IMPACT OF CYTOMEGALOVIRUS INFECTION ON AGEING OF THE IMMUNE SYSTEM

SARA P. H. VAN DEN BERG

Impact of cytomegalovirus infection on ageing of the immune system

Sara van den Berg

Impact of cytomegalovirus infection on ageing of the immune system

Het effect van cytomegalovirus-infectie op de veroudering van het immuunsysteem
(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de
Universiteit Utrecht
op gezag van de
rector magnificus, prof.dr. H.R.B.M. Kummeling,
ingevolge het besluit van het college voor promoties
in het openbaar te verdedigen op

woensdag 18 november 2020 des middags te 2.30 uur

door

Sara Petra Hendrika van den Berg

geboren op 21 maart 1990
te Rheden

PROMOTOR:
Prof. dr. D. van Baarle

COPROMOTOREN:
Dr. J.A.M. Borghans
Dr. J. de Wit

Dit proefschrift werd (mede) mogelijk gemaakt met financiële steun van het Strategisch Programma RIVM ('Cytovite')

LEESCOMISSIE:

Dr. A.C.T.M. Vossen
Prof. Dr. E.J.H.J. Wiertz
Prof. Dr. J.H.H. van de Wijgert
Prof. Dr. P. Moss
Prof. Dr. R.A.W. van Lier

PARANIMFEN:

Josien Lanfermeijer
Lyanne Derksen

COLOFON

Impact of cytomegalovirus-infection on ageing of the immune system
Sara P. H. van den Berg
ISBN/EAN: 978-94-6416-172-4

Cover design by Birgit Vredenburg, persoonlijkproefschrift.nl
Layout and design by Rutger van Aken, persoonlijkproefschrift.nl
Printed by Ridderprint | www.ridderprint.nl

TABLE OF CONTENT

1

General introduction and scope of this thesis

AGEING OF THE IMMUNE SYSTEM: IMMUNOSENESCENCE

The worldwide population is ageing rapidly. In 2050, one in six people in the world will be over the age of 65, while in 2019 this was still one in eleven individuals. An increase in the proportion of older adults poses important public health challenges. With age, new diseases arise. The immune system is no exception to this: as 'Immunological resistance' wanes with age, susceptibility for auto-immunity, cancer and infectious diseases increase with age [1-3]. Specifically respiratory infections cause a large disease burden in older adults, with influenza inducing most morbidity and mortality [4]. Ageing of the immune system is often popularly termed 'immunosenescence'. Although the use of the term immunosenescence differs between researchers, a strict definition contains an age-related *functional* impairment of the immune system. This entails a decreased response to infectious diseases as well as a diminished vaccine response in older adults. Paradoxically, the means by which we aim to provide protection to infectious diseases in the elderly, vaccination, thus *also* declines in effectiveness with age. This results in suboptimal protection of an already vulnerable population.

HALLMARKS OF T-CELL CHANGES WITH AGE

Characteristics of ageing of the immune system are well-described and primarily affect the adaptive immune system, specifically the composition of the T-cell pool. The production of naive T-cells by the thymus decreases dramatically with age [5]. As a result of this, and because naive T-cells are gradually recruited into the memory T-cell pool, naive T-cells decrease in number with age [6, 7]. This will likely affect *de novo* immune responses in aged individuals. In contrast, memory T-cells increase in number because of antigen challenges with different pathogens during life. Memory T-cells are heterogeneous and can be divided into distinct differentiation states. In older individuals, more memory T-cells are of the 'late-stage differentiated phenotype' and express higher levels of so-called senescence-associated markers, including the markers CD57 and KLRG-1 [8]. This expression pattern is indicative of T-cells lacking the ability to proliferate, and therefore these cells are thought to respond less well to recall immune challenges [8, 9]. Furthermore, the overall diversity of T-cells, marked by specific T-cell receptors, is thought to decrease with age [10, 11] causing a less broad immune response at old age [12]. Large clonal expansions of memory T-cells, consisting of a lot of cells expressing identical T-cell receptors, are more abundant in older adults. These changes are predominantly present in the $CD8^+$ T-cell pool, but have also been observed in the $CD4^+$ T-cell pool. Overall, these changes in the T-cell pool that appear with age are thought to contribute to a large extent to reduced protection to infectious diseases and reduced vaccine responses in older adults. Immunosenescence is thereby contributing to increased mortality at older age.

CMV-ENHANCED IMMUNOSENESCENCE: THE RISE OF A THEORY

In the early 2000s, two longitudinal studies [13, 14] from Sweden were published that formed the basis of a new theory: the ability of latent cytomegalovirus (CMV) infection to enhance immunosenescence. CMV is a common herpes virus, characterized by life-long persistence

in the host, that infects the majority of the population world-wide (40–100% of adults) and establishes latency in myeloid cells and epithelial cells [15]. Latent CMV infection was identified as part of the so-called 'immune risk profile' for all-cause mortality [13, 14]. Infection with CMV rarely causes clinical symptoms in healthy individuals, and viral reactivation from latency is thought to occur regularly during life. Therefore, CMV triggers frequent activity of the immune system leading to large expansions of differentiated T-cells. When the immune system is severely compromised, for example due to the use of immunosuppressive drugs after organ transplantation, CMV infection can no longer be controlled. The resulting reactivation of CMV leads to virus production, viral spread and severe CMV disease, and sometimes even mortality. In healthy individuals, controlling the attempts of the virus to reactivate leads to large expansions of differentiated T-cells which require large contribution of the immune system's resources. Importantly, these changes in the T-cell profile, for example a decreased $CD4^+/CD8^+$ T-cell ratio and increased numbers of late differentiated memory T-cells, were independently associated with the increased mortality risk [16]. The theory of life-long CMV infection gradually exhausting the immune system and accelerating immunosenescence was born. The CMV-enhanced immunosenescence theory relies on the hallmarks of the CMV-specific immune response and is supported by several lines of evidence. Namely 1) CMV establishes similar T-cell changes as ageing, 2) first studies suggested a negative association between CMV and the immune response to influenza and 3) epidemiological evidence links CMV to increased mortality (**Figure 1**). The CMV-specific immune response with age and these three lines of evidence became subject to further investigation in subsequent years.

THE CMV-SPECIFIC IMMUNE RESPONSE

Seroprevalence of CMV increases gradually with age. A gradual pattern is not typical for a contagious pathogen in a primary infection phase, in which a quicker saturation of the whole population is expected. The gradual increase with age requires periodically infectious reactivation events, slowly affecting more and more individuals [19]. Indeed, CMV, just like its 'sibling' herpes-simplex virus-1 causing episodes of cold sores, is thought to reactivate periodically during life. Indeed, viral load in plasma and urine are sporadically detected in healthy adults [20], especially in older adults [21, 22]. CMV thus seems to establish a rather 'dynamic' state of latency instead of true silent latency. This characteristic of CMV is thought to be key in the immune response to CMV.

Despite the overall decline in function of the immune system with age, CMV specific antibody levels correlate positively with age in large-scale cross-sectional studies [21, 30, 31]. Periodic CMV reactivation is thought to cause increases in the CMV-specific immune response over time [19, 21]. CMV-specific antibody levels are therefore often interpreted as a surrogate marker for experienced CMV reactivation, as well as to identify CMV-seropositive elderly with poor control of the virus who are at increased risk of adverse clinical outcomes [17, 32-37]. In line with this, CMV-specific antibody levels are positively associated with the frequency of occasional shedding of CMV in urine and saliva in older adults [21]. *However, there is little*

evidence from longitudinal studies that CMV-specific antibody levels indeed increase over time within individuals. Whether CMV-specific antibody levels are a good marker for CMV-reactivation is still under debate.

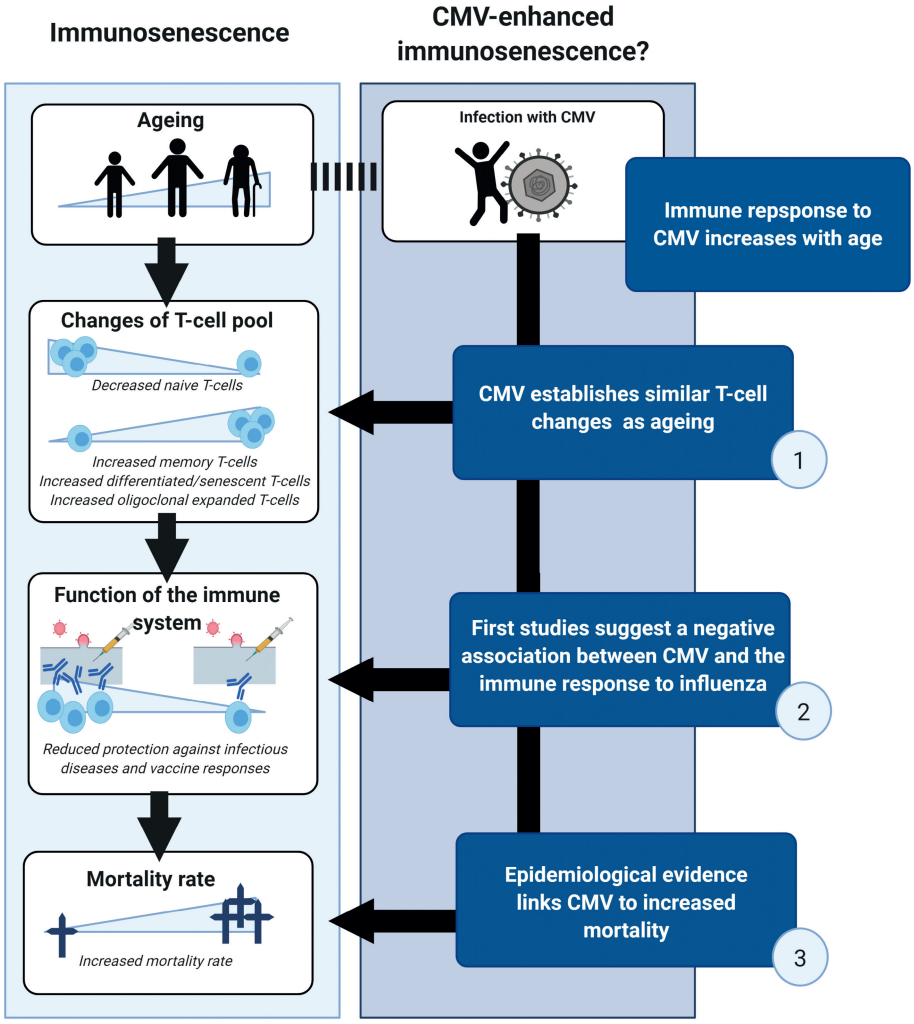


Figure 1. Rise of a theory: CMV-enhanced immunosenesence. Left panel: process of ageing of the immune system (immunosenescence). With age, changes in the T-cell pool arise, which are thought to be the basis for reduced function of the immune system and subsequently mortality. Right panel: the influence of CMV infection on immunosenescence. Paradoxically, the CMV-specific immune response does not decrease, but increases with age. This is thought to gradually exhaust the immune system based on the following lines of evidence: (1) CMV infection establishes similar changes to the T-cell pool as healthy ageing (Komatsu et al. 2003, Weinberger et al. 2007, Derhovanessian et al. 2011, Pera et al. 2016, van der Heiden et al. 2016, Hassouneh et al. 2017), (2) the first studies suggested a negative association between CMV and the immune response to influenza (Trzonkowski et al. 2003, Derhovanessian et al. 2014, Frasca et al. 2015, Merani et al. 2018), and (3) epidemiological evidence links CMV to increased mortality (Gkrania-Klotsas et al. 2012, Mathei et al. 2015).

(1) CMV establishes similar T-cell changes as ageing

CMV infection is associated with large changes in the T-cell pool. CMV infection leads to increased numbers of effector memory (T_{EM}) T-cells and effector memory T-cells reexpressing RA (T_{EMRA}) [38]. Memory T-cells in CMV-infected individuals also express higher levels of senescence markers, such as CD57 and KLRG-1, as compared to CMV-seronegative individuals [29, 39-43]. These cells are often referred to as terminally differentiated T-cells. The average telomere length in the total circulating T-cell pool was also shown to be reduced in CMV-seropositive individuals, suggesting they have experienced enhanced T-cell proliferation [44]. Furthermore, the number of (oligo)clonal expansions of T-cells is increased in CMV-infected individuals [45], leading to a less diverse T-cell repertoire [10]. Taken together, the effects of CMV on the T-cell pool resemble the general age-associated changes that occur in absence of CMV (**Figure 1**, number 1). Evidence is lacking what explains the CMV-induced changes in the T-cell pool. These changes might – at least in part – be explained by the presence of extremely large numbers of CMV-specific T-cells. *Thus, if something more is going on in the CMV-induced changes in the T-cells pool needs more research.*

(2) First studies suggesting a negative association between CMV and the immune response to influenza

The above described CMV-induced T-cell changes are linked to a worse outcome of immune challenge. For example an inverted $CD4^+/CD8^+$ T-cell ratio and the presence of oligoclonal T-cell expansions have been identified as part of the immune risk profile [13, 14, 16] and frequencies of $CD8^+CD28^-$ cells have been found to be inversely correlated with vaccine responses [46]. Therefore, The clinical consequences of CMV-induced changes for the function of the immune system were also investigated. Most of these studies have focused on immune responses against influenza in older adults. The risk for serious complications of influenza infection and hospitalization increase with age [47]. Most western countries recommend seasonal influenza vaccination to reduce disease burden. Several studies reported a negative effect of CMV on the influenza vaccine response [48-51] (**Figure 1**, number 2). However, there is no unequivocal evidence for a negative effect, as another study reported no effect [52] and even beneficial effects of CMV have been reported [53, 54]. *Thus, although the current dogma is a negative effect of CMV on the influenza vaccine response, the issue remains debated.*

(3) Epidemiological evidence links CMV to increased mortality

The association between CMV infection and health of older people was further investigated over the last decade. Many studies have reported an association between CMV infection and frailty as well as increased risk for mortality [18, 34-37, 55-59], although again not all studies found such associations [60]. CMV infection was shown to decrease life-expectancy with as much as 3.7 years [59]. Especially individuals with high CMV-specific antibody levels were shown to have lower influenza vaccine responses [32, 48], and more cardiovascular disease [17], frailty and overall mortality [17, 18] (**Figure 1**, number 3). The association between CMV and mortality may be explained by effects on the immune system, or alternatively, by affecting

the risk of (severe) cardiovascular disease. Indeed, CMV infection has been associated with a higher prevalence and severity of several cardiovascular diseases, for example coronary heart disease and ischemic heart disease [17]. *Thus, the increased risk of mortality induced by CMV infection is probably not solely explained by CMV-enhanced immunosenescence.*

Potential mechanisms of CMV-enhanced immunosenescence

Although there is a clear link between CMV infection and several T-cell characteristics, the mechanisms by which CMV-related changes in the T-cells pool worsen the outcome of a heterologous immunological challenge are largely unclear. Two main hypotheses have been proposed. First, the presence of large numbers of CMV-specific T-cells may hamper the induction of other T-cell responses. Both the recruitment as the maintenance of large numbers of CMV-specific T-cells could compete with other memory T-cells, specific for heterologous infections. In this way, CMV-specific T-cells would fill the 'immunological space' at the cost of other immunological memories [61]. This is not likely to be competition for a physical space, but rather competition between cells for homeostatic survival factors. This phenomenon has previously more generally been described as 'memory attrition' [62]. Secondly, the continual triggering of the immune system by persistent and 'dynamic latent' CMV infection may lead to a general low-grade inflammation state, thereby affecting subsequent immune responses. The increase of a more pro-inflammatory immune state with age, which is thought to hamper the function of the immune system, is often summarized as 'inflammaging'. *Both speculated underlying mechanisms of CMV-enhanced immuno-senescence lack solid evidence, especially in humans, and thus remain controversial.*

SCOPE OF THIS THESIS

In this thesis, we aim to address two gaps in the study of CMV-enhanced immunosenescence.

PART I: DOES CMV-INFECTION HAMPER THE IMMUNE RESPONSE TO INFLUENZA?

Part I of this thesis focuses on the immunological outcome of the CMV-enhanced immunosenescence theory in humans. We investigate if CMV is a threat to the immune response to influenza vaccination and infection.

Chapter 2 investigates the effect of age and CMV infection on the antibody response to a novel influenza vaccine strain in humans. We address the hypothesis that CMV causes enhanced immunosenescence in adults, leading to lower antibody responses upon an immune challenge. We hypothesize that the lack of consensus in the literature on the association between CMV infection and antibody responses to influenza vaccination may be due to the fact that pre-existing immunity to influenza can be a disturbing confounder. We therefore focussed on the response to vaccination against the pandemic influenza strain of 2009, thereby aiming to bypass the role of pre-existing immunity. We find no evidence for CMV-induced impairment of the antibody response to this novel influenza strain vaccine in adults. If anything, our data

even suggest that there might be a beneficial effect of latent CMV infection on the protection rate (titer≥40) after novel influenza vaccination against this novel influenza strain.

Since the conclusion of **chapter 2** seems to contradict the general view in the literature, in **chapter 3** we performed a systematic review and meta-analysis on the effect of latent CMV infection on the antibody response to influenza vaccination. Our primary outcome reveals no clear evidence for an effect of CMV-seropositivity on the influenza vaccine response in young or old individuals. We also found evidence for a publication bias in the literature favouring publications that report a negative effect of CMV. In combination with chapter 2, this work supports the view that CMV infection may not directly form a threat for influenza vaccine responses in older adults.

As the effect of CMV on the immune system is most prominent for T-cells, in **chapter 4** we study the effect of CMV on the $CD8^+$ T-cell pool**.** We studied the hypothesis that CMV-induced immunosenescence affects the T-cell response to influenza virus infection in older adults, either through competition for 'limited immunological space' or by the induction of a chronic low grade inflammation-state. A large cohort of influenza-infected individuals was analysed for CMV-specific antibodies and T-cells, influenza T-cell responses, cytokine levels and severity of symptoms. Our results question the impact of CMV-induced immunosenescence on the immune response to influenza virus infection.

PART II: CMV-INDUCED CHANGES OF THE T-CELL POOL IN OLDER ADULTS

Part II of this thesis aims at gaining insights into CMV-specific memory T-cells.

Chapter 5 reviews the hallmarks of CMV-specific T-cell differentiation. We study whether the phenotype of CMV-specific $CD8^+$ T-cells is unique in comparison to T-cell responses to other chronic viruses. We also discuss the possible impact of antigen exposure and aging on the advanced differentiation state of CMV-specific $CD8^+$ T-cells. We conclude that the increased presence of the late-stage differentiated effector memory (T_{EM}) T-cell state is a rather unique feature of CMV infection.

A unique phenomenon of CMV-specific T-cells is their maintenance at high numbers for long periods of time. We wondered whether this requires altered dynamics in terms of production and loss rates of these T-cells. In **chapter 6** we investigate the underlying cellular dynamics that maintain CMV-specific T-cells, and compare the dynamics of the total $CD4^+$ and $CD8^+$ T-cell pool in CMV- and CMV+ older adults. We assessed this by investigating the expression of senescence, proliferation and apoptosis markers, as well as by an *in vivo* deuterium labelling study in CMV- and CMV+ older adults to deduce the production and loss rates of T-cells *in vivo*. We find that both CMV-specific T-cells and the total $CD4^+$ and $CD8^+$ T-cell pools in CMV+ individuals tend to express a late-stage differentiated phenotype. This phenotype is associated with reduced production and loss rates. Nevertheless, CMV

infection is not related to altered $CD8^+$ T-cells dynamics *in vivo*, neither of the CMV-specific T-cells themselves, nor as an effect on other T-cell specificities. $CD4^+$ memory T-cells in CMV+ individuals show an increased expression of late-stage differentiation markers and a trend towards lower T-cell production rates as compared to their counterparts in CMV- individuals.

In **chapter 7**, we had the unique opportunity to assess the effect of time since CMV infection in a 27-year longitudinal cohort. We address the contribution of duration of CMV infection on the immune system and general health. We asses CMV-specific antibody levels longitudinally and find that within individuals CMV-specific antibodies increase slightly over time. However, age is more strongly associated with CMV-specific antibody levels, regardless of duration of CMV infection. We also investigated whether T-cell 'memory inflation', i.e. an increase in CMV-specific T-cell numbers over time, occurs in humans. We find no convincing evidence for this process in humans. Older age at seroconversion is associated with increased $CD4^+$ T-cell responses. Finally, we investigate if CMV infection and duration leads to adverse health outcomes in older adults. Interestingly, CMV-seropositivity and duration are not associated with adverse health, while higher age at CMV-seroconversion is. Our results suggest a model in which *duration* of CMV infection has less impact, and *age at the moment of CMV seroconversion* has more impact on the immune system than was previously appreciated.

Chapter 8 summarizes the findings presented in this thesis and discusses them in a broader perspective.

REFERENCES

1. Boots, A.M., et al., *The influence of ageing on the development and management of rheumatoid arthritis.* Nat Rev Rheumatol, 2013. 9(10): p. 604-13.
2. Muller, L., S. Di Benedetto, and G. Pawelec, *The Immune System and Its Dysregulation with Aging.* Subcell Biochem, 2019. 91: p. 21-43.
3. Derhovanessian, E., et al., *Immunity, ageing and cancer.* Immun Ageing, 2008. 5: p. 11.
4. Fleming, D.M. and A.J. Elliot, *The impact of influenza on the health and health care utilisation of elderly people.* Vaccine, 2005. 23 Suppl 1: p. S1-9.
5. Bodey, B., et al., *Involution of the mammalian thymus, one of the leading regulators of aging.* In Vivo, 1997. 11(5): p. 421-40.
6. Wertheimer, A.M., et al., *Aging and cytomegalovirus infection differentially and jointly affect distinct circulating T-cell subsets in humans.* J Immunol, 2014. 192(5): p. 2143-55.
7. Arnold, C.R., et al., *Gain and loss of T-cell subsets in old age--age-related reshaping of the T-cell repertoire.* J Clin Immunol, 2011. 31(2): p. 137-46.
8. Pangrazzi, L. and B. Weinberger, *T-cells, aging and senescence.* Exp Gerontol, 2020. 134: p. 110887.
9. Kared, H., et al., *CD57 in human natural killer cells and T-lymphocytes.* Cancer Immunol Immunother, 2016. 65(4): p. 441-52.
10. Qi, Q., et al., *Diversity and clonal selection in the human T-cell repertoire.* Proc Natl Acad Sci U S A, 2014. 111(36): p. 13139-44.
11. Yoshida, K., et al., *Aging-related changes in human T-cell repertoire over 20years delineated by deep sequencing of peripheral T-cell receptors.* Exp Gerontol, 2017. 96: p. 29-37.
12. Nikolich-Zugich, J., M.K. Slifka, and I. Messaoudi, *The many important facets of T-cell repertoire diversity.* Nat Rev Immunol, 2004. 4(2): p. 123-32.
13. Olsson, J., et al., *Age-related change in peripheral blood T-lymphocyte subpopulations and cytomegalovirus infection in the very old: the Swedish longitudinal OCTO immune study.* Mech Ageing Dev, 2000. 121(1-3): p. 187-201.
14. Wikby, A., et al., *Expansions of peripheral blood CD8 T-lymphocyte subpopulations and an association with cytomegalovirus seropositivity in the elderly: the Swedish NONA immune study.* Exp Gerontol, 2002. 37(2-3): p. 445-53.
15. Stempel, M., B. Chan, and M.M. Brinkmann, *Coevolution pays off: Herpesviruses have the license to escape the DNA sensing pathway.* Med Microbiol Immunol, 2019. 208(3-4): p. 495-512.
16. Wikby, A., et al., *The immune risk profile is associated with age and gender: findings from three Swedish population studies of individuals 20-100 years of age.* Biogerontology, 2008. 9(5): p. 299-308.
17. Gkrania-Klotsas, E., et al., *Higher immunoglobulin G antibody levels against cytomegalovirus are associated with incident ischemic heart disease in the population-based EPIC-Norfolk cohort.* J Infect Dis, 2012. 206(12): p. 1897-903.
18. Mathei, C., et al., *No relation between CMV infection and mortality in the oldest old: results from the Belfrail study.* Age Ageing, 2015. 44(1): p. 130-5.
19. van Boven, M., et al., *Infectious reactivation of cytomegalovirus explaining age- and sex-specific patterns of seroprevalence.* PLoS Comput Biol, 2017. 13(9): p. e1005719.
20. Huang, Y., et al., *Cytomegalovirus Shedding in Healthy Seropositive Female College Students: A 6-Month Longitudinal Study.* J Infect Dis, 2018. 217(7): p. 1069-1073.

21. Stowe, R.P., et al., *Chronic herpesvirus reactivation occurs in aging.* Exp Gerontol, 2007. 42(6): p. 563-70.
22. Furui, Y., et al., *Cytomegalovirus (CMV) seroprevalence in Japanese blood donors and high detection frequency of CMV DNA in elderly donors.* Transfusion, 2013. 53(10): p. 2190-7.
23. Sylwester, A.W., et al., *Broadly targeted human cytomegalovirus-specific CD4+ and CD8+ T-cells dominate the memory compartments of exposed subjects.* J Exp Med, 2005. 202(5): p. 673-85.
24. Gordon, C.L., et al., *Tissue reservoirs of antiviral T-cell immunity in persistent human CMV infection.* J Exp Med, 2017. 214(3): p. 651-667.
25. Klenerman, P., *The (gradual) rise of memory inflation.* Immunol Rev, 2018. 283(1): p. 99-112.
26. Klenerman, P. and A. Oxenius, *T-cell responses to cytomegalovirus.* Nat Rev Immunol, 2016.
27. Bajwa, M., et al., *CMV-specific T-cell responses at older ages: broad responses with a large central memory component may be key to long-term survival.* J Infect Dis, 2017.
28. Khan, N., et al., *Herpesvirus-specific CD8 T-cell immunity in old age: cytomegalovirus impairs the response to a coresident EBV infection.* J Immunol, 2004. 173(12): p. 7481-9.
29. Komatsu, H., et al., *Population analysis of antiviral T-cell responses using MHC class I-peptide tetramers.* Clin Exp Immunol, 2003. 134(1): p. 9-12.
30. Korndewal, M.J., et al., *Cytomegalovirus infection in the Netherlands: seroprevalence, risk factors, and implications.* J Clin Virol, 2015. 63: p. 53-8.
31. Parry, H.M., et al., *Cytomegalovirus viral load within blood increases markedly in healthy people over the age of 70 years.* Immun Ageing, 2016. 13: p. 1.
32. Alonso Arias, R., et al., *Intensity of the humoral response to cytomegalovirus is associated with the phenotypic and functional status of the immune system.* J Virol, 2013. 87(8): p. 4486-95.
33. Turner, J.E., et al., *Rudimentary signs of immunosenescence in Cytomegalovirus-seropositive healthy young adults.* Age (Dordr), 2014. 36(1): p. 287-97.
34. Wang, G.C., et al., *Cytomegalovirus infection and the risk of mortality and frailty in older women: a prospective observational cohort study.* Am J Epidemiol, 2010. 171(10): p. 1144-52.
35. Araujo Carvalho, A.C., et al., *Association between human herpes virus seropositivity and frailty in the elderly: A systematic review and meta-analysis.* Ageing Res Rev, 2018. 48: p. 145-152.
36. Vescovini, R., et al., *Intense antiextracellular adaptive immune response to human cytomegalovirus in very old subjects with impaired health and cognitive and functional status.* J Immunol, 2010. 184(6): p. 3242-9.
37. Roberts, E.T., et al., *Cytomegalovirus antibody levels, inflammation, and mortality among elderly Latinos over 9 years of follow-up.* Am J Epidemiol, 2010. 172(4): p. 363-71.
38. Weltevrede, M., et al., *Cytomegalovirus persistence and T-cell immunosenescence in people aged fifty and older: A systematic review.* Exp Gerontol, 2016.
39. Weinberger, B., et al., *Healthy aging and latent infection with CMV lead to distinct changes in CD8+ and CD4+ T-cell subsets in the elderly.* Hum Immunol, 2007. 68(2): p. 86-90.
40. Derhovanessian, E., et al., *Infection with cytomegalovirus but not herpes simplex virus induces the accumulation of late-differentiated CD4+ and CD8+ T-cells in humans.* J Gen Virol, 2011. 92(Pt 12): p. 2746-56.
41. Hassouneh, F., et al., *Differential Effect of Cytomegalovirus Infection with Age on the Expression of CD57, CD300a, and CD161 on T-Cell Subpopulations.* Front Immunol, 2017. 8: p. 649.
42. van der Heiden, M., et al., *Differential effects of Cytomegalovirus carriage on the immune phenotype of middle-aged males and females.* Sci Rep, 2016. 6: p. 26892.

43. Pera, A., et al., *CMV induces expansion of highly polyfunctional CD4+ T-cell subset coexpressing CD57 and CD154.* J Leukoc Biol, 2016.
44. van de Berg, P.J., et al., *Cytomegalovirus infection reduces telomere length of the circulating T-cell pool.* J Immunol, 2010. 184(7): p. 3417-23.
45. Khan, N., et al., *Cytomegalovirus seropositivity drives the CD8 T-cell repertoire toward greater clonality in healthy elderly individuals.* J Immunol, 2002. 169(4): p. 1984-92.
46. Goronzy, J.J., et al., *Value of immunological markers in predicting responsiveness to influenza vaccination in elderly individuals.* J Virol, 2001. 75(24): p. 12182-7.
47. McElhaney, J.E., et al., *The unmet need in the elderly: how immunosenescence, CMV infection, co-morbidities and frailty are a challenge for the development of more effective influenza vaccines.* Vaccine, 2012. 30(12): p. 2060-7.
48. Trzonkowski, P., et al., *Association between cytomegalovirus infection, enhanced proinflammatory response and low level of anti-hemagglutinins during the anti-influenza vaccination - An impact of immunosenescence.* Vaccine, 2003. 21(25-26): p. 3826-3836.
49. Merani, S., et al., *Influenza vaccine-mediated protection in older adults: Impact of influenza infection, cytomegalovirus serostatus and vaccine dosage.* Exp Gerontol, 2018. 107: p. 116-125.
50. Derhovanessian, E., et al., *Latent infection with cytomegalovirus is associated with poor memory CD4 responses to influenza A core proteins in the elderly.* J Immunol, 2014. 193(7): p. 3624-31.
51. Frasca, D., et al., *Cytomegalovirus (CMV) seropositivity decreases B cell responses to the influenza vaccine.* Vaccine, 2015. 33(12): p. 1433-9.
52. den Elzen, W.P., et al., *Cytomegalovirus infection and responsiveness to influenza vaccination in elderly residents of long-term care facilities.* Vaccine, 2011. 29(29-30): p. 4869-74.
53. Furman, D., et al., *Cytomegalovirus infection enhances the immune response to influenza.* Science Translational Medicine, 2015. 7(281).
54. Strindhall, J., et al., *Humoral response to influenza vaccination in relation to pre-vaccination antibody titres, vaccination history, cytomegalovirus serostatus and CD4/CD8 ratio.* Infect Dis (Lond), 2016. 48(6): p. 436-42.
55. Strandberg, T.E., K.H. Pitkala, and R.S. Tilvis, *Cytomegalovirus antibody level and mortality among community-dwelling older adults with stable cardiovascular disease.* JAMA, 2009. 301(4): p. 380-2.
56. Thomasini, R.L., et al., *Aged-associated cytomegalovirus and Epstein-Barr virus reactivation and cytomegalovirus relationship with the frailty syndrome in older women.* PLoS One, 2017. 12(7): p. e0180841.
57. Feinstein, L., et al., *Does cytomegalovirus infection contribute to socioeconomic disparities in all-cause mortality?* Mech Ageing Dev, 2016. 158: p. 53-61.
58. Haeseker, M.B., et al., *Association of cytomegalovirus and other pathogens with frailty and diabetes mellitus, but not with cardiovascular disease and mortality in psycho-geriatric patients; a prospective cohort study.* Immun Ageing, 2013. 10(1): p. 30.
59. Savva, G.M., et al., *Cytomegalovirus infection is associated with increased mortality in the older population.* Aging Cell, 2013. 12(3): p. 381-7.
60. Collerton, J., et al., *Frailty and the role of inflammation, immunosenescence and cellular ageing in the very old: cross-sectional findings from the Newcastle 85+ Study.* Mech Ageing Dev, 2012. 133(6): p. 456-66.
61. Franceschi, C., M. Bonafe, and S. Valensin, *Human immunosenescence: the prevailing of innate immunity, the failing of clonotypic immunity, and the filling of immunological space.* Vaccine, 2000. 18(16): p. 1717-20.

62. Selin, L.K., et al., *Attrition of T-cell memory: selective loss of LCMV epitope-specific memory CD8 T-cells following infections with heterologous viruses.* Immunity, 1999. 11(6): p. 733-42.

