-γ [61].This phenomenon, known as 'trained immunity', is particularly evident that even the innate arm of the immune system, where the exposure to CMV results in the upregulation of the Ly49H receptor on NK cells[62] (**Figure 1-3**). The existence of innate memory is also supported by the findings in plants and invertebrates, where exposure to a pathogen facilitates a better response to re-infections[63, 64].
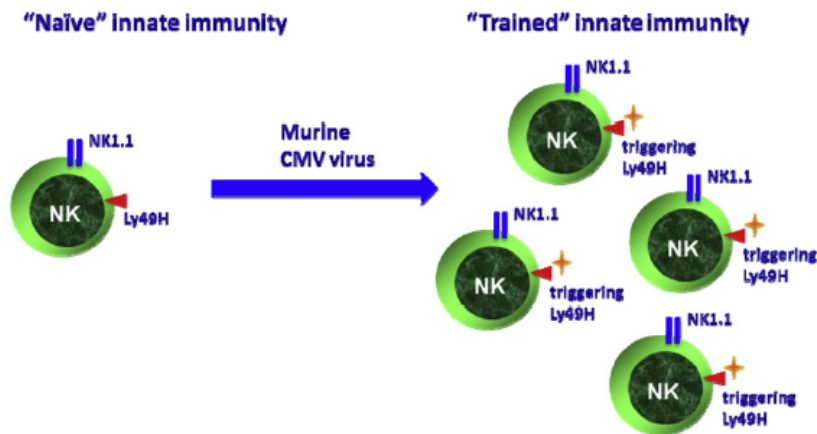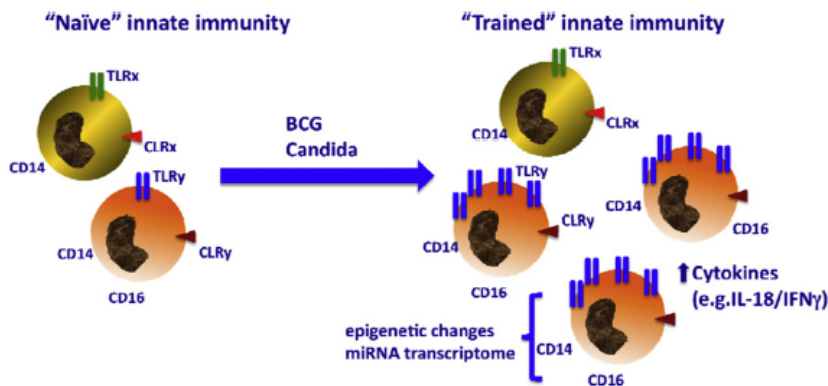
Figure 1-3 **Trained Immunity**

A) The Innate immunity could be trained through NK cells against viruses via higher activation of the Ly49H receptor. **B)** The trained immunity against bacteria of fungi might be facilitated through putative population changes in monocytes/macrophages or via altered expression of pathogen recognition receptors (PRR). Figure adopted from [62]

1.5.2 Immunosenescence in innate immunity

Most microorganisms enter the body via mucosa of the gut, the respiratory system and any breach in the external tissues (mostly skin). The first immune cells they encounter are resident macrophages in submucosal tissues. Macrophages, upon encounter of pathogen, are soon reinforced by large numbers of infitrating neutrophils [65]. Both these cells types are active phagocytic cells capable of internalizing and killing of a pathogen. After engulfing the pathogen, macrophages and neutrophils promote their killing by producing reactive and toxic species, such as antimicrobial peptides, H_2O_2, nitric oxide or superoxide anion[66]. Neutrophils are short lived cells, whereas macrophages are long lived and can produce multiple cycles of phagocytosis, killing multiple pathogens[67].

When macrophages recognize and internalize a pathogen, it triggers an inflammatory response characterized by pro-inflammatory cytokine and chemokine production, the latter attracting other cells to enhance the inflammatory response[68]. Functionality of both macrophages and neutrophils have been reported to decrease in elderly[69, 70]. Both types of cells lose the effective ability of phagocytosis[71]. Furthermore, the ability to kill engulfed pathogens is reduced due to less efficient production of ROS. Other innate immune cells like natural killer cells and antigen presenting cells are also influenced by aging[72-74]. DCs in the elderly often display impaired phagocytosis and reduced antigen presentation ability[75]. The same study also showed that DC migration via CCR7 receptor engagement is impaired [75], while other reports indicate that DCs from elderly people secrete increased levels of IL-6 and TNF-α[74].

1.5.3 Immunosenescence in adaptive immunity

While the age-related functional alterations in DC and other cells of the innate branch might affect the function of the immune system, the most pronounced deteriorating alterations associated with age are observed in the adaptive branch. This applies in particular for the T cell population.

1.5.3.1 Memory T cell development

Alterations in the subset composition of memory T cells and memory T cell expansion are considered as the major hallmark of Immunosenescence[76]. When memory T cell were first described it was reported that naïve T cells expressed the long isoform of CD45 – CD45RA. Cells with memory features, which respond faster to secondary antigen challenge, switch expression of CD45RA to the short isoform CD45RO. Until now it is unclear how this switch affects the cells functionality, (partly because the ligand of CD45 is unknown) but CD45 influences the TCR signaling, in some cases lowering and in some increasing this threshold of TCR responsiveness. Later it was shown that memory T cells may be distinguished by the expression of the lymph node-homing chemokine receptor CCR7 [77]. CCR7 expression allowed the discrimination of early memory and central memory (CM) from late memory effector memory (EM), which have higher effector functions supported by a lower activation threshold and lose CCR7 expression in order to leave the lymph nodes. The CD45 isoform segregation of memory and naïve cells has been challenged by the discovery that late memory cells re-express CD45RA (TEMRA). These cells are also termed as terminally differentiated (TE, an abbreviation used throughout this thesis).More recently, it was shown that, upon differentiation, memory cells gradually lose the co-stimulatory molecules CD28 and CD27[78]. The combination of CD28 and CD27 is also used now to further subdivide

populations of previously defined memory cells (such as EM1 EM2 EM3 etc.) based on the gradual loss of these molecules[79].

Also other markers have been reported to show altered expression with differentiation status, such as the homeostatic IL-7 receptors, the chemokine receptors CX3CR1 and CXCR4 [80, 81], the adhesion molecules CD62L or CD11b, cytotoxic molecules such as granzymes A and B or perforin[82, 83], and cytokines such as IL-2, IFN-γ[77]. Notably, at the late stage of memory, cells display expression of senescence-related markers such as CD57 or KLRG-1[84]. Other molecules, such as CD95, have been used to identify additional memory subsets. CD95 has been shown to be expressed at low levels on naïve (N) and at high levels on memory cells and, together with CD28, may be used to delineate basic memory populations [85].

It has also recently been shown that T cells with memory phenotype specific to a nominal antigen, so called virtual memory cells (VM), are present in mice regardless, of the previous exposure to a pathogen. These cells have high affinity against self antigens and develop in the periphery upon the response to IL-15. VM cells also express adhesion, chemotactic and effector molecules that facilitate a response to inflammatory stimuli. The combination of these attributes make VM cells suited for mediating both antigen-specific and bystander protective immunity, even against infectious challenges that have never previously been encountered. Possible human equivalent memory subset have been identified as well.

A general problem in studying the memory cell lies in the accurate phenotypic identification of these cells. Even though the above mentioned molecules share a similar pattern of expression upon memory differentiation (for instance the loss of CCR7 and CD28 upon memory differentiation) there are not entirely exclusive. Therefore some discrepancies in studies may be found if different surface markers are utilized to identify a certain memory T cell population.

Telomere length proved to be a good marker of replication history[86]. Earlier studies suggest a nearly linear development from N->CM->EM->TE. However recent studies show that previously thought terminally differentiated cells which were believed are the last stage of differentiation, actually have longer telomeres than EM[87]. This suggests that the linear model is not absolute and that the terminal differentiation stage might develop also through other mechanisms rather than only after excessive replication. The replicative senescent phenotype might instead be induced already on the memory population level for functional and regulatory purposes. This is supported by the discovery of reversible replicative senescence and functionality relationship, as blocking of p38MAPK, which drives T cell senescence[88] and PD-1 signaling, increased proliferative capacity and abrogated the production of TNF-a [89].

As the apparent heterogeneity of memory T cells creates now a more complicated picture. For instance, it is probable that stem cell memory cells (SCM) differentiate as primary memory populations and subsequently give rise to CM[90, 91]. However there is evidence that they may additionally give rise to EM[90, 91]. Also the existence of transitional T cells (TM), which lay somewhere between CM and EM in the differentiation hierarchy, further underlines the complexity of memory T cells[90, 91]. TM cells lose CCR7 expression however maintain the expression of the costimulatory molecule CD28 suggesting that, unlike EM, they still require the costimulatory signal from B7 molecules on DC. It is also hypothesized that all the cells may be differentiated as follows: N->SCM->CM->TM->EM->TE suggesting that this differentiation occurs through activated effector from each previous memory population[90].

Although the linear model of differentiation is challenged by the longer telomere length of TE, it is still the prevailing concept. It will therefore also serve as the basis for the memory T cell subsets in this study. A summary of the associated markers is provided in **figure 1-4**.
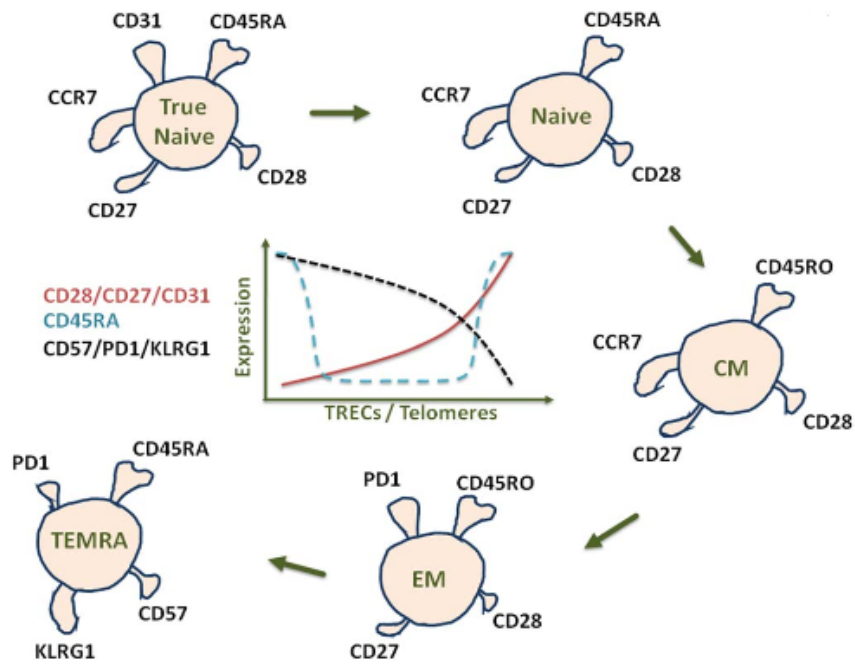
Figure 1-4 **Differentiation and development of memory T cells**

The gain of expression of CD57, PD-1 and KLRG-1 and loss of telomere length are major features of senescent T cells. Memory development from naïve to CM and subsequently EM is accompanied with gradual loss of activation and gain of senescent/inhibitory molecules, each T cell development stage characterize by its own sets of surface marker, which correspond to the gradual attrition of telomere length. This model is adopted from [92].

While the composition of these memory subsets strongly depends on the biological age it is also influenced by the type and extent of pathogen exposure. During chronic infection, the majority of CMV specific T cell acquire the EM and TE phenotype (**Figure1-5**) [78, 93]. EBV and HIV specific CD8 T cells, in contrast show almost no expansion of later stage memory T cells. The fact that CMV specific T cells occupy the very late and mature end of memory differentiation was further confirmed by deeper study by CyTOF analysis[93].

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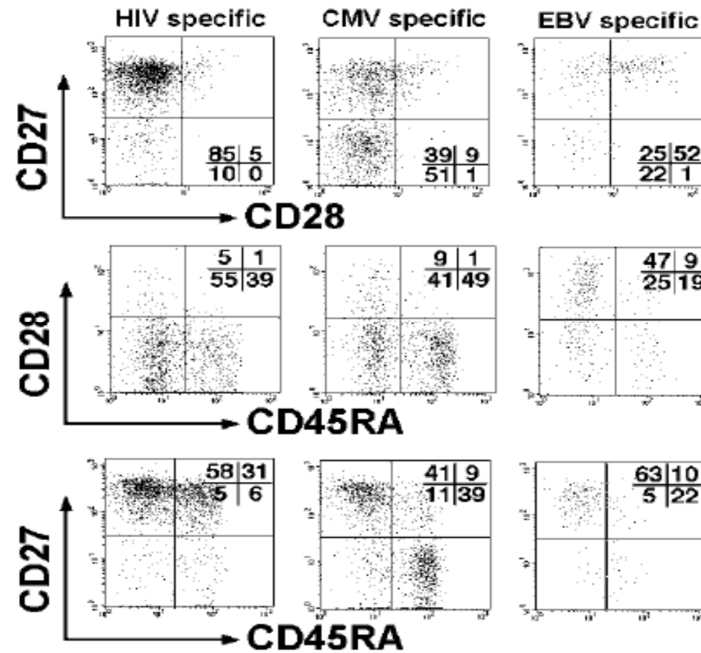
Figure 1-5 **Memory phenotypes of T cells of different viral specificity**

Virus-specific CD8+T cells, identified by tetramers loaded with respective peptide antigens, were co-stained with memory T cell markers (compare figure 1-4). The combination of markers indicates that CMV-specific CD8 T cells show high level of differentiation ($\rightarrow$TE state), which is less pronounced in HIV and EBV respectively. T cells from Influenza do not progress beyond the EM stage at all, while most of EBV specific T cells remain in naive stage. Figures adapted from [78].

1.5.3.2 Naive T cell repertoire narrowing

Naive T cells are maintained by so called homeostatic proliferation (HP). HP is a mechanism where existing clones of naive cells divide in order to preserve the number of naive cells [94]. HP however also leads to a narrowing of the TCR repertoire. In humans, memory cells are maintained by balanced levels of IL-7 and IL-15. IL-7 is also believed to function as a survival signal for naive T cells. HP will, on a purely stochastic basis, cause a TCR repertoire narrowing where some of the clones would randomly receive survival/cytokine signal and

some would not [95]. This phenomenon is further accompanied by the fact that expanding memory cells may simply occupy the "immunological" space of the naïve cells and compete for the maintenance cytokines IL-7 and IL-15. Alongside a gradually deteriorating thymic output this leads to reduced pool of naïve cells with different TCR.

When we examine the T cell population in elderly individuals, we also observe drastic changes at the memory cell level. The memory population contains low numbers of CM, moderate numbers of early EM and high numbers of late stage EM and especially TE. Probably the most striking feature of the late stage memory cells is an extreme expansion of only a few clones, where in some cases one clone reaches up to 80% of all TE. Such an observation was made especially in CMV+ elderly individuals [96]. This suggests that factors other than aging may lead to immune senescence

As discussed earlier, the development of T cell memory and subsequent senescence is influenced by numerous stimuli. One aspect of T cell immunosenescence is the accumulation of late stage memory and TE cells at the expense of naive cells. Allegedly, every primary infection elicits an immune reaction and induces development of CM. Every secondary infection furthermore induces additional memory T cell development. The more frequent the infections are, the more accelerated and pronounced T cell memory development might be.

Thus, living in an environment with high prevalence of infections supposedly drives T cells towards senescence faster than clean environment. That this is actually the case was shown in young infants in Africa [97].The study also shows the influence antenatal and postnatal conditions have on the responsiveness of the infants to BCG vaccine. Other studies in Gambia show a significant expansion of memory T cells upon CMV infection, which occurs around the age of 8weeks. This expansion persists even after 2 years follow up and the CMV specific clones remained responsive although their population did not decrease [50].

A dominant persistent infection could also lead to clonal expansion. In fact in CMV+ elderly patients, a significant fraction of T cells (up to 50% of all CD8^{+} T cell) and specifically of TE cells are specific to CMV epitopes [96]. Such observation of TE clonal expansion has also been made in the case of EBV infection [98]. However EBV specific TE cells expand to a smaller extent[78]. Furthermore, in patients with dual EBV and CMV infection, EBV specific T cell clonal expansion was abrogated suggesting CMV to be "dominant" over EBV for this phenomenon of expansion[99]. The influence of CMV on T cell memory skewing might thus be driven solely by the viral reactivations.

However, we may also consider that this phenomenon might be a consequence of direct alterations of T cell populations caused by the virus. An example of direct pathogenic influence on the distribution and balance of particular T cells is Human immunodeficiency virus (HIV) infection. HIV infects CD4^{+} T cells and induces their continuous extinction. Eventually, the CD4^{+} T cell count critically drops and this results in a massive failure of the immune system and death by other infections. Indeed HIV+ patients display changes at the T cell level similar to those observed in CMV+ individuals. For this reason HIV is considered to induce accelerated aging of the immune system, although many patients with HIV are also co-infected with CMV. The changes observed in elderly T cell populations *in vivo* are depicted in (**Figure 1-6**).
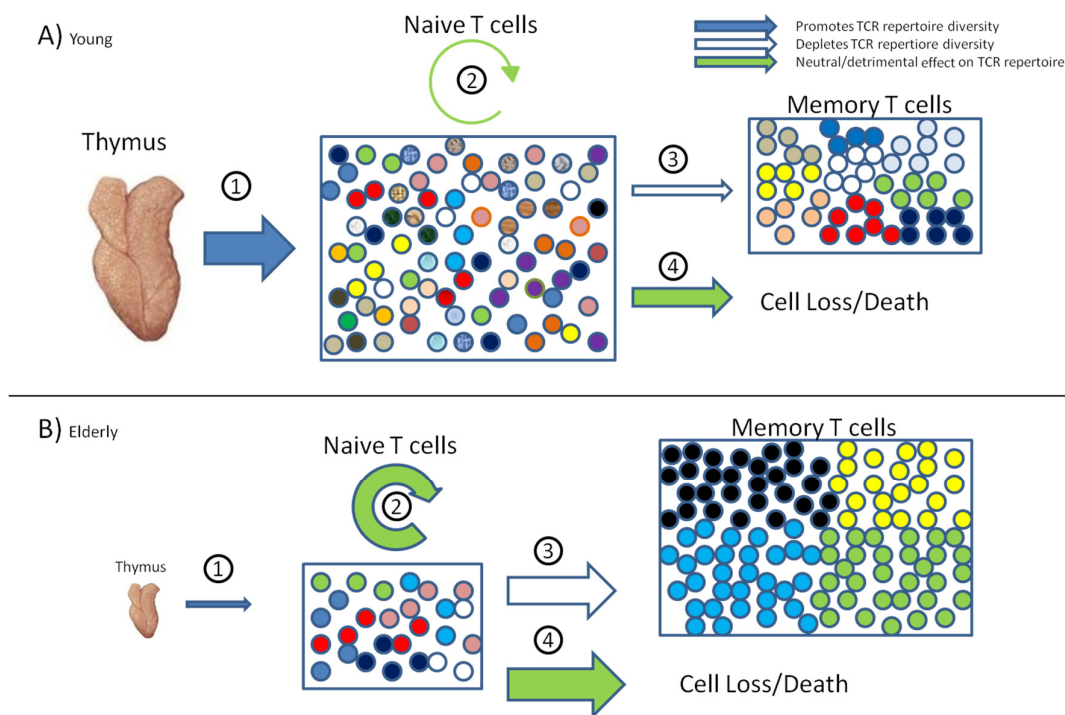
Figure 1-6 **Homeostasis of T cells in young and elderly individuals**

A) In young adult humans, with a steady supply of naïve cells from the thymus high T cell numbers remain stable with a vast diversity of TCR clone repertoire which is ready to responds to variety of antigens. Relatively small population of memory cells display equal numbers of different memory T cell clones. Homeostatic proliferation (HP) likely plays only a minor role in the maintenance of the naïve T cell pool in the young. **B)** T cell populations in elderly individuals show massive changes. Radical thymic involution is accompanied by shrinkage of the naïve T cell pool and drastic narrowing TCR repertoire diversity. HP plays a major role in maintaining, to a certain extent, the numbers of naïve T cells in elderly people. Memory T cell populations account for the majority of T cells and it displays a massive clonal expansion, where few clones of the memory cells numbers significantly exceed other clones. In general, processes which lead to lowered TCR repertoire diversity (HP, cell death, memory development) are increased in the elderly, which is accompanied by deteriorated naïve T thymic output. All these changes described above account for low plasticity of T cell immunity in the elderly and probably for poor responsiveness to primary infections and vaccinations. (Figure idea adopted from *D.L. Lamar et al*).

As the immune system is a very reactive and plastic system, it can be marked, often for life, by the exposures to which it responds. As after every primary infection there is a memory T cell development, continuous stimulation with the same antigen, which in the case of chronic

infection occur far more often than in the case of re-infection of non-chronic pathogens, the numbers and differentiation state of the memory cells are likely to increase. Furthermore, chronic stimulation may lead to a chronic inflammatory response which in turn triggers non-specifically other immune responses and immune cell differentiation. It has been shown that numerous chronic infections are associated with higher incidence of secondary infections [100]. These include all Helminths, bacteria, viruses and *Protozoae*. It has also been shown that numerous infections decrease the ability to respond to other either primary infections or chronic co-infections [101-103]. The mechanism of this phenomenon is unknown but it may be related to the many possible alterations in immune cell populations involved in important phases of the immune response. Thus, the chronic infections in general are likely contributing to the deterioration of the immune system.

1.5.5 Cytomegalovirus

The prevalence of CMV infection ranges from 20-95% worldwide dependent on age of an individual and the socioeconomic status of the inhabitant area. In general, the prevalence increases significantly with age and is significantly higher in third world countries. Interestingly, in Singapore which is deemed to be a highly developed country, the prevalence of CMV in people over the age of 65years is 95%. Elderly individuals with CMV infection suffer from a higher risk of mortality[104], cardiovascular disease[105], poor vaccine responsiveness[106] and enhanced chronic-low grade systemic inflammation[107].

This virus is from the *Herpesviridae* family. Its large genome, 230kbps, contains approximately 170 protein-coding regions and 16 putative mi-RNAs. More than half of the gene products have unknown functions. The order *Herpesvirales* consist of three families: *Herpesviridae*, *Alloherpesviridae* and *Malacoherpesvirida*. The families have a long evolution history, as their common ancestor is thought to have predated the divergence of vertebrates

from invertebrates, some 900 million years ago[108]. The long co-evolution explains the high adaptability of CMV against the host's immune response. Peculiarly, CMV has a very high promiscuity of infecting target cells types, which are fibroblasts, epithelial cells and monocytes, endothelium, smooth muscle cells, T and B cells and probably other cell types [109-111]. Little is known of the exact mechanisms of CMV infection.

Similar to other Herpes viruses, CMV is transmitted via body fluids (blood, saliva, cervical fluid or semen[112]). Scarce information suggested EGFR as an entry receptor [113]. Approximately half of the virus coded proteins have been identified and these include genes directly transforming or influencing the immune responses. IL-10 virus-coded homologue inhibits immune responses [114] while UL11 is a protein expressed on the surface of CMV infected cells and inhibits anti-CMV T cells by interaction with CD45 [115]. CMV gene products contain chemokines, which attract especially monocytes to the site of infection [116]. Attracted monocytes are infected by CMV and are believed to be the major reservoir of latent CMV[117]. It is probable that CMV is hiding in other cell types and, unless reactivated, remains invisible to the immune system. CMV lowers MHC class I complex expression and therefore prevents the infected cells from presenting sufficient amount of viral peptides on its surface [118]. The lowered expression of MHC I is often recognized by NK cells and the cells are killed, however other CMV-coded proteins expressed on the cell surface directly inhibit NK cells [119]. Upon primary infection, a general immune response is elicited against CMV including the IFN-γ pathway [120], NK cell cytotoxicity [121] and γ/δ T cell responses with subsequent development of an adaptive response [122] (**figure 1-7**). As with other herpesviruses, CMV primary infection is silent in the vast majority of cases [123]. Nevertheless, CMV is known for its massive, live threatening reactivation and expansion of virion particles in immunocompromised individuals, such as transplant [124] or AIDS patients[125]. This suggests that high resources are required in order for the immune system to

keep CMV under control. CMV has been associated with several co-morbidities, such as cardiovascular diseases[126], decreased cognitive functions[127] (which is a hallmark of aging), prolonged hospitalization[128] and immunomodulations in the elderly[129] such as previously mentioned inflamm-aging[130]. CMV drives clonal T cell expansion and TE cell accumulation in the elderly contributing to immunosenescence[131].
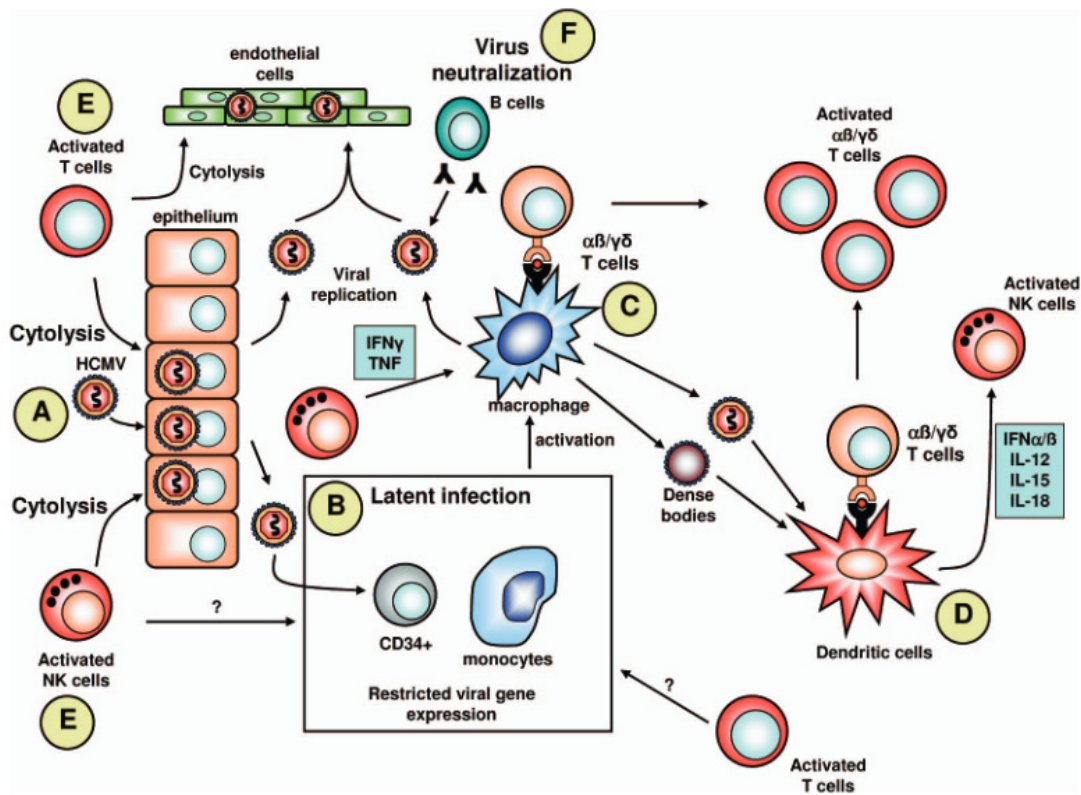
Figure 1-7 **Immune response against CMV**

Immune control of HCMV by innate and adaptive immunity. HCMV typically initiates its replication in mucosal ephitelium (A), after which the virus disseminates to monocytic cells, which are attracted to site of infection by viral coded chemokines and where it establishes latent infection (B). The differentiation of these virus-infected monocytes into macrophages can initiate productive infection (C). Virus particles can be processed by professional antigen-presenting cells (e.g., DCs), which can stimulate antigen-specific T cells (D). Additionally, these activated DCs secrete cytokines/chemokines, which activate the innate arm of the immune system (e.g., NK cells) (D). Virus-infected macrophages can also directly stimulate antigen-specific T cells (C). These activated T cells and NK cells can directly lyse virus-infected cells by cytolysis or block virus replication through the secretion of cytokines such as IFN-γ and/or TNF-α (E). B cells furthermore control extracellular virus through antibody-mediated neutralization (F). Figure is adapted from [132].