2

Negative effect of age, but not of latent Cytomegalovirus infection on the antibody response to a novel influenza vaccine strain in healthy adults

2018 Frontiers in Immunology, doi: 10.3389/fimmu.2018.00082

S. P. H. van den Berg[1,2], A. Wong[3], M. Hendriks[1], R.H.J. Jacobi[1], D. van Baarle[1,2], J. van Beek[1]

1 Center for Infectious Disease Control, National Institute for Public Health and the Environment, Bilthoven, the Netherlands
2 Laboratory of Translational Immunology, Department Immunology, University Medical Center Utrecht, Utrecht University, the Netherlands
3 Department of Statistics, Informatics and Mathematical Modelling, National Institute for Public Health and the Environment, Bilthoven, the Netherlands

ABSTRACT

Older adults are more vulnerable to influenza virus infection and at higher risk for severe complications and influenza-related death compared to younger adults. Unfortunately, influenza vaccine responses tend to be impaired in older adults due to ageing of the immune system (immunosenescence). Latent infection with cytomegalovirus (CMV) is assumed to enhance age-associated deleterious changes of the immune system. Although lower responses to influenza vaccination were reported in CMV-seropositive compared to CMV-seronegative adults and elderly, beneficial effects of CMV infection were observed as well. The lack of consensus in literature on the effect of latent CMV infection on influenza vaccination may be due to the presence of pre-existing immunity to influenza in these studies influencing the subsequent influenza vaccine response.

We had the unique opportunity to evaluate the effect of age and latent CMV infection on the antibody response to the novel influenza H1N1pdm vaccine strain during the pandemic of 2009, thereby reducing the effect of pre-existing immunity on the vaccine-induced antibody response. This analysis was performed in a large study population (n=263) in adults (18-52 years old). As a control, memory responses to the seasonal vaccination, including the same H1N1pdm and an H3N2 strain, were investigated in the subsequent season 2010-2011.

With higher age, we found decreased antibody responses to the pandemic vaccination even within this age range, indicating signs of immunosenescence to this novel antigen in the study population. Using a Generalized Estimating Equations (GEE) regression model, adjusted for age, sex and previous influenza vaccinations, we observed that CMV infection in contrast did not influence the influenza virus-specific antibody titer after H1N1pdm vaccination. Yet, we found higher residual protection rates (antibody level ≥40) in CMV-seropositive individuals than in CMV-seronegative individuals six months and one year after pandemic vaccination. In the subsequent season, no effect of age or CMV infection on seasonal influenza vaccine response were observed.

In conclusion, we observed no evidence for CMV-induced impairment of antibody responses to a novel influenza strain vaccine in adults. If anything, our data suggest that there might be a beneficial effect of latent CMV infection on the protection rate after novel influenza vaccination.

INTRODUCTION

Ageing of the population poses an important public health problem. With age, the function of the human immune system declines, a phenomenon also referred to as immunosenescence [1]. Profound changes of the immune system include the gradual loss of naïve cells, increase of memory cell numbers and decreased diversity of the T-cell and B cell repertoire [1-3]. These changes contribute to reduced protection against infectious diseases and reduced vaccine responses in older adults. Indeed, the incidence of influenza virus infections is increased and accompanied with more complications and higher mortality in older adults [4, 5]. Most developed countries recommend yearly influenza vaccination in individuals above 60 or 65 years of age [6], in order to prevent influenza virus infection by the induction of protective antibodies [4, 7]. However, the antibody response to influenza vaccination in older adults is impaired, causing a suboptimal protection in this vulnerable group [7-9].

Accumulating evidence indicates that latent cytomegalovirus (CMV) infection is associated with age-related changes of the immune system, and might enhance immunosenescence [2, 10, 11]. CMV is a common b-herpesvirus with a prevalence of 45-100% worldwide, which increases with advancing age [12]. CMV infection causes morbidity and mortality in severely immunocompromised patients, while the virus rarely causes clinical symptoms in healthy individuals. Despite the ability of the immune system to control primary infection, the virus establishes a latent infection, with episodes of viral reactivation during lifetime [13]. The frequent reactivation of CMV causes continuous antigenic stress for the immune system [3]. Anti-CMV IgG levels increase with age [14-16] and are thought to increase after viral reactivation episodes, thereby reflecting the amount of experienced CMV antigenic stress during lifetime [12, 14, 17]. The profound effect of CMV infection on the immune system is especially shown by the progressive large expansion of oligoclonal CMV-specific CD8 T-cells and, to a lesser extent, CD4 T-cells. Furthermore, CMV-seropositivity is strongly associated with an inverted CD4/8 ratio [18], bias of the TCR repertoire [19] and increase of highly differentiated T-cells [20].

It has been suggested that CMV-enhanced immunosenescence could impair the immune response to influenza vaccination [21, 22]. Indeed, in several studies CMV-seropositivity or a high anti-CMV IgG titer was associated with lower antibody responses to influenza vaccination in both adults [23-25] and older adults [25-28]. However, others did not find an effect of CMV infection [29, 30], or reported even an enhanced antibody response to influenza vaccination in both young [31, 32] and older CMV-seropositive individuals [33].

The overall impact of latent CMV infection on the antibody induction by influenza vaccines remains controversial and depends, among other factors, on pre-existing immunity to influenza virus [34]. Most studies investigated the antibody response to seasonal influenza vaccination; a yearly recommended trivalent influenza vaccine that often contains overlapping influenza vaccine strains in consecutive years. Natural exposure to influenza virus and previous vaccination causes preexisting immunity, which influences the consecutive vaccine

response. Higher pre-vaccination antibody titers (pre-titers) indeed were shown to result in lower post-vaccination antibody titers to subsequent vaccination [7, 35]. Furthermore, one could expect a larger effect of immunosenescence on *de novo* immune responses [36, 37]. A seasonal influenza vaccination is therefore a suboptimal study setting to investigate the effect of latent CMV infection on influenza vaccine antibody response.

We hypothesize that the effect of latent CMV infection on the antibody response to influenza vaccination can best be studied when a novel influenza virus strain is introduced into a naïve population. In this study, we had the unique opportunity to investigate the effect of latent CMV infection on the antibody response during the pandemic season of 2009 to the novel H1N1pdm vaccine strain in a large study population and at multiple time points after vaccination. This allowed a sophisticated study design to test the effect of latent CMV infection on a de novo influenza vaccine response by minimizing pre-existing immunity due to previous exposure by vaccination or natural infection. As a control, the influence of latent CMV infection on the memory antibody response to the vaccination in the subsequent year was also investigated, which included both the same H1N1pdm vaccine strain and an H3N2 vaccine strain.

MATERIAL AND METHODS

Study population and design

The current study is embedded in a trial that evaluated the immune responses to pandemic and seasonal influenza vaccination that was conducted in 2009-2011 (the Pandemic influenza vaccination trial, Netherlands Trial Register NTR2070). This study was carried out in accordance with the recommendations of Good Clinical Practice with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Central Committee on Research Involving Human Subjects of the Netherlands. Healthy individuals, between 18 and 52 years of age, were recruited among health care workers in the Utrecht area in the Netherlands. Individuals over 52 years of age were not included because of potential pre-existing immunity due to exposure to the influenza/A/H1N1 strain that circulated until 1957 [38]. Serum samples and questionnaires were used from the vaccine group of the Pandemic influenza vaccination cohort.

Vaccines

In the pandemic season, individuals received two doses of the monovalent MF59-adjuvanted influenza vaccine containing influenza A/California/7/2009(H1N1pdm09) with a three-week interval (Focetria, Novartis, Italy). Blood samples were collected before vaccination (T1), three weeks after vaccination at which also the second pandemic vaccine dose was given (T2), six weeks after the first vaccination (T3), 26 weeks after the first vaccination (T4) and if participants continued with the study during the 2010-2011 season, also 52 weeks after the first vaccination (T5) (**Figure 1**). Self-reported vaccine history (2006-2009) was extracted from the questionnaires. If study subjects received seasonal trivalent vaccination in 2009-2010

(Solvay, the Netherlands), it took place at least three weeks prior to the study or at the end of visit at time point 3 of the study. In season 2010-2011, individuals received the seasonal trivalent subunit vaccine Influvac 2010-2011, containing the influenza A vaccine strains A/California/7/2009(H1N1pdm09) and A/Perth/16/2009(H3N2) (Solvay, the Netherlands). Blood was collected before vaccination (T1), three weeks after vaccination (T2) and 20 weeks after vaccination (T3).

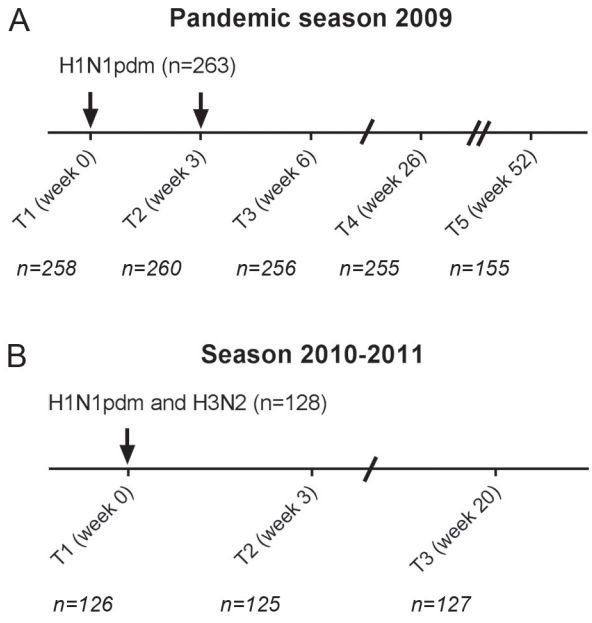


Figure 1. Study schedule. Participants received in the pandemic season two monovalent influenza H1N1pdm vaccinations with a three week interval **(A).** In total, 263 CMV-seropositive and CMV-seronegative individuals were vaccinated. 155 participants continued for the subsequent year in the study (T5 season 1). In the season 2010-2011, 128 individuals were vaccinated (T1 season 2) with the seasonal trivalent influenza vaccination which contained among others the same H1N1pdm vaccine strain and an H3N2 vaccine strain **(B)**. Arrows (↓) indicate the moment of vaccination. Time points (T) indicate the moment of blood withdrawal. For each time point, the number (N) of individuals with data of influenza antibody levels are indicated.

Assessment of serum anti-CMV antibody titers

Anti-CMV IgG antibody concentrations were measured using a commercial ELISA assay (IBL international GMBH, Hamburg, Germany) according to manufacturer's instructions. Participants with a CMV antibody level of ≥ 12U/ml or higher were considered CMV-seropositive, a level of ≤ 8U/ml were considered CMV-seronegative and a level between 8U/ml and 12U/ml was considered equivocal and these participants were excluded for further analysis. CMV-seropositive individuals were divided in low anti-CMV levels (≤ 30U/ml), medium anti-CMV levels (> 30U/ml, ≤ 93U/ml) or high anti-CMV levels (> 93U/ml) according to the standards in the CMV ELISA kit.

Hemagglutination-inhibition (HI) assay

HI assays were performed in the pandemic season for A/California/7/2009(H1N1pdm09) and in season 2010-2011 for A/California/7/2009(H1N1pdm09) and A/Perth/16/2009(H3N2) to determine influenza virus-specific antibody titers before and after vaccination. Briefly, a dilution series of cholera filtrate-treated serum samples was incubated with four Hemagglutinin Units (HAU) of influenza virus for 20 minutes, 0.25% turkey erythrocytes for 45 minutes and scored for agglutination [39]. The influenza antibody titer is the inverse of the last dilution of the serum that completely inhibited hemagglutination. A detectable influenza antibody body titer is defined as > 5 HAU.

Statistical analysis

Antibody responses to H1N1pdm influenza vaccination in the pandemic season were expressed in two different ways: (1) influenza antibody titer and (2) protection rate (antibody titer ≥ 40 HAU). For all statistical analyses, influenza antibody titers were log (base 2) transformed, and presented as geometric mean titer (GMT) with 95% confidence interval (CI) in the figures.

First, a two-tailed Student's t-test (for two groups) or one-way ANOVA (for three or more groups) was used to explore group differences in influenza antibody titers (e.g. between low, medium and high CMV IgG groups). For the two-tailed T test, equality of variances was tested with Levene's test for equality. Group differences in categorical variables were compared with the Fisher exact test.

Second, we investigated the effect of latent CMV infection in a multivariate context; the effect of CMV infection on influenza antibody titers was adjusted for potential confounders using a Generalized Estimating Equations (GEE) regression model (**Supplementary Table 1**) [40]. This model takes repeated measurements for the same individuals into account. For influenza antibody titer the normal distribution and for protection the binomial distribution of the model was used. The effect of CMV infection was investigated in two ways; (a) CMV-serostatus: CMV-seropositive individuals were compared to CMV-seronegative individuals, and (b) anti-CMV IgG groups: low, medium and high anti-CMV IgG levels were compared within CMV-seropositive individuals. Confounders included were age, sex, time and various variables concerning vaccination history (see **Supplementary Table 1**). The model yielded a beta regression coefficient for each variable, that reflects how a category (e.g. highest age group) compares to the reference category (e.g. lowest age group). Regression coefficients of the GEE models are given in **Supplementary Table 2-7**. The model also yielded adjusted results (i.e. influenza antibody titers or protection rates) for each time point at which comparisons between CMV-serostatus or anti-CMV IgG group were performed, by including an interaction term between time and CMV-serostatus or anti-CMV IgG group in the GEE models. The adjusted outcomes of the models and pairwise comparisons are presented in the figures. These analyses were also performed for the influenza vaccine response in season 2010-2011 for H1N1pdm and H3N2. P values of ≤ 0.10 were considered a trend and of ≤ 0.05 were

considered significant. Data were analyzed using SPSS statistics 22 for Windows (SPSS Inc., Chicago, IL) and R 3.4.0 (https://www.r-project.org/).

RESULTS

Characteristics of the study population

In total 288 individuals were vaccinated with the pandemic influenza vaccine in the pandemic season (**Figure 1**). CMV-serostatus was determined and 25 individuals with an equivocal CMV status were excluded from further analysis. Of the remaining 263 individuals, 171 were CMV-seropositive (65%). Groups of CMV-seropositive and CMV-seronegative individuals were comparable for sex, age and previous influenza vaccinations (**Table 1**). In season 2010-2011 128 of the 263 participants were vaccinated with the seasonal vaccination of which 76 (59.4%) were CMV-seropositive. Also, in the subsequent season no differences in sex, age and previous influenza vaccinations between CMV-seropositive and CMV-seronegative individuals were observed (**Table 1**).

	Pandemic season				Season 2010-2011			
	Total (n=263)[a]	CMV+ (n=171)	CMV- (n=92)	Signifi-cance	Total (n=128)	CMV+ (n=76)	CMV- (n=52)	Signifi-cance
Age (mean and SD)	39.9 (7.8)	39.3 (8.6)	39.5 (8.5)	P=0.88	41.3 (8.1)	41.7 (7.7)	40.63 (8.6)	P=0.43
Sex (% men)	45.2%	51.1%	42.1%	P=0.19	48.4 %	46.1%	51.9%	P=0.59
Previous influenza vaccination before pandemic season	49.0%	49.7%	47.8%	P=0.80	65.6%	63.2%	69.2%	P=0.57
Seasonal vaccination 2009-2010 before study	23.6%	23.4%	23.9%	P=1.00	87.1%[b]	77.6%	78.8%	P=1.00
Seasonal vaccination 2009-2010 during study	37.3%	38.0%	35.9%	P=0.79				

Table 1. Characteristics of study population for pandemic season and season 2010-2011. CMV-seropositive and CMV-seronegative group are compared with Student's t-test for age and with the Fischer exact test for categorical variables. [a] Time point 5, 52 weeks after pandemic influenza vaccination, blood was collected of 155 participants who continued in the study for season 2010-2011. [b] Seasonal vaccination in 2009 before study or during study combined.

Negative effect of age on influenza titers after de novo pandemic influenza vaccination

We investigated if there was an effect of age on the induction of antibodies to the pandemic influenza vaccination in our study population. After pandemic vaccination, H1N1pdm influenza virus-specific antibody titers were negatively correlated with age at all time points post-vaccination except wT5 (see **Supplementary Table 8**). Representative data are depicted for T2 in **Figure 2A** (T2, p=0.0013 R -0.198)**.** Individuals are divided in three age groups for further

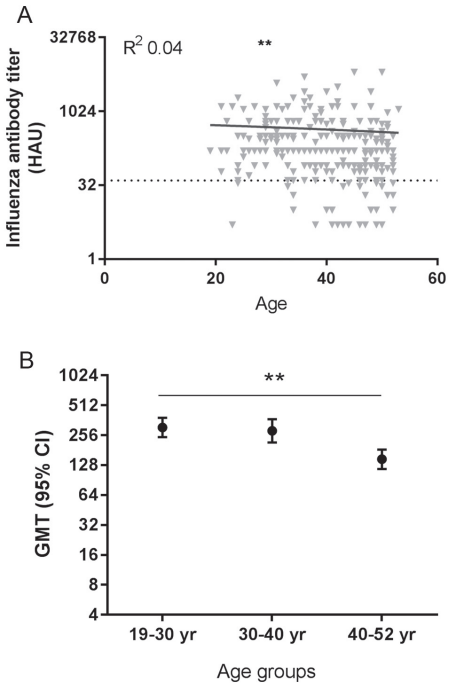


Figure 2. Effect of age on influenza virus-specific antibody titers after influenza vaccination. A representative figure of influenza H1N1pdm antibody titers after pandemic vaccination plotted against age (T2) (**A**). Dotted horizontal line represents a protective influenza titer of 40 HAU. The geometric mean of the influenza antibody titer (**B**) after vaccination are given for different age groups after vaccination with H1N1pdm for T2 (p=0.016 ANOVA). Correlations are tested with Pearson correlation. Differences between 2 age groups are tested with Student's t-test for log-transformed influenza antibody titers. ** p < 0.010. GMT = geometric mean titers.

analysis by approximately 10-year intervals. Significant differences were also observed between age groups in the H1N1pdm titers (e.g. T2, p=0.016), with lower responses in the oldest age group compared to the youngest two age groups (e.g. T2, 19-30 year versus 40-52 year p=0.007) (**Figure 2B**) (see **Supplementary Table 8**). Similar results were observed for the different age groups when analyzing the data by protection level, defined by reaching a titer of ≥40 HAU (data not shown). These data indicate that there are already signs of immunosenescence-driven impaired vaccine responses to a novel antigen challenge in middle-aged individuals.

No effect of CMV-seropositivity on antibody titers after pandemic influenza vaccination

Next, the effect of latent CMV infection on the influenza virus-specific antibody response to the vaccine with the newly introduced H1N1pdm influenza vaccine strain was investigated. CMV-seropositive individuals were compared to CMV-seronegative individuals for influenza titers before and after vaccination. No differences between CMV-seropositive and CMV-seronegative individuals in influenza titer at any time point in both seasons were found (**Supplementary Figure 1A**). Some individuals did already show a detectable pandemic titer before vaccination, although on average the pre-titer was very low (GMT 9.4 HAU). To correct for this and other potential confounders, influenza titers of CMV-seropositive and CMV-seronegative individuals were analyzed adjusted for pre-titer, sex, age and previous influenza vaccinations with a Generalized Estimation Equation (GEE) model (**Supplementary Table 2**). No significant differences were found between CMV-seropositive and CMV-seronegative individuals in antibody titers at each individual time point (**Figure 3A)**. So although age shows a negative effect on the novel pandemic H1N1pdm antibody response indicative of immunosenescence to *de novo* response (**Figure 2**), no effect of CMV-serostatus on the influenza virus titer is observed after pandemic vaccination in adults (**Figure 3A**).

Higher residual protection rates after pandemic influenza vaccination in CMV-seropositive individuals than in CMV-seronegative individuals

Subsequently, we investigated whether there was an effect of CMV-serostatus on the protection rate, as defined by antibody titer ≥ 40 HAU, against influenza virus after influenza vaccination [41]. Shortly after vaccination, no effect of CMV-serostatus on the protection rate was observed. However, CMV-seropositivity was associated with enhanced six months and one year protection rates after pandemic vaccination. The percentage protected individuals is significantly higher for CMV-seropositive individuals than for CMV-seronegative individuals, both 26 (p=0.047) and 52 weeks (p=0.044) after pandemic vaccination (**Figure 3B**) (unadjusted data in **Supplementary Figure 1B**). Together, this suggests that latent CMV infection did not impair the protection rate after influenza vaccination, but if anything, might be beneficial for persistence of protection after the *de novo* influenza vaccination.

High anti-CMV IgG levels as surrogate marker of CMV reactivation are not associated with impaired pandemic influenza vaccine response in CMV-seropositive individuals

To study in the CMV-seropositive individuals whether the frequency of CMV reactivation has a negative effect on the influenza antibody responses, anti-CMV IgG levels were used as a surrogate marker of CMV reactivation [25, 42] and associated with the influenza antibody response to vaccination. To this end, CMV-seropositive individuals with low anti-CMV IgG levels (≤ 30U/ml), medium anti-CMV IgG levels (> 30U/ml, ≤ 90U/ml) or high anti-CMV IgG levels (> 90U/ml) were compared for their influenza antibody titer and protection rate both unadjusted (**Supplementary Figures 1C, D**) and with the GEE model (**Supplementary Table 3**). No differences were observed between anti-CMV IgG groups in the H1N1pdm influenza titers or protection rate after the pandemic vaccination (**Figure 3 C, D**). This indicates that despite a negative effect of age on the antibody response to the pandemic vaccination (**Figure 2**), no signs of impairment by CMV reactivation were observed. Also this shows that the positive effect of CMV-status on long-term protection after pandemic influenza vaccination (**Figure 3B**) could not be explained by differences in anti-CMV IgG groups within CMV-seropositive individuals.

No effect of age or CMV-serostatus on seasonal influenza vaccination with H1N1pdm and H3N2

The same analyses for the effect of age and latent CMV infection on influenza vaccination were performed for the 128 individuals that continued with the study and were vaccinated in season 2010-2011 with the seasonal influenza vaccination containing the same H1N1pdm strain and an H3N2 strain. A trend of a negative effect of age on the H1N1pdm memory response was observed, but no significant differences in antibody titers for H1N1pdm or H3N2 were found between age groups at any time point after vaccination in season 2010-2011 (e.g.T2 respectively p=0.101 and p=0.434 (**Figure 4A**). Both the influenza antibody titer and the protection rate did not differ between CMV-seropositive and CMV-seronegative individuals (**Figure 4B, C, D; Supplementary Table 4, 6**). Surprisingly, influenza antibody

titers and protection rate after the seasonal vaccination were higher for both H1N1pdm and H3N2 in the high anti-CMV IgG levels group compared to low anti-CMV IgG levels group within the CMV-seropositive individuals at most time points after vaccination (**Figure 4, E, F, G; Supplemental table 5, 7**). In summary, although no clear effect of age or CMV-serostatus, high-anti CMV IgG levels seem to be associated with high influenza antibody titers and protection rate in CMV-seropositive individuals.

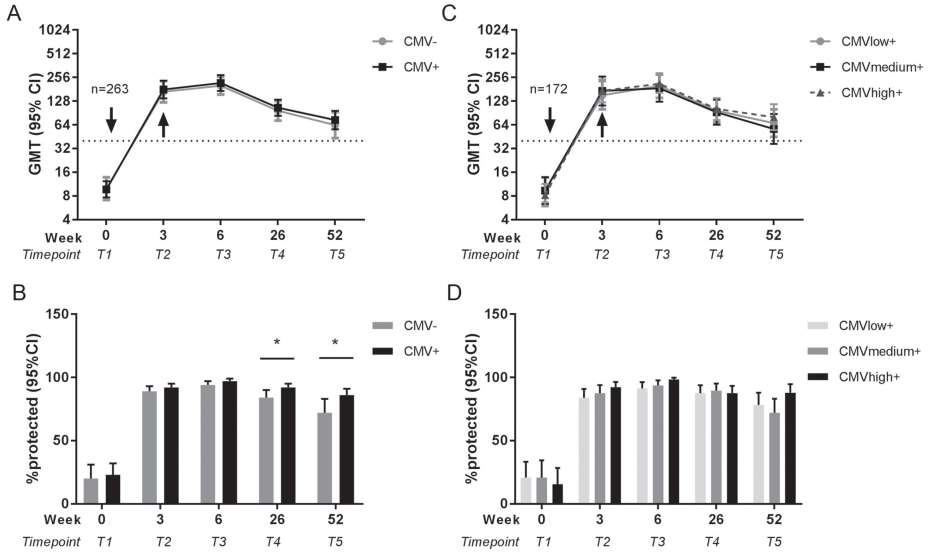


Figure 3. Effect of latent CMV infection on influenza virus-specific antibody titer and protection rate to pandemic influenza infection. Geometric mean and 95% CI of influenza antibody titers and the percentage protected (defined as a titer ≥ 40) are shown for CMV-seropositive and CMV- seronegative individuals (**A, B**) and for CMV-seropositive individuals with low, medium and high anti-CMV IgG levels (**C, D**) before and after pandemic vaccination with H1N1pdm in 2009. Arrows (↓) indicate the moment of vaccination. Dotted horizontal line represents a protective influenza titer of 40. Results are adjusted for sex, age group and previous influenza vaccinations by a Generalized Estimation Equation regression model. Significant differences are tested by pairwise comparison between CMV-seropositive and CMV-seronegative individuals or anti-CMV IgG group high and low per separate time point. * $p < 0.05$

DISCUSSION

In this study, we investigated the effect of age and latent CMV infection on the antibody response to a novel influenza vaccine strain in healthy adults. We found evidence of immunosenescence in these adults from the age of 40. However, latent CMV infection did not impair the antibody responses to a *de novo* influenza vaccine response. Interestingly, indications for the contrary were observed: CMV-seropositive individuals even showed a higher long-term influenza protection rate after pandemic influenza vaccination. These results suggest that latent CMV infection does not always further weaken age-related impaired immunity, but if anything, might be beneficial.

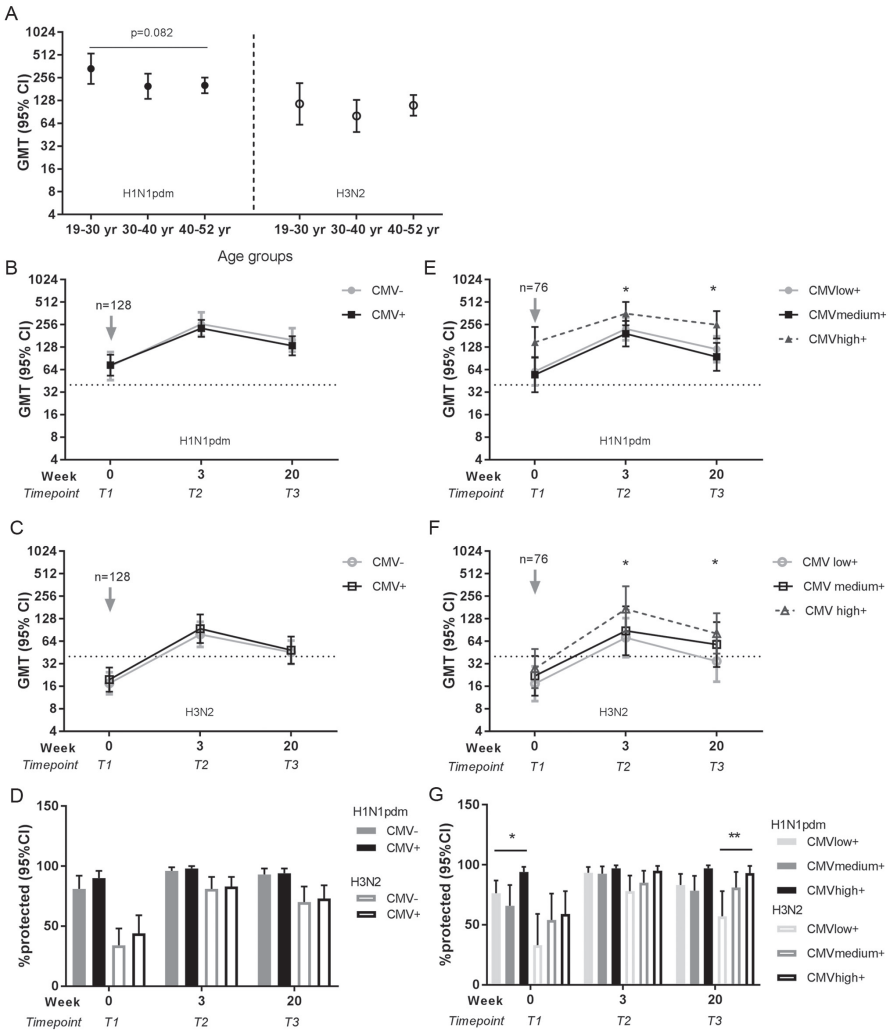


Figure 4. Effect of age and latent CMV infection on influenza virus-specific antibody titer and protection rate to seasonal influenza infection. Geometric mean and 95% CI of influenza antibody titers are shown per age group for H1N1pdm (left panel) and H3N2 (right panel) for the representative time point T2 (3 weeks) after seasonal influenza vaccination 2010-2011 (**A**). Geometric mean and 95% CI of influenza antibody titers (**B, C**) and the percentage protected (defined as a titer ≥ 40 HAU) (**D**) are shown for CMV-seropositive and CMV-seronegative individuals for H1N1pdm and H3N2 strain before and after seasonal vaccination 2010-2011. For CMV-seropositive individuals with low, medium and high anti-CMV IgG levels geometric mean and 95% CI of influenza antibody titers (**E, F**) and the percentage protected (defined as a titer ≥ 40) (**G**) are shown for H1N1pdm and H3N2 strain before and after seasonal vaccination 2010-2011. Arrows (↓) indicate the moment of vaccination. Dotted horizontal line represents a protective influenza titer of 40 HAU. Results for the effect of latent CMV infection are adjusted for sex, age group and previous influenza vaccinations by a Generalized Estimation Equation regression model. Significant differences are tested by pairwise comparison between CMV-seropositive and CMV-seronegative individuals or anti-CMV IgG group high and low per separate time point. Significant differences between age groups were tested with ANOVA and differences between 2 age groups are tested with Student's t-test for (log transformed) antibody titers. * $p < 0.05$

Our study showed no negative association between latent CMV infection and the antibody response to influenza vaccination. Other studies did report negative effects in adults [23, 25, 32] and older adults [25, 26, 28, 43]. However, most of these studies investigated the effect of latent CMV infection on the influenza vaccine response in the presence of pre-existing immunity. In one study, all subjects were even seroprotected (influenza antibody titer > 40 HAU) before influenza vaccination [26]. It is known that individuals with high pre-titers show a lower increase in influenza antibody response after influenza vaccination [7, 19, 35, 44]. Therefore, high pre-titers are associated with lower seroconversion (antibody titer ≥ 40 HAU and ≥ 4-fold increase) and higher protection rate (> 40 HAU). Furthermore, in all but two studies [25, 43], vaccine history was not taken into account, while previous vaccination is associated with lower seroconversion independently of pre-titers [7]. Not accounting for pre-existing immunity in influenza vaccine responses therefore may obscure findings and lead to different findings on the effect of latent CMV infection. Here, we controlled for pre-existing immunity by investigating the effect of latent CMV infection on pandemic vaccination for which pre-existing immunity was low, and by performing analysis adjusted for pre-titers and vaccine history. By doing so, we found that influenza vaccine responses are not impaired by latent CMV infection. If anything, signs of enhanced persistence of protection after influenza vaccination was observed in CMV-seropositive individuals. We observed similar results when we analyzed the effect of latent CMV infection on the seroconversion rate. No impairment by CMV-latent infection on the vaccine response was found, but CMV-seropositive individuals showed a higher seroconversion rate six months and one year after vaccination (T4 p=0.044, T5 p=0.02) (data not shown).

A beneficial effect of latent CMV infection on the immune system has been indicated [10] and is suggested to reflect higher activation status of innate cells after primary CMV infection or reactivation. Accordingly, an increased antibody titer short-term after influenza vaccination in young CMV-seropositive compared to young CMV-seronegative individuals was observed [31-33] and suggested to depend on boosting by low grade inflammation and high levels of circulating IFNγ in CMV-seropositive young individuals [31, 33]. A beneficial effect of latent CMV infection on the long-term persistence of protection after vaccination in adults has to our knowledge not been reported. Waning of protection is thought to be most significant in individuals above 65 years of age [45] and accelerated by latent CMV infection [46]. Our results might suggest a positive effect of CMV infection in adults on the protection rate. Thereby our data fit in a scenario in which latent CMV infection has a beneficial effect in adults and may become detrimental with ageing.