It has been shown that other infections (mainly viral) beside CMV have been shown to induce the increased numbers of memory T cells, mostly CD8+CD28- cells [44]. All these viruses, and possibly other bacterial or fungal pathogens, establish persistent infections, which trigger the expansion of memory cells, however to much lower extend than CMV. The viruses discovered to induce this phenomenon are the human deficiency virus (HIV), Epstein-Barr virus (EBV), Hepatitis B virus (HBV) and Hepatitis virus C (HCV). All emerging evidence however shows that CMV overpower these viruses significantly and is the strongest inducer of memory cell expansion so far discovered[96]. The chronic stimulation of immune system by persistent infection however results in different outcomes. While HBV or HCV specific memory clones become exhausted and impaired in their functions[133], CMV specific T cells maintain highly effective pro-inflammatory features. Individuals co-infected with CMV and EBV do not display expansion of EBV specific clones, suggesting strong CMV-specific immunodominance[99]. Moreover, while HIV, EBV and HCV infect mainly only CD4 T cells, B cells and hepatocytes, respectively, CMV has apparently many different types of target cells such as epithelium, hepatocytes, myeloid precursors, monocytes, and probably many others which remain to be identified.

1.5.5.1 EBV

Besides CMV also other viruses have been associated with significant clonal expansion. One of the pathogens establishing lifelong infection with particularly high prevalence(up to 90% worldwide) is EBV, a member of *herpesviridae*. In EBV infected individuals 5-10% of all CD8 T cells are specific for one of the EBV epitopes[134]. However, in contrast to CMV, EBV has not been associated with immunosenescence. Even though EBV is related to CMV (G-and B-herpesvirus, respectively) EBV is spreading mostly through induced replication of infected

cells. The immune response to EBV is mainly CD8 mediated, with some epitopes derived from the lytic cycle proteins dominating[135].

1.5.5.2 HIV

Human immunodeficiency virus is a retrovirus responsible for acquired immune deficiency syndrome (AIDS), which is manifested by the loss of CD4+ T cells (main target of HIV infection) leading to immunodeficiency. The main response against HIV is mediated via cytotoxic CD8 T cells and this response is mostly responsible for the decrease of CD4 T cells numbers [136]. Other mechanisms, such as the cytolytic HIV effect are contributing to this phenomenon. The expansion of multifunctional CD8 T cell improves the control of HIV, however over time these cells become exhausted which leads to a significantly lowered capacity of the immune system to slow down the progress of HIV infection and other infections as the accumulation of exhausted clones occur on the expense of other effective T cells.

1.5.6 T cell responses towards Cytomegalovirus

Upon infection, CMV alters the gene expression of the host cell as well as many immune-related processes including MHC I antigen-presentation[119]. It is however the type of T cell responses, which are induced by CMV that appear unique compared to other pathogens. To understand the shaping of T cell responses we must understand the interactions of T cells with CMV. Among other cells types it is evident that T cells are the main immune cell populations responsible for controlling CMV latency and preventing reactivation, as the abrogation of T cell response results in an increased replication of CMV[125]. An intriguing feature of CMV infection is the fact that the CMV-specific T cells have significant differences in terms of scale and phenotype with other antiviral T cell populations[137]. The majority of CMV specific T cell clones exhibit the phenotype of highly differentiated effector memory T

cells, whereas T cell specific to other viruses exhibit the phenotype of less differentiated

memory cells (EBV) or the phenotype of early stages of memory (Influenza) (**Figure 1-8**)
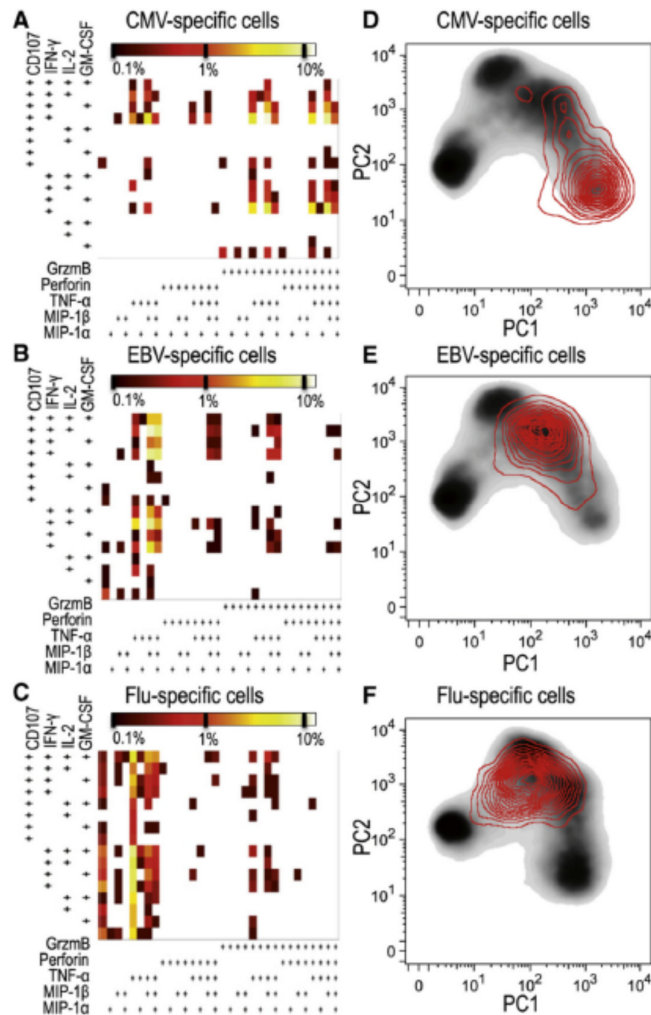
Figure 1-9 **Differentiated memory phenotypes of CMV specific T cells**

Virus-specific CD8 T cells, detected by tetramers loaded with antigens specific for CMV (A, D), EBV (B,E) or influenza virus (C, F)were analyzed after intracellular staining by CyTOF. Panels A to C display the detection of inflammatory and cytotoxic molecules (GrzmB, Perforin, TNF-a, MIP1a, MIP-1b, CD107, IFN-g, GM-SCF and IL-2), while panels D to F represent a principal component (PC) analysis. Figures adapted from [93].

T cell responses to individual CMV peptides can reach up to 5-10% of the total CD8 T cell population in the blood [96]. These responses are mostly targeted against peptides derived from HCMV 65kDa phosphoprotein (pp65) and immediate-early protein 1 (IE1) but varies between individuals. CMV-specific memory T cells seem to accumulate over time, as in elderly a dominant clone against one CMV epitope can comprise 25% of all CD8 T cells [96] . Some studies however suggest that the expansion does not follow a linear trend but rather rapidly increases in elderly individuals[138, 139]. Even though it has been believed that the CMV related memory inflation is induced by viral replication, it has been suggested that this is not the only mechanism and might even be of minor importance. As with all herpesviruses, CMV establishes latent infection via deposition of its genome in the infected cell nucleus. The viral genes are almost immediately silenced by histone binding and chromatin formation[140]. CMV reactivation therefore occurs preferably during cell maturation [141, 142], a stage in which the epigenetic modification and gene silencing is altered or diminished due to histone modification and chromatin re-structuring [143].

It is important to note that the term 'latency' does not refer to persistent infection, but rather toa viral state in which functionally intact, reactivation-competent viral genomes are maintained without completion of the productive replication cycle [144]. Therefore, no infectious viral progeny is formed or released. In contrast, persistent infection refers to continuous formation and release of infectious progeny (though with possibly very low level of occurrence). It is possible that low-level persistence exists in the case of CMV, but the formation and release of infectious viral particles might be occurring only during the absence of T cell immune response[125]. It is the stochastic continuous transcription and translation of certain genes and their proteins, which are presented on infected cells that stimulate the tissue patrolling TEM cells[145, 146]. Therefore the major immunodominant epitopes, against which we observe the major immune response resulting in memory inflation, are related to the

stochastic gene de-silencing after established latency[131, 147]. It is rather the position of these genes in the viral genome as well as the strength of the gene-related promotor[148] that might explain why in some individuals we observe completely altered immunodominance of viral epitopes.

CMV specific T cell clones are typically defined as TE, as they possess some unique features compared to CD8 T cells in other chronic viral infections. CMV specific CD8 T cells lose costimulatory molecules CD27 and CD28 and gain the replicative senescent molecule CD57 and lectin-like receptor subfamily G member 1 (KLRG1)(compare figure 1-5). Furthermore these cells have high expression of effector molecules such as granzyme B and perforin [78] (compare figure 1-X). It is generally believed that these features of memory T cell differentiation develop over time. However, it was shown that some mature effector features of CMV-specific T cells are acquired very early after the infection [149]. Some of the molecules are required or facilitate memory inflation, for instance the anti-apoptotic protein BCL2L1 co-stimulatory receptor 4-1BB. Moreover, CMV memory T cells express molecules typically associated with NK cells, such as CD56, killer cell immunoglobulin-like receptors (KIRs), and leukocyte immunoglobulin-like receptors (LIRs), as well as CD45RA instead of the RO isoform on other memory populations.

Extensively prolonged antigen stimulation during chronic infections by viruses like HIV, HBV or HCV often leads to a functional exhaustion[150], which is accompanied by the expression of inhibitory receptors such as programmed cell death protein 1 (PD1), lymphocyte activation gene 3 protein (LAG3), T cell immunoglobulin mucin receptor 3 (TIM3) CD160 or 2B4 [151]. Chronic CMV infection however does apparently not induce this exhaustion phenotype. This is due to the fact that, unlike highly replicating viruses, CMV reactivation occurs only sporadic [152]. Based on data in MCMV infection model, the continuous expansion of CMV-specific memory T cells is instead more likely to occur through the

differentiation of CM re-stimulation [152]. In this model, CMV specific CM T cell are not stimulated by normal APC, but respond to signals from non-haematopoetic cells[153]. This could explain the massive memory expansion without strong antigen stimulation. The non-haematopoietic cells are the major niches for CMV latent viral genomes [153]. Therefore, the production of proteins, rather than full reactivation of viral particles, could be enough to stimulate TCM and induce differentiation and expansion of EM and subsequently TE. This assumption is supported by data obtained with spread-defective single round virus (SDSR). The SDSR virus is unable to produce viral particles but can establish talent infection. The kinetics of memory inflation is comparable with the wild type MCMV infections.[154]

It is important to note that the initial dose of virus in primary infection seems to determine the magnitude of memory inflation after latency establishment [155]. The T cell inflation appears to be mainly occurring within the CD8 compartment however but also the fraction of CMV-specific CD4 T cells increases with age up to 30% [156]. There is however no evidence of the non-classical memory inflation as described in CD8 where expanded CMV clones do not undergo the usual contraction phase. Furthermore, the recent evidence of non-hematopoetic cell driven antigen stimulation of CMV clones gives new insights to the much more pronounced role of CMV in CD8 memory T cell expansion as compared to CD4. However, despite the less profound knowledge of CD4 T cell responses against CMV as compared to CD8, it is evident that direct expansion and differentiation of CD4 specific cells occurs in healthy adults. An extensive replication of these cells is indicated by the exhausted and replicative senescent phenotype of these cells [157].

Co-stimulation of CMV specific T CD8 cells in priming and inflation further delineates the differential response and development of these cells compared to the conventional memory cells. It has been shown that even though the CD28/B7 co-stimulation is important for the priming of CD8 CMV specific T cells it is dispensable for the further development of CM cell

and T cell inflation[158].By contrast, CD27/CD70 signaling or OX40 and 4-1BB are not only important during priming, but also during the inflation phase[159-162]. 4-1BB is furthermore important in the re-stimulation of HCMV-specific CD8 clones *in vitro*[163]

The T cell response is usually targeted to certain proteins and epitopes of a pathogen. It has been shown that during MCMV infection only some clones with certain antigen specificities undergo the memory inflation response and others follow the classical pattern including the contraction phase of an immune response [164]. Furthermore, the expanding clones display distinct memory phenotype and their inflation is dependent on the initial dose of the antigen [164]. The inflation inducing antigens are more expressed during latency than the antigens, which do not induce inflation of their putative T cell clones. Additionally, antigens inducing inflation are presented on the infected cells despite the presence of virus encoded genes blocking MHC I/peptide presentation [164]. This has not been clearly shown in humans but it is likely that HCMV specific CD8 T cells follow similar patterns as their phenotype and expansion are similar to their MCMV specific counterparts.

It has been shown that the MCMV-driven memory inflation is dependent on non-hematopoietic antigen presentation, most likely by the latently infected cells[153].Furthermore, it is also largely independent of cross-presentation via APCs[165] although variations in antigen processing may play a role. Upon stimulation by IFN, cells start expressing different subunits of the proteasome which results in the assembly of the so-called immunoproteasome[166]. Proteins processed by the immunoproteasome yield altered peptides as compared to the peptides derived from identical proteins processed by "classical" proteasome. In MCMV infection, the immunodominant protein M45 for clones undergoing classical memory expansion followed by contraction is processed via the immunoproteasome. By contrast, the epitope of protein M38 inducing memory inflation is independent of immunoproteasome processing [167]. The immunoproteasome is constitutively expressed in

APCs, such as DCs, however is induced in other cells types upon encountering inflammatory signals, which are most probably not present during latency.

Mice infected with MCMV expressing heterologous epitopes under the control of early promoters induced a dominating response and inflation of memory CD8 T cells specific to this epitope. When co-infected with a wild-type strain which infected different cells, the memory inflation was equal for both endogenous and heterologous epitopes [168]. Therefore the availability of antigen and competition of MHC antigen derived peptide presentation, which is a common factor for immunodominance of a response[169] plays a role in the establisment and shaping of the response against MCMV and related memory inflation. It has also been shown that T cell clones with high avidity for the antigen are preferentially triggered for inflation as compared to clones with lower antigen binding strength[170]. In humans restricted oligoclonal expansion has also been shown where all the expanded clones had very similar avidity for the putative antigen epitope [171]. The same study also showed that the clonal composition remains stable over time, however the follow-up was only 2 years and age of individuals ranged from 25-45 years old and as previously mentioned the inflation is established early on and subsequent significant "re-expansion" most likely occurs after 65 years of age[104].

In summary, aging is a very complex and multi-system process. The aging of the immune system is further enhanced by infection, especially chronic viral infection. CMV has been shown to significantly contribute to the alterations in the composition and function of memory T cells, which are hallmarks of immunosenescence. This relates in particular to the accumulation of highly differentiated, replicative senescent but not exhausted T cell populations. The general accumulation of presumably antigen-experienced T cells is manifested in the significant skewing of T cell populations towards memory. This is accompanied with a drastic decrease of the naïve T cells pool resulting in a significant shrinking of TCR repertoire. As the appearance of immunosenescence-markers seems to be directly

linked to CMV infection, a substantial medical problem may arise from CMV infection, since

prevalence of CMV is estimated to be 70-80% worldwide.

Project aim:

There is a strong relationship between the age-related erosion of the immune system and the susceptibility to infections, control of neoplasms as well as regulation of inflammation. Chronic infections have been shown to have a profound impact on T cell biology, which results in an acceleration of T cell differentiation and increased susceptibility to replicative senescence, and cytomegalovirus (CMV) infection seems to play a major role in this process. The hallmarks of immunosenescence are the alterations in the T cell compartment, evident in the strong expansion of memory T cells concomitant to the decrease in naïve T cells. The trajectory of this phenomenon is not well understood. I therefore focused my PhD studies on identifying early signs of immunosenescence driven by persistent CMV infection.

Specific aim of this study was:

a) to characterize T cell populations of CMV+ and CMV- individuals in blood samples obtained from a cohort of young Singaporean adults to identify early effects of CMV infection,

b) to determine their putative impact later in life by comparison with the data generated in the Singapore Longitudinal Aging Study (SLAS) for the older adults' cohort, and

c) to determine to what extent the CMV-associated phenotypic and functional variations in the T cell memory compartment of the young can account already for the typical characteristics of immunosenescence observed in the old.

We also focused on the detection of possible onset of low grade chronic systemic inflammation linked to CMV history that is observed in elderly (inflamm-aging) and in some cases correlated with higher morbidity and mortality. My project therefore aimed to contribute to the understanding of the role of CMV in inducing differentiation of T cells early in life and identify whether chronological aging per se could contribute to immunosenescence, as it is currently defined. Because our cohort study is cross-sectional and samples for primary infections in

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humans are difficult to obtain (CMV is mostly asymptomatic), I also aimed to develop a 'humanized' mouse model to longitudinally follow the effects of CMV primary infection on T cell populations.

2 Materials and Methods

2.1.1 Study participants

Healthy, young individuals of Chinese ethnicity were recruited from the National University of Singapore (mean age was 21 years). Exclusion criteria included recent infections, anti-inflammatory medication and recent hospitalization. Individuals with recurrent/chronic conditions unrelated to infections such as asthma, allergy and diabetes were not excluded. The female/male ratio was 53/47. The study has been approved by the National University of Singapore-Institutional Review Boards 04-140, and all participants gave written informed consent.

Home-dwelling healthy elderly (>65 years old) individuals were recruited for the Singaporean longitudinal aging study (SLAS) cohort. Elderly participants were identified from door-to-door census and eligible persons, excluding those with severe physical or mental disability, who could not participate in the extensive questionnaire interviews or physical and neurocognitive performance tests, were invited to participate in the research. The female/male ratio was 63/37. The study has been approved by the National University of Singapore-Institutional Review Boards, and all participants gave written informed consent.

2.1.2 Blood samples and PBMC preparation

Blood samples were collected in CPT tubes (BD Biosciences, San Jose, CA, USA) by venipuncture from fasting participants. After spinning at 1650 rpm for 20 minutes at RT, PBMCs and plasma were collected. Plasma was stored at -80ºC and cells were washed twice in PBS and cryopreserved in liquid nitrogen in 90% FBS containing 10% DMSO. Samples were processed and stored within 4 hours after blood draw. At the day of experiment, cells were rapidly thawed with 37°C RPMI-1640 containing 20% FCS. PBMC were then washed of

DMSO twice in PBS containing 10% FBS. Recovery of samples was generally >85% with the cell viability >95%. Cells were used for downstream experiments including immnuophenotyping, stimulation for intracellular cytokine determination, cell sorting and gene expression analysis.

2.1.3 Immune cell characterization by flow cytometry

Samples were thawed in batches of n=30 (randomly selected between CMV- and CMV+ donors) and PBMCs were stained in FACS buffer (5% FBS, 0.2µM EDTA, in PBS) using the following fluorophore-conjugated antibodies: aCD3 PECy5.5 (Beckman Coulter, Brea, CA, USA), CD4 PECy7 (BioLegend, San Diego, CA, USA), CD8 APCCy7 (BD Biosciences), CD27 AlexaFluor700 (BioLegend), CD28 ECD (Beckman Coulter), CD45RA eFluor605NC (eBiosciences, San Diego, CA, USA), CD57 Pacific Blue (BioLegend) and Live/dead staining (Invitrogen, Carslbad, CA, USA) for 20 minutes at4°C in the dark. For intracellular cytokine staining cells were stimulated with 5 ng/ml PMA (Sigma-Aldrich, St. Louis, MO, USA) and 1µM Ionomycin (Sigma-Aldrich) for 2 hours. Then 3ng/ml Brefeldin A (eBioscience) and 2µM Monensin (eBioscience) were added to the cells culture for another 2 hours. Subsequently, the cells were stained with surface markers for 20 minutes at 4°C. After washing with FACS buffer cells were fixed and prepared for intracellular cytokine staining with the BD Cytofix/Cytoperm kit (BD Biosciences). Cells were stained with anti-TNFα PE (eBiosciences) and anti-MIP1β FITC (BD Biosciences) for 30 minutes on ice. After staining all samples were washed and acquired on LSR Fortessa™ Cell Analyzer using BD Facs Diva software.

2.1.4 Calcium Flux measurement

For each staining 4 x 10^6 PBMCs were used per sample. Samples were resuspended in 250 µl RPMI + 10% FBS stained with 1µl of Indo-1 dye(working concentration = 1: 250) at 37°C for 40 min, subsequently washed with FACS buffer. Surface marker (SM) staining was performed in FACS buffer at 4°C for 20 min. After SM staining, samples were washed with RPMI+ 10% FBS and resuspended in 160 µl RPMI + 10% FBS. For acquisition and calcium fluxing, samples were warmed up at 37°C (for up to 30 min). The sample flow was adjusted to cover at least 5 minutes of data acquisition. The voltage of indo channels was adjusted to achieve an angle of approximately 45 degrees (Y axis: Blue, X axis: Violet) and the threshold adjusted to minimize debris collection. To control the temperature a small beaker with37°C water was placed under the tube in the SIT during acquisition. Samples were measured for 50 sec before 1 µl of ionomycin (stock concentration: 1mg/ml) was added to stimulate calcium flux. Samples were acquired a total of 6 min. Kinetic analysis was performed in FlowJo X through derived parameter (Indo Violet- 405nm/Indo Blue-450nm channels).

2.1.5 Surface marker screening of barcoded cohort samples by LEGENDScreenTM Human Cell Screening

45 frozen samples from the following cohorts were thawed as described above(15 samples each): Chinese old CMV+ (SLAS) (Mean age=74years) Chinese young CMV+ (Y+) and CMV- (Y-) (mean age both 21 years). M/F ratio was 0.25 in all 5 populations. Samples from each population were pooled, washed twice with PBS to remove any residual FBS and barcoded with either Carboxyfluoresceinsuccinimidyl ester (CFSE) or Far Red cell tracker(FR) of different concentrations(SLAS: no barcoding, Y+: Far Red 0.1uM, Y-: CSFE 0.1uM). For the barcoding, cells were resuspended at the concentration of 10^7 cells x ml^{-1} and stained in PBS with a corresponding cell tracker concentration at 37°C for 10 minutes. CFSE or FR were

then quenched with equivocal volume of FBS and were left for 1 minute at RT. Subsequently the solution was toped up with PBS and washed twice. After Cell tracker-barcoding all populations were washed with FACS buffer and pooled together with for the staining of surface markers (CD3, CD4, CD8, CD27, CD28, CD45RO, CD57, CD95, CCR7and Yellow L/D staining). Cells were stained for 45minutes on ice and subsequently washed with FACS buffer. The staining was checked on LSR Fortessa before proceeding to the next step. The expected T cell phenotypes were observed and served as staining control. The cells were then stained using the LEGENScreenTM Human Cell Screening PE Kit (BioLegend). The Kit consists of four 96-well plates, where each well contains lyophilised monoclonal antibodies conjugated with PE fluorochrome against one CD marker. 200 000 cells were added in 75 µl of FACS buffer into each well. The cells were then incubated for 20 minutes on ice. After staining cells were span at $500 \times g$ for 6 minutes. The supernatant was carefully discarded and cells resuspended in 200 µl of FACS buffer and washed again. Subsequently cells were resuspended in 160 µl and acquired by FACS. The total of 342 tubes took approximately 12 hours to acquire. Therefore, standard 8 peek fluorescent beads were used every 30 minutes to ensure stability of the flow cytometer laser/PMT voltage. Potentially false positive marker were removed from the analysis if less than 10% of the cells in the T cell memory stages N, CM, EM, TE were positive (unless those have been shown in literature to be expressed marginally on certain T cell memory subtypes). The cut off was arbitrarily chosen to account for the errors of antibody titration by the manufacturer. For further analysis we have used the R software. Clustering of the populations according to percentages of positive cells for a putative marker and together with (log10 transformed) were done using Pearson correlation.

2.1.6 CMV, HSV-2 and Helicobacter pylori serology

Measurement of specific IgG antibodies in participants' plasma was performed using commercially available reagents for CMV (Genesis Diagnostics, McAlfer, TX, USA), HSV-2 and H. pylori (Novatec, Temecula, CA, USA). Briefly, frozen plasma samples(stored at -80°C) were thawed to room temperature and diluted according to manufacturer's instructions (1:100). Diluted samples were added to 96-well plates containing respective antigens of CMV, HSV-2 or H. pylori. Samples were incubated for 1hour for CMV and 20 minutes for HSV-2 and H. *pylori*. After washing, samples were treated with anti-human IgG conjugates of horseradish peroxidase and incubated for 20 minutes for CMV, 30 minutes for HSV-2 and H. *pylori*. Subsequently, TMB substrate was added for 15 minutes for all three ELISA after which stop buffer was dispensed and wells were red immediately on 2104 EnVision® Multilabel Reader (PerkinElmer, Waltham, MA, USA).