Two studies that reported a short-term negative effect of latent CMV infection in adults, did take the factor pre-existing immunity into account by either correcting for antibody titers pre-vaccination [24] or by investigating the effect of latent CMV infection on the novel pandemic vaccine [23]. However, these studies differ from our study in terms of vaccine type and analysis of the antibody response. Turner *et* al studied the fold increase of influenza antibody titers to seasonal vaccination, corrected for pre-titers before vaccination [24]. They reported

a negative effect on the influenza antibody fold increase in one strain of the trivalent vaccine in CMV-seropositive adults with high anti-CMV IgG levels compared to CMV-seronegative adults. Wald *et al* [23] also reported a negative effect of CMV-seropositivity in adults, by investigating the same pandemic H1N1pdm vaccine response in 2009 as we did. However, they did not adjust for confounders in the analysis [23]. These differences in findings of the effect of latent CMV infection on the influenza vaccine response without pre-existing immunity are unexplained. We speculate that the vaccine dose and adjuvant use may be a reason for these differences. In Turner *et al* half the recommended dose was used [24]. Likewise, an unadjuvanted monovalent vaccine [47] was used in Wald *et al* while in our study the vaccine was adjuvanted. The use of MF59 adjuvant is expected to activate the CD4+ T-cells and further enhance antibody production, thereby eliciting a stronger immune response compared to an unadjuvanted vaccine. Taken together, it may be possible that only with less potent influenza vaccines, a short term negative effect of latent CMV infection is present.

The correlation of lower antibody response to the novel pandemic influenza vaccination with age points to an immunosenescence-driven weakened immune response. Typically, lower antibody responses to influenza vaccination are associated with high age (> 60 years old). Interestingly, we observed already an effect of age in this group of non-elderly (18-52 years of age), although small. This effect of age was due to a lower influenza antibody response from the age of 40 years onwards. It is suggested that differences between age groups to influenza vaccination responses might also explained by HA imprinting [48]. HA imprinting implicates that the immune response is skewed to the group of HA antigens of the influenza strain that is first encountered during childhood. However, this was not the case and HA imprinting could be excluded as an explanation for the age differences.

Similar analyzes were performed for the effect of age and latent CMV on the seasonal influenza vaccine response in season 2010-2011. Seasonal vaccination in 2010-2011 contained the same H1N1pdm strain of the pandemic season and the antigen-drifted H3N2 strain that overlaps in serological response to great extent with previous H3N2 strains [49]. Thus both seasonal strains elicit an immunological memory response. Immunosenescence mainly affects the *de novo* immune responses [36, 37]. In line with this, effects of age on an influenza vaccine response diminish after further vaccination with the same strain [34], explaining the different findings for the effect of age between the pandemic season and season 2010-2011. It was surprising to find that individuals with high anti-CMV IgG levels showed a higher influenza titer and protection rate to seasonal vaccination. We cannot exclude that these individuals might be high-antibody producers in general, as previously shown for Respiratory Syncytial Virus (RSV) and the response to other respiratory viruses [50]. Also, the total group that continued to season 2010-2011 with the study was smaller (n=128) and had a higher number of previous vaccinations than the group of the pandemic season (n=263), complicating the asjusted analysis. Different results were obtained for using seroconversion rate instead of protection rate as definition of responder on the seasonal vaccination. A

positive effect of high anti-CMV levels group was not observed on the seroconversion rate (data not shown). This shows the importance for correcting in our statistical model for these factors and strongly implies caution with interpretations of CMV-induced effects in small study groups or non-adjusted studies as reported in literature.

Important strengths of our study compared to others, are the use of a novel influenza vaccine strain, the relatively large groups of study subjects in the pandemic season and the adjusted analysis with the GEE model. Since aging and latent CMV infection are thought to affect the immune system both independently and by interacting with each other, separation of these factors in analysis is crucial [51]. A limitation of the study is that the study population consists of health care workers who received repeated previous influenza vaccinations. Individuals with repeated previous seasonal influenza vaccinations show in general higher pre-vaccination titers than first time vaccinated individuals [44]. Even in the pandemic season, cross reactivity was reported for the H1N1pdm strain [52, 53]. Together with potential natural exposure to the H1N1pdm strain just before the study, this may explain the detectable titers before pandemic vaccination in this study. The seasonal 2009 vaccination 3 weeks before the study in the pandemic season indeed increased the pandemic pre-titer (data not shown). However, vaccine history of the last years preceding the vaccine trial of the study subjects was reported and was adjusted for in the analysis. Importantly pre titers did not affect the study results, since individuals in our study without detectable pre-titers (n=203) for pandemic influenza vaccination, showed comparable results for the effect of CMV infection for the pandemic season (data not shown).

The influenza response in humans is complex and raises the question if influenza vaccination is the best model to investigate the effect of latent CMV infection on vaccine responses. A less complicated model, in which a vaccine for people that are truly naïve is used, might be a better study design for this question. However, we consider influenza vaccination represents the most relevant because of its high societal importance. Therefore, knowledge on the effect of CMV infection on the influenza antibody response is of great importance.

In conclusion, we used a novel influenza vaccine strain to investigate the effect of age and latent CMV infection on the *de novo* immune response to influenza. We found indeed already impaired antibody responses to vaccination in adults with increasing age, but latent CMV infection did not impair the influenza virus-specific antibody response. Thereby, we show that CMV infection does not per se enhance the age-related impaired immunity as assumed, but if anything might give opposite effects. A model in which CMV infection boosts the immune system during adulthood, while in older adults CMV infection enhances the ageing of the immune system, might be appropriate. These results are important in the decision to invest in preventing latent CMV infection in healthy individuals through strategies like CMV vaccination.

REFERENCES

1. Siegrist, C.A. and R. Aspinall, *B-cell responses to vaccination at the extremes of age.* Nat Rev Immunol, 2009. 9(3): p. 185-94.
2. Tu, W. and S. Rao, *Mechanisms Underlying T-cell Immunosenescence: Aging and Cytomegalovirus Infection.* Front Microbiol, 2016. 7: p. 2111.
3. Fulop, T., A. Larbi, and G. Pawelec, *Human T-cell aging and the impact of persistent viral infections.* Front Immunol, 2013. 4: p. 271.
4. McElhaney, J.E., et al., *T-Cell Immunity to Influenza in Older Adults: A Pathophysiological Framework for Development of More Effective Vaccines.* Front Immunol, 2016. 7: p. 41.
5. Loubet, P., et al., *Factors associated with poor outcomes among adults hospitalized for influenza in France: A three-year prospective multicenter study.* J Clin Virol, 2016. 79: p. 68-73.
6. van Essen, G.A., et al., *Influenza vaccination in 2000: recommendations and vaccine use in 50 developed and rapidly developing countries.* Vaccine, 2003. 21(16): p. 1780-5.
7. Goodwin, K., C. Viboud, and L. Simonsen, *Antibody response to influenza vaccination in the elderly: a quantitative review.* Vaccine, 2006. 24(8): p. 1159-69.
8. Pera, A., et al., *Immunosenescence: Implications for response to infection and vaccination in older people.* Maturitas, 2015.
9. Jefferson, T., et al., *Vaccines for preventing influenza in the elderly.* Cochrane Database Syst Rev, 2010(2): p. CD004876.
10. Arens, R., et al., *5(th) International Workshop on CMV and Immunosenescence - A shadow of cytomegalovirus infection on immunological memory.* Eur J Immunol, 2015. 45(4): p. 954-7.
11. Pawelec, G. and E. Derhovanessian, *Role of CMV in immune senescence.* Virus Res, 2011. 157(2): p. 175-9.
12. Cannon, M.J., D.S. Schmid, and T.B. Hyde, *Review of cytomegalovirus seroprevalence and demographic characteristics associated with infection.* Rev Med Virol, 2010. 20(4): p. 202-13.
13. Stowe, R.P., et al., *Chronic herpesvirus reactivation occurs in aging.* Exp Gerontol, 2007. 42(6): p. 563-70.
14. Korndewal, M.J., et al., *Cytomegalovirus infection in the Netherlands: seroprevalence, risk factors, and implications.* J Clin Virol, 2015. 63: p. 53-8.
15. Cannon, M.J., T.B. Hyde, and D.S. Schmid, *Review of cytomegalovirus shedding in bodily fluids and relevance to congenital cytomegalovirus infection.* Rev Med Virol, 2011. 21(4): p. 240-55.
16. Bhattacharyya, M.K. and A.J. Lustig, *Telomere dynamics in genome stability.* Trends Biochem Sci, 2006. 31(2): p. 114-22.
17. Lustig, A., et al., *Telomere Shortening, Inflammatory Cytokines, and Anti-Cytomegalovirus Antibody Follow Distinct Age-Associated Trajectories in Humans.* Front Immunol, 2017. 8: p. 1027.
18. Wikby, A., et al., *Expansions of peripheral blood CD8 T-lymphocyte subpopulations and an association with cytomegalovirus seropositivity in the elderly: the Swedish NONA immune study.* Exp Gerontol, 2002. 37(2-3): p. 445-53.
19. Hadrup, S.R., et al., *Longitudinal studies of clonally expanded CD8 T-cells reveal a repertoire shrinkage predicting mortality and an increased number of dysfunctional cytomegalovirus-specific T-cells in the very elderly.* J Immunol, 2006. 176(4): p. 2645-53.
20. Appay, V. and S.L. Rowland-Jones, *Lessons from the study of T-cell differentiation in persistent human virus infection.* Semin Immunol, 2004. 16(3): p. 205-12.

21. McElhaney, J.E., *The unmet need in the elderly: How immunosenescence, CMV infection, co-morbidities and frailty are a challenge for the development of more effective influenza vaccines.* European Geriatric Medicine, 2012. 3: p. S21-S22.

22. Saurwein-Teissl, M., et al., *Lack of antibody production following immunization in old age: association with CD8(+)CD28(-) T-cell clonal expansions and an imbalance in the production of Th1 and Th2 cytokines.* J Immunol, 2002. 168(11): p. 5893-9.

23. Wald, A., et al., *Impact of human cytomegalovirus (CMV) infection on immune response to pandemic 2009 H1N1 influenza vaccine in healthy adults.* J Med Virol, 2013. 85(9): p. 1557-60.

24. Turner, J.E., et al., *Rudimentary signs of immunosenescence in Cytomegalovirus-seropositive healthy young adults.* Age (Dordr), 2014. 36(1): p. 287-97.

25. Trzonkowski, P., et al., *Association between cytomegalovirus infection, enhanced proinflammatory response and low level of anti-hemagglutinins during the anti-influenza vaccination--an impact of immunosenescence.* Vaccine, 2003. 21(25-26): p. 3826-36.

26. Frasca, D., et al., *Cytomegalovirus (CMV) seropositivity decreases B cell responses to the influenza vaccine.* Vaccine, 2015. 33(12): p. 1433-9.

27. Derhovanessian, E., et al., *Latent infection with cytomegalovirus is associated with poor memory CD4 responses to influenza A core proteins in the elderly.* J Immunol, 2014. 193(7): p. 3624-31.

28. Alonso Arias, R., et al., *Intensity of the humoral response to cytomegalovirus is associated with the phenotypic and functional status of the immune system.* J Virol, 2013. 87(8): p. 4486-95.

29. den Elzen, W.P., et al., *Cytomegalovirus infection and responsiveness to influenza vaccination in elderly residents of long-term care facilities.* Vaccine, 2011. 29(29-30): p. 4869-74.

30. Haq, K., et al., *Cytomegalovirus Seropositivity Predicts a Decline in the T-cell But Not the Antibody Response to Influenza in Vaccinated Older Adults Independent of Type 2 Diabetes Status.* J Gerontol A Biol Sci Med Sci, 2016.

31. Furman, D., et al., *Cytomegalovirus infection enhances the immune response to influenza.* Sci Transl Med, 2015. 7(281): p. 281ra43.

32. Strindhall, J., et al., *Humoral response to influenza vaccination in relation to pre-vaccination antibody titres, vaccination history, cytomegalovirus serostatus and CD4/CD8 ratio.* Infect Dis (Lond), 2016. 48(6): p. 436-42.

33. McElhaney, J.E., et al., *Predictors of the antibody response to influenza vaccination in older adults with type 2 diabetes.* BMJ Open Diabetes Res Care, 2015. 3(1): p. e000140.

34. Mosterin Hopping, A., et al., *The confounded effects of age and exposure history in response to influenza vaccination.* Vaccine, 2016. 34(4): p. 540-6.

35. Beyer, W.E., et al., *Effects of repeated annual influenza vaccination on vaccine sero-response in young and elderly adults.* Vaccine, 1996. 14(14): p. 1331-9.

36. Michel, J.P. and P.O. Lang, *Promoting life course vaccination.* Rejuvenation Res, 2011. 14(1): p. 75-81.

37. Lang, P.O. and R. Aspinall, *Immunosenescence and herd immunity: with an ever-increasing aging population do we need to rethink vaccine schedules?* Expert Rev Vaccines, 2012. 11(2): p. 167-76.

38. Miller, E., et al., *Incidence of 2009 pandemic influenza A H1N1 infection in England: a cross-sectional serological study.* Lancet, 2010. 375(9720): p. 1100-8.

39. Luytjes, W., et al., *HI responses induced by seasonal influenza vaccination are associated with clinical protection and with seroprotection against non-homologous strains.* Vaccine, 2012. 30(35): p. 5262-9.

40. Zeger, S.L. and K.Y. Liang, *Longitudinal data analysis for discrete and continuous outcomes.* Biometrics, 1986. 42(1): p. 121-30.

41. Wijnans, L. and B. Voordouw, *A review of the changes to the licensing of influenza vaccines in Europe.* Influenza Other Respir Viruses, 2016. 10(1): p. 2-8.

42. Wang, G.C., et al., *T-cell receptor alphabeta diversity inversely correlates with pathogen-specific antibody levels in human cytomegalovirus infection.* Sci Transl Med, 2012. 4(128): p. 128ra42.

43. Derhovanessian, E., et al., *Cytomegalovirus-associated accumulation of late-differentiated CD4 T-cells correlates with poor humoral response to influenza vaccination.* Vaccine, 2013. 31(4): p. 685-90.

44. Huang, K.A., et al., *Antibody Responses to Trivalent Inactivated Influenza Vaccine in Health Care Personnel Previously Vaccinated and Vaccinated for The First Time.* Sci Rep, 2017. 7: p. 40027.

45. Castilla, J., et al., *Decline in influenza vaccine effectiveness with time after vaccination, Navarre, Spain, season 2011/12.* Euro Surveill, 2013. 18(5).

46. Reed, R.G., R.N. Greenberg, and S.C. Segerstrom, *Cytomegalovirus serostatus, inflammation, and antibody response to influenza vaccination in older adults: The moderating effect of beta blockade.* Brain Behav Immun, 2017. 61: p. 14-20.

47. Chen, W.H., et al., *Phase 2 assessment of the safety and immunogenicity of two inactivated pandemic monovalent H1N1 vaccines in adults as a component of the U.S. pandemic preparedness plan in 2009.* Vaccine, 2012. 30(28): p. 4240-8.

48. Gostic, K.M., et al., *Potent protection against H5N1 and H7N9 influenza via childhood hemagglutinin imprinting.* Science, 2016. 354(6313): p. 722-726.

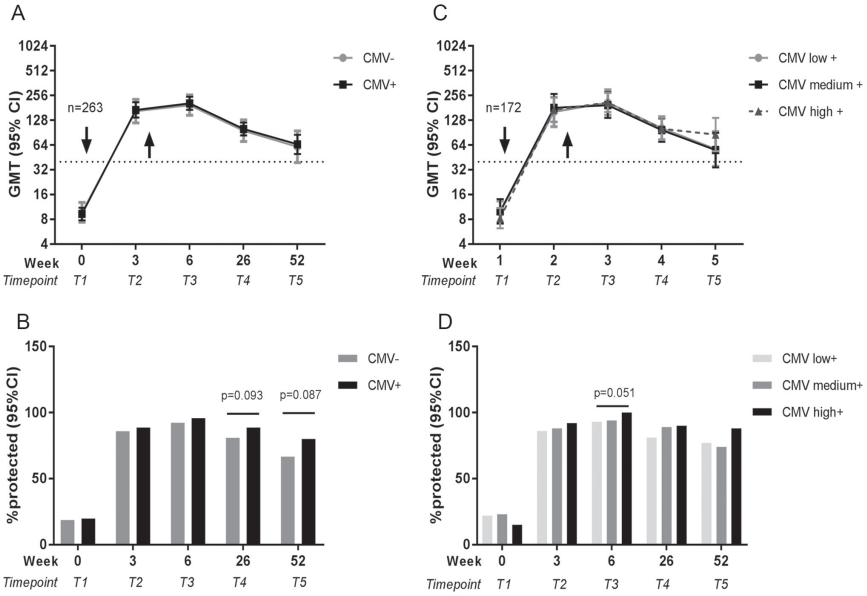
49. Ann, J., et al., *Molecular and antigenic evolution of human influenza A/H3N2 viruses in Quebec, Canada, 2009-2011.* J Clin Virol, 2012. 53(1): p. 88-92.

50. Orange, J.S., W. Du, and A.R. Falsey, *Therapeutic Immunoglobulin Selected for High Antibody Titer to RSV also Contains High Antibody Titers to Other Respiratory Viruses.* Front Immunol, 2015. 6: p. 431.

51. Wertheimer, A.M., et al., *Aging and cytomegalovirus infection differentially and jointly affect distinct circulating T-cell subsets in humans.* J Immunol, 2014. 192(5): p. 2143-55.

52. Wrammert, J., et al., *Broadly cross-reactive antibodies dominate the human B cell response against 2009 pandemic H1N1 influenza virus infection.* J Exp Med, 2011. 208(1): p. 181-93.

53. Centers for Disease, C. and Prevention, *Serum cross-reactive antibody response to a novel influenza A (H1N1) virus after vaccination with seasonal influenza vaccine.* MMWR Morb Mortal Wkly Rep, 2009. 58(19): p. 521-4.



Supplementary Figure 1. Effect of CMV infection on unadjusted influenza antibody titer and protection rate to pandemic influenza infection. Geometric mean and 95% CI of influenza antibody titers and the percentage protected (defined as a titer ≥ 40) are shown for CMV+ and CMV- individuals (**A, B**) and for CMV-seropositive individuals with low, medium and high anti-CMV IgG levels (**C, D**) before and after pandemic vaccination with H1N1pdm in 2009. Arrows (↓) indicate the moment of vaccination. Dotted horizontal line represents a protective influenza titer of 40 HAU. Significant differences are tested by T test for influenza antibody titers and by Fisher exact test between CMV-seropositive and CMV-seronegative individuals or anti-CMV IgG group high and low per separate time point.

Dependent variable	
Outcome GMT	2log titer
Outcome protection	titer ≥ 40 HAU or not
Outcome seroconversion	titer ≥ 40 HAU and 4-fold increase or not

Factor CMV effect	
Factor CMV-serostatus	CMV-serostatus positive / negative
Factor CMV IgG group in CMV + individuals	Anti-CMV IgG group Low (≤ 30U/ml) / medium (> 30 U/ml, ≤ 90 U/ml) / high (> 90 U/ml)

Other factors		
	Season 1 (H1N1pdm)	**Season 2 (H1N1pdm and H3N2)**
Sex	Sex Male / Female	Sex Male / Female
Age	Age group 18-30 season / 30-40 season / 40-52 season	Age group 18-30 season / 30-40 season / 40-52 season
Previous vaccinations	Previous influenza vaccinations in last 3 seasons Never / Sometimes / Always	Previous influenza vaccinations in last 3 seasons Never / Sometimes / Always
	Seasonal influenza vaccination 2009 before study Yes / No	Seasonal vaccination 2009 Yes / No
	Seasonal influenza vaccination 2009 during study Yes / No	

Supplementary Table 1. Factors in Generalized Estimation Equation regression model for adjusted analysis of the effect of latent CMV infection on the antibody response to influenza vaccination. GEE models to correct for sex, age and previous influenza vaccinations are performed with one CMV factor in it (either CMV-serostatus or anti-CMV IgG level group) for one dependent variable (either titer or protection), to investigate the effect of latent CMV infection on the antibody response to influenza vaccination.

	Influenza antibody titer			Protection		
Parameter	**B (beta)**	**Standard Error**	**Sig (P-value)**	**B (beta)**	**Standard Error**	**Sig (P-value)**
(Intercept)	3.783	0.3168	0	-1.199	0.3872	0.002
Age group 2 (40-52 year)	-0.629	0.2547	**0.013**	-0.59	0.3226	**0.068**
Age group 1 (30-40 year)	-0.014	0.2596	0.958	-0.023	0.3358	0.946
Age group 0 (18-30 year)	0a	.	.	0a	.	.
Sex male	0.161	0.1974	0.414	-0.074	0.247	0.765
Sex female	0a	.	.	0a	.	.
Previous influenza vaccinations yes	-0.583	0.3263	**0.074**	-0.607	0.4259	0.154
Previous influenza vaccinations sometimes	-0.459	0.2442	**0.06**	-0.381	0.346	0.27
Previous influenza vaccinations no	0[a]	.	.	0[a]	.	.
Seasonal 2009 vaccination before study yes	0.017	0.2758	0.952	0.803	0.3979	**0.043**
Seasonal 2009 vaccination before study no	0[a]	.	.	0[a]	.	.
Seasonal 2009 vaccination during study yes	-0.004	0.2612	0.989	-0.094	0.3367	0.779
Seasonal 2009 vaccination during study no	0[a]	.	.	0[a]	.	.
CMV-serostatus positive	-0.029	0.2395	0.903	0.189	0.3461	0.586
CMV-serostaus negative	0[a]	.	.	0[a]	.	.
Timepoint 5	2.679	0.29	**< 0.001**	2.375	0.3555	**< 0.001**
Timepoint 4	3.286	0.2388	**< 0.001**	3.071	0.3557	**< 0.001**
Timepoint 3	4.344	0.2405	**< 0.001**	4.148	0.415	**< 0.001**
Timepoint 2	4.089	0.2684	**< 0.001**	3.466	0.3828	**< 0.001**
Timepoint 1	0[a]	.	.	0[a]	.	.
CMV-serostatus positive * Timepoint 5	0.243	0.3385	0.473	0.63	0.4587	0.17
CMV-serostatus positive * Timepoint 4	0.155	0.2825	0.582	0.582	0.4633	0.209
CMV-serostatus positive * Timepoint 3	0.138	0.2892	0.632	0.613	0.6122	0.317
CMV-serostatus positive * Timepoint 2	0.122	0.3201	0.703	0.188	0.4869	0.7
CMV-serostatus positive * Timepoint 1	0[a]	.	.	0[a]	.	.
CMV-serostatus negative * Timepoint 5	0[a]	.	.	0[a]	.	.
CMV-serostatus negative * Timepoint 4	0[a]	.	.	0[a]	.	.
CMV-serostatus negative * Timepoint 3	0[a]	.	.	0[a]	.	.
CMV-serostatus negative * Timepoint 2	0[a]	.	.	0[a]	.	.
CMV-serostatus negative * Timepoint 1	0[a]	.	.	0[a]	.	.

Supplementary Table 2. Regression table effect CMV-serostatus on pandemic influenza vaccine response of H1N1pdm strain in the pandemic season. Bold: p value < 0.10 Bold and underlined: p value < 0.05. [a] reference category

	Influenza antibody titer			Protection		
Parameter	**B (beta)**	**Standard Error**	**Sig (P-value)**	**B (beta)**	**Standard Error**	**Sig (P-value)**
(Intercept)	3.844	0.3311	0	-0.794	0.419	0.058
Age group 2 (40-52 year)	-0.485	0.3022	0.109	-0.527	0.432	0.222
Age group 1 (30-40 year)	0.438	0.3215	0.173	0.606	0.443	0.171
Age group 0 (18-30 year)	0[a]	.	.	0[a]	.	.
Sex male	-0.226	0.2302	0.326	-0.495	0.333	0.137
Sex female	0[a]	.	.	0[a]	.	.
Previous influenza vaccinations yes	-0.394	0.3869	0.309	-0.689	0.571	0.228
Previous influenza vaccinations sometimes	-0.392	0.2664	0.142	-0.251	0.417	0.548
Previous influenza vaccinations no	0[a]	.	.	0[a]	.	.
Seasonal 2009 vaccination before study yes	-0.37	0.2902	0.203	0.110	0.459	0.810
Seasonal 2009 vaccination before study no	0[a]	.	.	0[a]	.	.
Seasonal 2009 vaccination during study yes	-0.055	0.2745	0.841	-0.454	0.402	0.258
Seasonal 2009 vaccination during study no	0[a]	.	.	0[a]	.	.
CMV IgG group high	-0.208	0.335	0.535	-0.387	0.533	0.468
CMV IgG group medium	-0.019	0.3543	0.957	0[a]	.	.
CMV IgG group low	0[a]	.	.	0[a]	.	.
Timepoint 5	2.83	0.3081	**< 0.001**	2.922	0.527	**< 0.001**
Timepoint 4	3.357	0.2884	**< 0.001**	3.643	0.573	**< 0.001**
Timepoint 3	4.404	0.326	**< 0.001**	4.075	0.605	**< 0.001**
Timepoint 2	4.012	0.3443	**< 0.001**	3.314	0.511	**< 0.001**
Timepoint 1	0[a]	.	.	0[a]	.	.
CMV IgG group high * Timepoint 5	0.459	0.4242	0.279	1.147	0.767	0.135
CMV IgG group high * Timepoint 4	0.278	0.3884	0.475	0.390	0.734	0.596
CMV IgG group high * Timepoint 3	0.293	0.4027	0.467	2.238	1.278	**0.080**
CMV IgG group high * Timepoint 2	0.388	0.4392	0.377	1.275	0.738	**0.084**
CMV IgG group high * Timepoint 1	0[a]	.	.	0[a]	.	.
CMV IgG group medium * Timepoint 5	-0.226	0.4282	0.597	-0.364	0.723	0.614
CMV IgG group medium * Timepoint 4	-0.048	0.3668	0.895	0.219	0.752	0.771
CMV IgG group medium * Timepoint 3	-0.084	0.4223	0.843	0.362	0.866	0.676
CMV IgG group medium * Timepoint 2	0.195	0.4474	0.663	0.330	0.703	0.638
CMV IgG group medium * Timepoint 1	0[a]	.	.	0[a]		
CMV IgG group low * Timepoint 5	0[a]	.	.	0[a]		
CMV IgG group low * Timepoint 4	0[a]	.	.	0[a]		
CMV IgG group low * Timepoint 3	0[a]	.	.	0[a]		
CMV IgG group low * Timepoint 2	0[a]	.	.	0[a]	.	.
CMV IgG group low * Timepoint 1	0[a]	.	.	0[a]	.	.

Supplementary Table 3. Regression table effect CMV IgG group on pandemic influenza vaccine response of H1N1pdm strain in the pandemic season. Bold: p value < 0.10 Bold and underlined: p value < 0.05. [a] reference category

	Influenza antibody titer			Protection		
Parameter	**B (beta)**	**Standard Error**	**Sig (P-value)**	**B (beta)**	**Standard Error**	**Sig (P-value)**
(Intercept)	7.139	0.3716	0	3.222	1.1526	0.005
Age group 2 (40-52 year)	-0.334	0.3094	0.281	-1.643	1.0299	0.111
Age group 1 (30-40 year)	-0.221	0.347	0.525	-1.385	1.0983	0.207
Age group 0 (18-30 year)	0[a]	.	.	0[a]	.	.
Sex male	-0.19	0.2621	0.468	-0.236	0.4394	0.591
Sex female	0[a]	.	.	0[a]	.	.
Previous influenza vaccinations yes	-0.934	0.481	**0.052**	-0.547	0.7594	0.471
Previous influenza vaccinations sometimes	-0.693	0.4209	0.1	-0.657	0.7267	0.366
Previous influenza vaccinations no	0[a]	.	.	0[a]	.	.
Seasonal 2009 vaccination yes	-0.315	0.4453	0.479	-0.467	0.8388	0.578
Seasonal 2009 vaccination no	0[a]	.	.	0[a]	.	.
CMV-serostaus positive	0.042	0.3729	0.911	0.685	0.4495	0.128
CMV-serostaus negative	0[a]	.	.	0[a]	.	.
Timepoint 3	1.158	0.2085	**< 0.001**	1.202	0.3643	**0.001**
Timepoint 2	1.864	0.2372	**< 0.001**	1.658	0.4823	**0.001**
Timepoint 1	0[a]	.	.	0[a]	.	.
CMV-serostatus positive * Timepoint 3	-0.298	0.2569	0.246	-0.526	0.4531	0.246
CMV-serostatus positive * Timepoint 2	-0.231	0.284	0.415	0.102	0.7427	0.891
CMV-serostatus positive * Timepoint 1	0[a]	.	.	0[a]	.	.
CMV-serostatus negative * Timepoint 3	0[a]	.	.	0[a]	.	.
CMV-serostatus negative * Timepoint 2	0[a]	.	.	0[a]	.	.
CMV-serostatus negative * Timepoint 1	0[a]	.	.	0[a]	.	.

Supplementary Table 4. Regression table effect CMV-serostatus on seasonal influenza vaccine response of H1N1pdm strain in the season 2010-2011. Bold: p value < 0.10 Bold and underlined: p value < 0.05. [a] reference category

	Influenza antibody titer			Protection		
Parameter	**B (beta)**	**Standard Error**	**Sig (P-value)**	**B (beta)**	**Standard Error**	**Sig (P-value)**
(Intercept)	7.321	0.3907	0	3.920744	1.127556	0.001
Age group 2 (40-52 year)	-0.969	0.4197	**0.021**	-1.06577	0.777988	0.171
Age group 1 (30-40 year)	-0.663	0.4203	0.115	#		
Age group 0 (18-30 year)	0[a]	.	.	0[a]	.	.
Sex male	-0.599	0.2927	0.041	-1.35677	0.665921	**0.042**
Sex female	0[a]	.	.	0[a]	.	.
Previous influenza vaccinations yes	-0.55	0.4719	0.244	0.066665	0.996880	0.947
Previous influenza vaccinations sometimes	-0.489	0.4355	0.261	-0.52934	0.897337	0.555
Previous influenza vaccinations no	0[a]	.	.	0[a]	.	.
Seasonal 2009 vaccination yes	-0.418	0.3913	0.286	-1.34266	1.088206	0.217
Seasonal 2009 vaccination no	0[a]	.	.	0[a]	.	.
CMV IgG group high	1.295	0.4562	**0.005**	1.76129	0.897221	**0.05**
CMV IgG group medium	-0.149	0.5024	0.767	-0.61748	0.765129	0.42
CMV IgG group low	0[a]	.	.	0[a]	.	.
Timepoint 3	0.986	0.2243	0	0.495826	0.364392	0.174
Timepoint 2	1.887	0.2159	0	1.62821	0.810095	**0.044**
Timepoint 1	0[a]	.	.	0[a]	.	.
CMV IgG group high * Timepoint 3	-0.209	0.3584	0.56	0.258459	1.355159	0.849
CMV IgG group high * Timepoint 2	-0.615	0.3293	**0.062**	-0.87393	1.531723	0.568
CMV IgG group high * Timepoint 1	0[a]	.	.	0[a]	.	.
CMV IgG group medium * Timepoint 3	-0.191	0.3379	0.573	0.262801	0.586560	0.654
CMV IgG group medium * Timepoint 2	-0.064	0.417	0.878	0.50072	1.231240	0.684
CMV IgG group medium * Timepoint 1	0[a]	.	.	0[a]		
CMV IgG group low * Timepoint 3	0[a]	.	.	0[a]		
CMV IgG group low * Timepoint 2	0[a]	.	.	0[a]		
CMV IgG group low * Timepoint 1	0[a]	.	.	0[a]		

Supplementary Table 5. Regression table effect CMV IgG group serostatus on seasonal influenza vaccine response of H1N1pdm strain in the season 2010-2011. Bold: p value <0.10 Bold and underlined: p value <0.05. # for age group 0 (18-30 year) all participants were protected after vaccination (outcome 1) wherefore the model did not run. This is solved merging age group 0 and 1 for this model, resulting in no separate regression coefficient for age group 1. [a] reference category

	Influenza antibody titer			Protection		
Parameter	**B (beta)**	**Standard Error**	**Sig (P-value)**	**B (beta)**	**Standard Error**	**Sig (P-value)**
(Intercept)	3.937	0.4387	0	-1.055	0.571	0.065
Age group 2 (40-52 year)	0.044	0.4029	0.913	-0.351	0.5248	0.504
Age group 1 (30-40 year)	-0.239	0.4554	0.6	-0.516	0.5764	0.371
Age group 0 (18-30 year)	0[a]	.	.	0[a]	.	.
Sex male	-0.023	0.2778	0.933	0.215	0.3255	0.508
Sex female	0[a]	.	.	0[a]	.	.
Previous influenza vaccinations yes	-0.224	0.4314	0.604	-0.069	0.4824	0.886
Previous influenza vaccinations sometimes	-0.06	0.3824	0.875	0.422	0.4741	0.373
Previous influenza vaccinations no	0[a]	.	.	0[a]	.	.
Seasonal 2009 vaccination yes	0.729	0.4627	0.115	0.868	0.4818	**0.072**
Seasonal 2009 vaccination no	0[a]	.	.	0[a]	.	.
CMV-serostaus positive	0.163	0.3143	0.605	0.425	0.3732	0.255
CMV-serostaus negative	0[a]	.	.	0[a]	.	.
Timepoint 3	1.377	0.2333	**< 0.001**	1.548	0.3552	**< 0.001**
Timepoint 2	2.175	0.2797	**< 0.001**	2.144	0.4236	**< 0.001**
Timepoint 1	0[a]	.	.	0[a]	.	.
CMV-serostatus positive * Timepoint 3	-0.065	0.3074	0.832	-0.278	0.4502	0.537
CMV-serostatus positive * Timepoint 2	0.088	0.3812	0.817	-0.271	0.5373	0.614
CMV-serostatus positive * Timepoint 1	0[a]	.	.	0[a]	.	.
CMV-serostatus negative * Timepoint 3	0[a]	.	.	0[a]	.	.
CMV-serostatus negative * Timepoint 2	0[a]	.	.	0[a]	.	.
CMV-serostatus negative * Timepoint 1	0[a]	.	.	0[a]	.	.

Supplementary Table 6. Regression table effect CMV-serostatus on seasonal influenza vaccine response of H3N2 strain in the season 2010-2011. Bold: p value <0.10 Bold and underlined: p value <0.05. [a] reference category

Parameter	Influenza antibody titer			Protection		
	B (beta)	Standard Error	Sig (P-value)	B (beta)	Standard Error	Sig (P-value)
(Intercept)	3.895	0.7973	0	-1.777	0.861	0.039
Age group 2 (40-52 year)	-0.347	0.7905	0.661	-0.428	0.817	0.601
Age group 1 (30-40 year)	-0.519	0.8256	0.53	-0.373	0.8499	0.66
Age group 0 (18-30 year)	0[a]	.	.	0[a]	.	.
Sex male	-0.093	0.3691	0.801	0.475	0.4621	0.304
Sex female	0[a]	.	.	0[a]	.	.
Previous influenza vaccinations yes	0.407	0.5556	0.464	0.81	0.6561	0.217
Previous influenza vaccinations sometimes	0.407	0.4929	0.409	1.406	0.6414	**0.028**
Previous influenza vaccinations no	0[a]	.	.	0[a]	.	.
Seasonal 2009 vaccination yes	0.566	0.6298	0.368	0.763	0.6701	0.255
Seasonal 2009 vaccination no	0[a]	.	.	0[a]	.	.
CMV IgG group high	0.674	0.3982	**0.09**	1.033	0.6058	**0.088**
CMV IgG group medium	0.35	0.5267	0.506	0.836	0.6501	0.199
CMV IgG group low	0[a]	.	.	0[a]	.	.
Timepoint 3	1.003	0.2659	**< 0.001**	0.989	0.3764	**0.009**
Timepoint 2	2.043	0.3249	**< 0.001**	1.965	0.5822	**0.001**
Timepoint 1	0[a]	.	.	0[a]	.	.
CMV IgG group high * Timepoint 3	0.554	0.4295	0.197	1.211	1.0022	0.227
CMV IgG group high * Timepoint 2	0.598	0.5711	0.295	0.741	1.1863	0.532
CMV IgG group high * Timepoint 1	0[a]	.	.	0[a]	.	.
CMV IgG group medium * Timepoint 3	0.387	0.5298	0.465	0.31	0.7386	0.675
CMV IgG group medium * Timepoint 2	-0.043	0.6415	0.946	-0.339	0.8217	0.68
CMV IgG group medium * Timepoint 1	0[a]	.	.	0[a]	.	.
CMV IgG group low * Timepoint 3	0[a]	.	.	0[a]	.	.
CMV IgG group low * Timepoint 2	0[a]	.	.	0[a]	.	.
CMV IgG group low * Timepoint 1	0[a]	.	.	0[a]	.	.

Supplementary Table 7. Regression table effect CMV IgG group serostatus on seasonal influenza vaccine response of H3N2 strain in the season 2010-2011. Bold: p value < 0.10 Bold and underlined: p value < 0.05 [a] reference category

Time point	Correlation age and influenza titer	Differences between age groups in influenza titer
T2	p=0.0013, R -0.198	p=0.016
T3	p=0.000, R= -0.269	p=0.000
T4	p=0.006, R= -0.172	p=0.015
T5	p=0.165, R=-0.112	p=0.714

Supplementary Table 8. Negative effect of age on titers after de novo pandemic influenza vaccination. Correlations between age and H1N1pdm influenza antibody titer is presented on all time points of the pandemic season (Pearson correlation). Differences H1N1pdm influenza antibody titers after vaccination (T2-T5) (log transformed) between age groups (18-30 year, 30-40 year, 40-52 year) are presented (ANOVA). P < 0.05 is considered significant.

3

Effect of latent cytomegalovirus infection on the antibody response to influenza vaccination: *a systematic review and meta-analysis*

2019 Medical Microbiology and Immunology, doi: 10.1007/s00430-019-00602-z

S.P.H. van den Berg[1,2], K. Warmink[1], J.A.M. Borghans[2], M.J. Knol[3], D. van Baarle[1,2]

1 Center for Infectious Disease Control, Center of Immunology of Infectious diseases and vaccines, National Institute for Public Health and the Environment, Bilthoven, the Netherlands
2 Laboratory of Translational Immunology, University Medical Center Utrecht, Utrecht, the Netherlands
3 Center for Infectious Disease Control, Epidemiology and Surveillance Unit (EPI), National Institute for Public Health and the Environment, Bilthoven, the Netherlands

ABSTRACT

Latent infection with cytomegalovirus (CMV) is thought to accelerate ageing of the immune system. With age, influenza-vaccine responses are impaired. Although several studies investigated the effect of CMV infection on antibody responses to influenza vaccination, this led to contradicting conclusions. Therefore, we investigated the relation between CMV infection and the antibody response to influenza vaccination by performing a systematic review and meta-analysis.

All studies on the antibody response to influenza vaccination in association with CMV infection were included (n=17). The following outcome variables were extracted: a) the geometric mean titer pre-/post-vaccination ratio (GMR) per CMV-serostatus group, and in addition b) the percentage of subjects with a response per CMV-serostatus group and c) the association between influenza- and CMV-specific antibody titers.

The influenza-specific GMR revealed no clear evidence for an effect of CMV-seropositivity on the influenza vaccine response in young or old individuals. Meta-analysis of the response rate to influenza vaccination showed a non-significant trend towards a negative effect of CMV-seropositivity. However, funnel-plot analysis suggests that this is a consequence of publication bias. A weak negative association between CMV antibody titers and influenza antibody titers was reported in several studies, but associations could not be analysed systematically due to the variety of outcome variables.

In conclusion, by systematically integrating the available studies we show that there is no unequivocal evidence that latent CMV infection affects the influenza antibody response to vaccination. Further studies, including the level of CMV antibodies, are required to settle on the potential influence of latent CMV infection on the influenza vaccine response.

INTRODUCTION

Age-related reduced function of the immune system, often referred to as "immunosenescence", is suggested to be influenced by cytomegalovirus (CMV) infection [1]. Main features of CMV-seropositivity include low percentages of naïve T-cells and reduced diversity in the T-cell repertoire, which may impair the ability to respond to heterologous infection or vaccination [2] and result in lower B-cell functions by lack of T-cell help [3, 4]. CMV-seropositivity has also been identified as a factor of the Immune Risk Profile (IRP) for mortality in the Swedish longevity studies [5].

CMV prevalence increases with age in the general population from 30% in children to above 90% at the age of 80 and older [6, 7]. Primary CMV infection and reactivation from latency can cause significant problems when the immune system is compromised or immature, but is usually asymptomatic in healthy individuals [8]. However, CMV frequently reactivates during life [9, 10], and can lead to detectable CMV DNA levels, mainly in the elderly [8, 11]. Control of CMV requires continuous immune surveillance and leads to large numbers of CMV-specific T-cells, up to 10-30% of CD8+ T-cells in the periphery. The lifelong need to control CMV is by many thought to take its toll and to hamper immune responses to heterologous infections or vaccination [3]. Indeed, in several mouse models the immune responses to heterologous infections was shown to be negatively affected by CMV [12-14]. However, other studies suggested a positive effect of CMV on the response to heterologous infections [12, 15]

In humans, the potential effect of CMV infection on a heterologous immune response are mainly studied in the context of influenza vaccination. Seasonal influenza vaccination is an effective means to prevent influenza infection [16-18]. However, effectiveness of influenza vaccination decreases with age, leaving older adults exposed to an increased risk of influenza infection [1]. In older adults, influenza infection more often leads to disease-related hospitalization, complications and mortality [17, 19-21]. Influenza vaccines are primarily focused on eliciting a strain-specific antibody response. Antibodies are important as they give rise to so-called sterilizing immunity; the immune status where the host immune response effectively blocks virus infection. The most widely used method to measure strain-specific influenza titers is the hemagglutination-inhibition (HI) assay, which reflects the ability of specific antibodies to bind influenza virus and inhibit viral agglutination of red blood cells [22, 23]. European medicine agency guidelines describe the analysis and presentation of influenza antibody data for development of influenza vaccines [24], stating as a minimum requirement that geometric mean titers (GMTs) (with 95% confidence intervals) and pre-/post-vaccination ratios (GMR), and response rates should be reported.

Clarification of the effect of CMV on influenza vaccine responses is of high importance. The current suboptimal immune response to influenza vaccination in elderly will become an increasingly large problem. By 2050, the population of older persons (defined by the United Nations as those aged 60 years and above) is expected to double in size compared to 2015. With an increasing life expectancy, the group of elderly at high-risk for influenza

complications will increase quickly and contribute to the rising challenges of public health. As latent CMV infection is highly frequent in the population, it is critical to elucidate whether CMV infection influences influenza vaccination responses, in order to be able to optimize vaccine strategies in the population.

Several studies investigated the effect of CMV infection on immune responses induced by influenza vaccination. The first study by *Trzonkowski et al.* reported a negative association between CMV infection and the response to influenza vaccination [25]. Some studies confirmed this result [26], but others did not find an effect of CMV infection on the influenza vaccine response [27]. In contrast, *Furman et al.* reported a positive effect of CMV infection on the immune response to influenza vaccination in adults [28]. To date, no consensus of the effect of latent CMV infection on the antibody response to influenza vaccination has been reached [4, 29, 30].

Here, we systematically reviewed studies on the effect of CMV infection on the antibody response to influenza vaccination in healthy individuals. The process of systematic reviewing the available evidence in literature was reported in line with the PRISMA criteria (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) [31]. We extracted three standardized outcome variables of 17 studies, in line with European Medicine Agency (EMA) [24] and Food and Drug Administration (FDA) guidelines [32]. Summarizing all extracted data on GMR to influenza vaccination revealed no clear difference between CMV-seropositive and CMV-seronegative individuals. In a meta-analysis, a small (but non-significant) trend was observed that CMV-seropositive participants responded less often to influenza vaccination than CMV-seronegative individuals. We show that this effect is likely explained by publication bias. In addition, we summarized reports on a possible correlation between CMV antibody titers and influenza antibody titers, which showed weak negative correlations between the two. Together, these analyses provide no unequivocal evidence that latent CMV infection affects the influenza antibody response to vaccination.

MATERIAL AND METHODS

Search strategy and selection criteria

The database EMBASE was systematically searched for articles on CMV and influenza vaccination, combined with a search on CMV and immune response to include articles that covered the subject but did not explicitly mention influenza vaccination. The full search strategy was performed on 27-06-2017 and is provided in **Supplementary Table 1**. Two authors independently performed the selection process (SB and KW), in which all identified articles were first screened based on title and abstract and the remaining articles were reviewed in-depth. Discrepancies regarding the inclusion or exclusion of an article between the authors were resolved by discussion. English articles that reported an immunological response to influenza vaccination and had included at least a CMV-seronegative group or a CMV-seropositive group were considered for inclusion.

No restrictions were placed on study design or publication date. Only human studies with *in vivo* influenza vaccination were included. No restriction was placed on the age of the study population. Studies on primary CMV infection, CMV disease or immunocompromised participants were not included, because the immune system is expected to operate differently in those cases.

Data extraction and risk of bias assessment

Data collected from the studies included study design, study population, the type of influenza vaccine and the reported outcomes. These data were extracted via a data-extraction form, which was developed by KW based on the Cochrane Data Extraction and Assessment Template [33]. The form was pre-tested on several articles by KW and MK and refined accordingly. The final form can be found in **Supplementary Table 2**. If influenza vaccine response outcomes of several studies were reported in one article, the studies were assessed as separate studies.

The quality of each individual study was investigated by assessment of the risk of bias based on the Newcastle Ottawa scale for cohort studies [34]. According to these guidelines, studies were awarded with "stars" for high quality choices in three categories: "selection of cohorts" (max 4*), "comparability of cohorts" (max 2*) and "assessment of outcome" (max 3*). Based on all the acquired information, a study could acquire a maximum of 9 stars and the overall quality of the study was rated as high (+) (≥8 stars), intermediate (+/-) (7 stars) or low (-) (≤ 6 stars).

Data analysis: statistical and narrative synthesis

As the outcome variables were heterogeneous, a combination of narrative and statistical approaches to data synthesis was applied. Three influenza antibody outcomes were systematically extracted from the studies (**Figure 1**), in line with European Medicine Agency (EMA) guidelines for handling of influenza antibody data. Following the EMA guidelines, whenever possible, we separately extracted the outcome variables per influenza strain. Since age is the most important confounder, we also extracted the three outcome variables separately for young and old individuals. Findings were reported per outcome variable.

The principal outcome variable that we studied was the influenza-specific geometric mean titer ratio (GMR) pre-/post-vaccination (outcome a) with corresponding 95% confidence interval (CI) in CMV-seropositive and CMV-seronegative participants. Studies that reported this outcome or reported the data required to calculate the outcome were included in a figure per age subgroup. Per study the definition of young and old individuals differed, leading to two age groups in this analysis: young (<65 years of age) and old individuals (>60 years of age). Studies of which corresponding 95% CI could not be extracted or calculated reliably, were summarized in a separate figure, also per age subgroup (young and old adults).

Secondly, the odds ratio (OR) with 95% CI for the association between a response to influenza vaccination and CMV-serotatus was investigated (outcome b). The ORs were calculated using the numbers of responders and non-responders presented in the studies, where response to vaccination was defined as a ≥ four-fold increase, a post-vaccination titer ≥ 40 hemagluttinating units (HAU), or both. Meta-analysis was performed in R 3.3.3 using the 'metafor' package [35], to compare and pool the ORs of the studies. Random effects meta-analysis was performed in R 3.3.3 using the 'metafor' package [35]. The pooled OR and 95% CI using the DerSimonian-Laird method was calculated for the total group and for the predefined subgroups of young (<60 years of age) and old (>60 years of age) participants [36]. Heterogeneity among studies was assessed by the χ^2-based Q test and I^2 statistics. To analyze the influence of the quality of the studies, a sensitivity analysis was done by calculating the pooled OR for the highest-quality studies which had least potential for bias and confounding. The presence of possible publication bias was assessed using funnel plot regression [37]. The possible presence of any undetected studies and an effect estimate adjusted for publication bias were calculated using the trim-and-fill function in the 'metafor' package [38] in R 3.3.3.

Thirdly, the role of CMV antibody levels, instead of CMV-serostatus, was investigated by extracting associations between influenza antibody titers and CMV antibody levels (outcome c). Correlations between CMV antibody levels and influenza antibody titers were tabulated, and outcomes of regression models incorporating CMV antibody levels and influenza antibody titers were narratively synthesized. No restriction for this outcome was placed on reporting post-vaccination titers or fold increase influenza titers to vaccination. Likewise, no restriction was placed on the performed statistics.

RESULTS

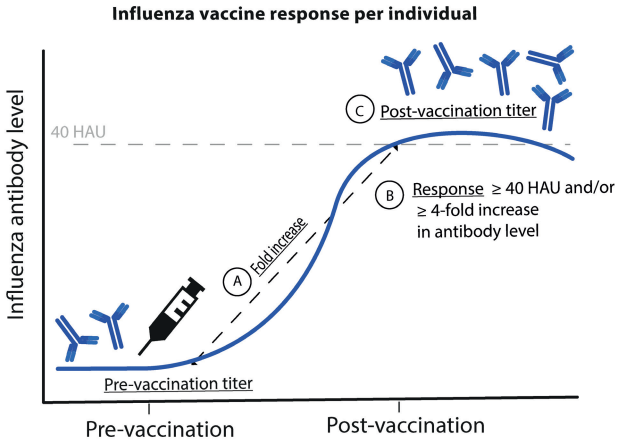
Selection and quality assessment of retrieved articles

Study selection and characteristics

The comprehensive EMBASE search for articles on CMV and influenza vaccination retrieved 689 individual publications (**Figure 2**). The first selection, based on screening of title and abstract reduced this to 83 articles. Reasons for exclusion were for example no reporting of influenza vaccination response, no CMV-serostatus measurement or no primary research article. The selected 83 articles were assessed in full text and 15 articles of these were included in this systematic review. Articles were excluded if the data came from acute CMV infection or CMV disease or from an immune compromised population, or when CMV infection was only studied *in vitro* or in an animal model. The agreement on the independently performed study selection between authors KW and SB was large with disagreement on only three studies. These differences could easily be resolved by discussion and judgement by a third person was not needed. References of the 15 included articles were checked for additional relevant articles, but none were found. One article (Furman *et al*), that contained the outcomes of three different study populations, was assessed as three individual studies, which brought the total number of studies up to 17. Whenever possible, study records were

stratified by influenza strains (H1N1, H3N2, Influenza B) and age groups (young and old), which led to multiple records per outcome variable for some studies (**Supplementary Figures 1-4**).

a



b

Outcome variables assessed in this systematic review

Influenza vaccine response	Parameter of CMV-infection
A Fold increase in geometric mean titer (GMT)	CMV-seronegative vs CMV-seropositive
B Percentage responders to influenza vaccination	CMV-seronegative vs CMV-seropositive
C Post-vaccination titer*	Correlation with CMV antibody level

Figure 1. Investigated influenza antibody outcomes. The influenza antibody vaccine response is investigated in the context of CMV infection in this review in three ways, based on the variables as indicated by A, B and C . Outcomes are a) the geometric mean titer pre-/post-vaccination ratio (GMR) per CMV-serostatus group, b) the percentage of subjects with a response per CMV-serostatus group and c) the association between the post-vaccination influenza antibody titers and CMV antibody titers. * 1 study reporting correlations (outcome c) did not correlate the post-vaccination titer, but the fold-increase. HAU = hemagglutination unit. 40 HAU = correlate of protection.

Characteristics of the retrieved studies are summarized in **Table 1**. Most studies were cohort studies, but some were primarily set up as a vaccine trial with a subgroup analysis for CMV-serostatus. For our research question, all studies could be considered as observational studies. The sample size differed between studies with a range from 37 – 731 participants, leading to a total of 2249 participants. The age groups differed between studies with a range of 19 – 97 years of age and in some studies both young and older adults were included [25, 26, 39-42]. The definition of older adults differed between studies. In this review, either >60 years or >65 years of age was used, as indicated per subgroup or outcome variable.

Quality assessment of studies

The risk of bias assessment led to an overall quality score per study. Twelve studies were rated as having a high quality, one an intermediate quality and four a low quality (**Table 2**). Most studies scored high on the selection process (1) with good representativeness of the

exposed cohort and selection of the non-exposed cohort from the same population. Many different ways of reporting the influenza vaccine outcome were used and the quality of the reported outcome varied between the studies. All cohort studies measured the outcome of interest also before vaccination (pre-vaccination titers), which granted them an additional star compared to the three cross-sectional studies. In five studies, influenza titers were assessed using ELISA, which is generally regarded a less reliable method than the hemagglutination inhibition (HI) assay [43]. All studies had an adequate follow-up time, which was considered to be more than two weeks for humoral immunity outcomes [44, 45].

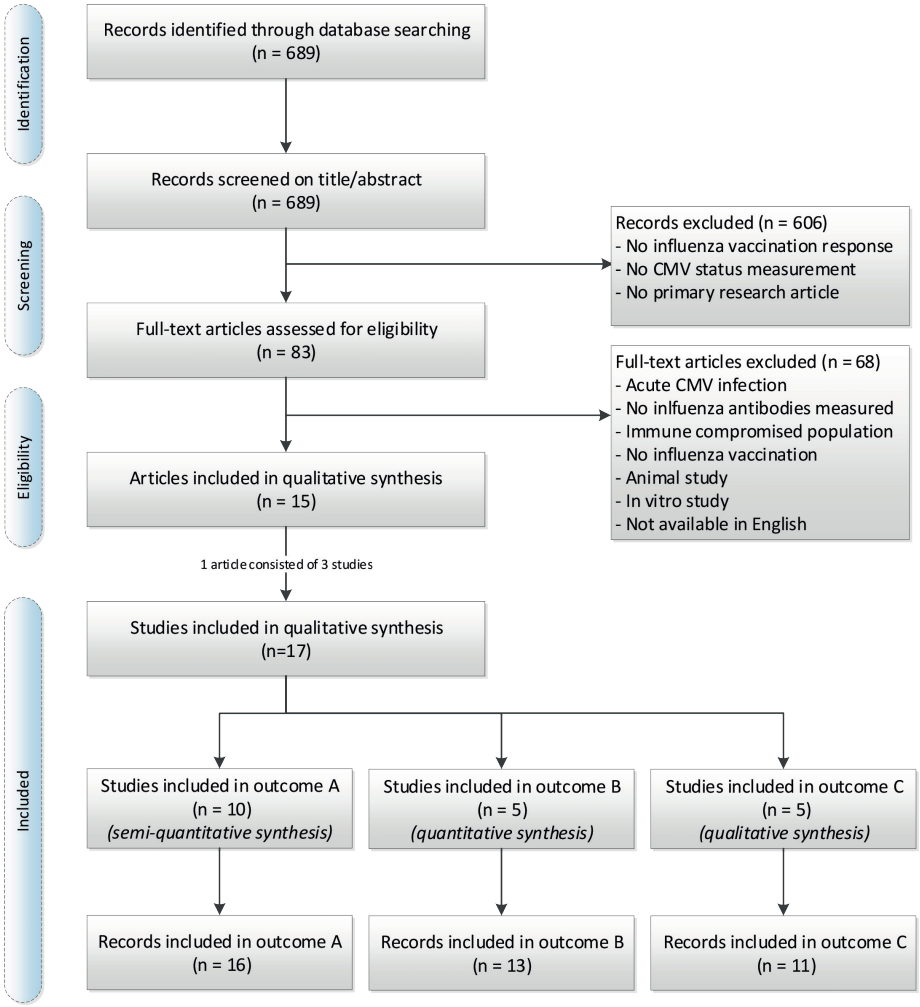
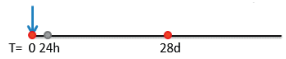
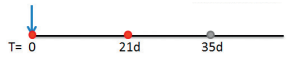
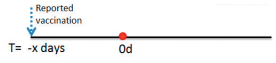
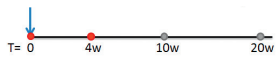
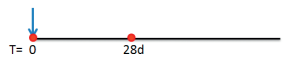


Figure 2. PRISMA-flow diagram of identification and selection of studies.

Studies (reference article)	Country	Primary study design	Design for research question	Sample size	Age (groups)	Timeline
Turner et al, 2014	UK	RCT	cohort	158	21 average	
Den Elzen et al, 2011	Netherlands	RCT	cohort	731	77-88	
Derhovanessian et al, 2012	Belgium	phase III trial	cohort	54	19-81	
Nielsen et al, 2015	UK	cross sectional	cross sectional	152	20-77	
Furman et al, 2015 – study 1	USA	cohort	cohort	91	20-32 and >60	
Furman et al, 2015 – study 2	USA	cohort	cohort	77	21-33 and >60	
Furman et al, 2015 – study 3	USA	cohort	cohort	37	19-44	
Haq et al, 2016	Canada	case-control	cohort	70	>65	
McElhaney et al, 2015	Canada	cohort	cohort	119	>65	

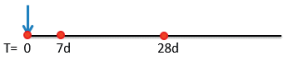
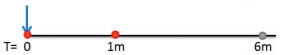
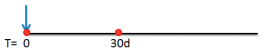
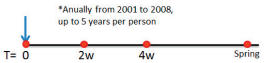
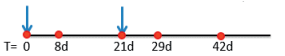
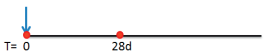
Frasca et al, 2015	USA	cohort	cohort	62	<59 and >60	
Trzonkowski et al, 2003	Poland	cohort	cohort	91	19-41 and >65	
Strindhall et al, 2015	Sweden	cohort	cohort	88	69	
Reed et al, 2016	USA	cohort	cohort	98	60-91	
Wald et al, 2013	USA	RCT	cohort	97	18-64 and ≥65	
Arias et al, 2013	Spain	case-control	cross sectional	162	20-50 and 68-97	
Moro-Garcia et al, 2011	Spain	cross sectional	cross sectional	100	69-97	
Guidi et al, 2014	Italy	cohort	cohort	62	Unknown	

Table 1. Characteristics of included studies Time line represents moment of vaccination (arrow) and blood withdrawn (red dot) on which influenza vaccine response is investigated or other information is gathered (grey dot). Note; studies *Moro-Garcia et al* and *Arias et al* seem to use a population of the same elderly cohort.

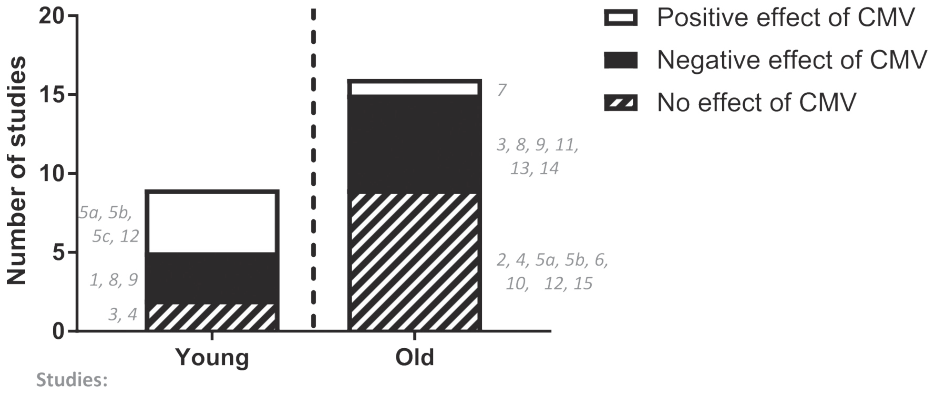
Study	Selection (max 4*)	Comparability (max 2*)	Outcome (max 3*)	Overall quality
Turner et al, 2014	****	**	***	+
Den Elzen et al, 2011	****	**	***	+
Derhovanessian et al, 2012	****	**	***	+
Nielsen et al, 2015	***	-	*	-
Furman et al, 2015 – study 1	****	**	***	+
Furman et al, 2015 – study 2	****	**	***	+
Furman et al, 2015 – study 3	***	**	**	+/-
Haq et al, 2016	****	**	***	+
McElhaney et al, 2015	***	**	***	+
Frasca et al, 2015	****	**	***	+
Trzonkowski et al, 2003	****	**	**	+
Strindhall et al, 2015	***	**	***	+
Reed et al, 2016	****	**	**	+
Wald et al, 2013	****	**	***	+
Arias et al, 2013	***	**	*	-
Moro-Garcia et al, 2011	***	*	*	-
Guidi et al, 2014	***	-	***	-

Table 2. Risk of bias and study quality of studies included for systematic assessment Risk of bias was analysed based on the Newcastle Ottawa scale for cohort studies. According to these guidelines, studies were awarded with "stars" for high quality choices in three categories: "selection of cohorts" (max 4*), "comparability of cohorts" (max 2*) and "assessment of outcome" (max 3*). Based on all the acquired information, a study could acquire a maximum of 9 stars and the overall quality of the study was rated as high (+) (≥8 stars), intermediate (+/-) (7 stars) or low (-) (≤ 6 stars)

Non-systematic summary of conclusions reported in the different studies

First, we summarized the conclusions on a possible effect of CMV infection on the antibody response to influenza vaccination reported in the different studies that we included (**Supplementary Table 3** and **Figure 3)** for young (<60 years of age) or old (>60 years of age) adults. In young individuals, two studies reported no effect, three a negative effect and four a positive effect of CMV-seropositivity. In old individuals, nine studies reported no effect, six a negative effect and one a positive effect of CMV-seropositivity on the humoral influenza vaccination response. Overall, more studies investigated old individuals than young individuals and a positive effect of CMV-seropositivity was mainly reported in young individuals. When reviewing the results based on strain-type or influenza antibody outcome variable, no clear conclusion could be drawn (data not shown).

Assessment of the statistical methods performed by the studies showed that the majority of the studies used appropriate statistics, but different methods were applied for the statistical testing of influenza antibody data (legend **Figure 3**). Normalization of HI data by log transformation and parametrical testing to compare the geometric titer is preferred [24, 46]. However, some studies performed non-parametric testing on raw antibody data to compare the median, which can be different from the geometric mean, especially in cases where the raw data is not natural log-distributed. In addition, influenza antibody outcome variables differed greatly between studies (**Table 3**). This hampers direct comparison of the results of the different studies in literature and a systematic comparison of the studies is necessary.



Studies:

1	Turner et al, 2014	5c	Furman et al, 2015 study 3[2]	11	Reed et al, 2016
2	Den Elzen et al, 2011	6	Haq et al, 2016	12	Wald et al, 2013[5]
3	Derhovanessian et al, 2012	7	McElhaney et al, 2015	13	Arias et al, 2013[6]
4	Nielsen et al, 2015[1]	8	Frasca et al, 2015[3]	14	Moro-Garcia et al, 2011
5a	Furman et al, 2015 study 1[2]	9	Trzonkowski et al, 2003[4]	15	Guidi et al, 2014
5b	Furman et al, 2015 study 2[2]	10	Strindhall et al, 2015		

Figure 3. Summary of conclusions from studies on latent CMV infection on the influenza antibody response. Conclusion per study, are shown for the effect of CMV infection on the influenza antibody response, separated for young (<65 years of age) or old (>60 years of age) individuals. A flow diagram of records available per reported conclusion out of the 15 articles is presented in supplementary figure 1. Note that the article of *Furman et al* contained the outcome of three study populations and was thus assessed as three individual studies. The study group in *Nielsen* et al had an age range of 21-77 years, covering both young and old adults, therefore the reported conclusion (no effect) was included in both young and old bar graphs. Statistics per study were performed by parametric tests on log-transformed influenza antibody data, unless indicated otherwise in footnotes. [1] A Mann Whitney test was performed on raw influenza antibody data (post-titer). [2] Data was presented as a geometric mean of three different influenza strains titer. Also, we could not verify how the geometric mean of three influenza strains per individual was handled in the measurement of spread on group level. [3] A Mann Whitney test was performed on fold increase of influenza antibody data. [4] Antibody data was analysed with non-parametric test (Spearman correlation) on non-log-transformed antibody data (for both CMV and influenza antibodies). [5] We could not verify what statistics were used, since it is stated in the paper that the GMT and 95%CI of day 21 post-vaccination are presented in figure 1 of the article, but the 95% CI showed equally distributed error bars on a linear scale. Also, it is stated that Mann-Whitney was used to compare GMTs, which is statistically not possible.

Impact of CMV on antibody titers after influenza vaccination

No effect of CMV-serostatus on influenza antibody titer to influenza vaccination (GMR) (outcome a)

We first examined the geometric mean titer pre-/post-vaccination ratio (GMR) to influenza vaccination for CMV-seropositive and CMV-seronegative individuals (outcome a). In 10/17 studies the GMR per CMV-serostatus could be estimated from figures [40] or could be recalculated with the reported data [26, 28, 47-51] (**Supplementary figure 2**). For only 5 of these 10 studies, the 95% confidence interval (CI) could be extracted [40, 47, 50-52]. These 95% CI were either estimated directly from figures or calculated based on depicted SD/SE. We refrained from calculating the ratios of the GMR of CMV-seropositive versus CMV-seronegative individuals since the measure of spread was estimated from figures, and thus

no meta-analysis was performed. Instead, we summarized the GMR with the 95% CI in an overview figure (**Figure 4A**). For the remaining 5 studies, we were unable to estimate a reliable 95% CI [26, 28, 49], either due to illegible charts [49] or due to lack of clarity surrounding reported measure of spread [26, 28] (**Figure 4B**).