2.1.7 Plasma/cell supernatant cytokine determination by Luminex

Culture supernatant from FACS-sorted CD8 TE cells was collected afterstimulationwith PMA/Ionomycin for 4 hours. The levels of cytokine in these supernatants as well as in plasma samples were then measured by Luminex®[172]. Briefly, 25 µl of thawed sample was used to incubate with Millipore multiplex kits. In our study, were measured EGF, Eotaxin, FGF-2, Flt-3L, Fractacline, G-CSF, GM-CSF, GRO, IL-6, IL-8, MIP-1α, MIP-1β, VEGF, sCD40L, sEGFR, sIL-2Ra, sIL-4R, sIL-6R, sRAGE, sTNFRI, sTNFRII, sVEGFR2, sVEGFR3, SGP130.

2.1.8 Cell sorting, gene-expression and data analysis

PBMCs from 6 CMV+ and 6 CMV- young individuals were thawed and stained with the following antibodies: CD8 APC (BD Biosciences, CD4 PECy7 (BD Biosciences), CD45RA FITC (BD Biosciences), CD28 ECD (Beckmann & Coulter) and Live/Dead Aqua

staining (Invitrogen) for 20 minutes at 4°C. Twenty thousand CD8+ T$_{TE}$ cells (CD8+CD4-CD28-CD45RA+) were sorted in 10% FBS 1640 RPMI medium. Post-sort quality control revealed >97% accuracy of the sorting process. After washing with the same medium, cells were stimulated with PMA at 5ng/ml (Sigma-Aldrich) and 1μM Ionomycin (Sigma-Aldrich) for 4 hours at 37°C. At the end of the stimulation time, cells were spun, dry pellet were re-suspended in 5 μl of RLT lysing buffer and frozen at -80°C until further processing. Frozen pellet in RLT buffer were thawed and processed as described [173]. We have used the nCounter® Human Immunology v2 kit (nCounter, Seattle, WA, USA). Flow cytometry data was analyzed withFACSDiva (BD Biosciences) and Flow Jo software (Treestar, Ashland, OR, USA). When indicated data was statistically analyzed using Mann-Whitney test. Anti-CMV IgG correlations were analyzed using the Pearson correlation. Network analysis of the gene expression was done using the String 9.1 software (Swiss Institute of Bioinformatics, NFF Center for Protein Research, European Molecular Biology Laboratory). All statistical analyses were done using the GraphPad Prism 6.0 (GraphPad, La jolla, CA, USA) and R v 3.1.2. (The R Foundation for Statistical Computing, General public license). Pathway/functional analysis of gene and surface markers expression were done using the Ingenuity Pathway Analysis 01-04 Software (QIAGEN, Redwood City, CA, USA).

2.1.9 Human foreskin fibroblasts (HFF)

HFF were kindly donated to me by Christine Neumann from the Institute of Medical Biology (IMB), A*STAR. HFF were thawed and cultured at 37°C in T175 flasks in 23ml of high Glucose 10% FBS DMEM (Gibco) until they reached confluence. Every 2-3 days ¾ of media was exchanged for a fresh medium. After reaching confluence cells were trypsinised with 7 ml of trypsin for 5 minutes at RT. Subsequently 7 ml of medium was added to stop the

reaction and cells were washed in 50ml Flacon tubes at 400rpm for 7 minutes at RT.HFF were split 1/5 for next culture. 2 passages were used to reach the sufficient number of cells for humanized mice infection. Freshly isolated foreskin was placed into complete K-SFM (17005042 + EGF + BPE; Gibco) containing gentamicin at a concentration of 5 µg/ml at 4°C for short-term storage. The tissue was then washed with PBS containing gentamicin at a concentration of 20 µg/ml 3 times. Excessive subcutanous tissue was removed and then cut into $0.5x1cm^2$ pieces, which were subsequently incubated overnight at 4°C in 0.25% (m/v) dispase in K-SFM. After the incubation epidermis was separated from dermis. Subsequently, dermis was washed in PBS once and cut into smaller pieces of $2x2mm^2$ size, which were then incubated at 37°C for 40 minutes in 2.5ml trypsin-EDTA (cat. No. 25300).Cells were dissociated from dermis by thorough pipetting. The suspension was centrifuged at 500rpm for 10 minutes at room temperature and subsequently washed with DMEM/10% FBS. The cell pellet was then gently resuspended in 5 ml of complete medium and seeded into 10 cm diameter petri dish and incubated in the cell culture incubator at 37°C in 10 ml medium per dish. The medium was changed the next day to wash away the floating dead cells and some small pieces. The medium was changed twice a week until the cells reached 80% confluence. The medium was then removed and cells washed once with PBS. 2.5 ml trypsin-EDTA was then added to the dish and incubated for 5 min at 37°C. Digested cells were collected and washed twice with DMEM/10% FBS at 500 rpm for 10 min at room temperature. Cells were then frozen down in 10% DMSO 90%FBS.

2.1.10 CMV infection

2.1.10.1   CMV

The CMV strain used for the humanized mouse infection was a recombinant HCMV strain (TB40) expressing enhanced green fluorescent protein (EGFP) fused to the C terminus

of the capsid-associated tegument protein pUL32. The UL32-EGFP-HCMV was kindly donated to us by Prof. Christian Sinzger, PhD from the Institute of Medical Virology, University of Tubingen, Tubingen, Germany. We multiplied the virus on HFF cells until the cythopathic effect (CPE) reached almost 100%. Cells were subsequently harvested and were subjected to 3 freeze/thaw cycles to release the viral particles. All supernatants were collected and viral particles were concentrated by ultracentrifugation.

2.1.10.2   Ultracentrifugation

30ml of supernatant with CMV particles were topped up carefully with 4ml of 20% sterile saccharose in high resistance ultracentrifugation tubes and were span at 28 000rpm for 4hours at 4°C. Before centrifugation, the tubes were weight balanced with a maximum deviation of 0.05g. After centrifugation the supernatant was discarded and the viral pellet was resuspended in PBS and left on ice for 15 minutes to let all viral particles detach from the tube into the PBS. Viral aliquots were frozen at -80°C.When the HFF cells reached confluence, they were infected with UL32-EGFP-HCMV at 0.01 Multiplicity of Infection (MOI). When CPE reached 70%, one T175 flask was harvested with trypsin as described above. The cells were subjected to 3 freeze/thaw cycles. The supernatant was subsequently titrated on HFF and the infection determined after 24 hours. The rest of the cultures were harvested and animals were injected intraperitoneally with approximately 700 000 plaque forming units (pfu) per animal in 300 µl of PBS, which represented approximately 6.5×10^6 HFF.

2.1.10.3   Titration of HCMV

HFF cells were seeded in 80 wells of a 96-well plate at 12,000 cells per well. Cells were grown in High glucose DMEM with 10%FBS for 24 hr at 37°C. The HCMV was diluted in

serial dilutions in 10 fold dilution steps from 10^{-1} to 10^{-8} and 10 wells for each dilution were infected with the virus and incubated for 24h at 37°C.After the incubation, cells were washed and the plaques were counted based on the eGFP signal using fluorescent microscope. The virus titer was measured as 50% Tissue Culture Infective Dose (TCID50). TCID50 dilution represents the amount of virus required to kill 50% of infected hosts or to produce a cytopathic effect in 50% of inoculated tissue culture cells. The TCID50 was calculated using a TCID50 calculator. Virus titer in pfu/ml was calculated as 0.7 x TCID50/ml.

2.1.11 Blood analysis of humanized mice

One week prior infection, one week post infection and then every 2 weeks blood was drawn from both infected and non-infected mice via retro-orbital bleeding. 30 µl of blood was drawn each time. Blood was lysed with ACK buffer twice for 10 minutes at RT. Subsequently lysed blood was washed with FACS buffer and stained with anti-human (CD3-A700, CD4-Pe-Cy7, CD8-APC, CD27-BV605, CD28-ECD, CD45-APC-Cy7, CD45RA-BV650, CD57-PB, CCR7-PerCp-Cy5.5, CD95-PECy5) and anti-mouseantibodies(CD45-BV786) as well as L/D Aqua. To establish the actual cell count, CountBrightTM Absolute Counting Beads were added (Molecular ProbesTM, Invitrogen).

2.1.12 Organ DNA isolation

Small pieces of spleen, lungs and liver were harvested and processed in order to isolate DNA. Approximately 30 mg were used for the DNA isolation using the DNeasy Blood & Tissue Kit. Tissues were homogenized and incubated in 180 µl of ACT buffer and 20 µl of protein kinase K in 56°C until compete dissociation of the tissue. During the incubation tubes were occasionally vortexed. After complete dissociation of the tissue 20 µl of absolute ethanol was added

2.1.13 The NSG humanized mouse

The NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ mice, commonly known as NOD scid gamma (NSG™), do not express the Prkdc gene nor the X-linked Il2rg gene. The Prkdc gene codes for a DNA-dependent protein kinase catalytic subunit which is involved in DNA non-homologous end joining (NHEJ) required for double strand break and therefore crucial for the VDJ recombination. The lack of IL2rg gene results in profoundly impaired cellular and humoral immunity, known as Severe combined immunodeficiency (SCID). The reason for the significant impairment of the immune system is the large number of interleukin receptors, which use the common subunit γ (receptors forIL-2, IL-4, IL-7, IL-9, IL-15 and IL-21). The variety of cytokine signaling affected results in impaired T-cells, B-cells, NK cells and the cells of monocyte lineage growth and differentiation, T cells cell proliferation and survival. The humoral activity is seriously impaired by the lack of antibody producing plasma cells which do not differentiate from the B cells precursors.NSG mice are viable, fertile, normal in size and do not display any gross physical or behavioral abnormalities. Histological examination of lymphoid tissues reveals absence of lymphoid cells and some cystic structures in the thymus, an absence of follicles in the spleen and markedly diminished cellularity of lymph nodes. NSG mice are deficient in mature lymphocytes, serum Ig is not detectable and natural killer (NK) cell cytotoxic activity is extremely low. These mice are resistant to lymphoma development even after sub-lethal irradiation treatment. These mutant mice have been shown to readily support engraftment of human CD34+ hematopoietic stem cells and represent a superior, long-lived model suitable for studies employing xeno transplantation strategies.

2.1.14 HSC Isolation and Construction of Humanized Mice.

Human CD34+ cells were freshly isolated from aborted fetuses at 15–23 weeks of gestation, in accordance with the institutional ethical guidelines of the KK Women's and Children's Hospital, Singapore. Fetal liver tissues were processed as described [228]. CD34+ cells were purified by magnetic-activated cell sorting using the EasySep CD34-positive selection kit (Stemcell Technologies) under sterile conditions, following manufacturer's protocol. The purity of the CD34+ cells was 90–99%. NSG mice were purchased from the Jackson Laboratories and bred in a specific pathogen free facility at Biological Resource Center (BRC) in Agency for Science, Technology and Research (A*STAR), Singapore. One to three days old NSG pups were sub-lethally irradiated at 1 Gy and transplanted with 2×10^5 CD34+ human fetal liver cells by intra-hepatic injections. The mice were bled at 8 weeks post-transplantation to determine the levels of human immune reconstitution. All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC). This work has been done by our collaborators in the humanized mouse unit in the Institute of molecular and cell biology, A*Star, Singapore.

2.1.15 Nested Polymerase chain reaction (PCR)

As described previously[174] a 332 bp region of the HCMV genome was amplified by nested PCR using HCMV-specific forward and reverse primers for exons 1 and 2 of the major immediate early (MIE) gene. The outer primer pairs used were 5'-GAGTCCTCTGCCAAGAGAAA-3' and 5'-GAGTTCTGCCAGGACATCTTT-3', and the inner primer pairs were 5'-GAGTTCTGCCAGGACATCTTT-3' and 5'-CTCGGGGGTTCTCGTTGCAAT-3'. The outerprimer pair-PCR reaction mixture (20 µl) consisted of 100 ng DNA, 1× ThermoPol buffer(New England BioLabs), 200 µM of each deoxynucleoside triphosphate (DNTP), 1 µM of eachprimer, and 0.2 µl of vent DNA

polymerase (New England BioLabs). 30 cycles of outerprimer PCR were performed, with each cycle consisting of denaturation (20 sec. at 94°C), annealing (50 sec. at 62°C), and extension (20 sec. at 72°C). For the inner primer pair PCR reactions, the same reaction mixture was used with 2 µl of the outer primer pair PCR reaction diluted 1:100. 30 cycles of inner primer PCR were performed, consisting of denaturation (20 sec. at 94°C) and combined annealing and extension steps (1 min. at 60°C).

3 Results

3.1 Humans studies

3.1.1 Seropositivity to CMV is associated with advanced T cell differentiation profile in young individuals

Serologic analysis of plasma samples our young cohort of individuals of Chinese ethnicity indicated that more than two third of them are chronically infected by CMV (CMV-: n=84 and CMV+:n=183). In order to determine if young CMV+ individuals show similar alterations of memory T cells, as has been associated with aging (i.e. memory T cell inflation and the decrease of naive cells) we analyzed biobanked PBMC samples by FACS. Upon differentiation, memory T cells progressively loose the expression of co-stimulatory molecules CD28 and CD27. Moreover, when reaching replicative senescence they are gaining the expression of CD57. In an initial approach we therefore investigated single expression of CD27, CD28 and CD57 on CD8 and CD4 T cells (Figure 3-1).

The analyzed data suggested that CMV+ individuals indeed exhibit a skewing of T cells towards differentiated memory (figure 3-1, left panels). In CMV+ young individuals we observed increased numbers of CD27- (10.2 $\pm$5.9% vs 7.4 $\pm$3%, p<0.0001), CD28- (1.8 $\pm$3.0% vs 0.32 $\pm$0.32%, p<0.0001) and CD57+ (3.5 $\pm$3.8% and 1.4 $\pm$0.9%, p<0.0001) CD4 T cells. We observe similar trends for CD8 T cells: CD27- (20.9$\pm$13.4% vs 11.5+7%, p<0.0001), CD28- (22.6+12% vs 14.7+7.1, p<0.0001), CD57+(22 $\pm$13.8% vs 13.4 $\pm$8.7%, p<0.0001). These results indicate the higher differentiation state of the memory T cell populations and point towards the possible early onset of the deterioration of immune system functions.

We next tested how these observations reflect the CMV-induced differentiation of the individual memory subtypes. We have therefore investigated 4 basic stages of T cell memory

populations N, M, EM, TE, as defined by CD28 and CD45RA in CD8+ and CD27 and CD45RA in CD4+ T cells (**Figure 3-1**, right panels). Notably, already at a young age of 21 years seropositivity to CMV is showing strong association with expansion of CD28-CD45RA-T_{EM} cells (8.5 ±5.7% vs 5.7 ±3.9% p=0.0001) as well as CD28-CD45RA+ T_{TE} cells (13.9 ±9.5% vs 8.9 ±5.2% p<0.0001). Despite identical age range and mean the two groups of CMV+ and CMV- individuals show a significant difference in frequency of naïve CD28+CD45RA+ CD8+ T_N cells (56.6 ±14.1% and 60.55 ±12.4%, p=0.0013). The CD4+ T cells seem to be similarly impacted by CMV infection for CD27- CD45RA- EM (9.7±5.4% vs 7.1±2.9%, p=0.0003) T_{TE} (0.8 ±2.2% vs 0.22 ±0.24%, p=0.023) and naïve T cells (53.1 ±10.8% vs 56 ±9.3%, p=0.059) although the difference did not reach significance.
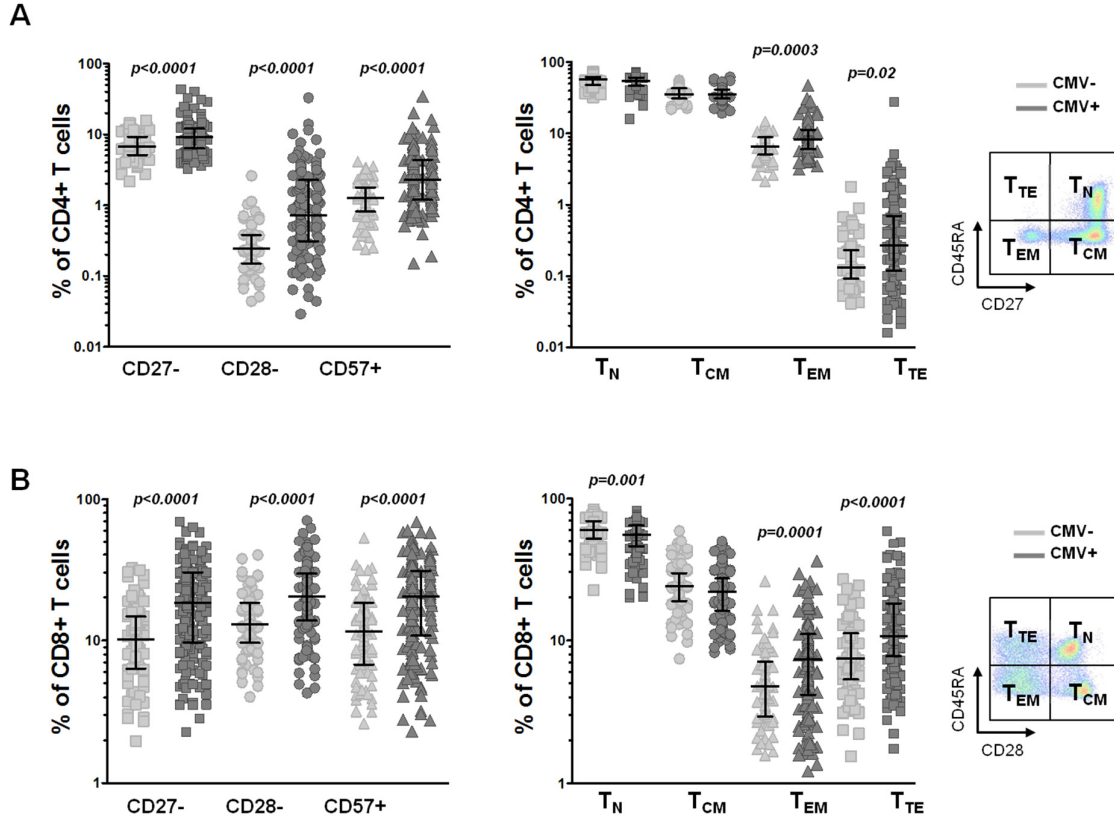
Figure 3-1 **Effect of CMV on T cell differentiation in young individuals**

Cryopreserved PBMCs were thawed and stained with anti-CD3, CD4, CD8, CD27, CD28, CD45RA and CD57 fluorophore conjugated antibodies. Frequencies of CD27-, CD28- and CD57+ (left panel) as well as the four main T cell subsets (right panel) Naïve (T_N) Central memory (T_{CM}) effector memory (T_{EM}) and terminally differentiated (T_{TE}) were measured for CD4+ (**A**) and CD8+ T cells (**B**). Data from CMV- (light grey) and CMV+ (dark grey) young individuals are shown. The gating strategy is shown on the right side.

It is known that in elderly the ratio between CD4 and CD8 T cells is usually skewed towards CD8. The decrease in CD4/CD8 ratio in elderly results from the expansion of CD8, mostly CD28- T cells and is also associated with the increased morbidity and mortality of elderly [175] .In a comparison between the CMV+ and CMV- individuals, we did not observe

any change in the CD4/CD8 ratio, which possibly rules out the overall expansion of CD8 T

cells in the CMV+ young (Figure 3-2).
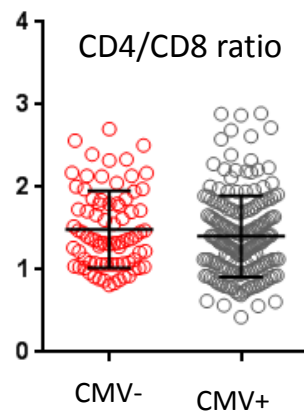
Figure 3-2 **The CD4/CD8 ratio**

CMV+ young individuals (grey circles) displayed no alterations in CD4/CD8 ratio (y
axis) as compared to CMV- (red circles) statistical analysis was done utilizing data from
cryopreserved PBMCs analyzed flow cytometry as described in Figure 3-1.

In

the blood naïve T cell emigrants that recently left the Thymus can be detected based on the

CD31 molecule. A low proportion of these cells is indicative of a higher homeostatic

proliferation of Naïve cells. In order to determine if CMV infection has an impact on this

process, we have determined the percentage of CD4+ recent Thymus emigrants within the

naïve T cells pool. While elderly people display a significant decrease of CD31+ CD4+ Naïve

T cells compared to young individuals, young CMV+ individuals display only a slightly

lowered percentage (Figure 3-3 **A**).However this observation was not significant. In young

CMV+ individuals the rate of homeostatic proliferation is therefore not substantially affected.

Notably, when we measured the Vβ repertoire in CD31+ and CD31- N cells of the

elderly we observed no significance difference in the TCR composition (Figure 3-3 **B**). It has

been proposed that homeostatic proliferation could, at least stochastically, affect the clone

distribution in elderly, favoring certain clones over other. This was however could not be

confirmed for our cohorts, although, due to the high prevalence of CMV in elderly in Singapore, we could not include a direct comparison with the CMV- elderly.
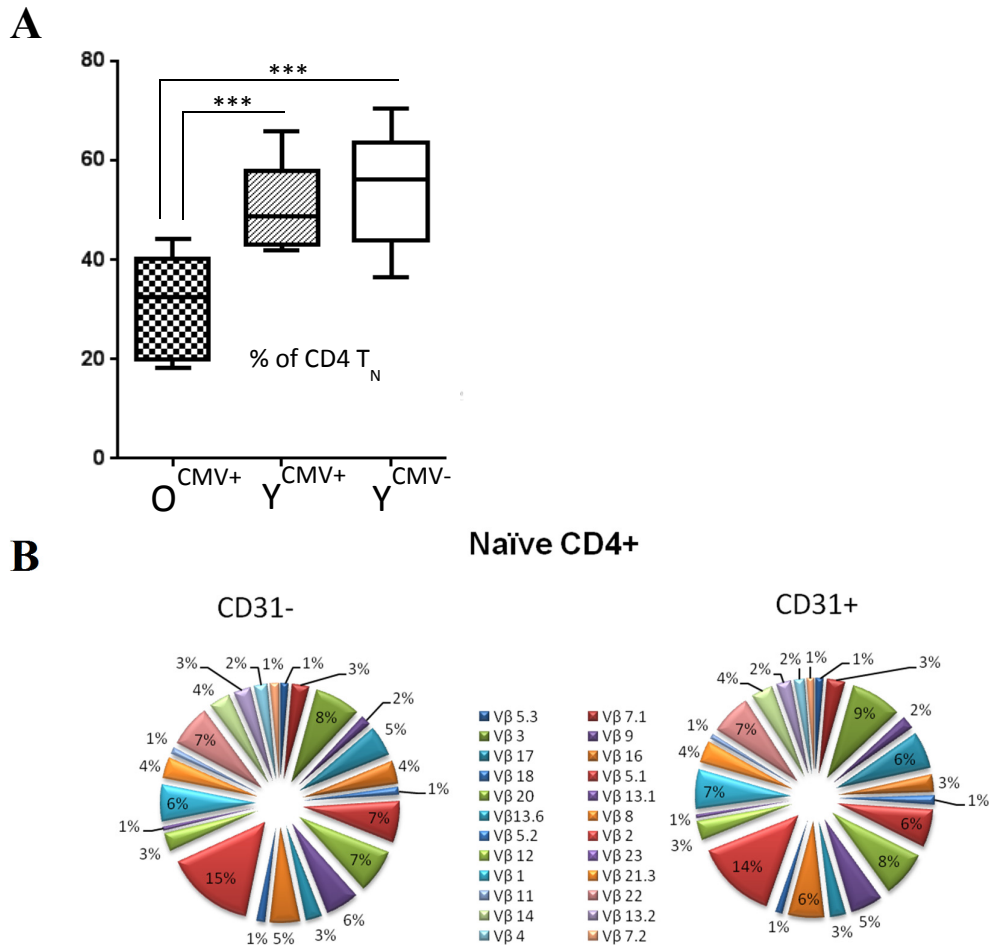
Figure 3-3 **Homeostatic proliferation of CD4 Naïve T cells**

Expression of CD31 was measured by flow cytometry on CD4 naïve T cells (**A**). Recent thymic emigrants are representing a significantly lower percentage of CD4 N cells in elderly (n=9) as compared to young CMV+ (n=9, p<0.001) and young CMV- (n=9, p<0.001). No significant differences have been observed between the young groups. (**B**) V-beta (VB) repertoire was measured by flow cytometry in elderly individuals (n=9). No significant differences were observed between CD31+ and CD31- CD4 N T cells.

In CMV+ young individuals, we observed a higher degree of differentiation of the T cells based on the enhanced percentage of CD27-, CD28- or CD57+ cells. To know if this reflects in the higher differentiation state of all memory subpopulations (including earlier stages of memory) we further investigated the antigen experience/stimulation history of the memory subpopulations as reflected by the loss of additional co-stimulatory molecule CD27 and CD28 for CD8 and CD4 cells respectively (**Figure 3-4**). In addition to the skewing in T cell development, CMV infection was also associated with changes in the phenotype of the expanded population that were indicative of differentiation and putatively antigen experience/replicative history. Based on the model (compare **figure 1-4**) we considered CD28+CD27+CD57- as the earliest and CD28-CD27-CD57+ as the most differentiated (replicative senescent) cells.