Overall, the influenza GMR differed greatly between studies: some records suggested a higher increase in the influenza-specific antibody titer in CMV-seropositive individuals compared to CMV-seronegative individuals, while other studies suggested the opposite (**Figure 4**). Even analyses restricted to young or old individuals only revealed contradicting results on the effect of CMV-serostatus. Likewise, even restricting the analysis to studies of good quality revealed no overall effect of CMV-seropositivity on the influenza-specific GMR after vaccination. In conclusion, the primary outcome of our systematic analysis revealed neither evidence for a negative nor for a positive effect of CMV-seropositivity on the influenza vaccine response in young or old individuals.

Meta-analysis: no significant differences in response to influenza vaccination between CMV-seropositive and CMV-seronegative individuals (outcome b)

Next, we investigated by odds ratio (OR) analysis whether there is any evidence for a positive (OR>1) or negative (OR<1) association between CMV-seropositivity (exposure) and response to influenza vaccination (outcome b). From five studies a clear definition of responders and non-responders could be extracted [40, 51-54] for different influenza strains and age groups, leading to a total of 13 OR records (**Supplementary figure 3**). Most studies defined a response to influenza vaccination as a ≥4 fold increase in antibody titer after vaccination; one study (*Strindhall et al*, 2016) used the stricter definition of a ≥4 fold increase in antibody titer *and* a post-titer of ≥ 40 HAU. Meta-analysis of all 13 records revealed a pooled OR of 0.65 (95% CI 0.40-1.08; I^2 = 33%; p=0.11). Although this OR indicates a trend that CMV-seropositive participants respond less often to influenza vaccination than CMV-seronegative individuals, this is not statistically significant. (**Figure 5**).

Stratified meta-analyses for the separate young and old groups and for the data in *Strindhall et al,* which used the stricter definition of response, also did not reveal any significant effects of CMV-serostatus on the influenza antibody response. A sensitivity analysis was done to assess the role of the quality of the studies on the pooled OR; meta-analysis restricted to high quality studies revealed an OR of 0.60 (95% CI 0.35-1.03), which did not markedly differ from the pooled OR of all study records. In conclusion, the average OR of 0.65 (95% CI: 0.40-1.08) suggests a (non-significant) trend that CMV-seropositive participants respond less often to influenza vaccination than CMV-seronegative individuals.

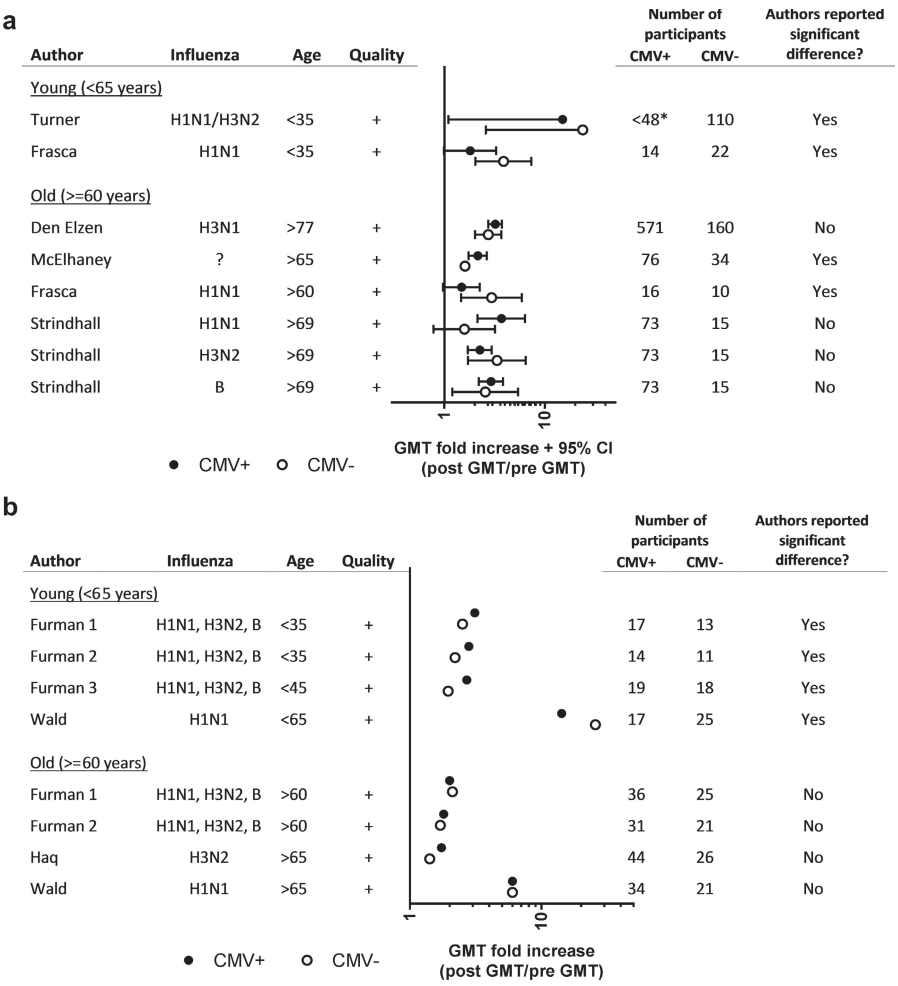


Figure 4 Influenza-specific geometric mean titer pre-/post-vaccination ratio (GMR) in CMV-seropositive versus CMV-seronegative participants. Studies are sorted by age of the study population (<65 and >60). Influenza strain, study quality and number of CMV-seropositive and CMV-seronegative participants are shown. For each outcome it is shown whether the authors reported a significant difference between the CMV-seropositive and CMV-seronegative groups. The GMR is shown per record for CMV-seropositive (black dot) and CMV-seronegative (white dot) participants, including 95% CI error bars (**A**) or without 95% CI (**B**). * Data for *Turnet er al* was not reported for CMV-seropositivity (n=48), but for different CMV-seropositive groups based on height of anti-CMV IgG level. Here, CMV-seropositive high individuals are shown (subgroup of n=48).

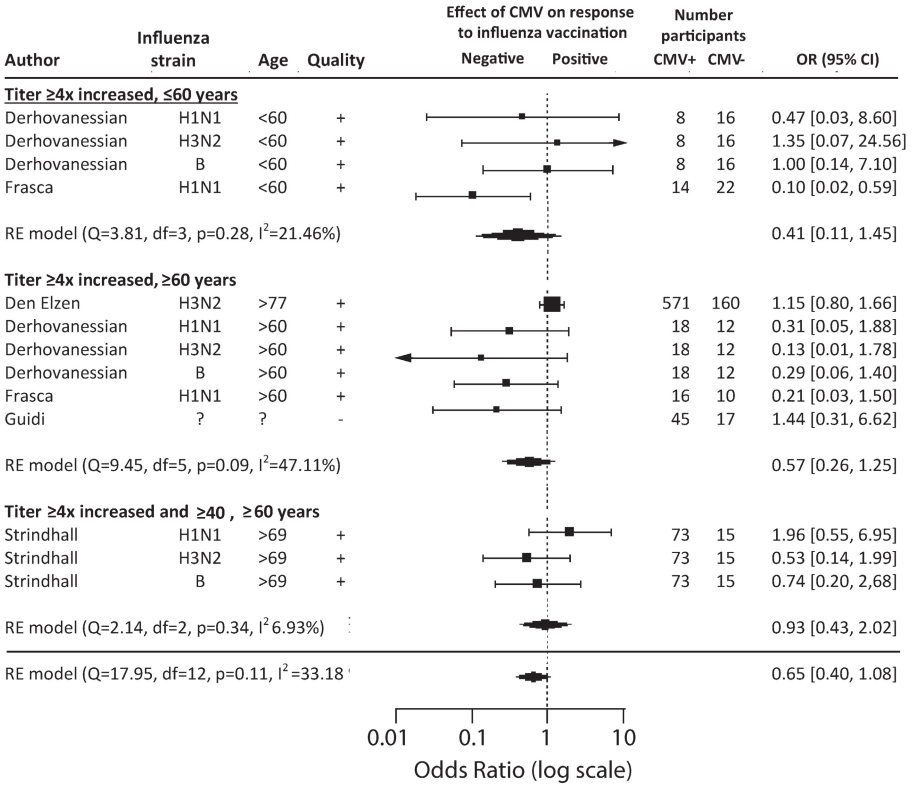


Figure 5. Effect of CMV serostatus on response to influenza vaccination. Results of the DerSimonian–Laird random effects model meta-analysis of five studies that included numbers of responders and non-responders to influenza vaccination. Odds ratios (diamonds) of the effect of CMV serostatus on responders to influenza and their 95% CI error bars (width of diamonds) are shown. Studies are split by age of the study population (< 60 or ≥ 60) and definition of responder that was used in the study: either ≥ four-fold increase or a four-fold increase in combination with a post-vaccination titer ≥ 40 hemagglutinating units (HAU). The influenza strain, number of study participants and overall study quality are noted for each study. I^2 (the percentage of variation across studies that is due to heterogeneity rather than chance), Q (the weighted sum of squared differences between individual study effects and the pooled effect across studies) and p values (to determine whether significant heterogeneity exists) are calculated for every subgroup separately and for all studies together. Arrows indicate error bars on the odds ratio extending beyond the scale.

Funnel-plot analysis suggests a publication bias in meta-analysis

Because a potential positive effect of CMV-seropositivity on influenza vaccine responses was only recently considered, we assessed whether there was any evidence for a publication bias in the studies included in our meta-analysis by performing a funnel-plot analysis [37]. Such an analysis is based on two assumptions. It assumes (1) that the OR of studies with a large study population are close to the true average OR, since they have the highest precision, while (2) the OR of studies with low precision (smaller study populations) should, based on chance, be spread evenly on both sides of the average OR. If this is not the case, there is a sign of bias in studies reaching publication. Funnel-plot analysis of the studies in our meta-analysis revealed that the low precision studies reported significantly more often a negative

than a positive effect of CMV (p=0.019, **Figure 6A**), indicating a publication bias. Importantly, influenza vaccine responses were initially investigated based on the assumption that CMV enhances immunosenescence and a positive effect of CMV on influenza antibody responses was only recently considered [28]. As a result, positive associations between CMV and the influenza vaccine response may have remained unpublished.

Based on the same two assumptions, the trim and fill method [38] was performed which removes the smaller studies causing the asymmetry, calculates the 'true center' of the studies and next replaces the omitted studies and missing 'counterparts' around the centre (filling). The trim and fill analysis estimated that five studies that revealed a positive effect of CMV infection on the influenza vaccination response have not been published in the literature (**Figure 6B**). Of note, in addition to the studies in our analysis, two more studies exist, of which the data was not published, but in which it was stated that no difference was observed [26, 47]. Interestingly, when the five hypothetical 'missing studies' were included in our analysis, the OR shifted up from 0.65 to 1.0, suggesting that in fact there is no effect of CMV-serostatus on the response to influenza vaccination. Together, these analyses suggest that publication bias underlies the trend to a negative effect of CMV-seropositivity on the response to influenza vaccination reported in the literature (**Figure 6**).

Correlation and regression models of CMV antibody levels and influenza antibody titers suggest a negative effect of CMV infection (outcome c)

The potential influence of latent CMV infection on the antibody response to influenza vaccination was also investigated by extracting the association between the CMV antibody level and the influenza antibody titer. Five studies reported this outcome variable, either as a correlation between the CMV antibody level and the influenza antibody titer post-vaccination or by using a regression model, in which additional factors than only CMV antibody level, such as age, were taken into account [25, 42, 47, 55, 56]. The five studies together reported 10 individual records (**Supplementary Figure 4**) and included younger and older adults (18-97 years) and different influenza strains (**Table 4**).

Unfortunately, different outcomes for associations were reported (**Table 3**) and raw data could not be extracted from studies to standardize the outcomes to a comparable outcome. Instead, reported associations were tabulated (**Table 4**). Of note, 6 out of 8 correlation outcomes came from a single study and were based on post-vaccination titers instead of the fold-increase, which can be influenced to a large extend by pre-vaccination titers. For one correlation outcome, influenza antibody data was divided for time elapsed since immunization, which is not a generally accepted method. Also, both CMV-seropositive and CMV-seronegative individuals were included in all associations. For relations between anti-CMV antibody levels and influenza antibody levels, only CMV-seropositive individuals should be included in our opinion, to focus on the height of CMV antibody level as a surrogate marker of reactivation.

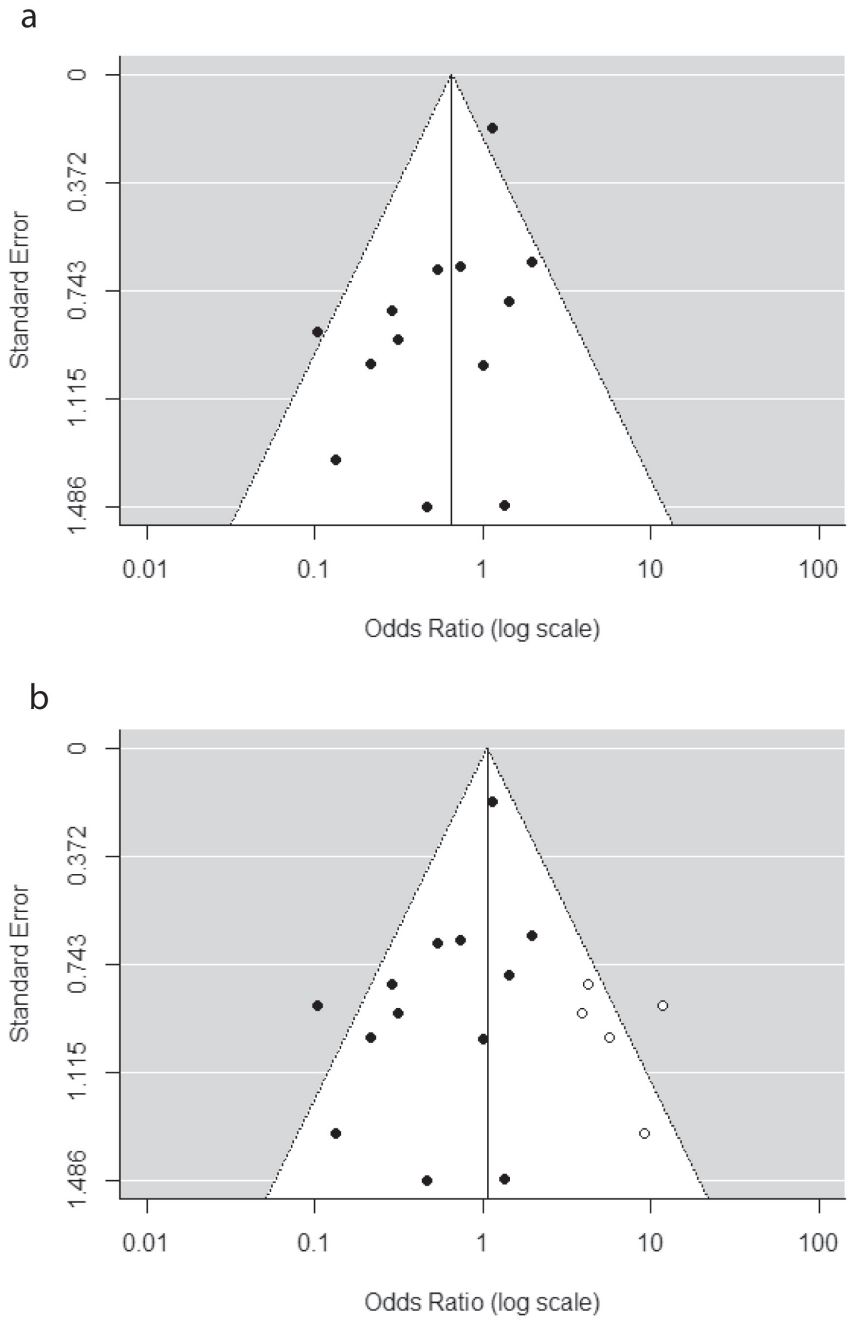


Figure 6. Analysis of publication bias among studies included in the meta-analysis investigating the effect of CMV serostatus on response to influenza vaccination. A. The funnel plot shows the standard error of each study on the vertical axis (precision) and the effect size of each study (odds ratio) on the horizontal axis to assess possible asymmetry indicating publication bias. Overall pooled OR is 0.65, as indicated by the vertical line. **B.** With help of the trim and fill method 5 possible unpublished studies were identified, shown as white dots. Including these hypothetical studies, the pooled OR shifts towards 1

All records reported a negative association between the CMV antibody level and the influenza antibody titer, and 8 out of 10 were reported to be significant. Reported correlation coefficients (8 outcomes) were on average r = -0,49 and varied from r = -0.16 to r = -0.77, showing mainly low to moderate negative correlations [57]. In addition, two models [42, 47] showed a significant negative association between CMV antibody levels and the influenza antibody titer to influenza vaccination. Overall, these correlation results indicate a small but significant negative association between CMV antibody levels and influenza antibody titers after vaccination.

DISCUSSION

This is the first systematic review investigating the association between latent CMV infection and the immune response to influenza vaccination. Almost two decades ago, CMV was associated with "immunosenescence" [58]. Since then, multiple studies on CMV-induced immunosenescence have been performed. The idea that CMV decreases the ability of the immune system to respond to other pathogens or vaccination [59, 60] is mainly based on studies investigating the influenza vaccine response [61]. Indeed, various studies reported a negative association between latent CMV infection and influenza vaccine responses, while other studies lacked to find an effect of CMV or even reported a positive effect of CMV. Thus, consensus on the effect of CMV is lacking. Nevertheless, an effect of CMV on the influenza vaccine response in the elderly is generally assumed [29, 61, 62]. By systematically reviewing and integrating the available studies, we here show that there is no unequivocal evidence for an impact of CMV on the influenza vaccine response.

We systematically selected studies on CMV and influenza vaccine responses and extracted three standardized influenza antibody outcome variables. The geometric mean titer ratio (GMR) pre/post influenza vaccination with (**Figure 4A**) and without (**Figure 4B**) 95% CI revealed no difference between CMV-seropositive and CMV-seronegative individuals (outcome a). Of note, also when only the post-vaccination geometric mean titer (post-GMT) was summarized, no overall trend for an effect of CMV-serostatus was observed (**Supplementary Figure 5**). We primarily assessed the GMR and not the post-GMT since the participants in the studies were not all influenza seronegative before vaccination. Pre-existing immunity is usually present in the case of seasonal influenza vaccination. Thus, post-vaccination titers as outcome will overestimate the vaccine antibody response and are therefore less meaningful. Linear regression analysis, as performed in some studies [42, 63], is the best method to correct for pre-vaccination titers [46], but this could not be analysed on the basis of the extracted data of the studies included for this review. Thus, with the GMR, the best outcome available, no effect of CMV-seropositivity on the influenza vaccine response is observed.

The meta-analysis of response rate to influenza vaccination (outcome b) (**Figure 5**) revealed a small (albeit non-significant) trend that CMV-seropositive participants respond less often

to influenza vaccination than CMV-seronegative individuals. Funnel plot analysis suggested that publication bias most likely underlies this trend in the literature (**Figure 6**).

Unfortunately, it was not possible to extract a standardized outcome for an association between CMV antibody level and the influenza antibody response to vaccination (outcome c), since the methods of the studies varied and no raw data was available. Overall, the reported correlation results (**Table 4**) of the studies indicated a small negative association between CMV antibody titers levels and influenza antibody levels after vaccination, suggesting that individuals who experienced multiple CMV reactivations during life may have impaired influenza vaccine responses. The tabulated correlations however should be interpreted with caution. CMV antibody levels increase with age and are thought to reflect experienced CMV reactivation or reinfection [7]. Therefore, high anti-CMV antibody levels may be related to enhanced CMV-induced immunosenescence and impaired influenza vaccine responses [42]. However, we noticed that in most studies CMV-seronegative individuals were included in the correlation of CMV antibody titers and influenza antibody titers, which may affect the correlation coefficient or the significance of the correlation. Of importance, in only one study CMV antibody levels were correlated with the fold increase in influenza antibody titers [47]; in all other studies it was correlated with the post-vaccination titer, thereby overestimating the vaccine response. Together, this questions the importance of the reported weak correlations between CMV antibody levels and influenza antibody titers to vaccination.

To illustrate the controversy in the literature, we also summarized the reported conclusions of various studies on the influenza antibody response (**Figure 3)**. Two previous reviews directly combined the various results in literature on the effect of latent CMV infection on the antibody response to influenza vaccination [4, 29]. *Frasca et al (2015)* and *Merani et al (2017)* refer to some of the studies included in our systematic review, and describe the effect of CMV on influenza antibody vaccine responses as controversial or ambiguous. Despite this, both reviews come to the conclusion that CMV does affect the immune response to influenza vaccination [4, 29]. In addition, *Merani et* al discuss possible methods to reduce the impact of immunosenescence on influenza vaccine responses by anti-CMV strategies [29]. The controversy in the literature and the difficulty to compare different influenza antibody outcomes in different studies highlights that a systematic approach is necessary.

The strength of our review lies in its systematic approach. This allowed us to synthesize all the available evidence (until 27th June 2017) on this particular question and to eliminate the effect of potential publication bias. Instead of merely summarizing the conclusions in literature, we extracted the published data in three standardized outcome variables of influenza antibody response, separated per age group. Furthermore, whenever possible, we assessed the data of each study per influenza strain. By this, we included multiple records per study and not only the record on which the authors' conclusion was based. To the best of our knowledge, only two new articles came out that investigated the effect of latent CMV infection on the influenza antibody response since the systematic search of this review (27th June 2017).

Merani et al (sept 2017) concluded that there is no difference in influenza GMR between CMV-seronegative and CMV-seropositive individuals, while CMV-seropositive individuals do show an impaired cellular granzyme B response to influenza virus challenge [64]. We published in *Van den Berg et al* (January 2018) that there is no negative effect of CMV infection on the antibody response to a novel influenza vaccine strain in adults [65]. Both studies will not change the conclusion of this systematic review that there is no unequivocal evidence for an effect of CMV infection on the antibody response to influenza vaccination.

In this review the direct association between CMV infection and the influenza antibody vaccine response is investigated. Several mechanisms of a potential negative effect have been postulated, based on the known effects of CMV infection on the immune system and the subsequent potential impact on the influenza vaccine response [47, 58]. CMV infection leads to increased pro-inflammatory cytokine levels, which in turn are associated with decreased influenza vaccine responses [29]. Likewise, CMV infection leads to increased differentiation of T-cells, which has been associated with poor influenza vaccination responses [25, 53]. It has also been reported that CMV infection is associated with decreased switched B cell percentages before influenza vaccination, and subsequently lower influenza vaccine responses [32]. In contrast, *De Bourcy et al* (2017) suggested a potential mechanism for the positive effect of CMV on the antibody influenza vaccine response reported in *Furman et al*: based on B-cell repertoire analyses, CMV infection is associated with more activated B cells after influenza vaccination.

This systematic review also has some limitations. Ideally, a systematic review is based on randomized controlled trials (RCT), but due to obvious ethical and practical reasons, no RCT's have been conducted to study the relation between CMV infection and influenza vaccine responses. Consequently, only observational studies were included. We assumed that the reported sizes of the study populations were correct for the duration of the studies, even if no statement was made on the number of participants that were lost to follow up. Furthermore, this systematic review was limited by the number of studies that was found to be eligible for inclusion, which led to a meta-analysis of only 5 studies leading to 13 records. Another limitation is the incomplete correcting for confounders. We only adjusted for pre-vaccination antibody levels by investigating the GMR and partially adjusted for age by separating the results for young and old adults. However, age is associated with different influenza antibody responses, not only because of immunosenescence, but also due to immunologic imprinting and different influenza exposure during lifetime. Thus, merely assigning individuals to a young and old age group might not be sufficient to adjust for age as a confounder. Research on the effect of CMV on the influenza antibody vaccine response is further complicated by different study populations, and different influenza strains, as summarized previously [29]. There is no biological basis for a differential effect of CMV on different influenza strains, but influenza vaccine responses vary a lot per season and subtype [66]. Unfortunately, data from *Reed et al* could not be incorporated in this review. They reported a negative association between CMV-seropositivity and influenza antibody vaccine response in a study including different

seasons and influenza strains. Their data could however not be extracted for analysis for one of the outcomes of this systematic review, since they were only reported as a result of a multi-factor model.

Universality in reported influenza antibody data in the CMV-immunosenescence field is necessary to reveal the potential effect of CMV on the antibody influenza vaccine response. We recommend further studies investigating the effect of CMV infection on the influenza antibody vaccine response to follow the EMA guidelines [24] and as an absolute minimum, to always report the influenza pre-GMT and post-GMT (with 95% CI) and the number of participants per group. It is important to take influenza strain and season into account by measuring and reporting the titers separately per influenza strain. A response rate is also of interest, but should not be the only outcome reported. The response rate can be defined in several ways [24] and the correlate of protection of 40 is based on adults, making the use in elderly questionable [24, 67]. In addition, a regression analysis to correct for pre-existing immunity is necessary. Especially when the effect of CMV infection on the influenza antibody response is small, correcting for confounders, like age (as continuous variable), pre-existing immunity, vaccination history, medicine use or comorbidities is highly recommended.

In conclusion, we show that based on the GMR, which in our perception is the best outcome available, there is no evidence for an effect of CMV-seropositivity on the influenza antibody vaccine response, and that publication bias probably explains the trend in the literature that CMV-seropositive individuals seem to respond less often to influenza vaccines than CMV-seronegative individuals. We suspect that in the past, several studies did not reach publication because they did not fit the prevailing idea that CMV induces immunosenescence. Our systematic review emphasizes that the effect of CMV infection on a clinically relevant immune function in humans, such as influenza vaccine responses, is not as black-and-white as previously suggested. Further large studies investigating the relation between CMV antibody levels and influenza vaccine responses with enough power to detect a potential small effect of CMV infection are needed, in which also confounding factors in addition to age are taken into account. Only if there is unequivocal evidence for CMV-associated impaired influenza vaccine responses, can we begin to address whether a CMV-impaired vaccine response in the elderly is merely a sign of immunosenescence, or whether CMV is causing immunosenescence.

ACKNOWLEDGEMENT

We thank Michiel van Boven for critically proofreading the article.

REFERENCES

1. Gruver, A.L., L.L. Hudson, and G.D. Sempowski, *Immunosenescence of ageing.* J Pathol, 2007. 211(2): p. 144-56.
2. Khan, N., et al., *Cytomegalovirus seropositivity drives the CD8 T-cell repertoire toward greater clonality in healthy elderly individuals.* J Immunol, 2002. 169(4): p. 1984-92.
3. Koch, S., et al., *Cytomegalovirus infection: a driving force in human T-cell immunosenescence.* Ann N Y Acad Sci, 2007. 1114: p. 23-35.
4. Frasca, D. and B.B. Blomberg, *Aging, cytomegalovirus (CMV) and influenza vaccine responses.* Hum Vaccin Immunother, 2015: p. 0.
5. Olsson, J., et al., *Age-related change in peripheral blood T-lymphocyte subpopulations and cytomegalovirus infection in the very old: the Swedish longitudinal OCTO immune study.* Mech Ageing Dev, 2000. 121(1-3): p. 187-201.
6. Cannon, M.J., D.S. Schmid, and T.B. Hyde, *Review of cytomegalovirus seroprevalence and demographic characteristics associated with infection.* Rev Med Virol, 2010. 20(4): p. 202-13.
7. Korndewal, M.J., et al., *Cytomegalovirus infection in the Netherlands: seroprevalence, risk factors, and implications.* J Clin Virol, 2015. 63: p. 53-8.
8. Crough, T. and R. Khanna, *Immunobiology of human cytomegalovirus: from bench to bedside.* Clin Microbiol Rev, 2009. 22(1): p. 76-98, Table of Contents.
9. van Boven, M., et al., *Infectious reactivation of cytomegalovirus explaining age- and sex-specific patterns of seroprevalence.* PLoS Comput Biol, 2017. 13(9): p. e1005719.
10. Parry, H.M., et al., *Cytomegalovirus viral load within blood increases markedly in healthy people over the age of 70 years.* Immun Ageing, 2016. 13: p. 1.
11. Furui, Y., et al., *Cytomegalovirus (CMV) seroprevalence in Japanese blood donors and high detection frequency of CMV DNA in elderly donors.* Transfusion, 2013. 53(10): p. 2190-7.
12. Redeker, A., et al., *The Contribution of Cytomegalovirus Infection to Immune Senescence Is Set by the Infectious Dose.* Front Immunol, 2017. 8: p. 1953.
13. Mekker, A., et al., *Immune senescence: relative contributions of age and cytomegalovirus infection.* PLoS Pathog, 2012. 8(8): p. e1002850.
14. Cicin-Sain, L., et al., *Cytomegalovirus infection impairs immune responses and accentuates T-cell pool changes observed in mice with aging.* PLoS Pathog, 2012. 8(8): p. e1002849.
15. Smithey, M.J., et al., *Lifelong CMV infection improves immune defense in old mice by broadening the mobilized TCR repertoire against third-party infection.* Proc Natl Acad Sci U S A, 2018. 115(29): p. E6817-E6825.
16. Saraswati, K., et al., *Scrub typhus point-of-care testing: A systematic review and meta-analysis.* PLoS Negl Trop Dis, 2018. 12(3): p. e0006330.
17. Sharifi Mood, B., F. Sharifi Mood, and R. Sharifi, *Seasonal Influenza and Prevention.* Int J Infect, 2016. 3(4): p. e35954.
18. Ayscue P, M.E., Uyeki T, Zipprich J, Harriman K, Salibay C, et al. , *Influenza-associated intensive-care unit admissions and deaths - California, September 29, 2013-January 18, 2014. .* MMWR Morb Mortal Wkly Rep. 2014;63(7):143-7.
19. Schanzer, D.L., et al., *Influenza-attributable deaths, Canada 1990-1999.* Epidemiol Infect, 2007. 135(7): p. 1109-16.
20. Thompson, W.W., et al., *Influenza-associated hospitalizations in the United States.* JAMA, 2004. 292(11): p. 1333-40.

21. Thompson, W.W., et al., *Mortality associated with influenza and respiratory syncytial virus in the United States.* JAMA, 2003. 289(2): p. 179-86.
22. Lambert, N.D., et al., *Understanding the immune response to seasonal influenza vaccination in older adults: A systems biology approach.* Expert Review of Vaccines, 2012. 11(8): p. 985-994.
23. Pedersen, J.C., *Hemagglutination-Inhibition Assay for Influenza Virus Subtype Identification and the Detection and Quantitation of Serum Antibodies to Influenza Virus*, in *Animal Influenza Virus*, E. Spackman, Editor. 2014, Springer New York: New York, NY. p. 11-25.
24. Agency, E.M., *Guideline on Influenza Vaccines.* 2016.
25. Trzonkowski, P., et al., *Association between cytomegalovirus infection, enhanced proinflammatory response and low level of anti-hemagglutinins during the anti-influenza vaccination--an impact of immunosenescence.* Vaccine, 2003. 21(25-26): p. 3826-36.
26. Wald, A., et al., *Impact of human cytomegalovirus (CMV) infection on immune response to pandemic 2009 H1N1 influenza vaccine in healthy adults.* J Med Virol, 2013. 85(9): p. 1557-60.
27. Den Elzen, W.P., et al., *Telomere length and anaemia in old age: results from the Newcastle 85-plus Study and the Leiden 85-plus Study.* Age Ageing, 2011. 40(4): p. 494-500.
28. Furman, D., et al., *Cytomegalovirus infection enhances the immune response to influenza.* Sci Transl Med, 2015. 7(281): p. 281ra43.
29. Merani, S., et al., *Impact of Aging and Cytomegalovirus on Immunological Response to Influenza Vaccination and Infection.* Front Immunol, 2017. 8: p. 784.
30. de Bourcy, C.F., et al., *Phylogenetic analysis of the human antibody repertoire reveals quantitative signatures of immune senescence and aging.* Proc Natl Acad Sci U S A, 2017. 114(5): p. 1105-1110.
31. Moher, D., et al., *Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement.* BMJ, 2009. 339: p. b2535.
32. Center for Biologics Evaluation and Research, U.S.D.o.H.a.H.S. and F.a.D. Administration, *Guidance for Industry Clinical Data Needed to Support the Licensure of Seasonal Inactivated Influenza Vaccines*
33. *Data Extraction and Assessment Template.* Cochrane Handbook, The Cochrane Public Health Group.
34. Wells, G.A.S., B.; O'Connell, D.; Peterson, J.; Welch, V.; Losos, M.; Tugwell, P., *The Newcastle-Ottawa Scale (NOS) for assessing the quality of nonrandomised studies in meta-analyses.* http://www.ohri.ca/programs/clinical_epidemiology/oxford.asp.
35. Viechtbauer, W., *'metafor': Meta-analysis package for R.* The Comprehensive R Archive Network (CRAN), 2016.
36. DerSimonian, R. and N. Laird, *Meta-analysis in clinical trials.* Control Clin Trials, 1986. 7(3): p. 177-88.
37. Egger, M., et al., *Bias in meta-analysis detected by a simple, graphical test.* BMJ, 1997. 315(7109): p. 629-34.
38. Duval, S. and R. Tweedie, *Trim and fill: A simple funnel-plot-based method of testing and adjusting for publication bias in meta-analysis.* Biometrics, 2000. 56(2): p. 455-63.
39. Furman, D., et al., *Cytomegalovirus infection enhances the immune response to influenza.* Science Translational Medicine, 2015. 7(281).
40. Frasca, D., et al., *Cytomegalovirus (CMV) seropositivity decreases B cell responses to the influenza vaccine.* Vaccine, 2015. 33(12): p. 1433-9.
41. Enani, S., et al., *Impact of ageing and a synbiotic on the immune response to seasonal influenza vaccination; a randomised controlled trial.* Clin Nutr, 2017.

42. Alonso Arias, R., et al., *Intensity of the humoral response to cytomegalovirus is associated with the phenotypic and functional status of the immune system.* J Virol, 2013. 87(8): p. 4486-95.
43. Kim, D.-K. and B. Poudel, *Tools to Detect Influenza Virus.* Yonsei Med J, 2013. 54(3): p. 560-566.
44. Pyhala, R., et al., *Early kinetics of antibody response to inactivated influenza vaccine.* Clin Diagn Virol, 1994. 1(5-6): p. 271-8.
45. He, X.S., et al., *Cellular immune responses in children and adults receiving inactivated or live attenuated influenza vaccines.* J Virol, 2006. 80(23): p. 11756-66.
46. Beyer, W.E., et al., *Seroprotection rate, mean fold increase, seroconversion rate: which parameter adequately expresses seroresponse to influenza vaccination?* Virus Res, 2004. 103(1-2): p. 125-32.
47. Turner, J.E., et al., *Rudimentary signs of immunosenescence in Cytomegalovirus-seropositive healthy young adults.* Age (Dordr), 2014. 36(1): p. 287-97.
48. den Elzen, W.P., et al., *Cytomegalovirus infection and responsiveness to influenza vaccination in elderly residents of long-term care facilities.* Vaccine, 2011. 29(29-30): p. 4869-74.
49. Haq, K., et al., *Cytomegalovirus Seropositivity Predicts a Decline in the T-cell But Not the Antibody Response to Influenza in Vaccinated Older Adults Independent of Type 2 Diabetes Status.* J Gerontol A Biol Sci Med Sci, 2016.
50. McElhaney, J.E., et al., *Predictors of the antibody response to influenza vaccination in older adults with type 2 diabetes.* BMJ Open Diabetes Res Care, 2015. 3(1): p. e000140.
51. Strindhall, J., et al., *Humoral response to influenza vaccination in relation to pre-vaccination antibody titres, vaccination history, cytomegalovirus serostatus and CD4/CD8 ratio.* Infect Dis (Lond), 2016. 48(6): p. 436-42.
52. Den Elzen, W.P.J., et al., *Cytomegalovirus infection and responsiveness to influenza vaccination in elderly residents of long-term care facilities.* Vaccine, 2011. 29(29-30): p. 4869-4874.
53. Derhovanessian, E., et al., *Cytomegalovirus-associated accumulation of late-differentiated CD4 T-cells correlates with poor humoral response to influenza vaccination.* Vaccine, 2013. 31(4): p. 685-90.
54. Guidi, L., et al., *Cytomegalovirus infection and response to influenza vaccination in inflammatory bowel disease patients on anti-TNF therapy.* Journal of Crohn's and Colitis, 2014. 8: p. S285.
55. Moro-García, M.A., et al., *Relationship between functional ability in older people, immune system status, and intensity of response to CMV.* Age, 2012. 34(2): p. 479-495.
56. Reed, R.G., R.N. Greenberg, and S.C. Segerstrom, *Cytomegalovirus serostatus, inflammation, and antibody response to influenza vaccination in older adults: The moderating effect of beta blockade.* Brain, Behavior, and Immunity, 2016.
57. Mukaka, M.M., *Statistics corner: A guide to appropriate use of correlation coefficient in medical research.* Malawi Med J, 2012. 24(3): p. 69-71.
58. Looney, R.J., et al., *Role of cytomegalovirus in the T-cell changes seen in elderly individuals.* Clin Immunol, 1999. 90(2): p. 213-9.
59. Pawelec, G. and E. Derhovanessian, *Role of CMV in immune senescence.* Virus Res, 2011. 157(2): p. 175-9.
60. McElhaney, J.E., et al., *The unmet need in the elderly: how immunosenescence, CMV infection, co-morbidities and frailty are a challenge for the development of more effective influenza vaccines.* Vaccine, 2012. 30(12): p. 2060-7.
61. Almanzar, G., et al., *Long-term cytomegalovirus infection leads to significant changes in the composition of the CD8+ T-cell repertoire, which may be the basis for an imbalance in the cytokine production profile in elderly persons.* J Virol, 2005. 79(6): p. 3675-83.

62. Frasca, D. and B.B. Blomberg, *Aging, cytomegalovirus (CMV) and influenza vaccine responses.* Human Vaccines and Immunotherapeutics, 2016. 12(3): p. 682-690.

63. Reed, R.G., R.N. Greenberg, and S.C. Segerstrom, *Cytomegalovirus serostatus, inflammation, and antibody response to influenza vaccination in older adults: The moderating effect of beta blockade.* Brain Behav Immun, 2017. 61: p. 14-20.

64. Merani, S., et al., *Influenza vaccine-mediated protection in older adults: Impact of influenza infection, cytomegalovirus serostatus and vaccine dosage.* Exp Gerontol, 2017.

65. van den Berg, S.P.H., et al., *Negative Effect of Age, but Not of Latent Cytomegalovirus Infection on the Antibody Response to a Novel Influenza Vaccine Strain in Healthy Adults.* Front Immunol, 2018. 9: p. 82.

66. Belongia, E.A., et al., *Variable influenza vaccine effectiveness by subtype: a systematic review and meta-analysis of test-negative design studies.* Lancet Infect Dis, 2016. 16(8): p. 942-51.

67. Committee for Medicinal Products for Human Use (1997) *Harmonisation of requirements for influenza vaccines*. European Medicines Agency. https://www.ema.europa.eu/en/harmonisation-requirementsinfluenza-vaccines. Accessed 20 Dec 2018

Number	Query results	Results
#27	'cytomegalovirus infection'/exp AND 'influenza vaccine'/exp OR ('cytomegalovirus infection'/exp AND 'influenza vaccination'/exp) OR ('cytomegalovirus infection'/exp AND ('influenza virus'/exp AND 'vaccine'/exp OR ('influenza virus'/exp AND 'vaccination'/exp))) OR ('cytomegalovirus'/exp AND 'influenza vaccine'/exp) OR ('cytomegalovirus'/exp AND 'influenza vaccination'/exp) OR ('cytomegalovirus'/exp AND ('influenza virus'/exp AND 'vaccine'/exp OR ('influenza virus'/exp AND 'vaccination'/exp))) OR ('cytomegalovirus'/exp/mj AND ('immune response'/exp/mj OR 'immune status'/exp/mj)) OR ('cytomegalovirus infection'/exp/mj AND ('immune response'/exp/mj OR 'immune status'/exp/mj)) AND 'human'/de	689
#26	'cytomegalovirus infection'/exp AND 'influenza vaccine'/exp OR ('cytomegalovirus infection'/exp AND 'influenza vaccination'/exp) OR ('cytomegalovirus infection'/exp AND ('influenza virus'/exp AND 'vaccine'/exp OR ('influenza virus'/exp AND 'vaccination'/exp))) OR ('cytomegalovirus'/exp AND 'influenza vaccine'/exp) OR ('cytomegalovirus'/exp AND 'influenza vaccination'/exp) OR ('cytomegalovirus'/exp AND ('influenza virus'/exp AND 'vaccine'/exp OR ('influenza virus'/exp AND 'vaccination'/exp))) OR ('cytomegalovirus'/exp/mj AND ('immune response'/exp/mj OR 'immune status'/exp/mj)) OR ('cytomegalovirus infection'/exp/mj AND ('immune response'/exp/mj OR 'immune status'/exp/mj))	837
#25	'cytomegalovirus'/exp/mj AND ('immune response'/exp/mj OR 'immune status'/exp/mj) OR ('cytomegalovirus infection'/exp/mj AND ('immune response'/exp/mj OR 'immune status'/exp/mj))	429
#24	'cytomegalovirus infection'/exp/mj AND ('immune response'/exp/mj OR 'immune status'/exp/mj)	227
#23	'cytomegalovirus'/exp/mj AND ('immune response'/exp/mj OR 'immune status'/exp/mj)	238
#22	'immune response'/exp/mj OR 'immune status'/exp/mj	92,574
#21	'cytomegalovirus'/exp/mj	14,375
#20	'cytomegalovirus infection'/exp/mj	16,489
#19	'immune status'/exp/mj	932
#18	'immune response'/exp/mj	91,662
#17	'cytomegalovirus infection'/exp AND 'influenza vaccine'/exp OR ('cytomegalovirus infection'/exp AND 'influenza vaccination'/exp) OR ('cytomegalovirus infection'/exp AND ('influenza virus'/exp AND 'vaccine'/exp OR ('influenza virus'/exp AND 'vaccination'/exp))) OR ('cytomegalovirus'/exp AND 'influenza vaccine'/exp) OR ('cytomegalovirus'/exp AND 'influenza vaccination'/exp) OR ('cytomegalovirus'/exp AND ('influenza virus'/exp AND 'vaccine'/exp OR ('influenza virus'/exp AND 'vaccination'/exp)))	414
#16	'cytomegalovirus'/exp AND ('influenza virus'/exp AND 'vaccine'/exp OR ('influenza virus'/exp AND 'vaccination'/exp))	122
#15	'cytomegalovirus'/exp AND 'influenza vaccination'/exp	41
#14	'cytomegalovirus'/exp AND 'influenza vaccine'/exp	134
#13	'cytomegalovirus infection'/exp AND ('influenza virus'/exp AND 'vaccine'/exp OR ('influenza virus'/exp AND 'vaccination'/exp))	47
#12	'cytomegalovirus infection'/exp AND 'influenza vaccination'/exp	72
#11	'cytomegalovirus infection'/exp AND 'influenza vaccine'/exp	203
#10	'influenza virus'/exp AND 'vaccine'/exp OR('influenza virus'/exp AND 'vaccination'/exp)	13,079
#9	'influenza virus'/exp AND 'vaccination'/exp	6,925
#8	'influenza virus'/exp AND 'vaccine'/exp	11,990
#7	'vaccine'/exp	286,298
#6	'vaccination'/exp	143,378
#5	'influenza virus'/exp	54,474

#4	'influenza vaccination'/exp	13,281
#3	'influenza vaccine'/exp	30,951
#2	'cytomegalovirus'/exp	34,775
#1	'cytomegalovirus infection'/exp	34,353

Supplementary Table 1. Literature search strategy for the effect of CMV infection on the immune response to influenza vaccination. Results represent the number of articles. Search was performed on 27 june 2017.

Data extraction form per study

Characteristics of studies (Summarized in Table 1)

Primary study design	Design for research question	Original paper URL	Conclusion/ summary	Population (sample size and age)	Exclusion criteria	Setting (country etc)	Timeline

Influenza vaccine outcomes (Summarized in Table 2)

Increase GMT (outcome 1)	Response (outcome 2)	Correlation (outcome 3)	Antibodies (another way)	Model (corrected)	Cellular

Conclusion articles (Summarized in Figure 3, and Supplementary table 3)

Influenza virus strain(s) antibodies measured	Reported effect of CMV Young study group	Reported effect of CMV Old study group	Statistical method

Risk of bias and study quality of studies included for systematic assessment (summarized in Table 3)

Study controls for	Statistical method appropriate	Selection (max 4*)	Comparability (max 2*)	Outcome (max 3*)	Overall score (+/-)

Influenza vaccination (summarized in Supplementary table 4)

Type vaccine	year and pandamic or seasonal	Dose	influenza virus strain(s)	Booster

Time and type of measurement of influenza and CMV antibodies (summarized in Supplementary table 5)

Influenza antibody measurement			CMV antibody measurement		
Time after vaccination	Assay of measurement	Vaccination history	Assay of measurement	Cut-off CMV-seropositive	Borderline handling

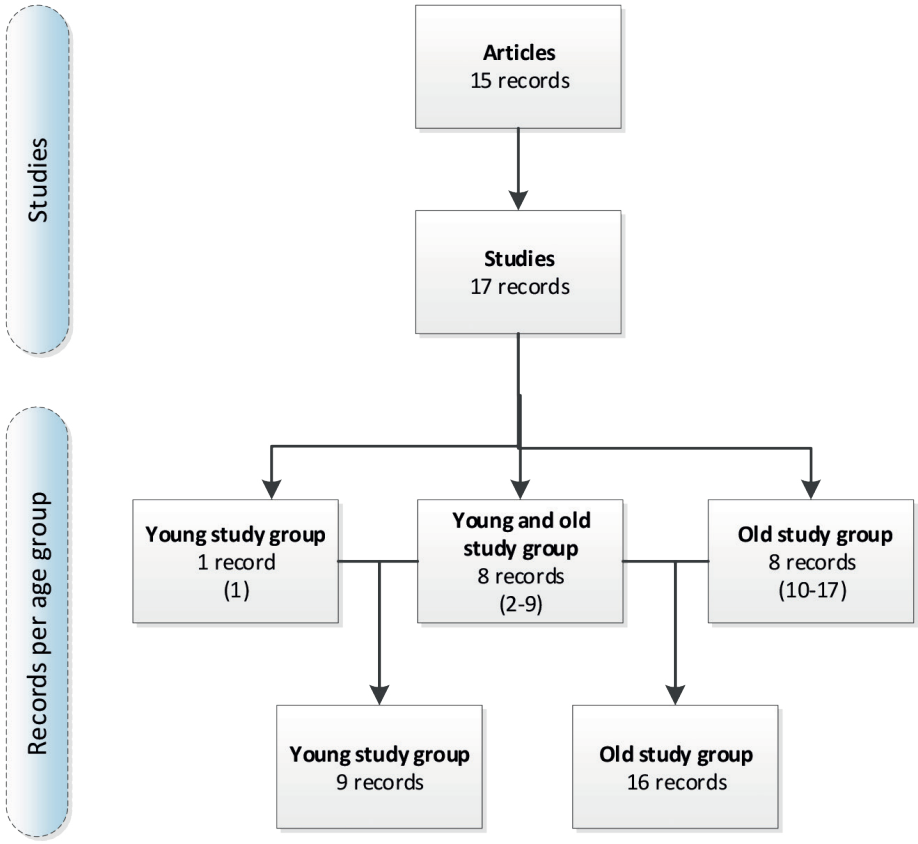
Supplementary Table 2. Data extraction form per study.

Study	Influenza virus strain(s) antibodies measured	Reported effect of CMV Young	Reported effect of CMV Old
Turner et al, 2014	A/H3N2 A/H1N1 A/H1N1 B Vic	**Negative** Brisbane H1N1 or H3N2* **No effect** Brisbane H1N1 or H3N2* B Vic	-
Den Elzen et al, 2011	A/H3N2	-	**No effect** A/H3N2
Derhovanessian et al, 2012	A/H1N1 A/H3N2 B Vic	**No effect** A/H1N1 A/H3N2 B Vic	**Negative** A/H3N2 **No effect** A/H1N1 B Vic
Nielsen et al, 2015	A/H1N1	**No effect** A/H1N1 (age range 21-77)	
Furman et al, 2015 – study 1 **	A/H1N1 A/H3N2 B Vic	**Positive** GM of the strains A/H1N1, A/H3N2, B Vic	**No effect** GM of the strains A/H1N1, A/H3N2, B Vic
Furman et al, 2015 – study 2 **	A/H1N1 A/H3N2 B Vic	**Positive** GM of the strains A/H1N1, A/H3N2, B Vic	**No effect** GM of the strains A/H1N1, A/H3N2, B Vic
Furman et al, 2015 – study 3 **	A/H1N1 A/H3N2 B Vic	**Positive** GM of the strains A/H1N1, A/H3N2, B Vic	**No effect** GM of the strains A/H1N1, A/H3N2, B Vic
Haq et al, 2016	A/H3N2	-	**No effect** A/H3N2
McElhaney et al, 2015	Unkown which strain was measured	-	**Positive** Unkown which strain
Frasca et al, 2015	A/H1N1 A/H3N2 B Vic	**Negative** A/H1N1 Others were not shown 'because low titers'	**Negative** A/H1N1 Others were not shown 'because low titers'
Trzonkowski et al, 2003	A/H1N1 A/H3N2 B Yam	**Negative** H1N1 **No effect** A/H3N2 B Yam (not significant: P=0.07 and P=0.08)	**Negative** A/H1N1 A/H3N2 B Yam2 1
Strindhall et al, 2015	A/H1N1 A/H3N2 B Vic	-	**No effect** A/H1N1 A/H3N2 B Vic
Reed et al, 2016	A/H1N1 A/H3N2 B (For each year performed)	-	**Negative** Averaged strains per year. On persistence and on peak in combination with other factors.
Wald et al, 2013	A/H1N1pdm	**Positive** A/H1N1pdm	**No effect** A/H1N1pdm
Arias et al, 201	Unkown which strain was measured	-	**Negative** Unkown
Moro-Garcia et al, 2011	Unkown which strain was measured	-	**Negative** Unkown

Guidi et al, 2014	A/H1N1 or A/H1N1pdm	-	**No effect** A/H1N1 or A/H1N1pdm

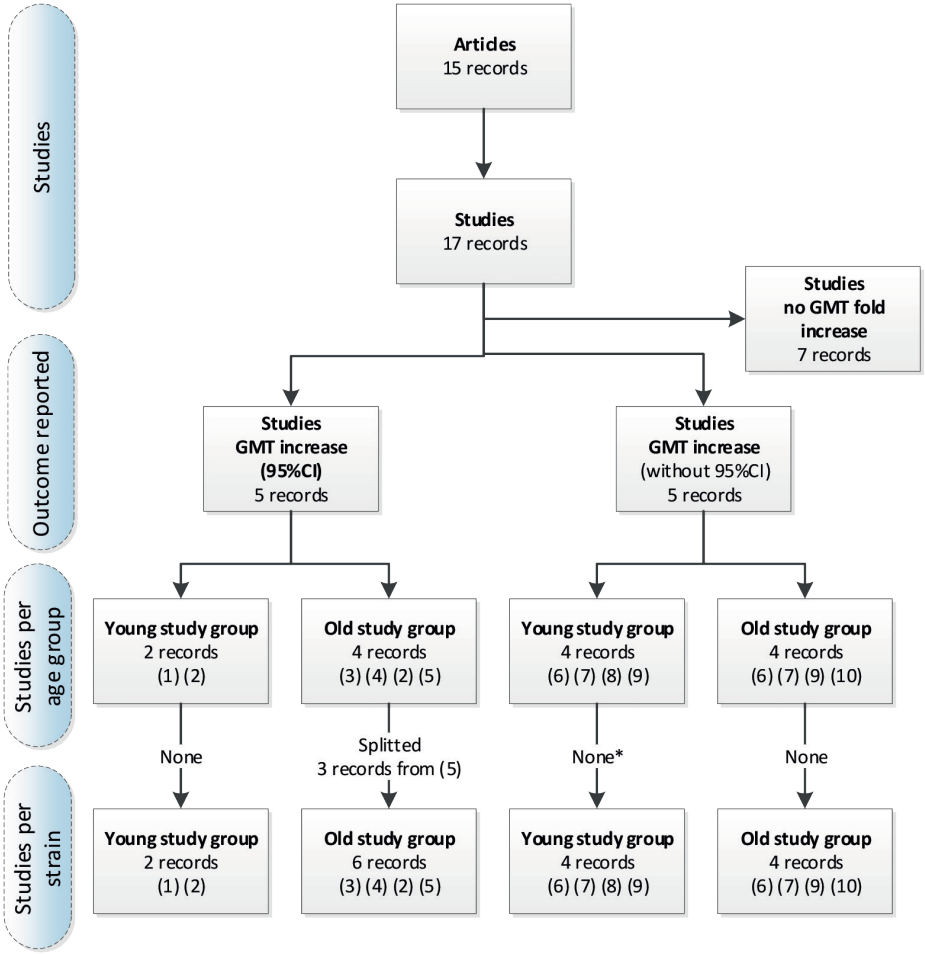
* Unkown for which influenza A/Brisbane strain a negative effect respectively no effect was found in this study (H1N1 or H3N2)
- not investigated in this study for this age group.
** Studies from Furman et al (1) reported a geometric mean of three influenza strains, no data of individual strains was reported.

Supplementary Table 3. Conclusion reported by articles.

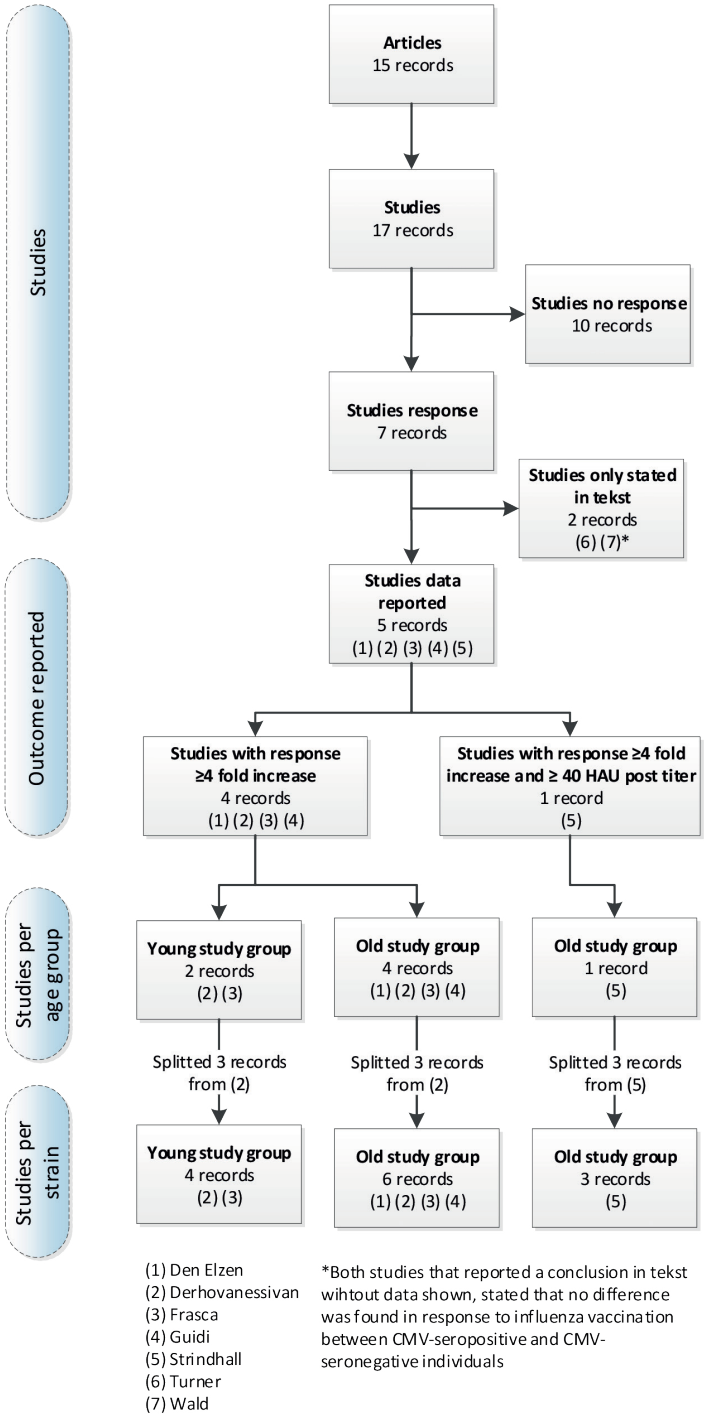


(1) Turner et al
(2) Nielssen
(3) Derhovanessivan
(4) Frasca
(5) Trzonkowski
(6) Furman 1
(7)Furman 3
(8) Furman 3
(9) Wald
(10) McElhaney
(11) Moro Gracia
(12) Arias
(13) Reed
(14) Guidi
(15) Den Elzen
(16) Haq
(17)Strindhall

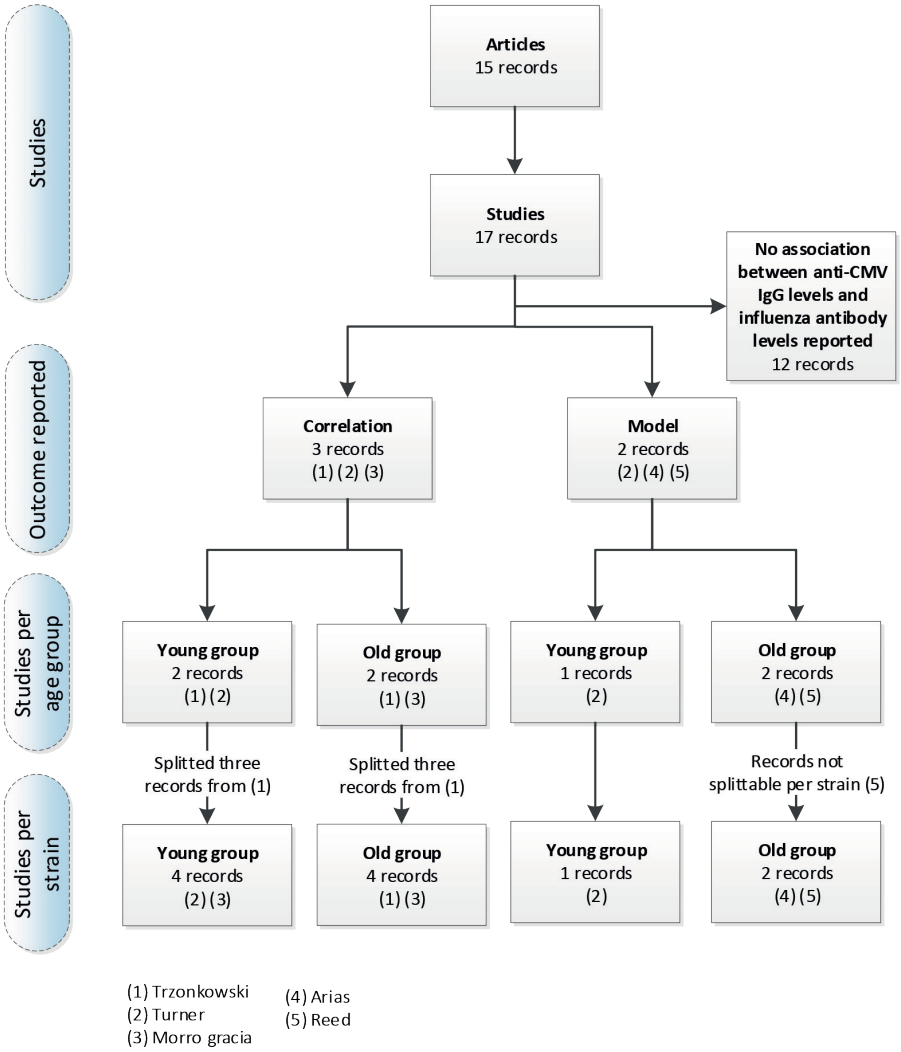
Supplementary Figure 1. Flow diagram of number of records available for reported conclusions of the effect of CMV on influenza antibody response in young (<60 years of age) or old (>60 years of age) adults.



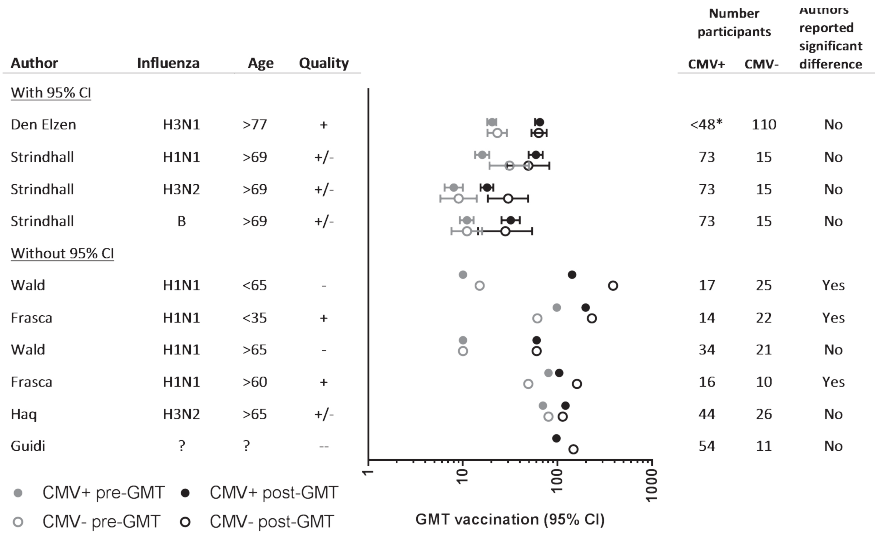
Supplementary Figure 2. Flow diagram of number of records available for influenza GMT fold increase (outcome A) with and without 95% confidence interval (CI).



Supplementary Figure 3. Flow diagram of number of records available for effect of CMV-serostatus on response to influenza vaccination (outcome B)



Supplementary Figure 4. Flow diagram of number of records available for correlation influenza antibody titers and CMV IgG levels (outcome C)



Supplementary Figure 5. Pre-vaccination geometric mean titer (pre-GMT) and post-vaccination geometric mean titer (post-GMT) in CMV-seropositive versus CMV-seronegative participants. * Data for *Turnet er al* was not reported for CMV-seropositivity (n=48), but for different CMV-seropositive groups based on height of anti-CMV IgG level. Here, CMV-seropositive high individuals are shown (subgroup of n=48).

4

Lower influenza virus-specific memory T-cell frequencies, but no impaired T-cell response to acute influenza virus infection by latent CMV infection

Manuscript in preparation

S.P.H. van den Berg[1,2], J. Lanfermeijer[1,2], R.H.J Jacobi[1], M. Hendriks[1], M. Vos[1], R. van Schuijlenburg[1], N.M. Nanlohy[1], J.A.M. Borghans[2], J. van Beek[1], D. van Baarle[1,2], J. de Wit[1]

1 Center for Infectious Disease Control, National Institute for Public Health and the Environment, Bilthoven, the Netherlands
2 Center for Translational Immunology, University Medical Center Utrecht, Utrecht University, the Netherlands

ABSTRACT

Latent infection with cytomegalovirus (CMV) is thought to contribute to the age-associated decline of the immune system. CMV induces large changes in the T-cell pool and may thereby affect other immune responses. This is thought to impact especially older adults, who are at higher risk of hospitalization and death upon influenza virus infection. Here, we investigated the impact of age and CMV infection on influenza virus-specific T-cell frequencies in healthy individuals (n=96), as well as the response to influenza virus infection in older adults (n=72). We assessed two major hypotheses of CMV-enhanced ageing: 1) impairment of the immune response due to the presence of large CMV-specific clonal expansions filling the 'immunological space', and 2) reduced immune responses as a result of a lingering low-graded level of inflammation by CMV.

We found that influenza virus-specific T-cell frequencies were decreased in healthy CMV+ older adults versus CMV- older adults. However, specific T-cell responses upon acute influenza virus infection were not negatively affected by CMV infection. We found no evidence for filling immunological space, as there was no negative association between influenza virus-specific T-cell frequencies, phenotype, or IFNγ responses with CMV-serostatus, CMV-specific antibody levels, or CMV-specific T-cell responses at any time point. We found no association between CMV infection and inflammatory cytokine levels in serum during influenza virus infection. Inflammatory cytokine levels were also not associated with the height of the influenza virus-specific T-cell response upon infection. Finally, CMV infection was not associated with increased severity of influenza-related symptoms. In fact, CMV infection was even associated with increased influenza virus-specific T-cell responses upon influenza virus infection. In conclusion, although associated with lower frequencies of influenza virus-specific memory T-cells in healthy individuals, CMV infection does not seem to form a threat for a proper T-cell response during acute influenza virus infection in older adults.