The analysis revealed that in CMV+ young individuals a significant expansion of differentiated cells on the expense of early stage cells was evident (**Figure 3-4 A**). Here we observed an increase of CD27-CD57+ CD8 T_{CM}, T_{EM} and T_{TE} cells (p=0.0004, p<0.0001 and p<0.0001 respectively) and a decrease of CD27+CD57- T_{CM},T_{EM} and T_{TE} cells (p=0.04,p<0.0001 and p<0.0001 respectively). We observed the same for CD4+ cells, where CD27- CD57+ T_{EM} and T_{TE} cells expand, while CD27+ CD57- T_{EM} and T_{TE} decrease (all p values <0.0001 (**Figure 3-4 B**).
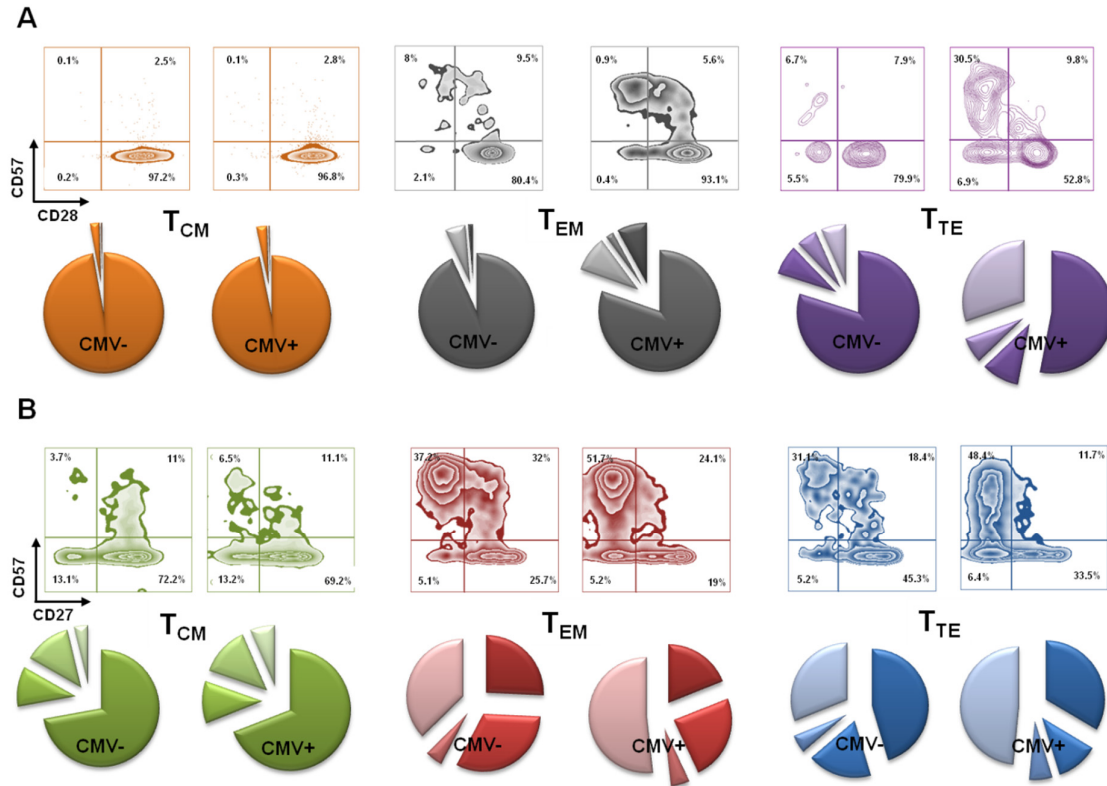
Figure 3-4 **Differentiation stage of memory T cell from young individuals**

A) CD8+ CM (green), EM (red) and TE (dark blue) cells from CMV+ and CMV- young individuals were compared. FACS plots depicts representative samples from each populations (Upper panel) Pie chart represent the portion of each quadrant (Q1=CD27+CD57-, Q2=CD27-CD57-, Q3=CD27-CD57+ and Q4=CD27+CD57+). The numbers in individual quadrants represent the means of percentages of corresponding subpopulations. **B)** Same analysis was done for CD4+ cells; CM (orange), EM (grey) and TE (purple).

3.1.2 T cells differentiation is pronounced in CMV+ elderly individuals

Our study is the first to report immune system alterations and T cell skewing towards memory in a Chinese population described before for other populations[21, 44, 48, 104, 176]. These changes are crucial hallmark of immunosenescence and the degree of these alterations strongly correlate with poor responsiveness to primary infections, vaccinations and higher morbidity and mortality[20, 21, 44, 48, 104, 176]. The analysis of data obtained for the elderly

Singaporean cohort indicated an expansion of memory CD8+ T cells(EM 35.9±16.9% and 7.6±5.8%, TE 19.4±14.2% and 12.1.3±9%) on the expense of naive cells (18.7±14% and 56.9±14% in CD8 (**Figure 3-5 A**). Alterations in CD4+ T cells were generally slightly less pronounced bur at least the CM population was affected in the same way (CM 52±12.2% and 36.5±7.8%, EM 19.1±9.5% and 8.6±4.3%, TE 3.5±4% and 0.4±0.7%), also here on the expense of naive cells (25.3±11.7% and 54.4±10% for elderly and young individuals, respectively).The p value for all comparisons was <0.0001.

In order to rule out possible data bias caused from much higher age span (65-90+) we have conducted statistical internal analysis in the elderly cohort, dividing the individuals in for age groups, 65-69y (G1), 70-74y (G2), 75-79y (G3) and 80+y (G4) (**Figure 3-5B**). We have observed only significant decrease of CD8 Naïve cells between G1 group as compared to all other groups (p=0.003, p=0.004, p=0.001 for G2, G3 and G4, respectively). Between these groups we observed significant decrease of CD4 naïve T cells in G1as compared to G3 and G4 (p=0.03 and p=0.01, respectively) and a significant increase of CD4 EM in G1 compared to G2 (p=0.03) and G3 (p=0.01).

Besides these differences, it was evident that the accumulation of age related T cell alterations seems to stagnate after reaching an age of late 60.Within the advanced elderly population, correlation analysis also showed no significant relationship between age and memory T cell expansion. The decrease in naïve cells with age however was significant (r=-0.237, p=0.007, r=-0.257, p=0.004 for CD4 and CD8 respectively) (**Figure 3-5C**). The alterations in naïve T cells pool are probably a consequence of continuous modification of thymic tissue, as the thymic involution is highly pronounced already at age of 65.
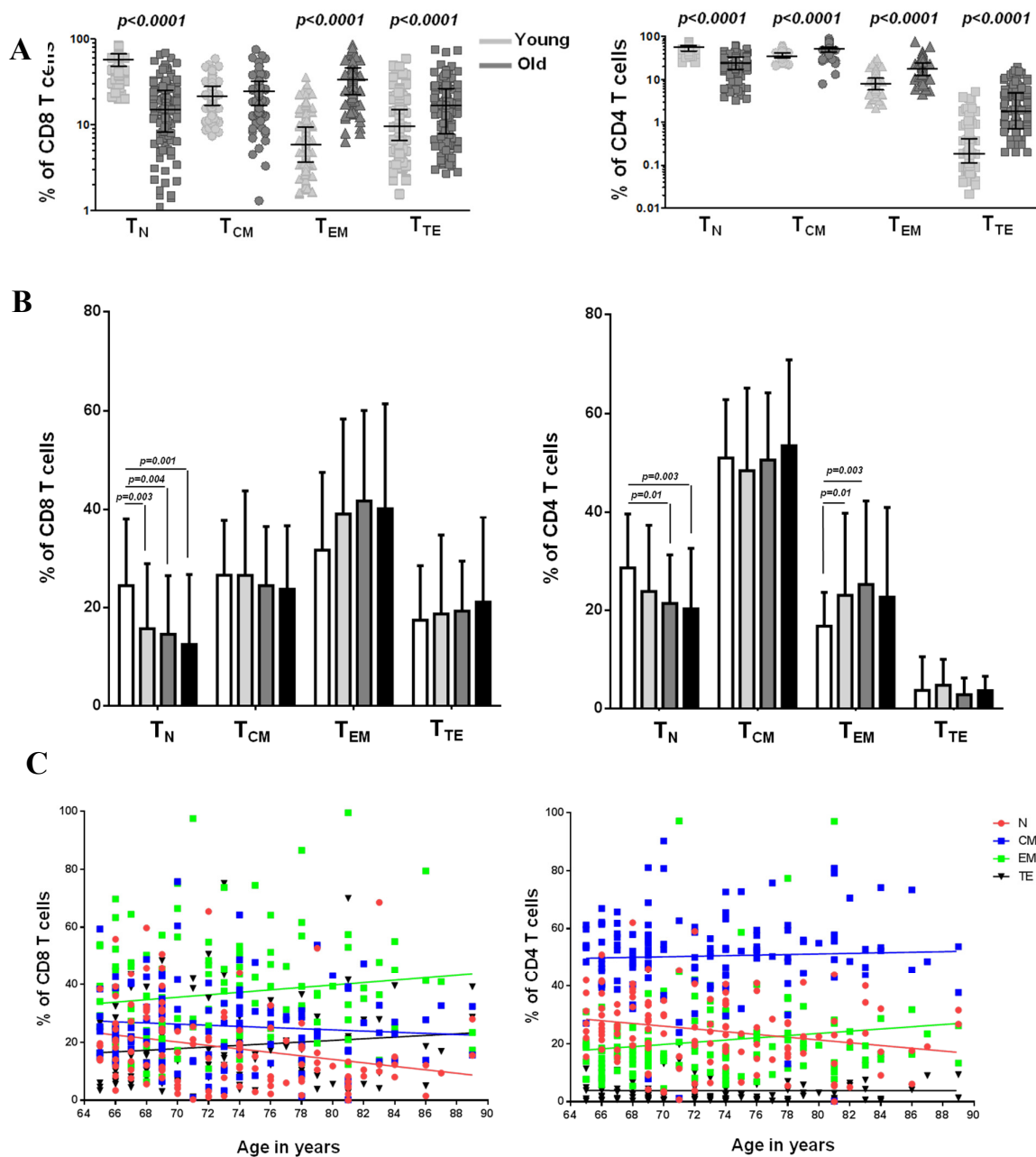
Figure 3-5 **Skewing of T cells towards memory in elderly individuals**

A) T cell populations form elderly (dark grey) were compared to T cells from young individuals (light grey) for CD8 (left graph) and CD4 (right graph). p <0.0001 for all. **B)** Age groups within the elderly cohort were compared. 65-69y (white), 70-74y (light grey), 75-79y (dark grey) and 80+y (black) for CD8+ (left panel) and CD4+ T cells (right panel). **C)** Correlations of the frequency of memory populations N (pink), CM (blue), EM (light green) and TE (black) with age showed no significance (CD8 left graph, CD4 in right graph).

While the incidence of CMV infection in the elderly cohort is very high (>95%), we have been able to identify a small number of CMV- elderly individuals (6 out of 729, mean age=66years, all females). Immunological phenotyping of their T cell populations revealed that especially in the CD8+ T cell population, the immune profile of CMV- elderly seemed very much like the immune profile of young individuals (**Figure 3-6**). Unlike CMV+ elderly, there was significantly lower expansion of CD8 EM (21.1 ± 14.1% and 9.2 ± 5.2%, p=0.0006) and TE (26.5 ± 9.1% and 8.5 ± 2.5%, p<0.0001). On the other hand, CM cells were significantly more expanded than in CMV+ individuals (27.8 ± 8.9% and 36.7 ± 6.8%, p=0.04). For CD4+ T cells we observed significantly lower expansion of EM cells (5.5 ± 4.6% and 1.3 ± 1.1%, p<0.0001) and TE cells(27.8 ± 8.9% and 36.7 ± 6.8%, p<0.0001).

The very distinct immune profiles of CMV+ and CMV- elderly suggest that CMV has a major impact on the expansion of memory T cells but not on the decrease of naïve T cell pool. It is important to note however that elderly people exhibit many additional co-morbidities with age. It is therefore not possible to rule out some confounding effects by other conditions, chronic infections or inflamm-aging on the observed alterations of T cells populations.
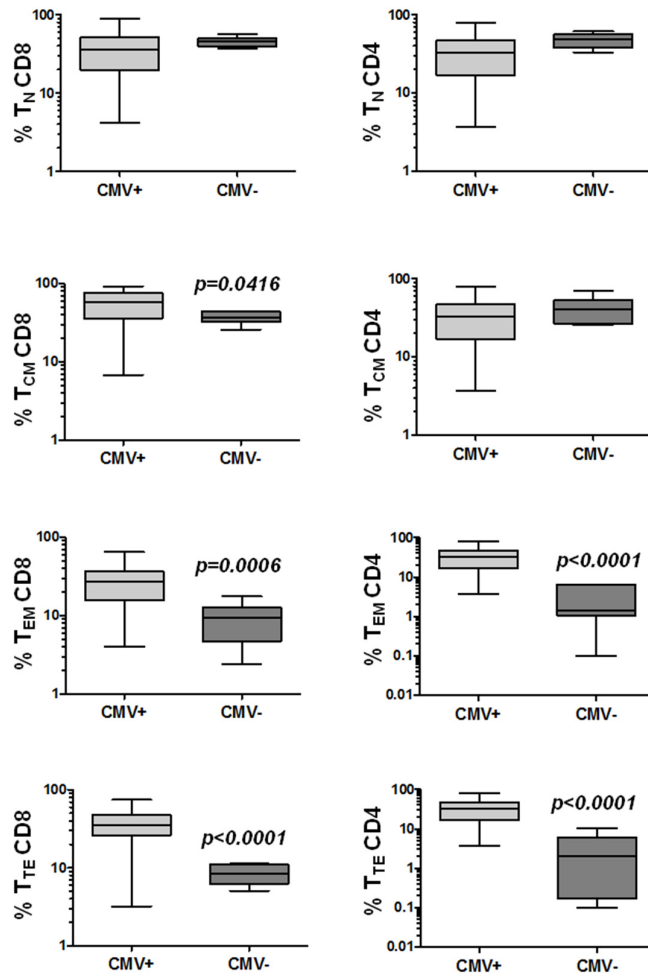
Figure 3-6 **T cells in elderly CMV- individuals**

The frequency of CD8+ (left panel) and CD4+ memory T cell subsets (right panel) from CMV+ (light grey) and CMV- elderly individuals (dark grey) were compared. CD45RO instead of CD45RA was used to define the subsets (N=CD45RO-CD28+, CM=CD45RO+CD28+, EM=CD45RO+CD28- and TE=CD45RO-CD28- for CD8; CD27 instead of CD28 was used for CD4 memory T cells). Due to the altered phenotype we used in this experiment the overall percentage of the Naive cells is lower and percentage of CM cells higher as compared to the values using CD45RA as defining marker because of the marginal overlap of expression of CD45RA and CD45R0 on these cells.

3.1.3 Anti-CMV IgG titre correlates with differentiation profiles of CD8+ and CD4+ EM and TE

So far, the initial phases of CMV primary infection has been described mostly in individuals at risk (organ transplant, HIV infection) but not much is known in healthy settings [177]. It has been postulated that the anti-CMV IgG titer correlates with the course of infection and/or with the viral reactivation [178-182], a notion supported by data on MCMV infection in mice[182]. The CMV-specific IgG titre appears to be influenced both by the time span of latent infection as well as by the initial dose of the virus and possibly also the rate or presence of reactivation. Furthermore the IgG titre has been shown to correlate with morbidity and mortality in elderly.

When we compared the anti-CMV titer in our young cohort, with the frequency of memory T cell subpopulations as well as the loss of CD27, CD28 and CD57 expression on CD4 and CD8,wefound a strong correlation with the CMV titre and the expansion of CD8CD28-, CD8CD27-, CD8CD57+, CD4CD28-, CD4CD27- and CD4CD57+ for both CD8+ and CD4+ T cells (Figure 3-7 **A**).The proportion of T_{TE} in CD4 and CD8 cells positively correlated with anti-CMV IgG titre (r=0.26, p=0.0006 and r=0.47, p<0.0001 respectively) (Figure 3-7 **B**). A similar correlation was observed for CD4T_{EM} (r=0.45,p<0.0001) and CD8 T_{EM} (r=0.34, p<0.0001). Furthermore, the course of CMV infection negatively correlated with naive and CM CD8 T cells (r=-0.29, p<0.0001 and r=-0.22, p=0.0032), suggesting an induced differentiation of memory cells rather than expansion of memory cells only. Thus, as shown before the expanded memory cell in CMV+ individuals displayed a phenotype of more antigen-experienced/replicative-senescent cells, CD4memoryCD28-CD57+(CD4M^{exp}) and CD8memoryCD27-CD57+(CD8M^{exp}).
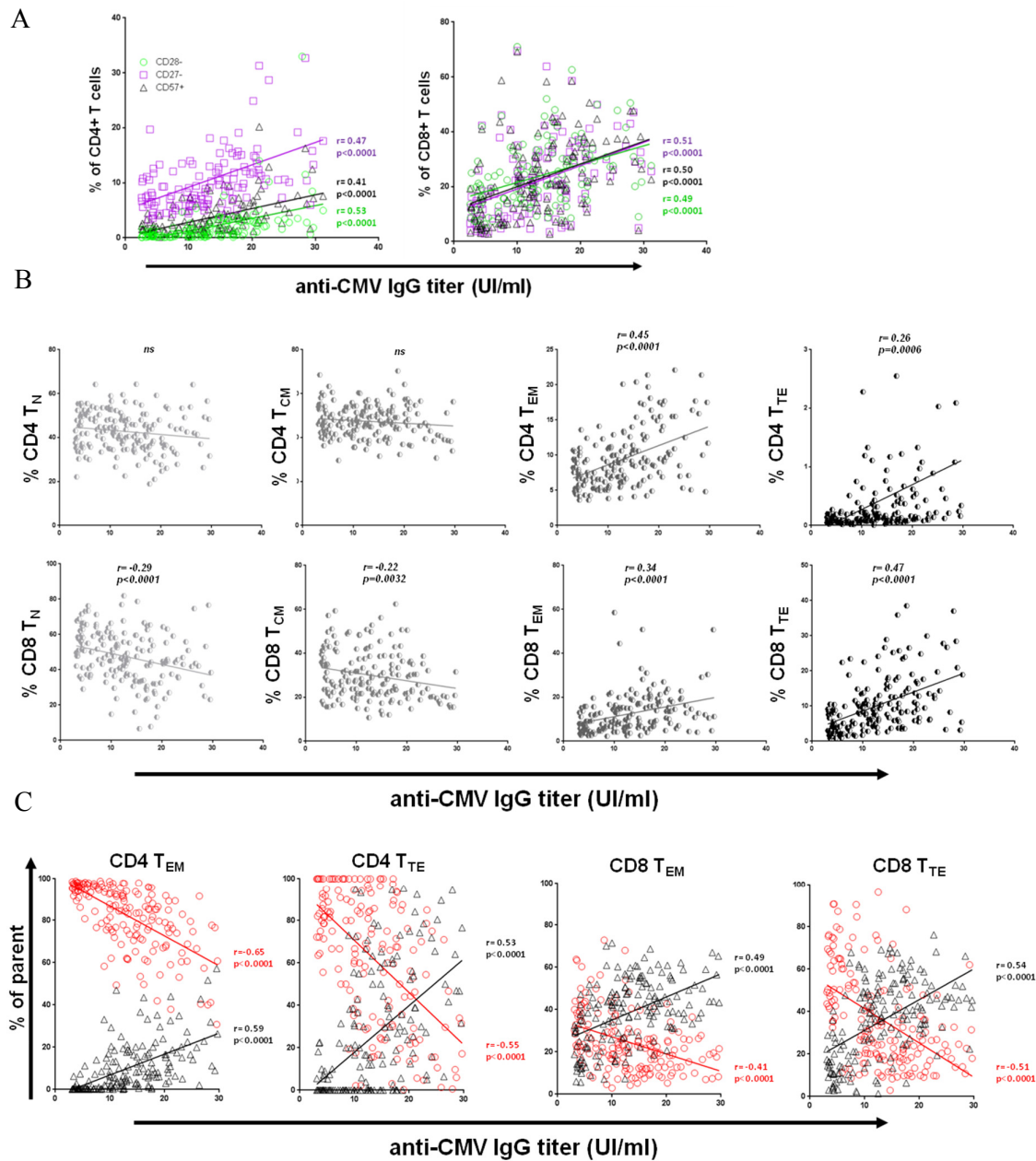
Figure 3-7 **Anti-CMV titre correlation with T cell memory differentiation**

A) Anti-CMV IgG titers from young individuals' plasma (measured by ELISA) were correlated with CD27- (purple), CD28- (green) and CD57+ expression (black) on CD8+ T cells (left panel) and CD4+ T cells (right panel). **B)** Memory T cell subpopulations were correlation with anti-CMV titers of CD4+ (top panel) and CD8+ T cells (bottom panel); from left to right: N->CM->EM->TE. **C)** The differentiation stage CD28+CD57- (red) vs CD28-CD57+ (black) were correlated with anti-CMV IgG titre. From left to right: CD4+ EM, CD4+ TE, CD8+ EM and CD8+ TE.

if the CMV infection is inducing expansion or rather forces differentiation of memory T cells in younger individuals, we also correlated the anti-CMV IgG titer with the frequency of CD4memoryCD28+CD57-(CD4M^{naive}) and CD8CD27+CD57-(CD8M^{naive}) cells. We discovered a strong positive correlation with the percentages of late-stage differentiated memory cells (**Figure 3-7 C**). The strongest correlation was observed for CD4 T$_{EM}$ CD28+CD57- (r=-0.65, p<0.0001), CD4 T$_{EM}$ CD28-CD57+ (r=0.59, p<0.0001) and CD8 T$_{TE}$ CD27+CD57- (r=-0.51, p<0.0001) and CD8 T$_{TE}$ CD27-CD57+ (r=0.54, p<0.0001). This correlation has never previously been tested before and thus represents a novel observation.

3.1.4 Memory cells from CMV+ young individuals and elderly differ in their cytokine production

T cells from elderly individuals were described as having a cytokine dysregulation among other aberrations [183].Elderly people often display low grade chronic systemic inflammation (inflamm-aging), which is manifested by elevated levels of pro-inflammatory cytokines such as TNF-α, IL-6, IL-1, IL-18. The reasons for this are not entirely clear but might be contributed by the higher percentage of circulation T cells secreting TNF-α, IL-6 or IFN-γ. Although the percentage of secreting cells remains similar to young individuals, the alteration in the cytokine profile in elderly also includes significantly decreased capability to secrete IL-2 and TGF-β. Moreover, it has been also shown that in elderly individuals a general shift from Th1 to Th2 cytokines is observed, as significantly higher percentage of their T cells produceIL-13, IL-10 and IL-4. We thus wanted to determine to what extent the secretion ability and relative fraction of cytokine producing T cells is influenced by CMV infection independent of age.

To address this question, we have stimulated sorted CD8 T$_{TE}$ cells from the young and the old cohort for 4h with PMA/Ionomycin and measured 40 cytokines and chemokines from

the supernatant via multiplex technology (Luminex) to cover a broad range of secreted molecules. We were able to detected 18 out of those 40 cytokines (**Figure 3-8 A**).Due to the high sensitivity of the Luminex technology, we could assume that the other 22 cytokines are not to be expressed by the stimulated cells. While the overall profile of CMV+ and CMV-young individuals was very similar, we observed a trend for an enhanced production of IL-17, TNF-α and MIP-1β in CD8 T_{TE} from CMV+ individuals. However, this was significant only for IL-17(p=0.029).

We have therefore conducted another experiment, in which we measured the intracellular cytokine amounts of TNF-α and MIP-1β by FACS (IL17 is difficult to detect by this technique). In this experiment we compared PBMC samples from young CMV- young individuals (n=15), CMV+ young individuals (n=10) and CMV+ elderly individuals (n=13) (**Figure 3-8 B**). While in all groups the production in CD4 T cells was unaffected by infection, a significantly higher percentage of CD8+ T cells produced both TNF-α and MIP-1β when comparing CMV+ elderly with young CMV+(p=0.01 and p<0.0001 for TNF-α respectively) and CMV-individuals (p=0.0063 and p<0.0001 respectively). This observation seemed to be related mainly to the expansion of double positive (TNF-α+MIP-1β+) CD8 T cells. We have also observed a weak trend in the increase of TNF-α+, MIP-1β+ and TNF-α MIP-1β double positive cells in CMV+ individuals' CD8 T cells, but this correlation was not significant.

While the production capacity of individual memory T cell subpopulations seemed unaffected, we observed enhanced capacity of CD4 TE cells from CMV+ elderly to produce MIP-1β as compared to young CMV+(p=0.03) and young CMV-individuals (p=0.002) (**Figure 3-8C**). We therefore further examined the TNF-α and MIP-1β production capacity of early stage memory and late replicative-senescent cells in the CD4 and CD8 compartments (CD4M^{naive}vs. CD4M^{exp}and CD8M^{naive}vs. CD8M^{exp})(**Figure 3-8D**). We have observed that both CD4M^{exp} and CD8M^{exp}were generally more potent in the production of TNF-α and MIP-

1β. This was true for all memory subsets except for the MIP-1β production by CD8EM^{exp} and the TNF-α production by CD4EM^{exp}.

When we examined the amount of cytokine produced by in both CD4 and CD8 in CMV+ and CMV- young individuals via Median Fluorescence Intensity comparison, we have found no significant difference (**Figure 3-8E**). The production of TNF-α was enhanced however, in both in the T cells from elderly individuals. The increase was significant however only for CD4 T cells (p=0.038). MIP-1β production, in turn, was significantly enhanced in CD8 T cells from elderly as compared to young CMV- and CMV+ individuals (p=0.0025 and p=0.0016 respectively).