INTRODUCTION

The worldwide population is ageing rapidly. With age, deleterious changes in the immune system arise, which impair responses against infectious diseases and vaccinations [1, 2]. This phenomenon is referred to as 'immunosenescence'. Age-related changes of the immune system, such as the increase in CD45RA+ memory T-cells [3, 4] and decreased diversity of the T-cell receptor repertoire [5, 6], are predominantly present in the CD8+ T-cell pool. About 2 decades ago, latent infection with cytomegalovirus (CMV) was implicated as a possible driving force of these age-related changes [7-9]. CMV-seropositivity was identified as part of the so-called 'immune risk profile', predictive of early mortality in older adults [9, 10]. Moreover, CMV-seropositivity is the largest non-heritable factor influencing differences among humans in the immune profile [11]. CMV is thought to establish this large effect on the immune system by its frequent attempts to reactivate, thereby gradually affecting the immune system [12]. Since the first signs of CMV-enhanced ageing of the immune system, it is often hypothesized that CMV might impair the immune response to a heterologous challenge [6, 12, 13].

With age, the risk for serious complications and hospitalization after influenza virus infection increases [14]. Vaccination is an important tool to prevent infection and reduce morbidity and mortality. Unfortunately, also the efficacy of influenza vaccination decreases with age, leading to suboptimal protection in older adults [2, 15]. As CMV is thought to contribute to the age-associated decline of the immune system, the role of CMV infection on influenza vaccination efficacy has been well studied and led to conflicting results and both a negative effect of CMV [16-18], as no effect of CMV on the vaccine response [19, 20] has been reported. A systematic review including a meta-analysis of our group showed no clear evidence for a negative effect of CMV infection on the antibody response to influenza vaccination [21].

Both CMV and ageing primarily affect the T-cell compartment. During influenza virus infection, an effect of CMV would therefore mainly be expected on the T-cell response. T-cells play an important role in clearance of influenza virus infection and protection against influenza-related disease [22]. Furthermore, T-cells responses are predominantly specific for the internal viral proteins, which are conserved among antigen drifted influenza strains [23]. Indeed, pre-existing and early influenza virus-specific T-cell responses are associated with lower disease severity of influenza [23, 24], while delayed T-cell responses to influenza are thought to induce prolonged inflammation and delayed viral clearance and recovery [24, 25]. The influenza virus-specific T-cell response is mostly directed against the conserved M1, NP and PA proteins [26]. Upon activation, influenza virus-specific CD8+ T-cells show increased expression of activation and proliferation markers [27, 28], produce proinflammatory cytokines, such as IFNγ and kill virus-infected cells by releasing perforin and granzyme B [26, 29]. In contrast to the attention for influenza vaccination in CMV-enhanced ageing research, the effect of CMV on the immune response to influenza virus infection is less well established.

Whether CMV infection attenuates the immune response to influenza virus infection in older adults remains unclear. Individuals with high CMV-specific antibody levels, assumed to reflect frequent episodes of viral reactivation, were suggested to experience the largest impact of CMV on the immune system. As CMV might not affect all CMV+ individuals equally, it is crucial to asses not only the effect of CMV-seropositivity, but also the hypothesized underlying mechanism. There are two major hypotheses explaining how CMV infection may affect the immune response to a heterologous virus. First, it has been proposed that large clonal expansions of terminally differentiated CMV-specific CD8+ T-cells, which are a hallmark of CMV infection and can take up to 30%-90% of the CD8+ T-cell memory pool [30-32], may fill the 'immunological space' and thereby hamper the induction of other immune responses [10, 13, 33]. Influenza virus-specific T-cells may thus be outcompeted by CMV-specific T-cells in their competition for proliferation and survival factors [13], which may hamper the immune response against influenza. Secondly, it has been suggested that CMV is linked to 'inflammaging', the lingering low-graded level of inflammation occurring with ageing [34]. The production of pro-inflammatory mediators has been shown to enable CMV reactivation [35, 36]. Upregulation of TNF-α, IL-6 and CRP have been observed in CMV-infected individuals, as well as increased production of IL-10 [11, 37, 38]. Especially the increase of IL-10 combined with a decrease of IFNγ has been associated with reduced cytolytic capacity of CD8+ T-cells responsible for clearing influenza virus from the lungs, which also fits with the observed lower levels of granzyme B [39-41]. Importantly, even though CMV has been suggested to diminish the T-cell response to influenza, there is no clinical evidence of a direct link between CMV infection and the T-cell response against influenza virus infection in humans.

We had the unique opportunity to study the effect of CMV infection on influenza virus specific T-cells with age and on the cellular immune response against influenza virus infection in a large group of older adults. We first investigated the effect of CMV infection on the presence of influenza virus-specific memory T-cells in healthy young and older individuals. Next we assessed the effect of latent CMV infection on the influenza virus-specific T-cell response in older adults undergoing an acute influenza virus infection. As CMV might only affect some individuals, we also assessed the association between 1) CMV-specific T-cells and influenza T-cell responses and 2) inflammatory cytokines and influenza T-cell responses in CMV- and CMV+ individuals. Our data show that CMV infection is associated with reduced frequencies of influenza virus-specific T-cells in healthy older adults. Nevertheless, CMV infection does not hamper the T-cell response during acute influenza virus infection. Our data suggest that CMV is unlikely to be detrimental for immune responses to influenza virus infection in older adults.

MATERIALS & METHODS

Study design

Healthy young and older adults

Samples of healthy individuals covering a broad age range were combined from two cohorts. Samples of young adults (n=34), between 18 and 52 years of age, from unvaccinated controls or pre-vaccination participants were used from a study carried out in 2009-2011 (the Pandemic influenza vaccination trial, Netherlands Trial Register NTR2070) [42]. The study was approved by the Central Committee on Research Involving Human Subjects of the Netherlands. Samples of older adults (N=62), ≥60 years of age, were control samples from a study carried out in 2014-2015 (Influenza-like-illness-3, Netherlands Trial Register 4818) (Van Kaaijk et al., submitted). This study was approved by the acknowledged ethical committee METC Noord Holland. Both studies were carried out in accordance with the recommendations of Good Clinical Practice with written informed consent from all subjects, in accordance with the Declaration of Helsinki.

Influenza virus A infected older adults during influenza virus infection

Laboratory-confirmed Influenza virus A infected older adults were selected from the same study as the healthy older adults. In this prospective observational study participants were monitored for influenza-like-illness (ILI) in the flu season of 2014-2015 (Influenza-like-illness-3, Netherlands Trial Register 4818). Participants were instructed about influenza-like-illness (ILI) symptoms according to the Dutch Pel criteria, defined by fever (≥37.8°C) with at least 1 other symptom of headache, muscle pain, sore throat, coughing, runny nose, or chest pain [43] and to report ILI as soon as possible after onset. Nasopharyngeal and oropharyngeal samples were obtained within 72 hours of reporting ILI by standard procedures [44]. Influenza virus A infection was laboratory confirmed, and subtyped by PCR and sequencing in n=72 individuals by methods described previously [44]. These 72 influenza virus A confirmed patients were included in the current study. The H3N2 strain was detected in the majority of patients (n=64, of which n=20 clade 3C.3b, n=37 clade 3C.2a, n=7 inconclusive clade), and the H1N1 strain in the remaining individuals (n=8). Blood samples were collected within the first 72 hours of fever onset, and followed up after 2 weeks and 8 weeks.

PBMC and serum isolation

Peripheral blood mononuclear cells were obtained by Lymphoprep (Progen) density gradient centrifugation from heparinized blood, according to the manufacturer's instructions. PBMCs were frozen in 90% fetal calf serum and 10% dimethyl sulfoxide at -135°C until further use. Serum was isolated out of tubes with clot-activation factor and stored at -80°C until further use.

Cytomegalovirus (CMV)-specific antibodies

Anti-CMV IgG antibody concentrations were measured either using a commercial ELISA or by a multiplex immunoassay developed in-house [45]. For healthy young adults, CMV-specific antibody levels were measured using a commercial ELISA (IBL international

GMBH) according to manufacturer's instructions. Participants with a CMV antibody level of ≥12 U/ml or higher were considered CMV+, those with a level of ≤8 U/ml were considered CMV-, and those with a level between 8 and 12 U/ml were considered inconclusive and hence excluded for further analysis. For older healthy adults and influenza virus A-infected individuals, CMV-specific antibody levels were measured in serum by an in-house-developed multiplex immunoassay [45]. Individuals with a CMV-specific antibody level of ≤4 arbitrary units/ml were considered to be CMV- and individuals with an antibody level > 7.5 RU/ml were considered CMV+, and those with a level between 4 and 7.5 arbitrary units/ml were considered inconclusive and hence excluded from further analysis. To reduce inter-assay variation, all samples from the same individual were measured on the same plate.

Antigen-specific T-cells by flow cytometry

Healthy individuals

HLA-A2 positive healthy individuals were selected based on availability from young and old healthy individuals for subsequent influenza virus-specific T-cell analysis, by staining PBMCs for expression of HLA-A2 with the HLA-A2(BB7.2)-V450 antibody (BD Bioscience). Of the HLA-A2 positive individuals, ±4 million PBMC's were stained using the HLA-class I tetramer for epitope GILG of the M1 protein of influenza virus (A*0201/GILGFVFTL-APC, Immudex) for 20 minutes at room temperature. Surface staining was performed for 30 minutes at 4 °C with the following antibodies: Fixable Viability Staining-780 (BD bioscience), CD3 (SK7)-AF700(BD bioscience), CD8(RPA-T8)-BrilliantViolet510, CD45RO(UCHL1)-BrilliantViolet711, CD27(O323)-BrilliantViolet786, CCR7(150503)-BrilliantUV395 (BD bioscience), KLRG-1(13F12F2)-PE-Cy7 (eBioscience), PD-1(EH12.2H7)-PerCP Cy5.5, CD95(DX2)-BrilliantViolet421 (BD Biosciences), CD127(A019D5)-BrilliantViolet650, CD57(HCD57)-PE and CXCR3(G025H7)-PE-Dazzle. All antibodies were purchased from Biolegend, unless stated otherwise. Acquisition was performed on a LSRFortessaX20 and data analysis was performed using FlowJo (Treestar). tSNE-analyses were performed using Cytobank (www.cytobank.org) [46]with for every donor 10.000 $CD8^+$ T-cells. Donors with less than 10.000 $CD8^+$ T-cells were exlcuded from t-SNE analysis (n=3).

Influenza virus A-infected individuals

Of all HLA-A2 positive individuals undergoing influenza virus infection (n=17), both influenza virus-specific T-cells and CMV-specific T-cells were assessed within the first 72 hours 2 and 8 weeks after fever onset. PBMCs were stained using the HLA-A2 tetramer for the GILG epitope of the M1 protein of influenza virus (A*0201/GILGFVFTL-APC, Immudex) (±8 mln PBMCs) and the HLA-A2 tetramer for the NLV epitope of the pp65protein of CMV (A*0201/NLVPMVATV)-APC (Immudex) (1 mln PBMCs) for 20 minutes at room temperature. Extracellular staining was performed for 30 minutes at 4 °C with the following antibodies: Fixable Viability Staining-780 (BD bioscience), CD3 (SK7)-AF700(BD bioscience), CD8(RPA-T8)-BrilliantViolet510 (Biolegend), CD45RO(UCHL1)-BrilliantViolet711 (Biolegend), CD27(O323)-BrilliantViolet786 (Biolegend), CCR7(150503)-BrilliantUV395(BD bioscience), KLRG-1(13F12F2)-PE-Cy7 (eBioscience), PD-1(EH12.2H7)-PerCP Cy5.5 (Biolegend),

CD95(DX2)-BrilliantViolet421 (BD Biosciences), CD127(A019D5)-BrilliantViolet650 (Biolegend), CD57(HCD57)-PE (Biolegend) and CXCR3(G025H7)-PE-Dazzle (Biolegend), CD38(HIT2)-PE-Dazzle (BD bioscience) and HLA-DR(TU39)-BrilliantUV737 (BD Bioscience). Analysis was performed on an LSRFortessaX20.

Influenza virus-specific and CMV-specific IFNγ T-cell response by ELISpot

Virus-specific T-cell responses were quantified using the IFNγ enzyme-linked immunospot (ELISPOT) assay at all three time points for those of which enough cells were available (respectively n=66, n=61, n=58 out of n=72). Briefly, 400.000 PBMCs were stimulated with a 15-mer peptide-pool with 11 amino acids overlap, covering the total influenza M1 protein (1 µg/ml) (JPT) and incubated for 16-20 hours at 37 °C on 96-well membrane-bottomed plates coated with anti-IFNγ mAbs. If indicated, IFNγ responses were corrected for the percentage of T-cells in lymphocytes based on flow cytometry data. For CMV responses, 100,000 PBMCs were stimulated with a 15-mer peptide-pool with 11 amino acids overlap, covering either the UL55 (1µg/ml) (JPT), the IE-1 (1µg/ml) (JPT), or the pp65 (1µg/ml) (JPT) CMV protein. The sum of the response to these three CMV peptide pools is presented in this study.

Cytokine and chemokine levels in serum

Cytokines and chemokine levels in serum were assessed for all influenza virus-infected individuals within 72 hours, and 2 and 8 weeks after fever onset. Levels were measured by bead-based multiplex LEGENDplex™ (BioLegend) according to the manufacturer's instructions. The pro-inflammatory cytokines IL-6, IFNy, IL-10 and CRP and chemokines CXCL-10, CXCL-9 and CXCL-11 were analyzed for this study. Reactions were performed in duplicate. Analysis was performed on a Canto II. Data were analyzed via Legendplex V8.0 software (Biolegend). All data were transformed into averages of the logarithms of two measurements, and each data point was corrected by subtraction of the within-run averages to correct for batch effects.

Severity of symptoms assessment

Symptom assessment was performed by the participants, by filling in a diary during influenza virus infection. Presence and duration (start date and end date) of the following symptoms were collected: fever (≥37.8 °C), cough, sore throat, runny nose, headache, pain while breathing and muscle pain. Symptoms were noted in hindsight maximum 10 days before onset of fever, as symptoms before then could not have been related to the influenza virus infection. Z-values, e.g. the number of standard deviations by which the value of the score of an individual is above or below the mean value were calculated for each symptom. Overall severity of symptoms during influenza virus infection was assessed as the average of the Z-values of the seven symptoms.

Statistical analysis

Differences between groups (for example CMV- compared to CMV+) were assessed using Mann-Whitney *U* test, and comparisons within the same individuals with the Wilcoxon

singed-rank test. Correlations were tested with Spearman's rank correlation coefficient. For all analyses p values < 0.05 were considered statistically significant. Data were analyzed using GraphPad Prism 8.3 and SPSS statistics 22 for Windows (SPSS Inc., Chicago, IL, USA).

RESULTS

Characteristics of study population

Healthy individuals were on average 59.2±19.1 years old (range 21-82 year) (n=96). They were categorized into young (21-52 years old) (n=34) and old (>60 years old) (n=62) individuals, of whom respectively 55.9% and 59.7% were CMV-infected (**Table 1**). No significant differences in age or sex were observed between CMV- and CMV+ individuals (**Table 1**). In addition to healthy individuals, older adults with confirmed influenza virus infection were included in this study (n=72). Also for the influenza virus-infected older adults, no significant differences in age or sex were observed between CMV+ and CMV- individuals (**Table 1**). The majority of individuals were infected with the H3N2 strain (n=64), while some were infected with the H1N1 strain of influenza (n=8). All individuals in the latter group turned out to be CMV-.

Healthy young adults				
	Total (n=34)	***CMV- (n=15)***	***CMV+ (n=19)***	***Statistics***
Age (mean±SD)	35.9 ±10.3	35.3±10.8	36.4±10.1	ns
Sex (% women)	61.8%	53.3%	68.4%	ns
CMV-serostatus (CMV+)	55.9%	.	.	.
Healthy older adults				
	Total (n=65)	***CMV- (n=25)***	***CMV+ (n=37)***	***Statistics***
Age (mean±SD)	71.8±6.5	70.9±6.8	72.5±6.5	ns
Sex (% women)	38.5%	33.3%	44.7%	ns
CMV-serostatus (CMV+)	58.5%	.	.	
Influenza virus infected older adults				
	Total (n=72)	***CMV- (n=35)***	***CMV+ (n=37)***	***Statistics***
Age (mean±SD)	69.9±6.1	69.2±5.3	70.4±6.9	ns
Sex (% women)	41.7%	37.1%	45.9%	ns
CMV-serostatus (CMV+)	51.4%	.	.	.
Influenza virus strain (%H1N1)	11.1%	22.9%	0.0%	P=0.002

Table 1. Characteristics of the study population

CMV induces an increase in senescence associated markers in the T-cell pool in older adults

We assessed the effect of latent CMV infection on the $CD8^+$ T-cell pool of all healthy individuals, by performing a cluster analysis (tSNE) based on memory T-cell markers CD27, CCR7, CD95, CD45RO, and CXCR3, and senescence-associated T-cell markers CD57 and KLRG-1, known to be altered in CMV infection [47]. Cluster analysis indeed conformed the large differences between the CMV-negative (n=40) and CMV-positive (n=56) group (**Figure 1A**), and six different clusters were identified. Clusters 1-3 containing non-senescent $CD27^{high}CCR7^{high}$ $CD57^{low}$ cells are predominantly expressed in CMV-negative individuals (**Figure 1B, Supplementary Figure 1A**). In contrast, clusters 4-6 containing the more

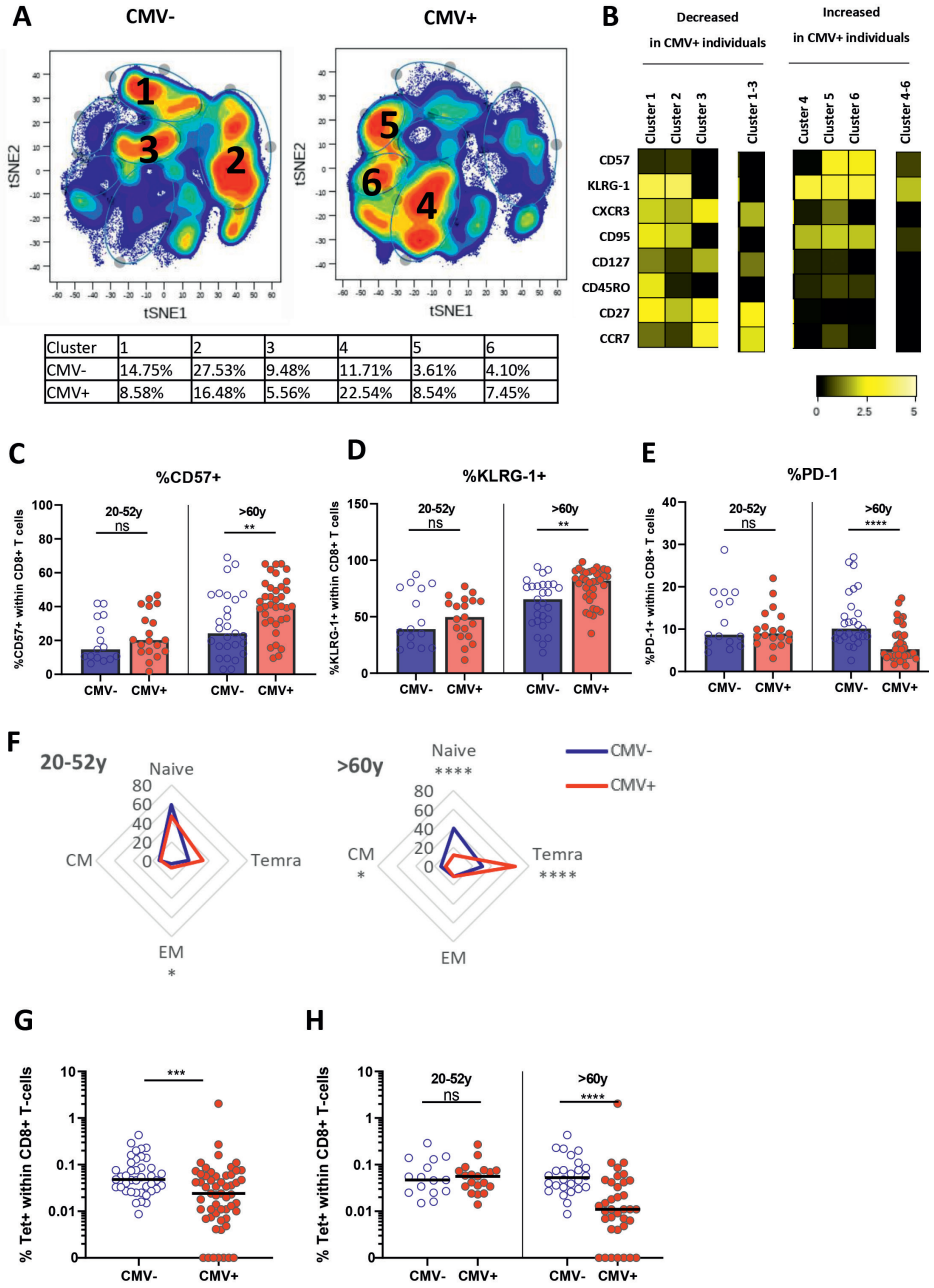
differentiated cells expressing KLRG-1^{high}CD57^{medium} were significantly increased in CMV+ individuals (**Figure 1B, Supplementary Figure 1A**). We next performed more detailed analysis on the expression of senescence-associated markers within the two age groups: young adults (20-52 years) and older adults (older than 60 years). CMV infection was associated with significantly increased expression of CD57 and KLRG-1 among older adults, but not in young adults (**Figure 1C,D**). Likewise, expression of PD-1 was only significantly reduced in CMV+ individuals (as compared to CMV-) in the older group, and not in the younger group (**Figure 1E**). In line with this, CMV infection was associated with increased frequencies of T_{EMRA} cells in older individuals, but not in young individuals (**Figure 1F**). Together, this indicates that CMV infection establishes large changes in the CD8$^+$ T-cell pool by inducing terminally differentiated and senescent T-cells mostly in older adults.

Frequency of influenza virus-specific T-cells is decreased in CMV+ individuals, but only in older adults

To investigate the hypothesis that CMV infection may negatively influence the immune response to other pathogens by outcompeting other antigen-specific T-cells, the frequency of influenza virus-specific T-cells in healthy individuals was determined using HLA-A2 tetramers containing the matrix protein-1 GILG-epitope. The frequency of influenza virus-specific T-cells was significantly lower in CMV+ compared to CMV- individuals (P=0.0005) (**Figure 1G**). Importantly, this lower percentage of influenza virus-specific T-cells was solely explained by a lower frequency in the older group, where some donors had no detectable HLA-A2 GILG-specific T-cells. Among the young adults no differences in the frequencies of influenza virus-specific T-cells between CMV- and CMV+ individuals were observed (**Figure 1H**). Thus, CMV infection results in lower frequencies of influenza virus-specific memory T-cells, but only in older adults.

Characterization of the T-cell response during influenza virus infection in older adults

To investigate the effect of CMV infection on the T-cell response during influenza virus infection in older adults, we characterized the T-cell response to influenza virus infection during the acute phase of infection, i.e. within 72 hours after start of fever, and 2 and 8 weeks later. The frequency of influenza virus-specific CD8$^+$ T-cells was determined by tetramer staining for the HLA-A2 GILG-epitope. Influenza virus-specific CD8$^+$ T-cell numbers were increased upon influenza virus infection at the 2 week time point compared to <72 hours after fever onset (median 0.03% to 0.15% of total CD8$^+$ T-cells respectively), after which the response contracted (median of 0.08/total CD8$^+$ T-cells) (**Figure 2A**). The increase in influenza virus-specific CD8$^+$ T-cell numbers was mainly explained by the expansion of influenza virus-specific effector memory T-cells (T_{EM}) (**Figure 2B**), while influenza virus-specific T-cell numbers in the other subsets (i.e. naïve (T_N), central memory (T_{CM}) and T_{EMRA}) did not increase (data not shown). IFNγ responses after in vitro stimulation of PBMCs with peptide pools covering the influenza matrix-protein-1 revealed similar results (**Figure 2C**).



Cluster	1	2	3	4	5	6
CMV-	14.75%	27.53%	9.48%	11.71%	3.61%	4.10%
CMV+	8.58%	16.48%	5.56%	22.54%	8.54%	7.45%

Figure 1. CMV is associated with a more differentiated and senescent CD8⁺ T-cell phenotype, especially in older adults, and less memory influenza-specific CD8⁺ T-cells. A) t-SNE analysis of total CD8⁺ T-cells based on MFI of CD57, KLRG-1, CXCR3, CD95, CD127, CD45RO, CD27 and CCR7 in CMV- and CMV+ individuals (total of n= 97). Equal amount of cells were used per donor. Six large clusters could be identified. **B)** Heatmap of expression of markers of t-SNE clusters. **C-E)** Percentage of CD57+ (C), KLRG-1+ (D) or PD-1+ (E) CD8⁺ T-cells in young and old CMV- and CMV+ individuals. **F)** Distribution of CD8+ T-cells over naïve and memory subpopulation for young CMV- and young CMV+ individuals (left panel) and old CMV- and old CMV+ individuals (right

panel). **G)** Percentage of influenza virus specific CD8⁺ T-cells in all CMV- and CMV+ individuals. **H)** Percentage of influenza specific CD8⁺ T-cells in CMV- and CMV+ individuals categorized in young and old individuals. Tetramer for matrix protein-1 GILG-epitope was used. All individuals were HLA-A2 positive. Median is presented in each figure. Differences between groups were compared by Mann Whitney U test.

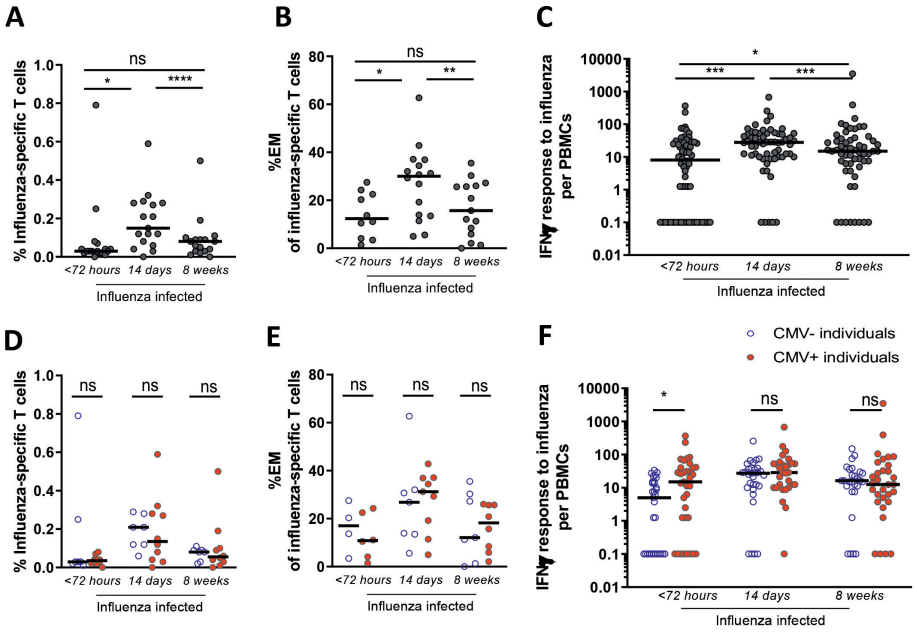


Figure 2. Characterization of the influenza virus-specific CD8⁺ T-cell response after infection shows no impairment by CMV-seropositivity, but enhancement of the early influenza virus-specific CD8⁺ T-cell response. A) Percentage of influenza specific CD8⁺ T-cells in HLA-A2 positive individuals upon influenza infection. Tetramer for matrix protein-1 GILG-epitope was used **B)** The percentage effector memory (EM) cells of influenza-specific CD8⁺ T-cells upon influenza infection. **C)** Influenza-specific IFNγ T-cell response upon influenza infection. **D)** Percentage of influenza specific T-cells in HLA-A2 positive individuals upon influenza infection for CMV- and CMV+ individuals. **E)** The percentage effector memory (EM) cells of influenza specific T-cells upon influenza virus infection for CMV+ and CMV- individuals. **F)** Influenza virus-specific IFNγ T-cell response upon influenza infection for CMV- and CMV+ individuals. Percentage of influenza specific CD8⁺ T-cells was measured using a tetramer for matrix protein-1 GILG-epitope. Influenza-specific T-cell response to overlapping influenza matrix-protein 1 peptide pools was measured by IFNγ ELISPOT spots. Wilcoxon test was used to compare T-cell responses of individuals in time and Mann-Whitney U test was used to compare CMV- and CMV+ individuals. Correlation between CMV-specific immune responses and IFNγ response to influenza virus was tested by Spearman correlation. T-cell IFNγ responses are presented per $1x10^6$ PBMCs.

When corrected for the percentage of T-cells among all lymphocytes, the influenza virus T-cell response was again higher at 2 weeks (p=0.022) compared to 8 weeks, albeit not significant (p=0.059) (**Supplementary figure 2A left panel**). The average increase in the IFNγ response to influenza was comparable between H3N2 influenza-infected and H1N1 influenza-infected individuals (data not shown)

T-cell response to influenza virus infection in older adults is not impaired by CMV, but increased in the early phase

To investigate whether the T-cell response during influenza virus infection is influenced by CMV infection, we analyzed CMV+ and CMV- individuals separately. No significant effect of CMV-seropositivity on influenza virus-specific $CD8^+$ T-cell percentages or on the percentage of T_{EM} cells among the influenza virus-specific T-cells was observed (**Figure 2D,E**). Likewise, influenza virus-specific IFNγ responses were not negatively associated with CMV-seropositivity (**Figure 2F**) or with CMV-specific antibody levels in CMV+ individuals at any of the time points investigated (**Supplementary Figure 2B**). Surprisingly, in the acute phase (<72 hours after fever onset), CMV+ individuals even showed a significantly higher influenza virus-specific IFNγ T-cell response than CMV- individuals (**Figure 2F**), also when corrected for the percentage of T-cells among all lymphocytes a trend was observed (**Supplementary Figure 2A, right panel).** Of note, this increased influenza T-cell responses in CMV+ individuals could not be explained by the moment of sampling within the 72 hours after fever onset (data not shown).

Large CMV-specific T-cell responses are not associated with impaired influenza virus-specific T-cell responses

As CMV might only affect the immune system in individuals with large CMV-specific T-cell responses, we next investigated the association between CMV infection and the T-cell response to influenza by taking into account the magnitude of the IFNγ CMV-specific T-cell responses within CMV+ individuals. No negative correlation between expanded CMV-specific T-cell responses and the height of the IFNγ T-cell response to influenza virus infection was observed at any of the three time points (**Figure 3A**). Surprisingly, we even observed a significant positive correlation between the height of the CMV-specific IFNγ T-cell response and the height of the influenza virus-specific IFNγ T-cell response at 2 and 8 weeks after fever onset (**Figure 3A**) (R: 0.52, p=0.016 and R:0.45, p=0.014 respectively). This argues against a competition between CMV-specific memory T-cells and influenza virus-specific T-cells during influenza virus infection.

Cytokine levels in serum of influenza-infected individuals are not affected by CMV and not associated with influenza virus-specific T-cell responses

We also investigated whether pro-inflammatory mediators may be associated with a negative impact of CMV infection on an immune response to a heterologous infection . Therefore, we investigated the levels of pro-inflammatory cytokines in serum of CMV+ and CMV- individuals, and their potential association with the influenza-specific T-cell response. At the early phase of infection, the inflammation-associated factors IL-6 and CRP were elevated in serum compared to 2 and 8 weeks later (**Figure 4A**). No significant difference in IL-6 and CRP levels was observed between CMV+ and CMV- individuals (**Figure 4A**). Also at the peak of the T-cell response (2 weeks later) and at steady state (week 8 after fever onset), CMV- and CMV+ individuals showed comparable cytokine profiles (**Figure 4A**). As it was suggested that a shift in the IFNγ:IL-10 ratio leads to a decline in influenza T-cell responses

with age and is associated with decreased protection against influenza [41, 48], we assessed this ratio. No difference was observed (**Figure 4B**) in the IFNγ:IL-10 ratio between CMV+ and CMV- individuals at any of the time points, despite that both IFNγ and IL-10 levels were elevated at the earliest timepoint (**Supplementary Figure 4**)**.** Although increased levels of the cytokines IL-6, CRP, IFNγ and IL-10 were observed in the acute phase of influenza virus infection, no association between these cytokine levels and the height of the influenza virus-specific T-cell response was observed at time point 1 (**Figure 4C**) or 2 weeks later (data not shown). Together, this suggests that CMV infection in older adults does not affect cytokine levels in serum thereby influencing the influenza virus specific T-cell response.