A

CD8 T$_{TE}$ CMV-

CD8 T$_{TE}$ CMV+

B

CD4+   CD8+

% of MIP-1β T cells

p=0.0063

p<0.0001

% of TNF-α T cells

p=0.01

p<0.0001

% of TNF-α/MIP-1β T cells

p=0.01

p=0.0003

CMV	-	+	+	-	+	+
YOUNG	+	+	-	+	+	-
OLD	-	-	+	-	-	+

C

CD4+ MIP-1β+   *CD4+ TNF-α+*   *CD8+ MIP-1β+*   *CD8+ TNF-α+*

Z-score

○ T$_{CM}$
□ T$_{EM}$
◇ T$_{TE}$

Young CMV-
Young CMV+
Old CMV+

D

% of parent

**** **** **** *** ****

T$_{CM}$ T$_{EM}$ T$_{TE}$ T$_{CM}$ T$_{EM}$ T$_{TE}$
CD4+ MIP-1β+   CD4+ TNF-α+

% of parent

**** **** * * ****

T$_{CM}$ T$_{EM}$ T$_{TE}$ T$_{CM}$ T$_{EM}$ T$_{TE}$
CD8+ MIP-1β+   CD8+ TNF-α+

63
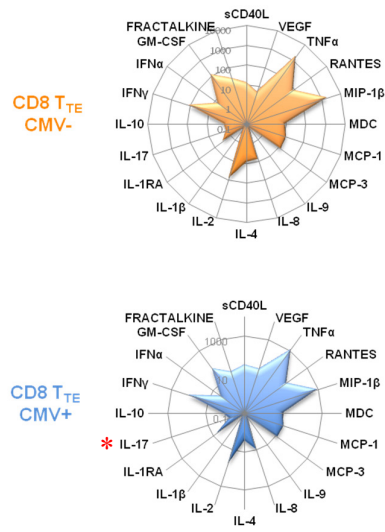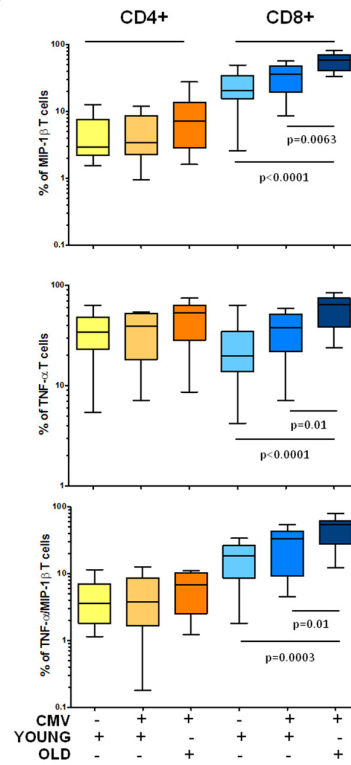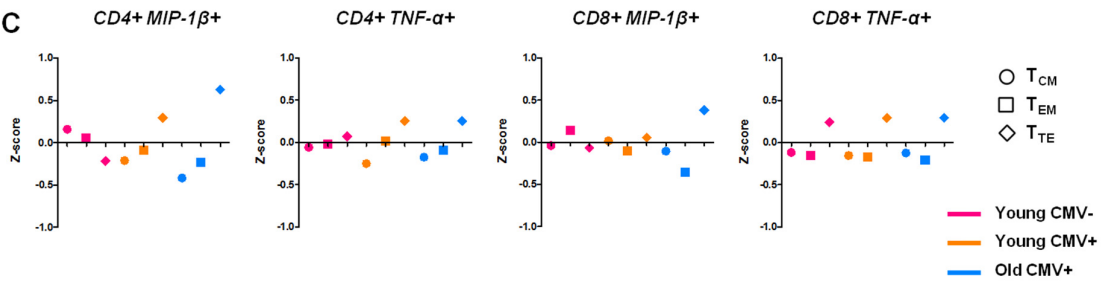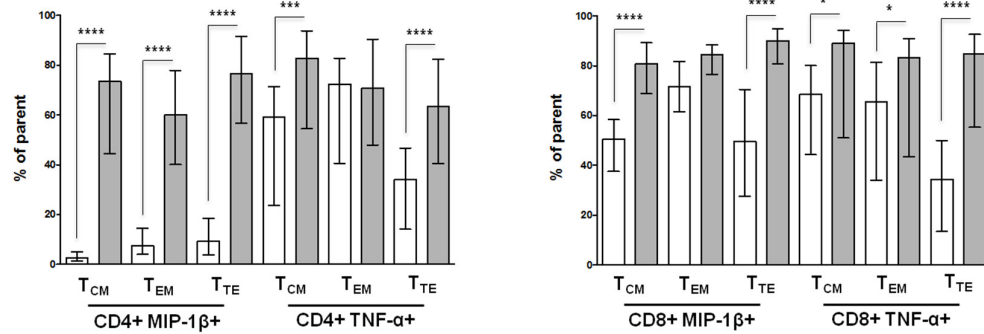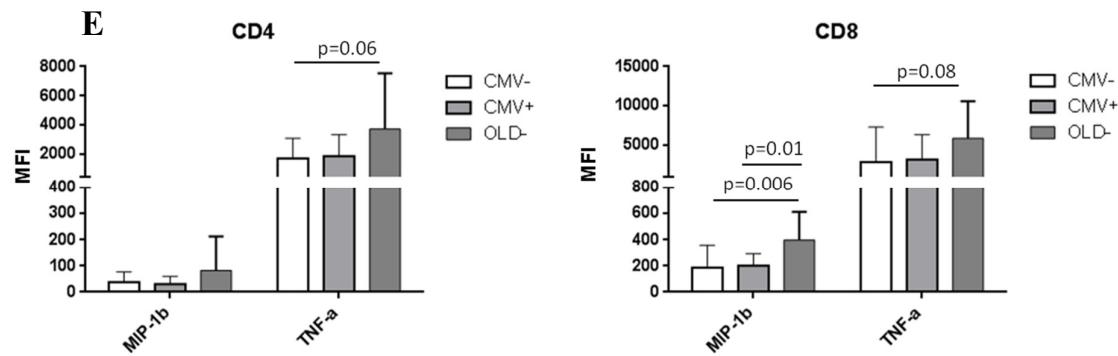
Figure 3-8 Cytokine production by memory T cells

A) Cytokine secretion in supernatant from sorted PMA/Ionomycin-stimulated CD8+TE cells. The cytokines were measured by Luminex from samples of CMV- young individuals (orange) and CMV+ young individuals (blue); * indicates p=0.029. **B)**Percentage of CD4 and CD8 T cells producing MIP-1β (top panel), TNF-α (middle panel) and double producing TNF-α+ MIP-1β+ (bottom panel). CD4+ T cells from young CMV- (yellow), young CMV+ (light orange) and elderly CMV+ (dark orange) and CD8+ T cells from young CMV- (light blue), young CMV+ (dark blue) and elderly CMV+ (denim blue) were analyzed. **C)** Cytokine production capacity of CD4+ and CD8+ CM (circle), EM (square) and TE (rhombus) T cells, was determined by intracellular FACS staining. The analysis covered CD4+ MIP-1β, CD4+ TNF-α, and CD8+ MIP-1β, CD8+ TNF-α. The capacity was compared between cells from young CMV- (pink), young CMV+ (orange) and elderly CMV+ (blue); results shown as a z score, mean of values from CM, EM and TE was normalized as 0. **D)** Cytokine production from differential stages of memory T cells. Early memory stages (white) from CM, EM and TE were compared vs late replicative senescent stages (grey) for CD4+ (CD28+CD57- vs. CD28-CD57+) as well as CD8+ T cells (CD27+CD57- vs CD27-CD57+). *, ***, **** indicate p values <0.05,<0.001, <0.0001, respectively. **E)** Cytokine production capacity (MIP-1β, TNF-α)of CD4+ (left graph) and CD8+ T cells (right graph) from young CMV- (white), young CMV+ (light grey) and elderly CMV+ (dark grey) was compared.

3.1.5 Gene expression in TE from young CMV+ individuals is altered as compared to those from their CMV- counterparts

CD8+ CD28- memory T cells (CD45RA-) isolated from healthy adults display gene expression changes as compared to their CD28+ counterparts [184]. These changes mainly affected genes of co-stimulatory molecules such as ICOS, BTLA, CTLA-4, PD-1, 4-1BB or SLAMF7. CD28- memory T cells also display variations in the expression of NK cell receptors such as KIR and NCR family or CD244. The physiological significance of this phenomenon remains poorly understood but the acquisition of NK receptors may contribute to the lowered threshold of activation of the memory T cells. Other differentially expressed genes include cytolytic molecules such as granzymes B,H and K as well as perforin as well as cytokines, chemokines and their receptors. These include IL-12 and IL-13, MIP-1b, IL-7R or CXCR1. Lastly, differential expression of transcription factor has been shown (TBX21, EOMES or MYC), which might be interlinked to the differential expression of other functional genes described above.

To establish the gene expression alterations in the highly differentiated T cells induced by CMV infection we measured the gene expression of CD8+TE derived from young CMV+ and CMV-individuals (**Figure 3-9A**). mRNA levels of 594 genes were quantified via nanostring technology after 4 hours of PMA/Ionomycin stimulation. Of these, 8 genes were differentially expressed with a p value <0.001. These genes were GNLY, TICAM1, IL18RAP, CD48, IL2RG, IGF2R, CHUK and CD58. Another 107 genes were differentially expressed with a p value <0.05. The fold-enrichment of other senescence and signaling related genes is displayed in **figure 3-9, B** and **C**, respectively.

To delineate the pathways, in which those genes are involved, we conducted an Ingenuity Pathway Analysis (IPA) (**Table 1**).The analysis suggested that the top canonical pathways involved in effector T cell differentiation are RAR activation (p=0.002), glucocorticoid receptor signaling(p=0.005), and various other signaling pathways involved in

cell proliferation, survival, differentiation, migration, and metabolism. We have also identified the prolactin signaling(p=0.03, not in top 10) pathway to be influenced. The IPA analysis also revealed common upstream regulators of differentially expressed genes, such as ULBP1 (p=0.0005),a stress-induced ligand for NKG2D (p=0.002),and IL-15 (p=0.0005), an important cytokine for maintaining memory T cells, as well as CCL5 (p=0.002), an important chemokine recruiting leukocytes into inflammatory sites.

Table 1 **IPA analysis of gene expression from CD8+ TE cells**

Ingenuity Canonical Pathways	p value	Upstream	p-value	Score	Top Diseases and Functions
RAR Activation	1.74E-03	TNFRSF8	1.60E-05	31	Cell-To-Cell Signaling and Interaction, Hematological System Development and Function, Immune Cell Trafficking
Glucocorticoid Receptor Signaling	6.03E-03	ULBP2	7.02E-05	24	Cell Death and Survival, Cellular Compromise, Cellular Development
Neuregulin Signaling	7.08E-03	U1 snRNP	7.02E-05	24	Cell Death and Survival, Gastrointestinal Disease, Hepatic System Disease
Thrombopoietin Signaling	7.08E-03	ID2	1.30E-04	22	Hematological System Development and Function, Tissue Morphology, Cellular Development
Adipogenesis pathway	7.76E-03	ID3	1.76E-04	20	Cell-To-Cell Signaling and Interaction, Hematological System Development and Function, Immune Cell Trafficking
Pyridoxal 5'-phosphate Salvage Pathway	8.71E-03	TCR	2.64E-04	16	Connective Tissue Disorders, Immunological Disease, Inflammatory Disease
Lymphotoxin β Receptor Signaling	1.00E-02	KLRB1	3.52E-04	12	Cellular Function and Maintenance, Hematological System Development and Function, Protein Synthesis
Crosstalk between Dendritic Cells and Natural Killer Cells	1.23E-02	ULBP1	4.08E-04	9	Cellular Movement, Hematological System Development and Function, Immune Cell Trafficking
Growth Hormone Signaling	1.29E-02	IL15	4.35E-04	7	Cell-To-Cell Signaling and Interaction, Cell-mediated Immune Response, Cellular Movement
PI3K/AKT Signaling	1.41E-02	CD3	4.36E-04	6	Cellular Movement, Hematological System Development and Function, Immune Cell Trafficking
Differential Regulation of Cytokine Production in Intestinal Epithelial Cells by IL-17A and IL-17F	1.62E-02	RELB	5.04E-04	3	Cell Cycle, Cell-To-Cell Signaling and Interaction, Cellular Growth and Proliferation
Antioxidant Action of Vitamin C	1.62E-02	CARD11	6.33E-04	3	Cellular Movement, Cell-To-Cell Signaling and Interaction, Immune Cell Trafficking
Regulation of IL-2 Expression in Activated and Anergic T Lymphocytes	1.66E-02	BCL10	6.33E-04	1	Cancer, Cell-To-Cell Signaling and Interaction, Cellular Movement

Table 1. Summary of the IPA analysis of the differentially expressed genes of CD8+ TE cells in CMV+ and CMV- healthy young adults.
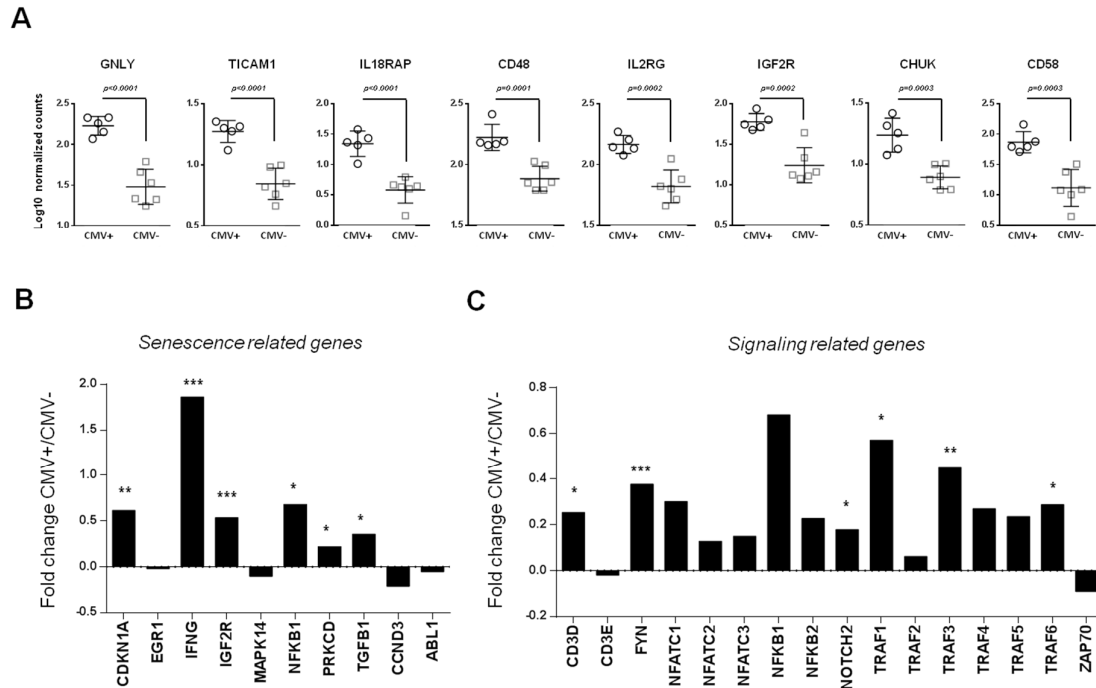
Figure 3-9 **Gene expression influenced by CMV infection in CD8 TE cells**

A) Expression level of the top 8 genes differentially expressed in CD8+ TE cells from CMV+ young individuals (black circles) as compared to their CMV- counterparts (grey squares). **B)** Fold change in expression in CD8+TE of genes related to senescence-development from CMV+ young vs. CMV- young. **C)** Fold change in expression of genes related to signaling in CD8+TE from CMV+ young vs. CMV- young.

3.1.6 Young CMV+ individuals do not display any detectable signs of inflamm-aging

Another phenomenon related to immunosenescence is inflamm-aging, the low grade chronic inflammation in elderly. It is characterized by elevated levels of inflammatory molecules in plasma (IL-6, TNF-a, IL-1, CRP)[185] and is believed to contribute to poor health, accelerated immunosenescence, as well as to the manifestation of medical conditions. We examined 20 cytokines related to inflamm-aging in plasma samples from CMV+ and CMV- young individuals by Luminex (Table 2). However, we observed no elevated levels of the molecules tested in any of the two groups. This observation suggests that inflamm-aging is not induced

by short-term CMV infection but rather needs a longer time of exposure to be manifested if

driven by chronic antigen challenge.

cytokine	CMV			HSV-2			H.pylori			anti-CMV IgG	
	p	-	+	p	-	+	p	-	+	p	r
EGF	0.47	0.62	0.78	0.41	0.73	0.81	0.38	0.86	0.76	0.69	-0.03
Eotaxin	0.86	1.46	1.48	0.47	1.50	1.52	0.07	1.47	1.50	0.90	0.01
FGF-2	0.45	1.01	1.15	0.10	1.11	1.23	0.41	1.25	1.12	0.88	0.01
Flt-3L	0.66	0.44	0.45	0.47	0.46	0.54	0.41	0.55	0.56	0.64	-0.04
Fractalkine	0.32	0.79	0.95	0.16	0.99	1.17	0.72	1.09	1.03	0.41	0.06
G-CSF	0.92	1.32	1.42	0.37	1.40	1.43	0.60	1.46	1.47	0.64	0.04
GM-CSF	0.54	0.65	0.64	0.22	0.66	0.77	0.90	0.76	0.74	0.96	0.00
GRO	0.68	1.65	1.69	0.17	1.68	1.74	0.55	1.72	1.72	0.83	0.02
IL-6	0.62	0.25	0.25	0.01	0.22	0.37	0.27	0.32	0.24	0.84	-0.02
IL-8	0.60	0.37	0.46	0.40	0.42	0.46	0.17	0.47	0.35	0.62	0.04
MIP-1a	0.50	0.47	0.60	0.15	0.51	0.61	0.04	0.63	0.49	0.57	-0.04
MIP-1b	0.98	1.33	1.33	0.01	1.32	1.47	0.13	1.41	1.31	0.26	-0.09
VEGF	0.46	1.47	1.58	0.03	1.54	1.73	0.18	1.63	1.57	0.90	-0.01
sCD40L	0.21	2.49	2.64	0.15	2.60	2.65	0.65	2.56	2.59	0.24	0.09
sEGFR	0.63	4.70	4.83	0.12	4.80	4.85	0.31	4.77	4.82	0.94	-0.01
sIL-1RII	0.54	3.61	3.76	0.57	3.74	3.75	0.19	3.71	3.76	0.37	-0.07
sIL-2Ra	0.78	2.54	2.64	0.96	2.62	2.63	0.72	2.59	2.62	0.20	-0.10
sIL-4R	0.84	3.13	3.24	0.04	3.21	3.24	0.33	3.21	3.23	0.97	0.00
sIL-6R	1.00	4.12	4.25	0.25	4.23	4.27	0.96	4.19	4.24	0.59	-0.04
sRAGE	0.34	1.41	1.48	0.50	1.49	1.49	0.15	1.47	1.54	0.10	0.13
sTNFRI	0.79	2.76	2.86	0.17	2.85	2.89	0.69	2.83	2.86	0.74	0.03
sTNFRII	0.85	3.43	3.56	0.22	3.54	3.59	0.33	3.51	3.55	0.75	0.02
sVEGFR2	0.43	3.95	4.06	0.32	4.04	4.07	0.83	4.01	4.05	0.99	0.00
sVEGFR3	0.35	1.72	2.14	0.27	1.96	2.06	0.91	2.16	2.06	0.11	-0.12
sgp130	0.71	5.01	5.17	0.63	5.14	5.17	0.82	5.11	5.16	0.97	0.00

Table 2 **Cytokines in plasma samples from young individuals.** Levels of plasma cytokines from young individuals seropositive and seronegative for CMV, HSV-2 and H.pylori are shown. Pathogen-specific antibodies were measured by luminex. Anti-CMV IgG titers, determined by ELISA, were correlated with the levels of the cytokines.

3.1.7 HSV-2 and Helicobater Pylori do not exhibit any visible influence on T cell memory populations

It is known that persistent stimulation during chronic infections contribute to the

expansion of memory and highly differentiated T cells. It is thus possible that also chronic

infections other than CMV might induce similar alterations of T cells. Such infections may

include HIV, EBV or HCV. However, as these infections are either rare in Singapore (HIV,

HCV), or present with the prevalence is almost 100% (EBV),we could not study them in our

large cohorts of young individuals. To assess the impact of persistent non-CMV infections, we therefore selected two other chronic human pathogens, *H. pylori* and Herpes-Simplex virus 2 (HSV2). *H.pylori* is a G- bacteria colonizing mucus layer in the stomach. The prevalence is approximately 50% in Singapore and it is a chronic life-long infection that has been associated with inflamm-aging. In contrast to HSV-1, which has a similarly high prevalence as EBV in young individuals,HSV-2is detected in approximately 20% of the young Singaporeans. It is a virus from the same family as CMV (Herpesviridae), which establishes life-latent infection.

For this study, we established the serologic state of the two pathogens in all individuals of our young cohort by ELISA and examined same aspects of T cells populations related to immunosenescence as we did with CMV. However, in contrast to CMV we observed no changes in the frequency of CD27- or CD28- or CD57+ T cells within both CD4 and CD8 T cells (**Figure 3-10A, B**). We further compared memory development stages and also found no differences in percentages within any of the four main stages (N, CM, EM, TE) (Figure 3-10**C, D**).We further analyzed both anti-HP and anti-HSV-2 IgG titres and found no correlation between the antibody levels and percentages of late EM and TE as shown before for the anti-CMV IgG titres. Moreover, in contrast to CMV also none of cytokines measured in plasma by luminex was found to be elevated in HP+ nor HSV-2+individuals (data not shown). Considering that HSV-2 virus evolved from the same viral ancestor as CMV, these observations, suggest that CMV exhibits a unique driving force on the T cell differentiation leading to immunosenescence.
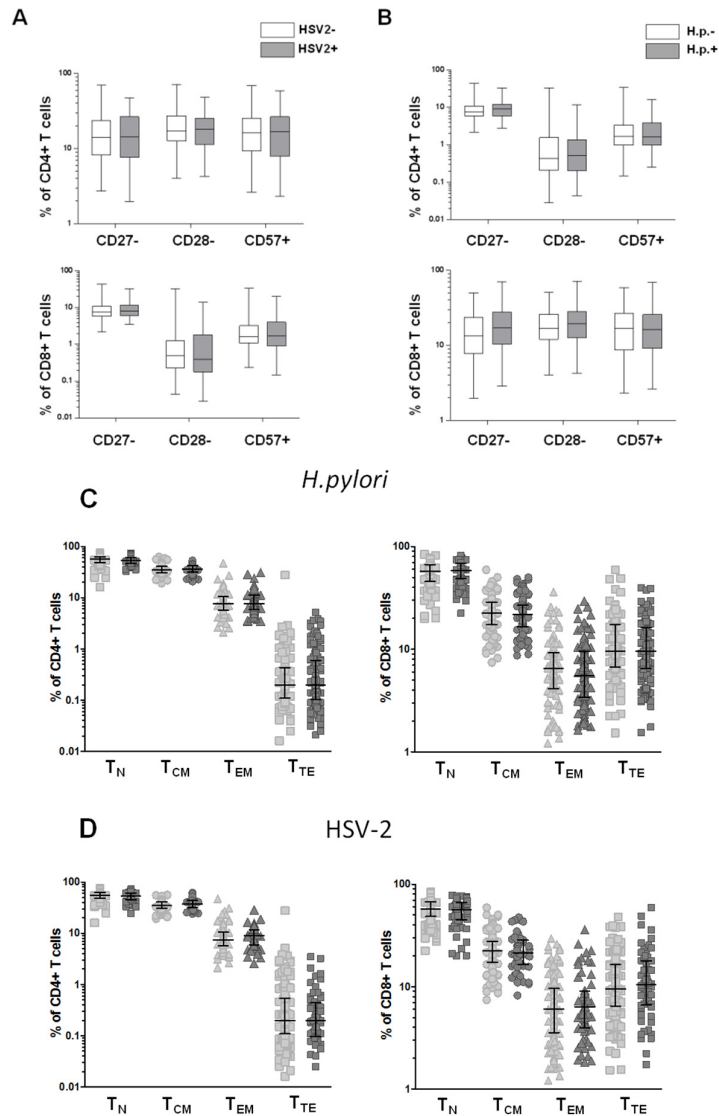
Figure 3-10 **Influence of H.Pylori and HSV-2 on T cell differentiation**

The same FACS measurement shown in Figure 3-1was used. **A)** Percentage of CD27-, CD28-CD57+ T cells of CD4+ lineage (top panel) and CD8+ lineage (bottom panel) were compared between HSV-2+ (white)and HSV-2- young healthy individuals (grey). **B)** Percentage in *H.pylori*+(white) and *H.pylori*+ individuals (grey). **C)**Percentage of memory cell populations in *H.Pylori*+ (dark grey) and *H.pylori*- young individuals (light grey). **D)** Percentage in HSV-2+ (dark grey) and *HSV-2*- young individuals (light grey).

3.1.8 Deep phenotyping of surface markers reveals the influence of age versus CMV on memory T cells development

As described previously in this thesis, the individual memory subsets differ in the expression of numerous surface molecules related to main cellular functions and properties. These include chemokine receptors, altering the chemotactic properties of the cells, adhesion molecules, which alter the cell-cell contact as well as vascularization, tissue migration and other pathways. For a comprehensive analysis we measured the expression of 332 CD markers on the surface of different memory T cell populations from both CD4 and CD8 T cells by deep phenotyping. Samples from young CMV+ and CMV- and elderly CMV+ individuals were barcoded and analyzed by FACS using the LegendScreen Human Cell Screening kit (described in Materials and Methods). After marker filtering, we have found 92markers to be expressed on T cells (**Table 3**).

Table 3 Surface marker expression detected on T cells

CD2	CD35	CD62L	CD146	DEC-205	HLA-DR	CD49d	CD132	CD140b	DNAM-1
CD6	CD39	CD66a/c/e	CD161	IL18Rα	NKp80	CD49e	CD96	CD143	Ly-9
CD7	CD41	CD69	SIRPγ	CD243	TCR Vα7.2	CD49f	CD97	CD148	CD277
CD8	CD49a	CD71	CD183	2B4	CD26	CD58	CD99	SLAM	ICOS
CD9	CD49f	CD73	CXCR4	NKG2D	CD29	CD59	BB27	ADAM-10	PD-1
CD11b	CD54	CD93	CCR5	CRACC	CD31	CD63	CD102	CD164	CD317
CD25	CD55	CD94	CD196	CX3CR1	CD38	CD82	IL-6Rα	CXCR1	NTB-A
CD27	CD57	CD95	CCR7	CXCR7	CD45RA	CD84	IL-7Rα	CCR3	CCR10
CD28	CD61	IFN-γRα	CD200R	FcRL6	CD45RO	CD100	OX40	IL-10R	Integrin β7
								IL-15Rα	LAP

Even though this data was obtained via a screening tool, we have observed the expression of some markers, which have never before shown to be expressed on T cells. The differences in the percentages of cells expressing a certain markers suggest an expansion of subpopulations of known memory populations. The MFI values of positive cells refer to the amount of marker expressed, which could lead to altered functions. Changes of expression of certain molecules was related to aging, if the alteration occurs in T cells from elderly only.

Changes related to CMV infection where identified as similar alterations in T cells from young (Y) and old O_ CMV+ positive individuals, while differential to cells from Y CMV-. In addition, some expression alterations were probably influenced by both CMV and age as the decrease or increase followed Y^{NEG} -> Y^{POS} ->O^{NEG}.

We were mainly interested in the CD4 EM and CD8 EM and TE cells as those display significant differences in differentiation between CMV+ and CMV- young people. To investigate the relationship of T cell differentiation through aging and CMV, we have conducted a Spearman clustering in the R software comparing the percentages of positive cells. To display the magnitude of expansion of positive cells we have compared the relative changes of expression among the 3 populations based on calculating y= $\left(\frac{x1}{\min(x)}, \frac{x2}{\min(x)} ... \frac{xn}{\min(x)}\right)$.

We have observed that CD8 TE cells from young CMV + individuals do cluster together with CD8 TE from elderly (Figure 3-11**A**). CD4 EM from CMV+ have altered expression as compared to their CMV- derived counterparts, while the CD4 EM from elderly were phenotypically more distant. (**Figure 3-11A**). When we further investigated the amount of each CD marker expressed on positive cells (via MFI) we observed same pattern (**Figure 3-11B**). The CD8 TE cells therefore appear to be more influenced by CMV infection than aging.CD4 EM, despite being altered by CMV, are less affected by the infection.
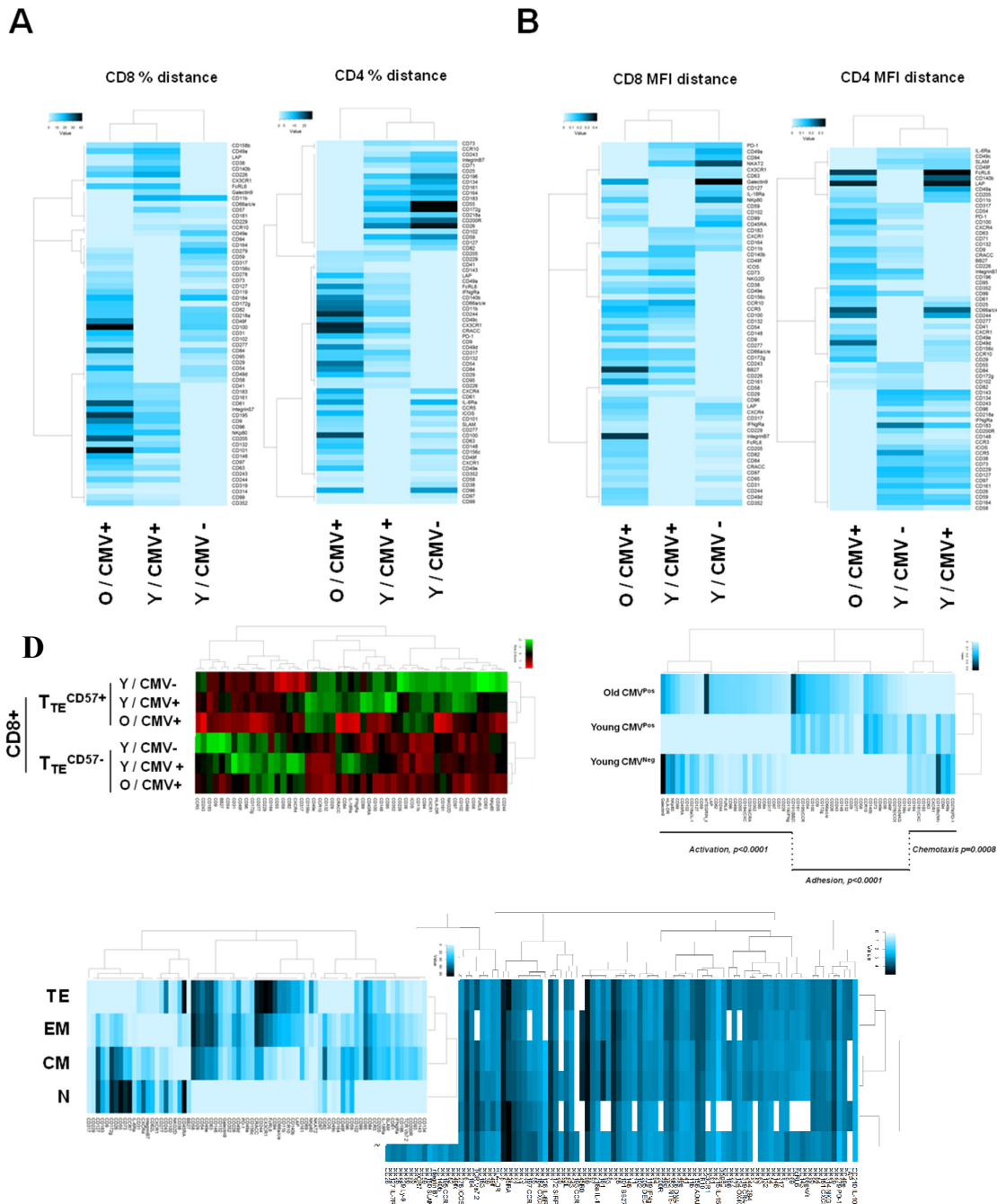
Figure 3-11 **Differential expression of 92CD markers on memory T cells**
15 samples from each young CMV+, CMV- and elderly were thawed from cryopreserved bank, tagged and stained with fluorescent antibodies to be subsequently utilized for the Human Screening kit (M&M). Spearmann clustering method was conducted via R software to measure distance of % **(A)** calculated as y= $\left(\frac{x1}{\min(x)}, \frac{x2}{\min(x)} ... \frac{xn}{\min(x)} \right)$ and distance of MFI **(B)** for CD8 TE and CD4 EM (right and left graphs respectively) from CMV+ and CMV- young individuals and elderly. **C)** Same analysis comparing CD57+ and CD57- CD8 TE from CMV+ and CMV- young individuals and elderly. Functional analysis done by String software reveal putative functional decrease in CD8 TE from the three populations **(D).** As a control of clustering method the expected heatmap viewing gradual development from N->CM->EM->TE was created **(E).** Similar observation was made when novel SCM and TM memory populations were added to the analysis**(F)**.

Interestingly we have found that CD57defines a very different phenotype of CD8 T_{TE} cells, as CD57+ and CD57- CD8 T_{TE} displayed major differences in the surface markers expression regardless of the population they were derived from. Furthermore, it is noteworthy that when the CD8 T_{TE} cells were subdivided according to their CD57 expression, the cells derived from CMV+ young and old have a closer relationship in phenotypes than CD8 T_{TE} cells derived from CMV- young (**Figure 3-11 C**). Among the differentially expressed markers we have identified numerous surface molecules related to adhesion, activation, migration and chemotaxis. Interestingly, each population of CD8 T_{TE} had a particular cluster of CD molecules on its surface relating to a particular function. We have found that T_{TE} from elderly had the lowest expression of CD markers related to chemotaxis, T_{TE} from CMV+ molecules related to activation, while T_{TE} from CMV- had the lowest expression of markers related to adhesion (**Figure 3-11 D**).

As a control for our clustering method we compared the CD8 N, CM, EM and TE and observed expected results: N+CM and highly differentiated EM+TE formed two separate clusters confirming the closer relationship of N and CM versus EM and TE (**Figure 3-11 E**). It is interesting to note that some of the proteins with enhanced expression (shown in our Nanostring data) were also expressed in higher levels on the surface of those cells. These were CD132, CD244, CCR5 or CD58.

The same was true for the extended memory populations N-SCM-CM-TM-EM-TE (**Figure 3-11 F**). These populations have been described in CD8 but not in CD4 [90]. We have therefore used same method to compare the possible differentiation path as well as comparison among different subpopulations, classical defined memory T cells or using differential marker to define the memory T cells. Our results suggest that there is linear development of these cells or, if reversible differentiation occurs, the cells obtain back their feature of previous stage.

While the measurement of more than 300 surface markers brings some inside into the process of induced T cell differentiation, the limitations are that each markers was measured as single expression. Therefore, specific marker combinations characterizing these subpopulations cannot be detected.

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3.1.8.1 The relationship of CD57 and surface marker expression

The depth of surface marker expression shown above has never been investigated in the context of aging. Therefore, it is unclear how much of difference/heterogeneity for instance confounds the potential use of the CD57 marker as memory differentiation marker[186]. We show that the expression of CD57 on CD8 TE displays is linked to unexpectedly large differences in many other markers (Figure 3-12 **A,B**)
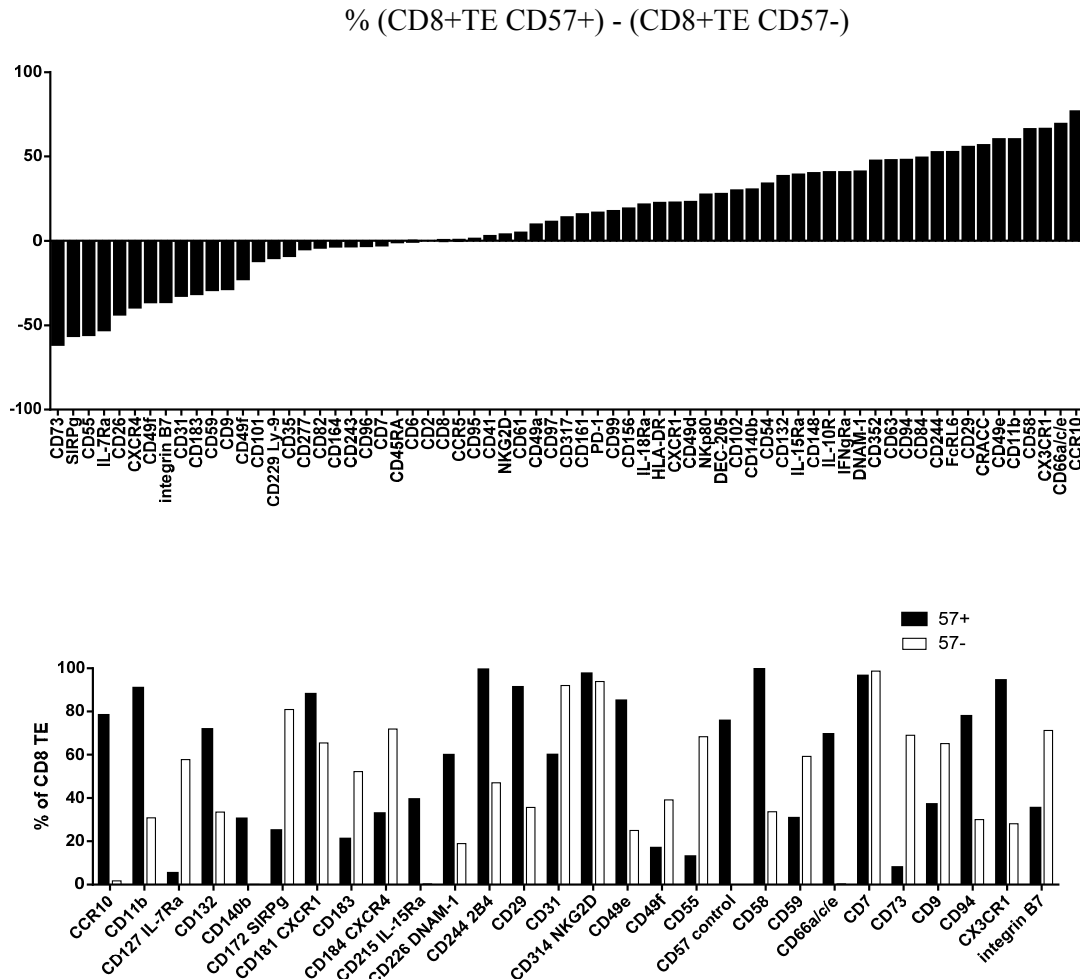
Figure 3-12 **CD57 expression depicts vastly differential expression of CD marker on CD8 TE cells**

A) Percentage of expression of all detected surface markers was compared as the deduction of CD8 TE CD57+ minus CD8 TE CD57-. Negative values represent markers, where the expression is higher on CD57- cells while positive values indicate enhanced expression on CD57+ cells. **B)** Frequency of CD8 TE cells expressing the indicated marker. Only significant association are depicted(p<0.05).

We looked at the relationship of markers differentially expressed between CD57+ and CD57- CD8TE and compared them to markers, which were clearly increasing/decreasing upon development of memory T cells (N->CM->EM->TE) (Figure 3-13 **A**).We identified 2 markers that were related to N/CM cells, which were significantly higher expressed on CD8TE CD57- cells (IL-7Ra and CXCR4). Vice versa, we found 8 markers increased significantly on CD57+

counterparts (CD54, CD63, CD94, CD244, FcRL6, CRACC, CD58 and CX3CR1) (Figure 3-13 **B**). This observation suggests that the CD57 expression not only marks the replicative senescence but significantly more pronounced differentiation in terms of memory.
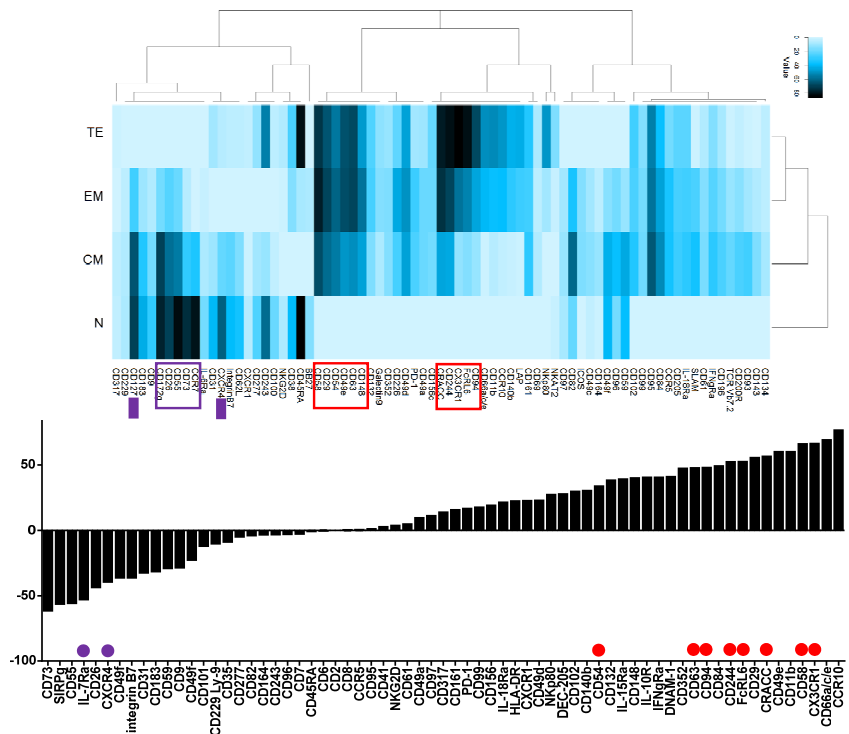
Figure 3-13 **CD57 and memory differentiation markers relationship**
CD markers which are closely related to either early (purple) or late (red) stages of memory differentiation **(A)** (as visualized by clustering heatmap) were compared to those highly differentially expressed in CD8+ TE CD57+ and CD57- **(B).**

3.1.9 Higher oxidative mitochondrial state in CD57+ memory T cells

Mitochondria are the energy generating power plants of all cells. We recognize 2 major metabolic pathways utilizing glucose, oxidative phosphorylation (OXPHOS) and glycolysis. OXPHOS is significantly more (approximately 16 times) efficient than glycolysis in ATP production, however is slower and requires oxygen. It has been shown that activated T cells switch their metabolism to glycolysis. Furthermore, in memory T cells it has been shown that

they have higher glycolytic capacity, which allows them to act very quickly upon stimulation both via executing effector functions and proliferating. The metabolic switch has however few negative impacts. Firstly, T cells utilizing only glycolysis are in a very high demand of glucose and it has been hypothesized that this might be the reason why malnutrition people have weakened immune system. Furthermore, glycolysis creates more ROS. This is favorable for inflammation and for oxidative burst in macrophages or neutrophils, but may be detrimental to the cell where increased ROS cause accumulation of damage and cell aging. This phenomenon has been hypothesized to be one of the cause of aging.

Highly metabolizing cells might contribute to tissue damage and enhanced turnover, which results in deterioration of function and might cause replicative senescence of surrounding somatic cells or tissue specific stem cells. Recently it has been shown that terminally differentiated T cells have higher ROS levels in their mitochondria as compared to other memory cells. The ROS has been shown to cause DNA damage [187]. Furthermore ROS can be actually responsible for senescence itself, so is the metabolic shift the cause of senescence [88].

We therefore measured the oxidative state of mitochondria in N, CM, EM and TE cells via staining with mitochondria sequestering probe MitoRedx. This probe is readily sequestered by mitochondria where it gets oxidized, which triggers its fluorescence. Therefore, the fluorescent signal reflects the oxidation state of the mitochondria and possibly reflects the shift from OXPHOS to glycolysis. We could show that different memory stages have different oxidative state. However when comparing young CMV+, young CMV- and elderly CMV+ we observed no significant differences in the oxidative status (Figure 3-14**A**).

We then looked at the CD8+TE cells expressing CD57 and observed that these were displaying significantly higher oxidative state in mitochondria as compared to their CD57-counterparts (Figure 3-14 **B**). These results show that CD57+ memory cells produce higher

amount of ROS and therefore might contribute to the oxidative damage of their surroundings. The accumulation of CD57+ cells therefore may be a positive loop for cell turnover and supports the theory that the accumulation of ROS related tissue damage being a cause of aging.

The higher oxidation state might be a consequence of high glycolytic metabolism. It was also shown that highly differentiated or senescent T cells have dysfunctional mitochondria, which are inefficient in energy productions[188]. It is therefore possible that the contribution of ROS-related damage caused by highly differentiated memory cells is a consequence of functional adaptation of being predisposed with high glycolytic activity to be able to act quickly and execute their effector functions. These findings are supported by a recent work of Akbar et al[88], where they show that senescent CD4 CD27-CD28- cells have higher ROS levels than their non-differentiated counterparts. Interestingly, in young individuals we observe that it is only the TE cells, which display high oxidative state in mitochondria, while conversely, in elderly all memory cells have higher OMS which is comparable among CM EM and TE. This observation suggest that cellular aging occurs upon memory differentiation but with advancing time the cellular aging affects even less differentiated cells.
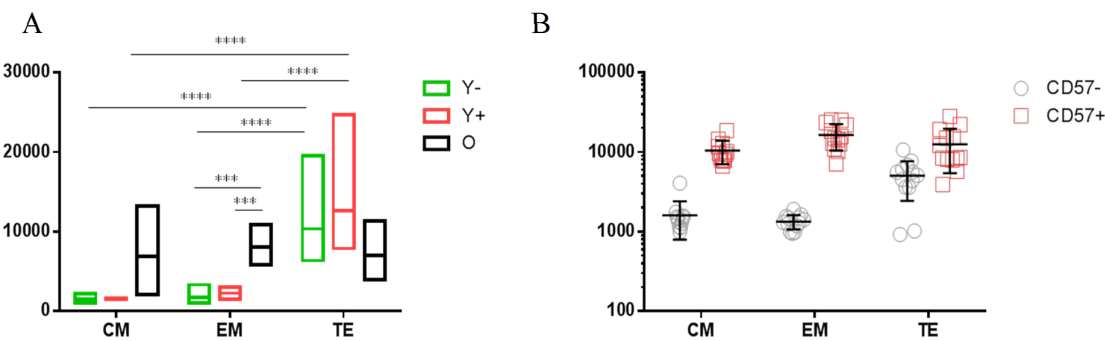
Figure 3-14 **mitochondrial oxidative stress in memory T cells**
A.)The level of oxidation of mitochondria was measured as the MFI signal of reduced Mitotracker probe. The values were compared from young CMV- (green), young CMV+ (red) and elderly CMV+ individuals (black). CM, EM and TE cells very compared. ***p<0.001, ****p<0.0001. **B**.) The mitochondrial oxidative state was compared between CD57+ (red) and CD57 – (grey) CM, EM and TE cells .

3.2 Mouse studies

TheCMV-related immune-senescence studies in human were followed up by studies in the mice. For this, the T cell phenotypes were traced over a time course of 4 months in NSG mice reconstituted with CD34+ human stem cells and CMV-infected human fibroblasts. After that period mice were sacrificed to harvested organs and isolate gDNA. Nested PCR reaction was employed to confirm a successful CMV infection.

3.2.1 Infection of human fibroblasts

CMV is a human virus. To introduce the virus into humanized NSG mice human foreskin fibroblasts (HFF) cells had firstly to be infect with CMV. A successful infection was detected via fluorescent microscopy, since the virus used for the experiments had the capsid protein UL32 fused with eGFP (**Figure 3-15).**
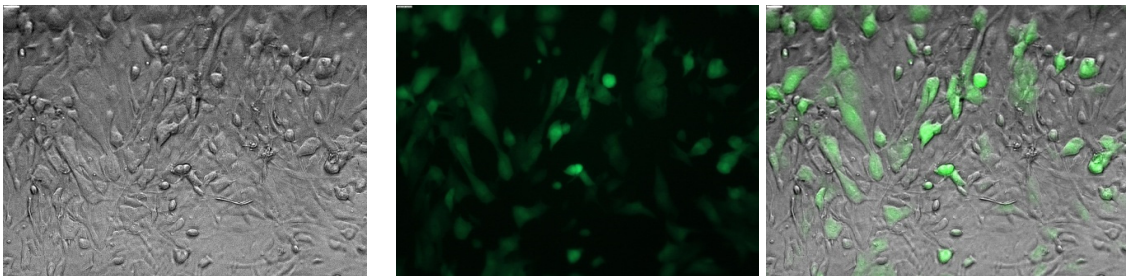
Figure 3-15 **CMV infection of HFF**

The microscope images of HFF infected with CMV show display the cellular pathology and detachment (left),the production of CMV particles, measured by GFP signal (middle) as well as an overlay of the two images (right).

3.2.2 Infection of NSG humanized mice

Since the viral particles are not detectable in blood, the confirmation of successful infection of the reconstituted NSG mice could only be done by detecting viral DNA in the organs (liver). This was done via nested PCR with DNA extracted from the mouse liver (**Figure 3-16**). Successful infection was indicated here by the appearance of a 332 bp fragment.
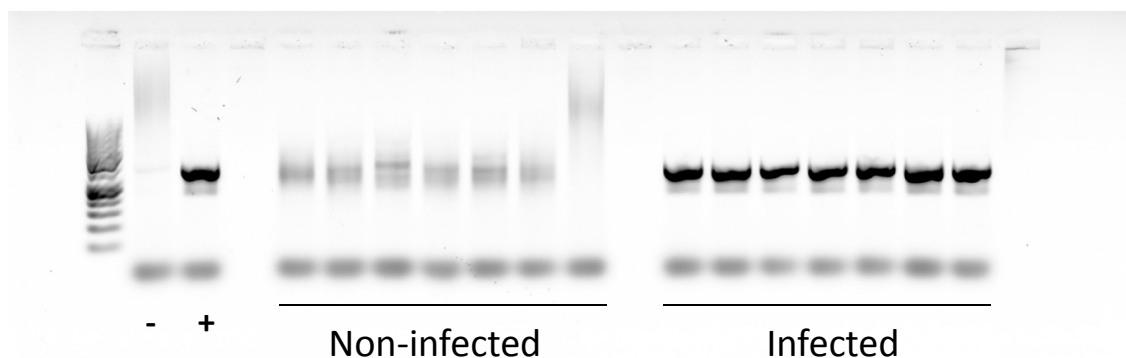
Figure 3-16 **Nested PCR of CMV DNA**
A gel separation of liver extracts of 7 infected and 7 non-infected mice is shown. The 50 bp DNA ladder was used to track the length of PCR products (CMV product was 332bp). Lanes for the negative control (-) and positive control of CMV gDNA (+) are indicated.

3.2.3 Primary CMV infection and memory inflation

The purpose of the mouse model was to mimic the primary CMV infection in humans, where the chronic CMV infection is accompanied with the expansion of differentiated memory T cells. To simulate primary CMV infection we peritoneally injected mice with CMV infected HFF or with HFF only as a control. After the injection, we followed the change in the T cell populations in blood via flow cytometry for 4 months.

Prior to the transfer of HFF, we confirmed that no significant differences existed between the 2 groups. The baseline was measured 1 week after the transfer of mice into the

non-pathogen free facility and we followed the mice roughly every 2 weeks from Primary infection starting at one week post-infection(P.I.) to observe any putative inflammation or excessive cellular expansion (Figure 3-17). Three weeks P.I. we observed significant expansion of CD8+CD28- (p=0.029) and CD8 TE cells (p=0.042) in CMV infected but not in non-infected mice. Ten weeks P.I. we observed significant decrease (approximately 2 fold) of human CD45+ cells in infected mice (p=0.01). At week 13 P.I. we observed significantly higher percentages of both CD8+ and CD4+ TE (p=0.04 and p=0.035 respectively). Lastly at week 16, the time point of sacrifice, we observed significant decrease of human CD45+ cells in CMV+ mice (p=0.02), increase of CD4+CD27- T cells (p=0.037), increase of CD8+CD57+ cells (p=0.039) and decrease of CD4+CM cells (p=0.017).

In **Figure 3-18** we show in more detail the last time point 4 months P.I. We have observed a general skewing towards memory and more differentiated cells in the infected mice, but only the above mentioned alterations were actually significant. A longer follow up of the mice might be needed to show more pronounced CMV-induced T cell differentiation and memory T cell inflation.
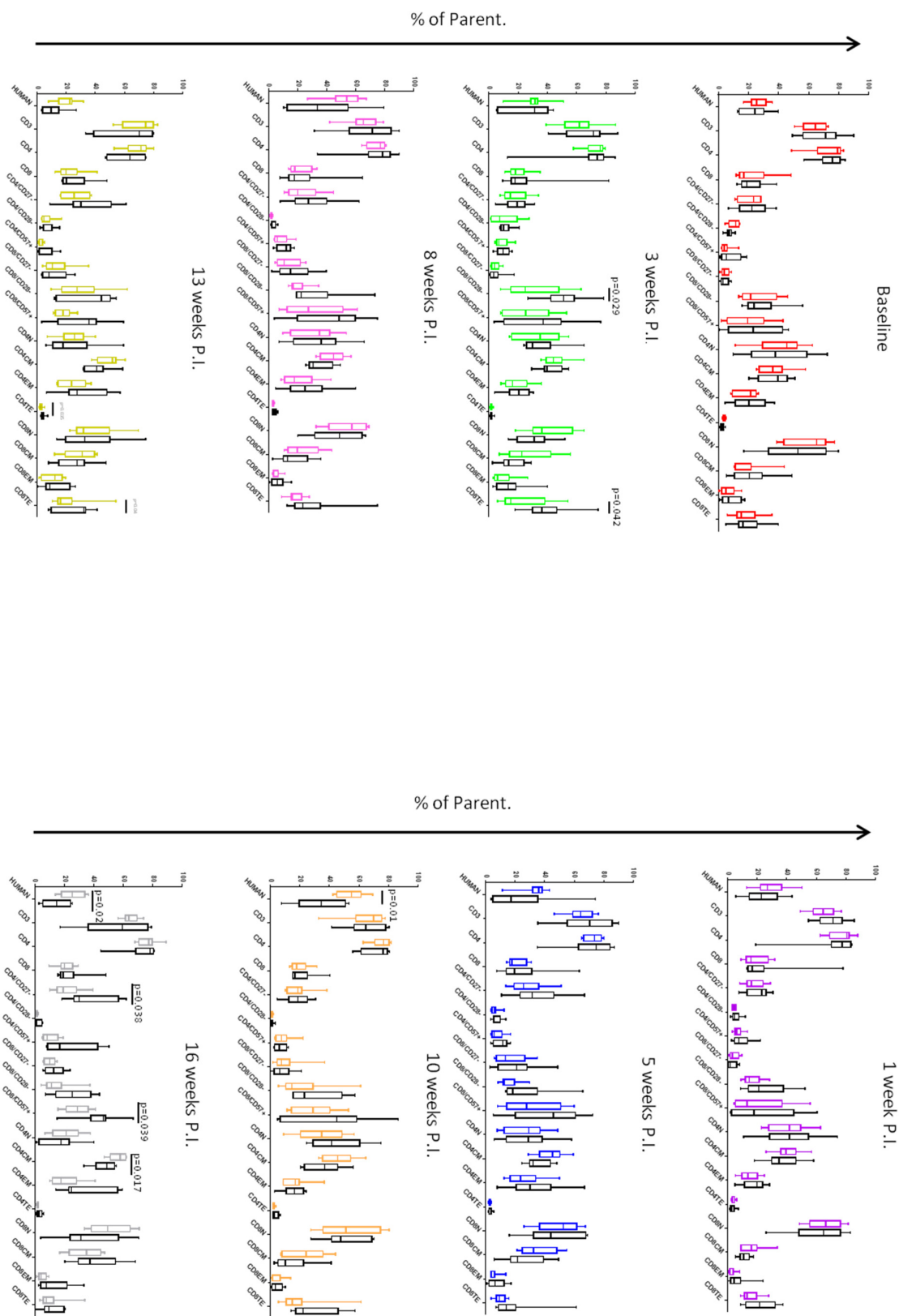
Figure 3-17 **comparison of T cell subpopulations from HFF-CMV and HFF mock infected mice**

We have phenotyped the blood cells from reconstituted humanized NGS mice before primary infection as baseline (CMV- as red) and subsequently at 1(purple), 3 (green), 5 (blue), 8 (pink), 10 (orange), 13 (yellow) and 16 weeks P.I.(grey). To phenotype the cell we used classical T cell memory markers CD3, CD4, CD8, CD27, CD28, CD45RA and CD57. We measured the percentage of human CD45+ cells, CD3+ lymphocytes, CD4+ and CD8+ T cells, CD27-, CD28- and CD57+ cells for both CD4 and CD8 N, CM, EM and TE. Three weeks P.I. we observed significant expansion of CD8+CD28- (p=0.029) and CD8 TE cells (p=0.042) in CMV infected mice. Ten weeks P.I. we observed significant decrease (approximately 2 fold) of human CD45+ cells in infected mice (p=0.01). At week 13 P.I. we observed significantly higher percentages of both CD8+ and CD4+ TE (p=0.04 and p=0.035 respectively). Lastly at week 16, the time point of sacrifice, we observed significant decrease of human CD45+ cells in CMV+ mice (p=0.02), an increase of CD4+CD27- T cells (p=0.037), an increase of CD8+CD57+ cells (p=0.039) and a decrease of CD4+CM cells (p=0.017).

3.2.4 The onset of memory T cell inflation after primary CMV infection

We have compared the memory T cell populations development at all time-points. T cells from both the infected and non-infected mice followed very similar pattern of memory differentiation (**figure 3-19**). It is interesting to note that at certain time points there was a major shift in certain population, such as the strong increase of CD4+CD28- cells at 5 weeks P.I. and then subsequent massive drop in the same population 2 weeks later. This observation might reflect the expansion and activation of effector T cell, as these temporarily loose the expression of CD28 co-stimulatory molecule. We have also observed variability between individual mice and this variability seemed to be more pronounced in CMV infected mice, which strongly compounded the analysis of the data.

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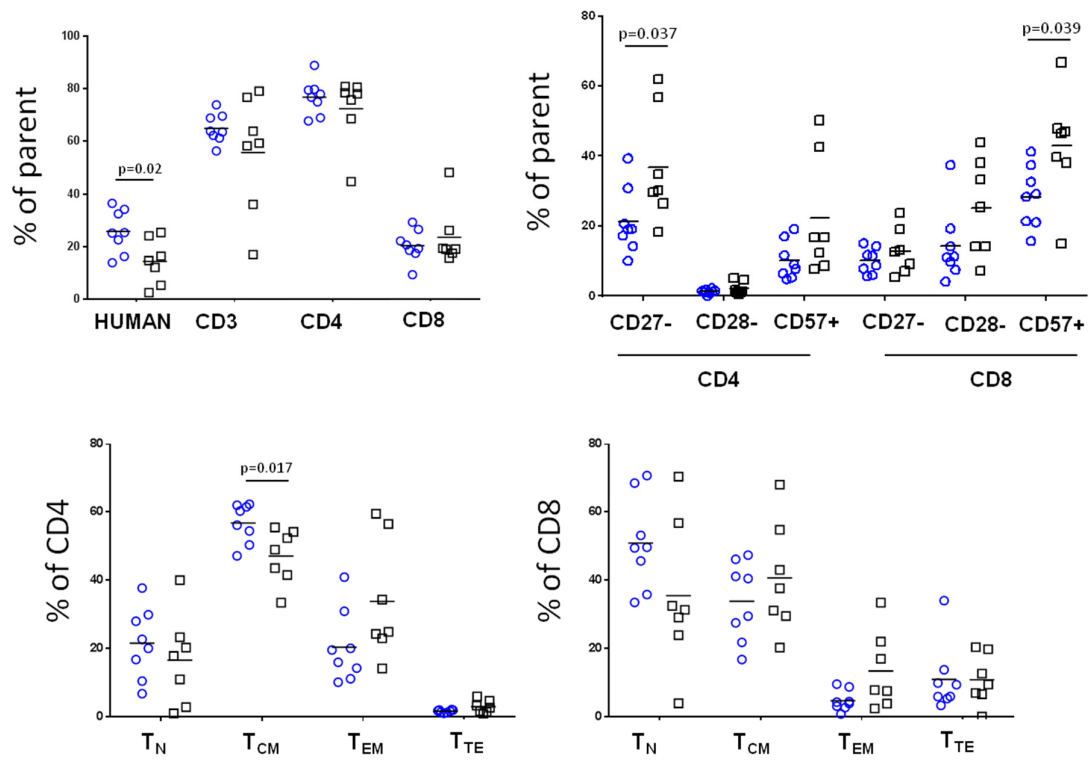
Figure 3-18 **T cell subpopulation alterations at 4 months P.I.**

Percentage values of individual mice are show to illustrate the T cell alterations 4months P.I. at the time of sacrifice. CMV- mice (blue), CMV+ mice (black). There was a significant decrease of percentages of human leukocytes (CD45+) in infected mice (p=0.02). However, we observed increased percentages of CD4 CD27- cells and CD8 CD57+ cells in infected mice (p=0.037 and p=0.039 respectively). There was a significant decrease of CD4 CM cells in infected mice (p=0.017). All other differences were not significant.
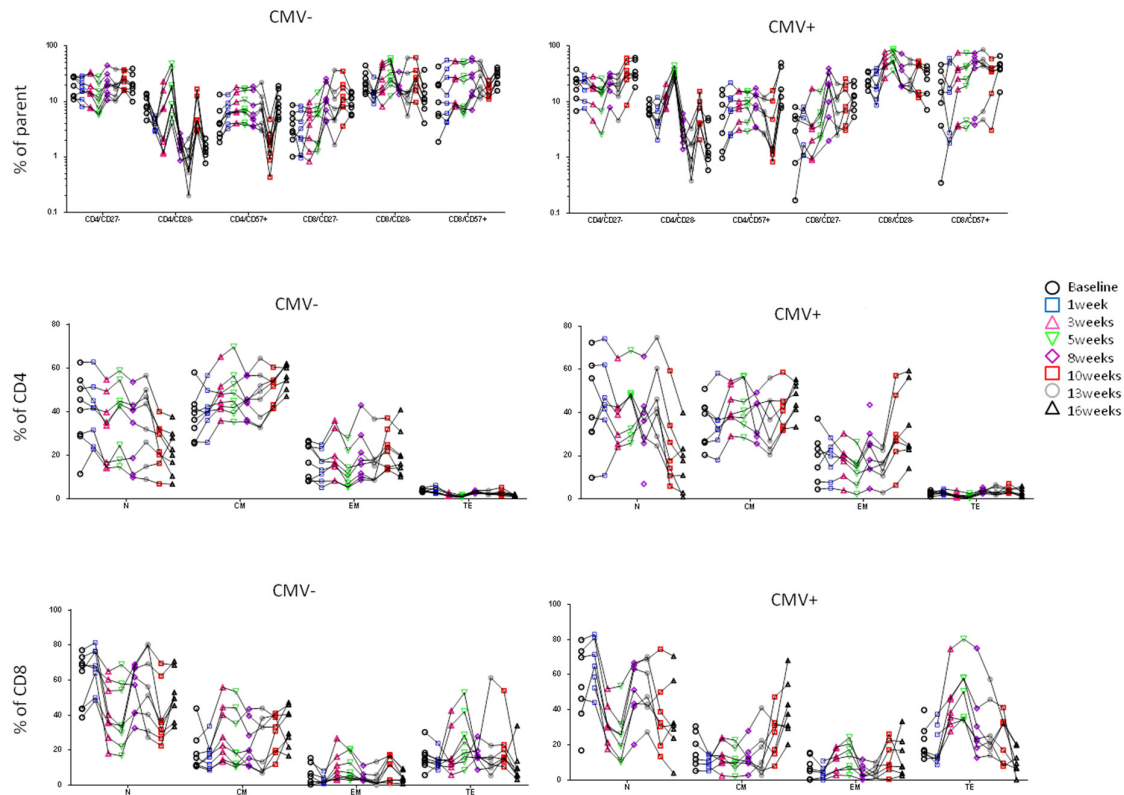
Figure 3-19 **Memory T cell alterations post primary infection**

Progression of reshaping of the T cell populations was observed for individual mice. Same markers as above were used in the measurement and determination of T cell populations. Single loss of CD27 or CD28 as well as gain of CD57 expression was measured (top panel). Main memory T cell population N, CM, EM and TE were followed in both CD4 (middle panel) and CD8 (bottom panel).

3.2.5 CCR7 expression follow similar pattern as CD28

Memory T cell population have been defined utilizing differential markers, which more or less correspond to each other's expression. Memory T cell development there is associated with a gradual loss of the co-stimulatory molecule CD28, but the acute downregulation of CD28 occurs also upon activation of effector T cells. To rule out the possibility that we are observing only the overall activation of T cells rather than their differentiation into memory cells, we have followed the memory cells utilizing the secondary lymph node homing chemokine receptor CCR7, which is only expressed on N and CM cells. However, based on

this marker we have not observed significantly altered pattern of memory T cell differentiation (**Figure 3-20**).
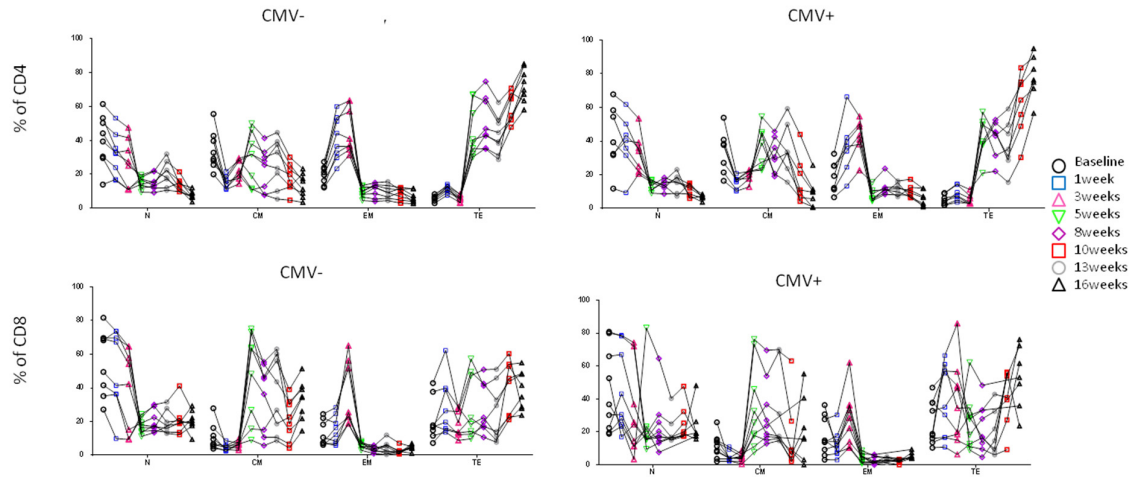
Figure 3-20 **CCR7 expression versus CD28 in phenotyping the memory**

The secondary lymphoid tissue homing chemokine receptor CCR7 was used to determine the memory populations instead of CD28 and CD27 in both CD8 and CD4 respectively. Data is shown for CMV-infected and non-infected reconstituted NSG mice.

3.2.6 The stability of human CD45+, CD3 T cells, CD4+ and CD8+ T lymphocytes over time

We already observed after 8weeks P.I. a significant 2 fold decrease of human CD45+ cells in infected mice. However, when we looked at the time course of these changes we observed that these alterations are not consistent. For human CD45+ we detected initial expansion followed by contraction (which was more pronounced in infected mice). We have observed decreasing trend of CD3 cells in infected mice as well as an increase of CD8. CD8 expansion was however followed by contraction at the time of sacrifice (**Figure 3-21**). It is important to note that the highest expansion of CD8 T cells was detected at the same time point

as the expansion of CD28- cells. The increase of CD8 T cell is therefore probably induced by
the expansion of CD28- cells (most likely by expanding effector cells).
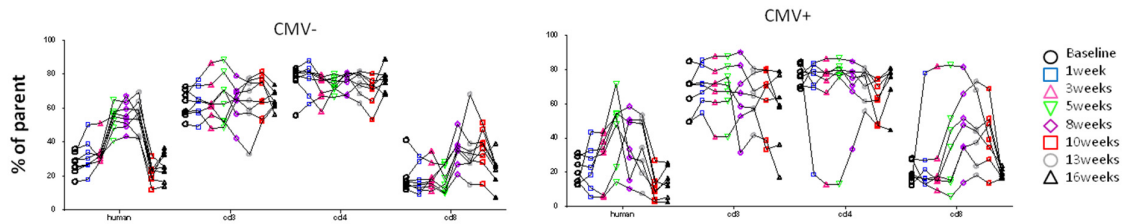
Figure 3-21 **The stability of human immune cells over time**
The frequency of human CD45+, CD3, CD4 and CD8 cells at all time points were measured to
display their stability in the humanized mice

3.2.7 Cell counts

Even though the relative frequency of the cell populations may differ, the absolute cell
count might follow alternative pattern. The total cell count of cell populations was therefore
determined by using the true-count beads. Interestingly almost all cell types have significantly
higher counts in non-infected mice, which was related to the substantial decrease of human
CD45+ cell in these mice (**Figure 3-22**).
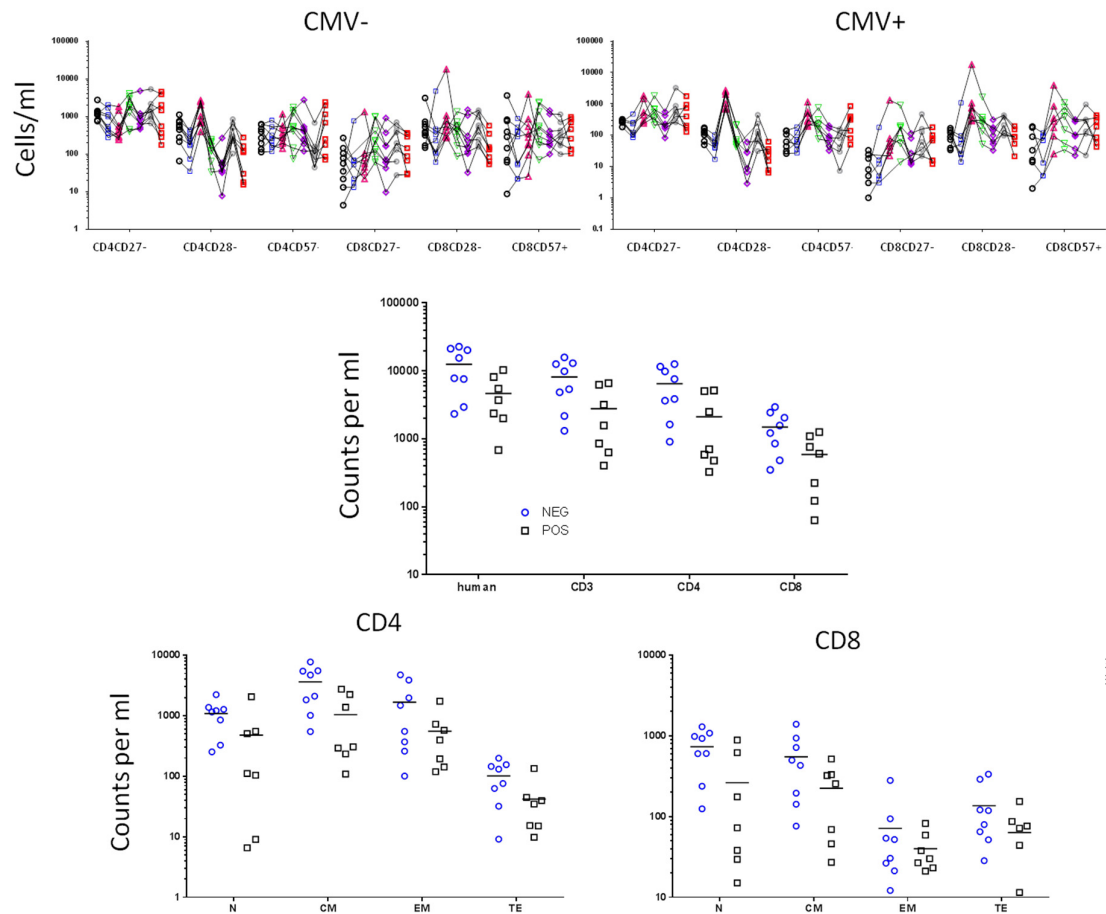
Figure 3-22 **Absolute counts of differentiated CD4 and CD8 cells**

The numbers of differentiated cells in both infected and non-infected mice follow very similar pattern, which is coherent with the changes of percentages (top panel) at the time of sacrifice 16 weeks after infection. We have observed that the total counts of human CD45+ cells have significantly decreased in the CMV+ (black) mice as compared to CMV- mice (blue)(middle panel). This was reflected in all memory population in both CD4 and CD8 T cells (bottom panel).

3.2.8 Grandparent % from human CD45+ cells show there is a decrease of CD3 T cells in infected mice

The general decrease of human blood cells in infected mice is probably the cause of the decreased cell counts in all T cell populations. However, the grandparent % of human CD45+, i.e. the portion of a certain population of human CD45+ cells, in infected mice the entire CD3 T cell population was decreasing within the CD45+ cell compartment (**figure 3-23**). This phenomenon might be due to an uneven expansion of non-T cell population in infected mice.
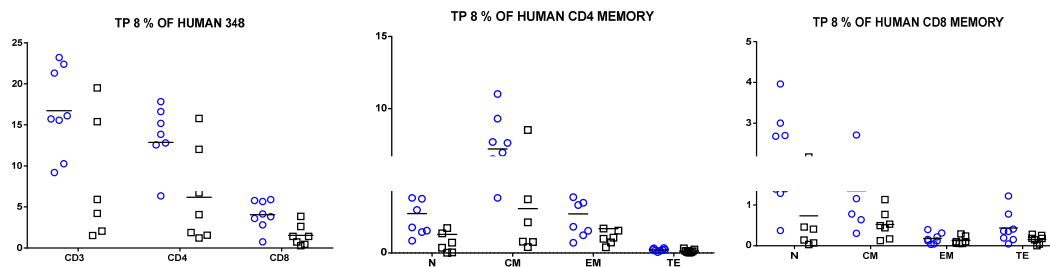
Figure 3-23 **Percentage of T populations of total human CD45+ human cells**
This figure reveals that there is also a significant decrease of CD3 T lymphocytes together with the overall loss of CD45+ lymphocytes in CMV+ mice (black squares) as compared to CMV- (blue circles). This could reflect a possible expansion of monocytes, the target cells of CMV. y axis depicts the percentage of CD45+ human cells.

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3.2.9 Anti-CMV IgG titres in the mouse plasma.

In young human individuals we could show a clear correlation of the anti-CMV IgG titre with T cell differentiation. In order to investigate this phenomenon in our model we measured this antibody level in mouse plasma at different time points. However, Even after 16 weeks P.I. we have observed no detectable levels of anti-CMV IgG in the mice plasma (not shown).

4 Discussion

The hallmarks of immunosenescence are the alterations in the T cell compartment, where massive expansion of memory T cells concomitant to the decrease in numbers of naïve T cells are the most characteristic events. The trajectory of this phenomenon is not well understood and I focused my PhD in identifying early signs of immunosenescence driven by persistent infections such as CMV.

The rational of my project is that we are experiencing a significant aging of the human population due to a major increase of life span in the past century. The number of elderly is increasing, which results in high demand of medical care from continuously increasing number of individuals. The complex deterioration of physiological functions with age include the worsening of immune responses and overall imbalance of the immune system. Aging of the immune system is potentially one of the more severe age-related deteriorations, since the major causes of death, cancer and primary infection, are clearly related to the immune system. The third major cause of death is cardiovascular disease and growing evidence shows that dysregulation of the immune system also has a major role in development in this type of diseases (reviewed in [189-192]).

Immunosenescence and the deterioration of immune functions is highly related to the large re-structuring of T cell populations, mainly the inflation of highly differentiated memory T cells. This phenomenon is further enhanced by chronic infections, which contribute to the T cell population-restructuring as well as to the low grade chronic inflammation in elderly. CMV has been shown to be the strongest player in the further deterioration of immune functions and memory cells inflation in elderly. It is therefore important to fully understand the degree of CMV-contribution to immunosenescence. In my project I have tried to identify the trajectory of T cell differentiation in young individuals infected with CMV. Moreover, I attempted to

further dissected the relationship of these cells with the cells expanded in elderly individuals and,by this, explored the differences in the trajectories of T cell differentiation caused by CMV and by aging. While we tested other chronic infections (*H. pylori* and HSV-2),we have not discovered any memory T cell inflation in these settings.

Although CMV infection is clearly linked to immune senescence, short-term exposure alone cannot account for the effect; no systemic inflammation was discovered in young CMV+ individuals. To complement our data we also tried to establish a humanized NSG mouse model to study primary CMV infection and to get an insight in the early differentiation of T cells triggered by this event. However, our data from the mouse model are only preliminary and still possesses many limitations.

4.1 CMV-infection induces T cell differentiation, memory inflation and cellular aging

The main discovery of this project is the restructuring of T cell population in young CMV+ individuals, which closely resembles the composition of the memory T cell populations in elderly. We have observed expansion of memory cells in both CD4 and CD8 (EM and TE) as well as the characteristic decrease of naïve cells. This primary observation suggests that CMV surpasses the role of aging in the differentiation of T cells, an assumption supported by our observation of "young" T cell populations in elderly individuals without CMV infection. While CMV- elderly still display significant decrease of naïve cells as compared to young, the inflation of memory cells is significantly lower than as observed in elderly CMV+ people. This observation was in our case only done on a small cohort of Chinese individuals due to a very high prevalence of CMV in Singapore. However, we further confirm same observation on a

Caucasian cohort, where the prevalence of CMV is significantly lower. It is important to note that different phenotyping strategies have been employed for Caucasian samples, where CCR7 was used to define the cell populations instead of CD28 and CD27. However, this altered phenotyping has no major effect on the precision of memory subsets definition.

Generally, the relationship between aging, CMV and immunosenescence remains only partially understood. To further investigate the role of CMV in immunosenescence independent of aging we studied those expanded memory cells in terms of their phenotypes, differentiation and gene expression profiles. It has been previously shown that certain pathogens induce disparate T cell memory phenotypes to the T cells bearing cognate TCRs [78]. Particularly CMV specific CD8 T cells mostly display the TE memory cell phenotype. We therefore focused on these cells as a model of CMV influence on the T cell differentiation.

It is not known whether a certain memory stage is altered as compared to non-specific T cells of the same memory population or if CMV merely induces expansion of certain memory stages which resembles their non-CMV specific counterparts. To investigate the possible influence of CMV infection on the development and expansion of senescent cells, we measured the gene expression of CD8 TE cells by nanostring technology. We measured 582 genes in T_{TE} from CMV+ and CMV- young individuals. 8 genes were differentially expressed with high significance (p<0.001): GNLY, TICAM1, IL18RAP, CD48, IL2RG, IGF2R, CHUK and CD58.We showed other 107 genes which expression was altered (p<0.05).

We verified the relevance of highly significantly expressed genes in literature and found some to be related to T cell differentiation, memory development or senescence.IL18RAP encodes IL18Rb protein which enhances significantly the affinity of IL-18R complex to IL-18. Importantly, it has been shown that IL-18RAP is a signature gene of T cell memory development. *Willinger et al*.[193] have shown that among other cytokine receptors, IL-18RAP expression increases upon memory T cell differentiation from N->CM->EM/TE.

CD48 and CD58 are members of the CD2 subfamily of immunoglobulin-like receptors and are expressed on lymphocytes and other immune cells. CD48 might be involved in the interaction of activated lymphocytes and is a part of build-up of the early TCR signalosome[194], that mediates cell-cell interactions including target cell adhesion. Furthermore, CD58 has been found to be highly expressed on memory T cells and is increased upon memory differentiation. The CHUK gene encodes for the IKKa molecule, which plays essential role in NF-κB signaling. NF-κB is a pleotropic end-point transcription factor, which is present in vast majority of cell types and is involved in rapid response to various cell stimuli and more importantly is a key factor in the immune response to infection.

TICAM1 gene encodes for an adaptor protein containing a Toll/interleukin-1 receptor(TIR) homology domain. TIR signaling mediates protein-protein interactions between the TLRs and other signal-transduction proteins. IL-2RG is a common cytokine receptor subunit for IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21 receptors and is absolutely crucial for lymphocyte development as a mutated version of the gene is a cause of x-linked combined severe immunodeficiency. Furthermore, the signaling the IL-2RG is crucial for IL-7 and IL-15 signaling, major pathways for memory T cell maintenance. It is possible that enhanced expression of this gene and subsequently higher surface expression facilitate those cells to better compete for the limited sources of cytokine survival signals. This notion is further supported by the observation of a higher surface expression of CD132 in steady state on CMV derived late stage memory T cells

IGF-2R is an insulin-like growth factor 2 receptor. Its common signal transduction is shared with TCR. IGF-2R has been shown to enhance the suppressive activity of Treg cells, which may imply that the expression of IGF-2R could influence effector functions of other T cells. GNLY encodes a cytotoxic molecule granulysin. Granulysin is an important cytotoxic molecule, which is a part of cytotoxic granules together with grandzymes or perforin. However,

granulysin has been mainly described to be a killer molecule targeted against microbes and not as a cytolytic molecule. It has been recently shown that intracellular bacteria is killed via granulysin forming the multimer pores in the bacteria to deliver granzymes, which cleave electron transport chain complex 1. The massive ROS generation induced by this event kills the bacteria [195]. However, the connection to a intracellular viral infection such as CMV is rather questionable, although we have found granzyme B to be significantly more expressed (p=0.02) in CMV+ derived TE.

Some of the genes described above do interact among each other and together contribute to the alterations of effector and other functions of the CD8 TE in CMV+ young individuals. For instance, it has been shown that CD48 interacts with IL-18 and IL-18Ra complex to facilitate binding of IL-18Rb [196]. The signaling of IL-18 induces the production of IFN-g in T cells and NK cells [197] and generally contributes to the inflammatory response through induction of TNF-a, GM-CSF or certain chemokines[198]. Signal transduction from IL-18R also involves NF-κB [199], p56 (lck) [200] and MAPK [200]. We may therefore anticipate that up-regulation of IL18RAP and CD48 may jointly lead to a higher responsiveness of CD8 TE cells to IL-18, which may have broad effects on the cell activation and effector function.

TRIF is a molecule involved in the NF-κB activating pathway. However, TICAM1 is a molecule from Toll-like receptors (TLR) signaling pathways. The expression of TLRs is mainly recognized to be on innate immune cells, mostly on APCs but it has been shown that TLR receptors may be expressed on murine [201, 202] as well as on human cells T cells [203].For instance, it has been discovered that CD8 T cells express TLR-1, TLR-2, TLR-6 and TLR-7 and probably other TLR receptors[201].This expression of TLRs in CD8 T cell has a functional impact as shown by *Cottalorda et al*. [201]. In their work, it has been demonstrated that the signaling through TLR-2 directly influences the threshold for optimal TCR signaling.

Most interestingly, it was shown that the signaling through TLR-2 significantly enhances the production of IL-2.The engagement of TLR-2 was sufficient, together with TCR stimulation, to induce effector functions via production of IFN-γ and affect proliferation and survival. Such impact of TLR-2 signaling was induced regardless of costimulatory signal from CD28. The similarity of TLR-2 and CD8 costimulatory signal might suggest that the enhanced expression of TLR-2 might, to a certain level, substitute the CD28 signals in CD28- cells. It has also been shown that TICAM1 plays an important role in MyD88-dependent TLR4 signaling[204, 205]. Furthermore, MyD88 has been shown to be a part of IL-18 signaling leading to NF-κB activation [206].Genes not directly linked to NF-κB signaling highlighted other possible functional alterations in CD8T$_{TE}$ from CMV+ young individuals.

To delineate the overall alteration in CD8 TE in young CMV+ individuals we have conducted an IPA analysis of115 genes found to be significantly altered in expression. The highest ranked pathway, in which these genes are involved, was the RAR signaling. The retinoic acid signaling has a broad effect on cells. In the context of immunity, RAR signaling can have a strong potential on inhibiting inflammation. This observation suggests that, even though many of the observed alteration suggest higher activity of the cells, there still is the enhancement of regulatory pathways. The glucocorticoid signaling has been shown to be significantly related to inflammation. Glucocorticoid receptor (GR) is translocated to the nucleus after its activation, where it functions as a transcription factor with important targets such as IL-1b, IL-6, IL-8, IL-12 or TNF-a. Furthermore GR directly interferes with NF-κB, AP1 or CREB.

Phosphatidylinositol 3-kinase/Akt signaling pathways exist in all mammalian cells and exert profound effects on multiple diverse processes including cell proliferation, survival, differentiation, migration, and metabolism. Importantly, the PIK3/Akt pathway has been shown to be important in CD8 effector functions and memory development as well as terminal

differentiation. This pathway is also activated via IL-7 and IL-15 cytokines, the master homeostatic factors of memory T cells. PI3K/Akt pathway is also involved in T cell metabolism facilitating the important shift from effective but slow oxidative phosphorylation to ineffective but fast glycolysis.

The top upstream regulators we have found were directly related to memory T cells. Important molecules were found related both in function and senescence of T cells, including IL-15, a master homeostasis regulator of memory T cells. Furthermore ULBP1 was identified, which is a stress-induced ligand for the NKG2D receptor (NK cells activation receptor). The receptor has been shown to be upregulated during HCMV infection (although CMV counteracts the upregulation by expression of viral UL16)[207]. The upstream regulators should thus be further investigated with the regard to the functional response and gene expression after triggering of downstream pathways.

All these observations suggest that the CMV driven terminally differentiated T cells are profoundly altered in terms of metabolism, responsiveness to cytokines and functionality. They have also probably reached higher degree of memory differentiation and are closer to terminal differentiation. The data on altered gene expression suggest that CD8 TE cells from CMV+ young individuals have a higher functional potential as compared to their CMV- counterparts. However, we only analyzed mRNA levels and the actual functionality of the gene products still has to be tested. The elevation of mRNAs might be compensation for lower translation at least in some cases, or for higher threshold of responsiveness to translated proteins. Further functional studies will enlighten the relationship with enhanced transcription of certain genes and the activity of the TE cells where further more we can better understand how are these alteration driven and if they eventually have any significant impact on the overall activity, development, differentiation and survival of the senescent/memory T cells.

The main limitation of our study is that we have examined T cells in total and not determined the behavior of CMV-specific T cells. We therefore are not able to account for the magnitude of the expansion of CMV-specific versus non-specific cells. This limitation is partially due to the fact that detecting the CMV specific clones requires the staining of TCR receptors through tetramers (dextramers). However, the available molecules will not cover the entire plethora of possible CMV specific clones. As previously mentioned the CMV specific clones respond mainly to peptides from E1 and pp65 proteins, but the pool of these clones might account for approximately 50-60%[208]. Furthermore individuals with different HLA alleles utilized different peptides from the immunodominant or other proteins.

Another approach to identify CMV specific T cells is CMV peptide stimulation assay, where the responsive clones are identified through production of certain cytokines (IFN-g, IL-2 etc.) or expression of activation molecules (CD154). However not all CMV specific clones will respond as they might be exhausted or are in the requirement of another stimuli. Nonetheless these experiments should be done in order to find out how CMV specific cells behave and how certain clones might be influenced significantly more than others. To establish the portion of CMV specific expanded memory cells would also help to understand how CMV infection drives this expansion, if it is more specific or rather bystander.

4.2 CMV surpasses aging in terms of T cell differentiation

By flow cytometry phenotyping we have shown that the expanded memory T cells EM and TE in young CMV+ individuals display significantly higher degree of differentiation as compared to their CMV-counterparts. This was measured by the subsequent loss of co-stimulatory molecules as well as the gain of expression of CD57, a marker of replicative senescence. We further investigated the very detailed surface marker expression on all CD4

and CD8 T cells (332 out of 364CD markers assigned to the nomenclature via the human screening technology). We have compared young CMV+ and CMV- together with elderly CMV+ individuals. We have discovered that 92 markers were to some extent expressed on the surface of T cells (some populations would expressed different set of markers as compared to others) we have investigated the surface expression on all memory cells types including the recently discovered SCM and TM, but mainly focused on CD8 TE and CD4 EM cells (The cells types mostly influenced by CMV infection in terms of phenotype and CD57 defined replicative senescence). We have conducted a Spearman correlation clustering to identify the relative distance of CD8 TE and CD4 EM derived from different groups of individuals. When we compared the percentage distance of memory cells expressing measured markers, we have found that the CD4 EM cells from young individuals have closer relative proximity than those from elderly. This was also true when we compared the amount of markers expressed based on MFI. In the case of CD8 TE cells we have found that cells from young CMV+ are in close proximity to cell from elderly, as compared to cells from young CMV-. Therefore, we suggest the CMV infection is strongly influencing both expansion of certain subpopulations and increase their expression of particular markers. Adhesive and chemotactic molecules seem to be influenced by the CMV infection as similar percentage of cells expressed these molecules on the other hand molecules related to activation were mostly expressed on CMV+ young derived cells. This suggests that T cells derived initially from CMV infection are in an activated state and over time become more exhausted. To support the reliability of our clustering method and the surface markers measurement we have conducted a control clustering on CD8 N, CM, EM and TE cells. As expected, N and CM formed one cluster and EM, TE formed another, which is in concordance with previous gene expression studies [193]. Our results also suggest the existence of CD8TE subpopulations, which might not necessarily have to be CMV specific, as it is clear that not all expanded clones (both in young and elderly) are CMV specific and that

probably CMV induces the clonal expansion by a bystander effect as well. One might point out the rather weak statistic background for our surface marker investigation, but we employed markers, whose expression pattern is known for each memory T cell subpopulation (CD27, CD28, CD57 and CD197). Moreover, we also confirmed the expected expression; CD27 and CD28 surface expression down-regulates upon T cells differentiation, CD197 is expressed only on N and CM and CD57 is expressed and up-regulated on late-stage memory cells/replicative senescent cells.

These observations suggest significant alterations in the cellular functionality of same memory populations derived from CMV+ or CMV- individuals, but functional studies are still needed to confirm the biological relevance of this observation. As mentioned above, the CMV+ clones display a rare phenotype, as the majority of these cells have the terminally differentiated or late effector memory phenotype. Therefore, it is possible our observation is actually the expansion of CMV specific clones.

4.3 Chronic systemic low-grade inflammation in elderly is not induced by CMV in young individuals

We have investigated the immunosenescence-related alterations in young CMV individuals on the cellular level, but we also wanted to have some insight into the putative systemic alterations CMV might induce in young people. We have therefore studied the possible presence of inflammatory molecules in the plasma of young individuals, as these are signs the chronic low grade systemic inflammation (inflamm-aging) in elderly. We have investigated the levels of EGF, eotaxin, FGF-2, Flt-3L, fractacline, G-CSF, GM-CSF, GRO, IL-6, IL-8, MIP-1α, MIP-1β, VEGF, sCD40L, sEGFR, sIL-2Ra, sIL-4R, sIL-6R, sRAGE, sTNFRI, sTNFRII, sVEGFR2, sVEGFR3, SGP130. We have observed no elevation or

decrease in CMV+ individuals as compared to CMV-. This is partially contrary to the data by *Turner et al*. where they show elevated levels of IL-6 in CMV+ young individuals (mean age was identical as in our study, i.e. 21 years). However, the reported elevation was only mild (0.6pg/ml vs 0.75pg/ml) [209].

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4.4 Other chronic infections do not induce alterations in T cell to a degree surpassing aging

Two other life-long chronic infections, *Helicobacter pylori* and HSV-2, show no signs of immunosenescence in our cohort. This further highlights the uniqueness of CMV and its role in the immune system exhaustion. Clearly other candidates causing chronic infections may be considered. However both *H.Pylori* and HSV-2 were well chosen candidates. *H.Pylori* has been associated with inflamm-aging and HSV-2 is a virus from the same family as CMV. Other viruses like EBV or HSV-1 might have been considered, but the prevalence of EBV in Singapore reaches 99% and therefore was not a good candidates. Furthermore, as the vast majority of CMV- and CMV+ individuals were both HSV-1+ and EBV+ we could observe the dominant effect of CMV infection and no real effects of these herpesviruses.

4.5 The course of CMV infection significantly correlated with the degree of differentiation of T cell in young individuals

It has been postulated that the anti-CMV IgG titer correlates with the course of infection and/or with the viral reactivation [178-182]. This has recently been shown on mice for MCMV infection [182]and is supported by the breadth and level of promiscuous gene expression[131, 147]. We have shown that the anti-CMV IgG titer significantly correlates with the expansion

of memory cells in young CMV+ individuals and the degree of differentiation of these cells. It is impossible to know the time span of CMV infection in the young individuals in our study, but there is a clear relationship in the intensity of CMV infection and immunosenesce-related alterations in young individuals and this observation further signifies the impact of CMV on immunosenescence.

Even though we observed the positive correlation of the anti-CMV IgG levels and the memory T cells expansion, we have not tested whether there is any correlation between the antibody level and the CMV-specific T cell responses. It is interesting to note that such correlation has been observed in elderly, where the higher IgG titer correlated with a higher percentage of pp65 specific T cells as well as with their higher multifunctionality (as measured by secretion of Il-2, IFN-γ, TNF-α, perforin or the expression of CD107a)[210].

4.6 Cytokine production by memory T cells from young CMV positive versus CMV negative individuals

It has been previously shown that late differentiated T cells remains functional or even multifunctional, but other studies show the deterioration of functionality of T cells from elderly as compared to young individuals[211, 212]We already show highly differential surface marker expression from TE cells from young CMV positive individuals as compared to CMV negative counterparts. We then compared the cytokine production by TE cells. After sorting and stimulation with PMA/Ionomycin we measured 40 cytokines (18 were detectable) from the supernatant via Luminex technology to cover a broad range of functional molecules secreted by those cells. We have found a trend of increase of for a number of cytokine in TE cells form CMV+ individuals of which only the increase for IL-17 was significant. We conducted then an intracellular staining (ICS)of cytokines and investigated TNF-a and MIP-

1b, which showed the highest(albeit non-significant) differences in the Luminex study for sorted CD8 TE. With this approach we show that significantly higher percentage of CD8 T cells from elderly producing TNF-α and MIP-1β as compared to young individuals. In CMV+ young patients we observed a similar trend in comparison to CMV-, but it didn't reach significance.

The phenomenon of altered production of TNF-α and MIP-1β in CD8 T cells from elderly is not reflected on the memory subpopulations level (CM, ME and TE) as these memory populations contain similar portion of cytokine producing cells. This observation suggests that the difference we observe in the overall CD8 population is caused by the expansion of memory cells in elderly. Possibly the same phenomenon would also be observed on larger cohort of young CMV+ individuals, or individuals in their 3rd decade of life. This, however, needs to be further tested. In CD4 T cells we have not identified any differences in TNF-α and MIP-1β production that putatively was caused by CMV or aging (as compared among three populations of CMV+ young, elderly and CMV+ young individuals). However we observed a significantly higher percentage of CD4 TE cells in elderly that produced MIP-1β as compared to young. This observation might be simply reflect the fact that CD4 TE cells exist only in some individuals and are believed to be present mostly in elderly.

Memory T cells are phenotypically defined by the loss of additional co-stimulatory molecule (either CD27 or CD28) and the subsequent gain of the expression of CD57. To understand the functional link of the further differentiation of we investigated the TNF-α and MIP-1β production of cells with low (CD27+/CD28+ CD57-) and high (CD27-/CD28- CD57+) antigen experience for CD4 and CD8 T memory T cells. To establish an effect of the phenotypic change independent of the memory cell type we compared values from CM, EM and TE together. The pooled results revealed a significant difference between CD27+CD57- and CD27-CD57+ CD8 T cells, where the later population contained substantially higher

percentage of cytokine producing cells. The same was true for CD4 cells (CD28+CD57- vs CD28-CD57+). This phenomenon was very similar for all memory cells types and therefore may be directly related to the differentiation phenotype of all CM, EM and TE cells.

A limitation in these cytokine secretion experiments may be the relatively small numbers of individuals in the ICS study (n= 15CMV- young, n= 10CMV+ young and n= 13 old CMV+ individuals). When investigating the production capacity of memory cells types from old and young (CMV- and CMV+), we observed a trend, especially in the elderly, of increased percentage of cytokine producing cells. While close to significance, the high individual variability in humans preventing us from discovering a significant difference and the experiment should be repeated on larger cohorts of patients.

The majority of studies have shown immunosenescence-related alterations in T cells of elderly individuals. In young CMV+ individuals we show a significant expansion of cells, which have been identified as the hallmarks of immunosenescence and are reportedly correlating with morbidity and mortality of elderly individuals[47, 48]. There are only 2 robust previous studies revealing some rudimentary signs of immunosenescence in young [209] or the relationship between aging, CMV and immunosenescence[213]. Other studies, concerning CMV and aging have been done on small numbers of individuals with a basic flow cytometry phenotyping only[214, 215]. Our study is the only one employing cohorts of Chinese ethnicity with a thorough examination of inflamm-aging, cytokine secretion assays, deep phenotyping, T cell differentiation and gene expression of CD8 TE in young individuals.

It is impediment to notice that these 3 studies (including ours) produced similar results but also several discrepancies. This might be due to factors such as the different genetic background of the cohorts, number of individuals, distinct immuno-phenotyping of memory T cells or the high variability existing among humans. It is now becoming evident that CMV is an aging-independent driving factor towards immunosenescence, but still more studies have to

be done. It is also not clear whether the immunosenescence-related alterations we observe in T cells have any consequence on the functioning of the immune system or if they are an indication for an earlier onset of immunosenescence in CMV+ individuals. The important role of CMV is in our study highlighted by the observation that the rare CMV- elderly populations show only mild or no alterations of the T cells and we were able to confirm this observation on a larger Caucasian population.

Lastly, it has been shown that different strains and genomic mutations in CMV might influence the virus virulence [216]. Therefore, different content of CMV mutants and/or strains might impact the immune system a distinct manner, when comparing human populations from geographically remote places. The limitation of our study as compared to *Turner et al*. [209] and to *Wertheimer et al.* [213] is that we compare relative percentages rather than absolute cell numbers. However, the observation of unaltered CD4/CD8 ratio suggest that our observations of memory T cells expansion are not caused by the overall expansion of CD8 T cells(unless there is a concomitant expansion of CD4 cells, which has not been shown to occur in elderly). Still, more functional studies and gene expression experiments related to the onset of cellular senescence, such as metabolism, DNA damage or oxidative stress related genes have to be done on memory cells derived from CMV+ individuals.

Wertheimer et al. [213] studied a cohort of 152 CMV- individuals aged 21-101 and 239 CMV+ individuals aged from 21-96 years. They showed that CMV and aging jointly and differentially affect the circulating T cells subsets in humans. In their study, they analyzed three T cell subsets, naive, CM and EM, defined by the surface molecules CD28 and CD95. They showed that CMV is related to the CM and EM inflation independent of aging in both CD4 and CD8 T cells. Furthermore, they showed that decrease in naive cells is only related to aging and it only happens in CD8 T cells. This is contrary to our study where we show that decline in naive cells occurs in young CMV+ individuals in both CD4 and CD8 T cells. These

discrepancies might be caused by few factors: Wertheimer, Bennett et al subdivided their cohorts into 3 age groups based on age(age < 40, 50 ≤ age < 65, and age ≥ 65). In our cross-sectional study, we compare 2 age groups of different cohorts with a mean age of 21 and 71, respectively. In our study, there is a clear cut off in the age difference with very low age variability. We also excluded a major influence of biological aging, which has been shown to have a substantial effect in some individuals already in the third and fourth decade of life [19]. It is to note that *Wertheimer et al.* [213]in their study did not specify the median age of each group and therefore it cannot be excluded that results observed are not influenced by the possible variability of age distribution in each group.

The second study from *Turner et al.* [209] shows rudimentary signs of immunosenescence in young individuals while employing similar approach of cohort study as we did. However no direct comparison with the old cohort has been made. They used smaller cohort of 158 individuals of which 48 were CMV+. Employing the memory cell markers CD28, CD27 and CD45RA they show that both CD4 and CD8 that EM and TE cells are significantly expanded in CMV+ individuals as compared to CMV-. They also show the expansion of CM cells and decrease of naive cells For CD8 T cells. Similar results are also shown by us, but in our study we could further show decrease of naive CD4 cells while the expansion of CD8 CM cells was not significant. Due to a deeper phenotyping by flow cytometry we could further show that in CMV+ individuals the expanded memory cells are mostly late differentiated and CD57+,which has not been shown before. However. *Turner et al.* [209] show that CMV+ status is associated with expansion of CD27-CD28- CD8 T cells, while we assign the late-differentiated stage to both EM and TE cells (and CD8 CM) with further CD57+ expansion in both CD4 and CD8.

We also confirm the correlation of anti-CMV IgG titer with memory cells as previously observed by *Turner et al.* but our results differ slightly; *Turner et al.* show a positive correlation

with CD8 TE and CD4 late differentiated cells, whereas we show positive correlations with CD8 EM and TE as well as negative correlation with CD8 N and CM cells. Importantly, we show that late differentiated CD28-/CD27- CD57+ EM and TE in both CD4 and CD8 highly correlates with the anti-CMV IgG titer, which further highlights the role of CMV in expansion and differentiation of memory T cells.

4.7 The inflation of memory T cell by CMV infection in the NSG humanized mouse model

In human, the primary infection of CMV is difficult to detect, as it is typically an asymptomatic infection. It is unknown whether the expansion of memory cells is established early after the primary infections followed by a slow maintenance or whether the inflations occurs consistently in a slow pace. Recently it has been shown for MCMV infection in mice that T cells responding towards MCMV do not undergo strong contraction after the expansion of effector cells. Furthermore, the remaining memory T cells have a phenotype of EM cells, which are believed to be more developed than CM. To mimic the primary infection in humans we therefore tried to utilize the NSG humanized mouse model for CMV infection. CMV NSG humanized mouse model has been previously introduced by *Smith et al.*[217] and we have successfully replicated the CMV infection with distinct CMV strain but without stimulation of G-SCF.

When employing the mouse model, we observed a massive drop (2fold) in the human lymphocyte numbers after 7weeks post infection (PI) in CMV infected humanized mice as compared to mock infected mice. This drop remained until the sacrifice at 4 months PI. Even though we observe similar pattern of T cell alterations in both infected and non-infected cells at the time of sacrifice 4 month P.I., we observe a more pronounce expansion of memory and

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late differentiated cells as well as a decrease of naive cells in the infected mice. This however was significant only for CD4+CD27-, CD8+CD57+ and CD4 CM (where these early stage memory cells were more expanded in non-infected mice) and we have observed no other significant differences in the alterations of the memory cells populations.

Percentages of cells might not reflect the actual increase or decrease of absolute number of cells. We therefore measured the absolute cell count in the blood samples. Also with this approach, we only observe alterations between CMV- and CMV+ mice at the 7week PI time point. This was however due to the general drop of human lymphocytes in the infected mice. By investigation of percentage of T cells in reference to the total CD45+ population of human cells, we observed that there is also a significant drop in the percentage of CD3 T cells in infected mice. This observation could possibly reflect an expansion of monocytes (or other non-CD3 T cells) and could be caused directly by CMV as monocytes are target cell for CMV infection and latency establishment.

The humanized NSG mouse HCMV infection model represents several limitations and artificial conditions decreasing the value of the observed phenomena with regard to the 'real' T cell response against CMV in human. Firstly, CMV infects numerous cell types and in some of them establishes latency. NSG humanized mice only harbors human CD45+ leukocytes and certain number of human hepatocytes, which are probably the source of CMV infection and latency in our model. Furthermore, the administration of CMV into our mouse model is strongly artificial as infected human foreskin fibroblasts (HFF) are injected intra-peritoneally. It has been previously shown that the route of infection, together with the initial dose of virus, plays a major role in the T cell inflation[170].The data we show here are also only preliminary and are not sufficient to conclude how the CMV driven memory T cell inflation may occur.

To develop this into a true model of primary CMV, we would have to overcome the many limitations our model has. The humanized mice only have human lymphocytes (with a

different percentage of cell types as in humans, especially lowered myeloid compartment) and only certain percentage of human hepatocytes, which is a result of contamination via fetal liver CD34+ stem cells injection for the human immune system reconstitution. However, for the course of CMV infection in humans, of the immune cells it is the myeloid cell (monocytes and myeloid cells progenitors) that is infected by CMV and infected epithelial cells are probably responsible for the expansion of memory cells. However, our NSG humanized mouse model does not have human epithelial cells. This might be the reason why we do not observe any significantly enhanced T cell inflation. Even though we have observed certain alterations in infected mice as compared to non-infected our study suggests that the primary infection is not yet robust enough.

Conclusion

The mechanism, by which CMV induces immunosenesce related Immune system alterations, must be further studied and well understood in order to lessen the medical and economic burden induced by this virus. Our study contributes to the understanding and importance of CMV as a driving force towards T cell differentiation, which may be pushed into a replicative senescent state, and sheds new light into the understanding of the aging of the immune system. As we focused on the general characterization of the different memory T cell subsets, the follow-up research should now define how many of these clones are actually CMV-specific and how much is attributed to a bystander memory T cell differentiation. Our findings further suggest that CMV is a major health problem in terms of immune system aging. However we do not know yet whether these changes really possess any negative effect to the young individuals, as it has been shown that CMV infection might be actually beneficial for its host in several cases.[59-64]. It is possible that the beneficial effect of CMV infection in young age is manifested via trained immunity. Later in life they may have a negative impact on the host due to the accumulation of differentiated pro-inflammatory cells, as it has been shown that the memory T cell inflation, low grade inflammation and CMV infection contributes to higher morbidity and mortality[49, 57].

Furthermore we show this on a large Asian cohort, which has never been studied before. We studied the complex phenotypic features of memory T cells derived by aging or CMV, suggesting a strong role of CMV in the differentiation of T cells relatively early after the primary infection also for this ethnic group. In the future, deep functional and phenotypic studies will help to understand the influence of CMV on the immune system and the possible onset of shaping the immune system towards immunosenescence. Due to the complexity of the virus it is very unlikely to develop an efficient drug, rather it would be more feasible to develop a vaccine for early childhood vaccination. It is however impossible to depict the exact role and

course of CMV infection and the aging of the immune system, as this would require a large longitudinal study over decades. Even though it has been shown that in some cases CMV is a mutualistic symbiont rendering protection of its host against other infections, other research show CMV infection being related to the decrease of the immune system in young individuals. For now, we hypothesize that the expansion of multifunctional memory T cells in young CMV+ individuals might be beneficial early on but become detrimental as the individual ages. More research on this need including the development of a reliable primary CMV infection model. Our data from the NSG mouse suggests that this might possible, although our model has many limitations and the data gathered are only preliminary.

Establishing the NSG mouse model could be one way to shed some light to the development of memory T cells upon primary infection. However, still more research must be done in the field of immunosenescence to fully understand this phenomenon. In order to be able to slow down its pace or prevent certain detrimental outcomes of the reshaping of the immune system, CMV infection has to be considered as an important player in the aging of the immune system and possibly aging itself.

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