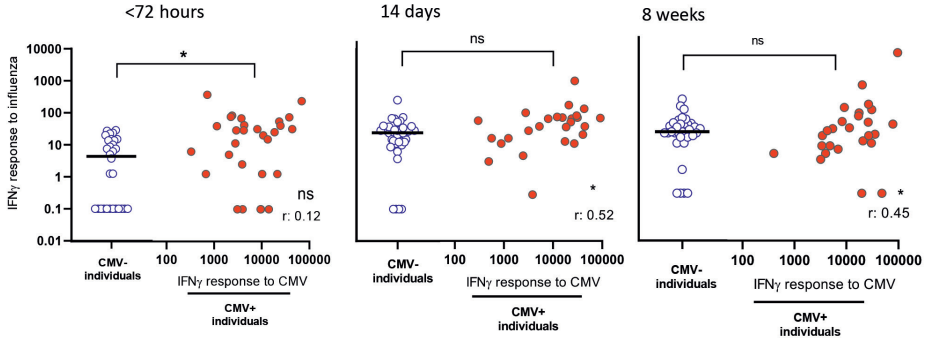


Figure 3. No reduced influenza-specific T-cell response in number of T-cells. Influenza virus-specific IFNγ T-cell response in CMV- individuals and CMV+ individuals differentiated on the CMV-specific T-cell response. Influenza-specific IFNγ T-cell responses are depicted in blue open circles in scatter plot for CMV- individuals and in red solid circles in correlation of CMV-specific IFNγ T-cell responses for CMV+ individuals**.** CMV-specific T-cell responses were measured by ELISPOT for IFNγ spots to overlapping pp65, IE-1 and UL55 peptide pools. Differences were tested using Mann-Whitney to compare CMV+ and CMV- individuals. Correlation between CMV-specific T-cell responses and IFNγ response to influenza was tested by Spearman correlation. T-cell IFNγ responses are presented per $1x10^6$ PBMCs.

Severity of symptoms of influenza virus infection is not increased by CMV

Since early T-cell responses during influenza virus infection play an important role in limiting disease severity [24, 25], we also investigated whether the height of the influenza virus-specific T-cell response was associated with severity of symptoms of influenza virus infection. In influenza virus-infected older adults, the number and duration of seven symptoms was assessed (fever (≥37.8 °C), cough, sore throat, runny nose, headache, pain while breathing and muscle pain). The severity of symptoms of influenza virus infection was positively associated with the height of the influenza virus-specific T-cell response at timepoint 3 (8 weeks after start of fever) (**Figure 5A**), but not at time point 1 (<72 hours after start of fever) or 2 (2 weeks after start of fever). The positive association between the height of the T-cell response against influenza at week 8 after start of fever with severity of symptoms was mainly based on the number of symptoms, and not on duration of symptoms (**Supplementary Figure 5**) and not associated with CMV-specific antibody levels(data not shown). Thus, a T-cell response 8 weeks after infection, and not an early T-cell response, is linked to the severity of symptoms of the influenza virus infection in this cohort.

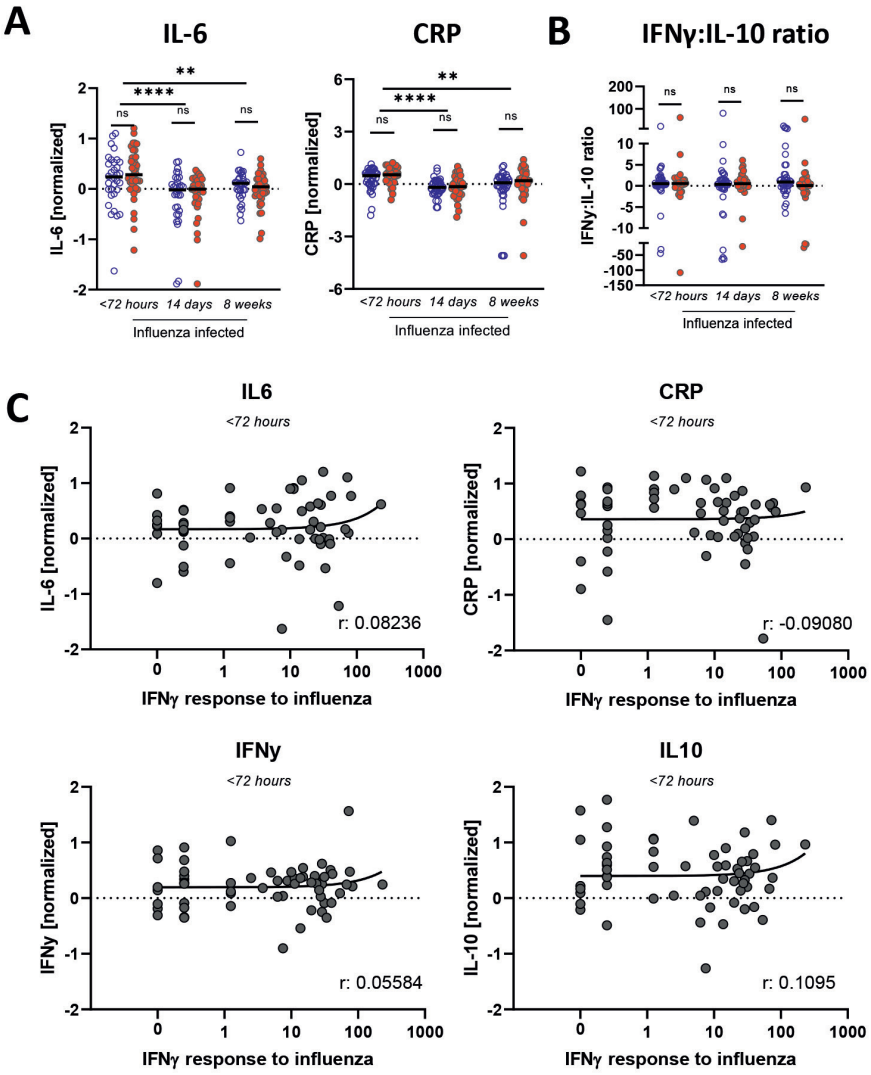


Figure 4. No sign of increased inflammation in CMV+ individuals based on cytokines levels in serum nor in relation to the influenza-specific T-cell response. A) Serum levels of "inflammaging markers" CRP and IL-6 upon influenza infection for CMV+ and CMV- individuals at <72 hours after fever onset, and 2 and 8 weeks later. **B)** The IFNγ:IL-10 ratio was calculated for CMV- and CMV-individuals upon influenza infection. **C)** Magnitude of the influenza virus-specific IFNγ response at <72 hours upon infection with influenza virus are not associated with the level of CRP, IL-6, IFNγ or IL-10 in serum. Serum levels of the cytokines were measured by multiplex assays and normalized based on subtracting the mean per plate. Differences were tested using unpaired T-test to compare CMV- and CMV+ individuals. Influenza-specific T-cell responses were measured by ELISPOT for IFNγ spots to overlapping influenza matrix-protein 1 peptide pools. Correlations between influenza-specific IFNγ response and the different cytokines levels in serum were assessed by Spearman correlation. T-cell IFNγ responses are presented per $1x10^6$ PBMCs.

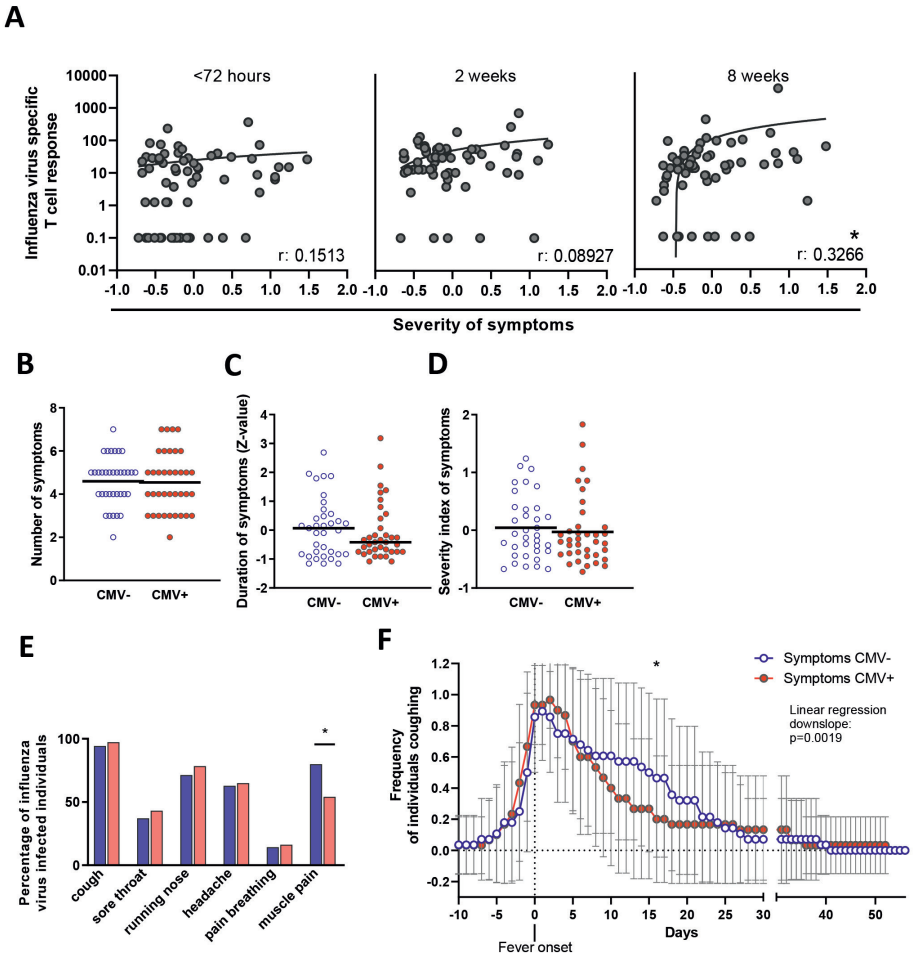


Figure 5. No negative effect of CMV infection on number and duration of symptoms of influenza virus infection. A) Influenza specific T-cell responses upon influenza virus infection at <72 hours after fever onset, and 2 and 8 weeks later was associated with the severity of symptoms of influenza infection. Association was tested by Spearman correlation**. B)** Number of symptoms during influenza virus infection in CMV- and CMV+ individuals. Due to study design, participants had a minimal of two symptoms; fever (≥37.8 °C) and at least 1 other symptom, either cough, sore throat, running nose, headache, pain while breathing or muscle pain. Statistical differences between CMV- and CMV+ were tested by Chi-square test. **C)** Duration of having symptoms, regardless which one and how many, of influenza virus infection in CMV- and CMV+ individuals. Duration in days was calculated to Z-values. Difference between CMV- and CMV+ individuals was tested by student-t test. **D)** Severity of symptoms, taking along both duration and number of symptoms was assessed by taking the mean of Z-values of the six symptoms (cough, sore throat, runny nose, headache, pain while breathing or muscle pain. **E)** Percentage of CMV- and CMV+ individuals suffering from respectively cough, sore throat, running nose, headache, pain while breathing and muscle pain. Statistical differences between CMV- and CMV+ were tested by Chi-square test. **F)** Frequency of CMV- and CMV+ individuals coughing during influenza virus infection. Difference between CMV- and CMV+ individuals was tested by linear regression analysis on the downslope (starting at day of fever onset, day 0 on the x-axis) and comparison of the slope of CMV- individuals and CMV+ individuals.

We next investigated whether CMV infection was associated with more severe symptoms during influenza virus infection compared to CMV- individuals. No significant differences between CMV- and CMV+ individuals were observed in the number of symptoms during influenza virus infection (**Figure 5B**), total duration of symptoms (**Figure 5C**), or severity of symptoms as assessed by a combination of duration and number of symptoms (**Figure 5D**). When investigating the influenza virus infection symptoms individually, CMV+ individuals did suffer less often from muscle pain (**Figure 5E)** and although coughing was not different between CMV+ and CMV-, CMV+ tended to cough less long compared to CMV- individuals (data not shown). When the frequency of individuals coughing was plotted over time, we indeed found a faster decline in the frequency of coughing individuals amongst CMV+ individuals compared to CMV- individuals (**Figure 5F**). Of note, both the increased frequency of muscle pain and the longer persistence of coughing among CMV- individuals could not be ascribed to the difference in virus strain infection between CMV- and CMV+ individuals (data not shown). Together, this suggests that CMV infection does not worsen the number and duration of influenza virus infection symptoms, and may actually be slightly beneficial.

DISCUSSION

In this study we tested the role of CMV infection on the immune response to influenza virus infection at older age. We found that CMV infection induces a more differentiated and senescent phenotype in the $CD8^+$ T-cell pool. Importantly, CMV+ older individuals had lower frequencies of influenza virus-specific CD8+ T-cells compared to CMV- older individuals. However, these lower frequencies of influenza virus-specific T-cells in CMV+ older individuals did not hamper the induction of a T-cell response during active influenza virus infection. Also, severity of influenza-associated symptoms was not negatively affected by CMV infection. Investigating two hypothesis of CMV-enhanced ageing, we did not find evidence supporting a negative effect of CMV infection in some CMV+ individuals by 'limited immunological space' or by presence of pro-inflammatory mediators. In contrast, a small positive association of CMV infection might be present, as T-cell responses against influenza were slightly increased in CMV+ individuals early after influenza virus infection (<72 hours after fever onset) and positively associated with the height of the CMV-specific T-cell response 2 weeks and 8 weeks after influenza virus infection.

Pre-existing T-cells and timing of the influenza virus-specific CD8+ T-cell response are thought to play an important role in the reduction of severity of influenza-related symptoms [23, 24]. An early T-cell response is thought to reduce the level of inflammation and to accelerate viral clearance, whereas delayed and prolonged T-cell response leads to high and prolonged levels of inflammation and increased severity of disease [24, 25]. Unfortunately, pre-existing T-cell responses could not be investigated in this study. Our data suggest that CMV+ individuals have an increased early or accelerated influenza virus-specific T-cell response compared to CMV- individuals. We speculate that this may lead to earlier viral clearance, and thereby might explain the faster recovery of coughing and decreased frequency of muscle pain. When we assessed severity of symptoms by the number and

duration of symptoms, we found a significant positive correlation between the magnitude of the influenza-specific T-cell response and severity of symptoms 8 weeks after fever onset, and not early after fever onset or 2 weeks later. This might suggest that indeed a prolonged T-cell response, still present 8 weeks after fever onset, is associated with severity of symptoms of influenza virus infection.

To the best of our knowledge, we are the first to investigate the potential effect of CMV infection on a heterologous infection in human in a larger group of individuals. A negative effect of CMV infection on the functioning of the immune system is often intuitively explained by competition between T-cells for 'limited immunological space'. Indeed, CMV infection has a profound impact on the composition of the overall CD8+ T-cell pool, by increasing the number of highly differentiated memory cells, especially in older adults [49]. We show that CMV infection leads to a decrease in the frequency of memory influenza virus-specific T-cells, as measured by GILG-tetramer staining, in healthy older adults. Unfortunately, absolute T-cell numbers could not be investigated in this study, leaving the possibility that the decreased frequency of influenza virus-specific T-cells might only be relative and merely reflecting a relative increase in CMV-specific T-cells in older adults. This might explain why decreased influenza virus-specific T-cell frequencies in healthy older adults would not result in reduced responses in acute influenza virus infection. Alternatively, the influenza-virus specific T-cells are indeed lower, also in number, in CMV+ older adults compared to CMV- older adults, but can still probably be boosted by a influenza virus infection. A similar effect has previously been reported for EBV-specific T-cells [31]. Relatively low frequencies of influenza virus-specific T-cell in older adults compared to younger adults have been reported before [50, 51] and are considered to be a key determinant of a diminished T-cell response in influenza virus infection [52]. The T-cell response to influenza virus infection in the context of CMV infection was to the best of our knowledge not investigated before in humans. During influenza virus infection, we observed that influenza virus-specific T-cells in CMV+ and CMV- individuals respond equally well. Previously, other studies reported impaired influenza virus-specific T-cell responses to influenza vaccination [17, 53, 54]. In contrast, other groups showed a positive effect of CMV on the vaccine-induced influenza virus-specific T-cell response in humans [55]. In addition, in mice, MCMV infection was shown to enhance the diversity of the T-cell repertoire against an unrelated acute infection [56]. We also showed that CMV T-cell responses were positively associated with the magnitude of the T-cell response 2 weeks and 8 weeks after fever onset of influenza virus infection. Together, this suggests that CMV+ individuals have a sufficient amount of influenza virus-specific T-cells responding to influenza virus infection.

As CMV infection was suggested to induce a more inflammatory environment [57], we also studied the potential association between CMV and inflammatory markers and how this might be related to influenza virus specific T-cell responses. Although the levels of pro-inflammatory cytokines and chemokines measured here were significantly increased in the acute phase of influenza virus infection (<72 hours after fever onset), they were similar between CMV+ and

CMV- individuals. Furthermore, these levels did not seem to be associated with the influenza virus specific T-cell response at any time point. Of course, we cannot rule out the possibility that other immune modulatory factors may be influenced by CMV infection and may act on the influenza-specific T-cell response.

Most studies claiming a negative effect of CMV on a immune response to a heterologous challenge have been performed in mice infected with murine CMV (MCMV). Several mouse studies have shown that only lifelong infection with MCMV leads to decreased immunity against heterologous infections [58-60]. The magnitude of the effect of CMV infection might thus be linked to the duration of CMV infection and the experienced amount of viral reactivation in an infected host. Here, we found no evidence for decreased immunity against a heterologous infection in humans infected with CMV. Also, high CMV-specific antibody levels were not associated with the height of the influenza virus-specific T-cell response, as was previously suggested for influenza antibody vaccine responses [37, 61]. One of the reasons for the observed differences between mice and men, could be the order of infections by CMV and influenza. In mouse studies, mice are typically first infected with MCMV long before they are challenged with a heterologous acute infection. Many humans, in contrast, may have undergone their first influenza virus infection before they were infected with CMV, which may lead to the presence of influenza-specific memory T-cells before the CMV-specific immune response is established. A potential harmful effect of CMV might be less pronounced in a host who already has a proper immune response against influenza. Furthermore, as mouse models of CMV are almost exclusively done in specific pathogen free mice, and humans are exposed to dozens of infections and triggers during life, it might be that the small effect of CMV is magnified in mice. Even if CMV can modulate other immune responses, there is no substantial evidence that CMV impacts the function of the immune system by hampering immune responses against heterologous infections in humans.

The increased CD8+ T-cell response to influenza virus infection that we observed in CMV+ older individuals remains partially unexplained. Previously, enhanced influenza vaccine responses in humans and mice were explained by an increase in IFNγ in serum [1]. As we did not observe a difference in IFNγ levels in serum between CMV+ and CMV- individuals, this could not explain the small positive association with CMV infection in our study. We cannot exclude the possibility that CMV infection may only affect CD4+ T-cells, as this effect was observed in IFNγ ELIspot assays, in which CD4+ T-cells may contribute as well. Another explanation for the difference in the early influenza virus-specific T-cell response between CMV- and CMV+ individuals could lie in the migratory capacity of the responding T-cells. We observed a trend towards enhanced CXCR3 expression of influenza-virus specific CD8+ T-cells in CMV- individuals, which may lead to early migration towards tissues such as the lung. Since the presence and function of tissue-resident T-cells are crucial in respiratory diseases such as influenza, the effect of CMV on influenza virus-specific T-cell responses at the site of infection instead of the blood would be of great interest, and requires further research.

In conclusion, identification of the driving forces that induce age-related changes in the immune system is important to protect the growing population of older adults against infectious diseases. Especially influenza leads to more increased disease burden in older adults, e.g. severe symptoms and higher risk of complications, hospitalization and mortality. Our study shows that despite the lower frequency of influenza virus-specific memory T-cell responses in older adults, CMV infection does not seem to impair the T-cell response against acute influenza virus infection. This work supports the view that CMV acts as an immune modulatory mediator, rather than having a negative impact on the immune system.

REFERENCES

1. Furman, D., et al., *Cytomegalovirus infection enhances the immune response to influenza.* Sci Transl Med, 2015. 7(281): p. 281ra43.
2. Goronzy, J.J., et al., *Value of immunological markers in predicting responsiveness to influenza vaccination in elderly individuals.* J Virol, 2001. 75(24): p. 12182-7.
3. Almanzar, G., et al., *Long-term cytomegalovirus infection leads to significant changes in the composition of the CD8+ T-cell repertoire, which may be the basis for an imbalance in the cytokine production profile in elderly persons.* J Virol, 2005. 79(6): p. 3675-83.
4. Chidrawar, S., et al., *Cytomegalovirus-seropositivity has a profound influence on the magnitude of major lymphoid subsets within healthy individuals.* Clin Exp Immunol, 2009. 155(3): p. 423-32.
5. Hadrup, S.R., et al., *Longitudinal Studies of Clonally Expanded CD8 T-cells Reveal a Repertoire Shrinkage Predicting Mortality and an Increased Number of Dysfunctional Cytomegalovirus-Specific T-cells in the Very Elderly.* The Journal of Immunology, 2006. 176(4): p. 2645-2653.
6. Nikolich-Zugich, J., *The twilight of immunity: emerging concepts in aging of the immune system.* Nat Immunol, 2018. 19(1): p. 10-19.
7. Looney, R.J.F., A, Campbell, D. Torres, A. Kolassa, J. Brower, C. McCann, R. Menegus, M. McCormick, K. Frampton, M. Hall, W, Abraham, G.N. , *Role of Cytomegalovirus in the T-cell Changes Seen in Elderly Individuals.* Clinical Immunology, 1999. 90(2): p. 213–219.
8. Koch, S., et al., *Cytomegalovirus infection: a driving force in human T-cell immunosenescence.* Ann N Y Acad Sci, 2007. 1114: p. 23-35.
9. Olsson, J.W., A. Johansson, B. Lofgren, S. Nilsson, B. Ferguson, F.G. , *Age-related change in peripheral blood T-lymphocyte subpopulations and cytomegalovirus infection in the very old: the Swedish longitudinal OCTO immune study.* Mechanisms of Ageing and Development, 2000. 121: p. 187–201.
10. Derhovanessian, E., A. Larbi, and G. Pawelec, *Biomarkers of human immunosenescence: impact of Cytomegalovirus infection.* Curr Opin Immunol, 2009. 21(4): p. 440-5.
11. Brodin, P., et al., *Variation in the human immune system is largely driven by non-heritable influences.* Cell, 2015. 160(1-2): p. 37-47.
12. Aiello, A.E., Y.L. Chiu, and D. Frasca, *How does cytomegalovirus factor into diseases of aging and vaccine responses, and by what mechanisms?* Geroscience, 2017. 39(3): p. 261-271.
13. Tu, W. and S. Rao, *Mechanisms Underlying T-cell Immunosenescence: Aging and Cytomegalovirus Infection.* Front Microbiol, 2016. 7: p. 2111.
14. McElhaney, J.E., et al., *T-Cell Immunity to Influenza in Older Adults: A Pathophysiological Framework for Development of More Effective Vaccines.* Front Immunol, 2016. 7: p. 41.
15. Reber, A.J.C., T. Kim, J.H. Cao, W. Biber, R. Shay, D.K. and Sambhara, S.*, *Immunosenescence and Challenges of Vaccination against Influenza in the aging population.* Aging and Disease, 2011.
16. Wald, A., et al., *Impact of human cytomegalovirus (CMV) infection on immune response to pandemic 2009 H1N1 influenza vaccine in healthy adults.* J Med Virol, 2013. 85(9): p. 1557-60.
17. Merani, S., et al., *Influenza vaccine-mediated protection in older adults: Impact of influenza infection, cytomegalovirus serostatus and vaccine dosage.* Exp Gerontol, 2018. 107: p. 116-125.
18. Frasca, D., et al., *Cytomegalovirus (CMV) seropositivity decreases B cell responses to the influenza vaccine.* Vaccine, 2015. 33(12): p. 1433-9.
19. den Elzen, W.P., et al., *Cytomegalovirus infection and responsiveness to influenza vaccination in elderly residents of long-term care facilities.* Vaccine, 2011. 29(29-30): p. 4869-74.

20. van den Berg, S.P.H., et al., *Negative Effect of Age, but Not of Latent Cytomegalovirus Infection on the Antibody Response to a Novel Influenza Vaccine Strain in Healthy Adults.* Front Immunol, 2018. 9: p. 82.

21. van den Berg, S.P.H., et al., *Effect of latent cytomegalovirus infection on the antibody response to influenza vaccination: a systematic review and meta-analysis.* Med Microbiol Immunol, 2019. 208(3-4): p. 305-321.

22. Wilkinson, T.M., et al., *Preexisting influenza-specific CD4+ T-cells correlate with disease protection against influenza challenge in humans.* Nat Med, 2012. 18(2): p. 274-80.

23. Sridhar, S., et al., *Cellular immune correlates of protection against symptomatic pandemic influenza.* Nat Med, 2013. 19(10): p. 1305-12.

24. Wang, Z., et al., *Recovery from severe H7N9 disease is associated with diverse response mechanisms dominated by CD8(+) T-cells.* Nat Commun, 2015. 6: p. 6833.

25. Grant, E.J., et al., *Human influenza viruses and CD8(+) T-cell responses.* Curr Opin Virol, 2016. 16: p. 132-142.

26. Kreijtz, J.H., R.A. Fouchier, and G.F. Rimmelzwaan, *Immune responses to influenza virus infection.* Virus Res, 2011. 162(1-2): p. 19-30.

27. Koutsakos, M., et al., *Human CD8(+) T-cell cross-reactivity across influenza A, B and C viruses.* Nat Immunol, 2019. 20(5): p. 613-625.

28. Wang, Z., et al., *Clonally diverse CD38(+)HLA-DR(+)CD8(+) T-cells persist during fatal H7N9 disease.* Nat Commun, 2018. 9(1): p. 824.

29. Jansen, J.M., et al., *Influenza virus-specific CD4+ and CD8+ T-cell-mediated immunity induced by infection and vaccination.* J Clin Virol, 2019. 119: p. 44-52.

30. Khan, N., et al., *Cytomegalovirus Seropositivity Drives the CD8 T-cell Repertoire Toward Greater Clonality in Healthy Elderly Individuals.* The Journal of Immunology, 2002. 169(4): p. 1984-1992.

31. Khan, N., et al., *Herpesvirus-specific CD8 T-cell immunity in old age: cytomegalovirus impairs the response to a coresident EBV infection.* J Immunol, 2004. 173(12): p. 7481-9.

32. van de Berg, P.J., et al., *A fingerprint left by cytomegalovirus infection in the human T-cell compartment.* J Clin Virol, 2008. 41(3): p. 213-7.

33. Pawelec, G.A., A. Caruso, C. Grubeck-Loebenstein, B. Solana, R. Wikby, A. , *Human immunosenescence: is it infectious?* Immunological Reviews, 2005. 205: p. 257–268.

34. Franceschi, C. and J. Campisi, *Chronic inflammation (inflammaging) and its potential contribution to age-associated diseases.* J Gerontol A Biol Sci Med Sci, 2014. 69 Suppl 1: p. S4-9.

35. Jenkins, C., et al., *Immunomodulatory properties of a viral homolog of human interleukin-10 expressed by human cytomegalovirus during the latent phase of infection.* J Virol, 2008. 82(7): p. 3736-50.

36. Pawelec, G., D. Goldeck, and E. Derhovanessian, *Inflammation, ageing and chronic disease.* Curr Opin Immunol, 2014. 29: p. 23-8.

37. Trzonkowski, P., et al., *Association between cytomegalovirus infection, enhanced proinflammatory response and low level of anti-hemagglutinins during the anti-influenza vaccination—an impact of immunosenescence.* Vaccine, 2003. 21(25-26): p. 3826-3836.

38. Prosch, S.S., K. Stein, J. Liebenthal, C. Stamminger, T. Volk, H. Kurger, D.V. , *Stimulation of the Human Cytomegalovirus IE Enhancer/Promotor in HL-60 Cells by TNFa Is mediated via Induction of NF-kb.* Virology, 1995. 208: p. 197-206.

39. McElhaney, J.E., et al., *The unmet need in the elderly: how immunosenescence, CMV infection, co-morbidities and frailty are a challenge for the development of more effective influenza vaccines.* Vaccine, 2012. 30(12): p. 2060-7.

40. McElhaney, J.E., et al., *Granzyme B: Correlates with protection and enhanced CTL response to influenza vaccination in older adults.* Vaccine, 2009. 27(18): p. 2418-25.

41. McElhaney, J.E., et al., *T-cell responses are better correlates of vaccine protection in the elderly.* J Immunol, 2006. 176(10): p. 6333-9.

42. Rosendahl Huber, S.K., et al., *Immunogenicity of Influenza Vaccines: Evidence for Differential Effect of Secondary Vaccination on Humoral and Cellular Immunity.* Front Immunol, 2018. 9: p. 3103.

43. Pel, J.Z.S., *Proefonderzoek naar de frequentie en de aetiologie van griepachtige ziekten in de winter 1963-1964.* Huisarts en Wetenschap 1965. 86: p. 321.

44. van Beek, J., et al., *Influenza-like Illness Incidence Is Not Reduced by Influenza Vaccination in a Cohort of Older Adults, Despite Effectively Reducing Laboratory-Confirmed Influenza Virus Infections.* J Infect Dis, 2017. 216(4): p. 415-424.

45. Tcherniaeva, I., et al., *The development of a bead-based multiplex immunoassay for the detection of IgG antibodies to CMV and EBV.* J Immunol Methods, 2018. 462: p. 1-8.

46. Kotecha, N., P.O. Krutzik, and J.M. Irish, *Web-based analysis and publication of flow cytometry experiments.* Curr Protoc Cytom, 2010. Chapter 10: p. Unit10 17.

47. Derhovanessian, E., et al., *Infection with cytomegalovirus but not herpes simplex virus induces the accumulation of late-differentiated CD4+ and CD8+ T-cells in humans.* J Gen Virol, 2011. 92(Pt 12): p. 2746-56.

48. Skowronski, D.M., et al., *Immuno-epidemiologic correlates of pandemic H1N1 surveillance observations: higher antibody and lower cell-mediated immune responses with advanced age.* J Infect Dis, 2011. 203(2): p. 158-67.

49. Wertheimer, A.M., et al., *Aging and cytomegalovirus infection differentially and jointly affect distinct circulating T-cell subsets in humans.* J Immunol, 2014. 192(5): p. 2143-55.

50. Deng, Y., et al., *Age-related impaired type 1 T-cell responses to influenza: reduced activation ex vivo, decreased expansion in CTL culture in vitro, and blunted response to influenza vaccination in vivo in the elderly.* J Immunol, 2004. 172(6): p. 3437-46.

51. Nguyen, T.H.O., et al., *Perturbed CD8+ T-cell immunity across universal influenza epitopes in the elderly.* J Leukoc Biol, 2017.

52. Murasko, D.M.B., E.D. Gardner, E.M. Gross, P. Munk, G. Dran, S. Abrutyn, E. , *Role of humoral and cell-mediated immunity in protection from in¯uenza disease after immunization of healthy elderly.* Experimental Gerontology, 2002. 37: p. 427-439.

53. Derhovanessian, E., et al., *Latent infection with cytomegalovirus is associated with poor memory CD4 responses to influenza A core proteins in the elderly.* J Immunol, 2014. 193(7): p. 3624-31.

54. Merani, S., et al., *Impact of Aging and Cytomegalovirus on Immunological Response to Influenza Vaccination and Infection.* Front Immunol, 2017. 8: p. 784.

55. Theeten, H., et al., *Cellular Interferon Gamma and Granzyme B Responses to Cytomegalovirus-pp65 and Influenza N1 Are Positively Associated in Elderly.* Viral Immunol, 2016. 29(3): p. 169-75.

56. Smithey, M.J., et al., *Lifelong CMV infection improves immune defense in old mice by broadening the mobilized TCR repertoire against third-party infection.* Proc Natl Acad Sci U S A, 2018. 115(29): p. E6817-E6825.

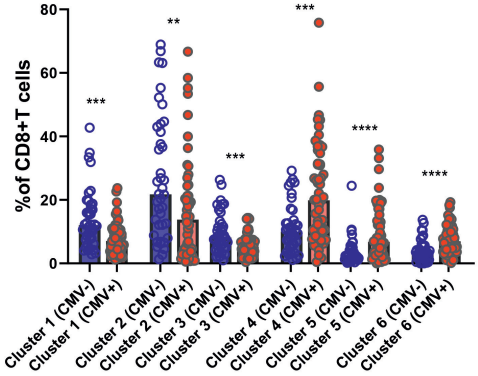
57. Tavenier, J., J.B. Margolick, and S.X. Leng, *T-cell immunity against cytomegalovirus in HIV infection and aging: relationships with inflammation, immune activation, and frailty.* Med Microbiol Immunol, 2019. 208(3-4): p. 289-294.

58. Redeker, A., et al., *The Contribution of Cytomegalovirus Infection to Immune Senescence Is Set by the Infectious Dose.* Front Immunol, 2017. 8: p. 1953.

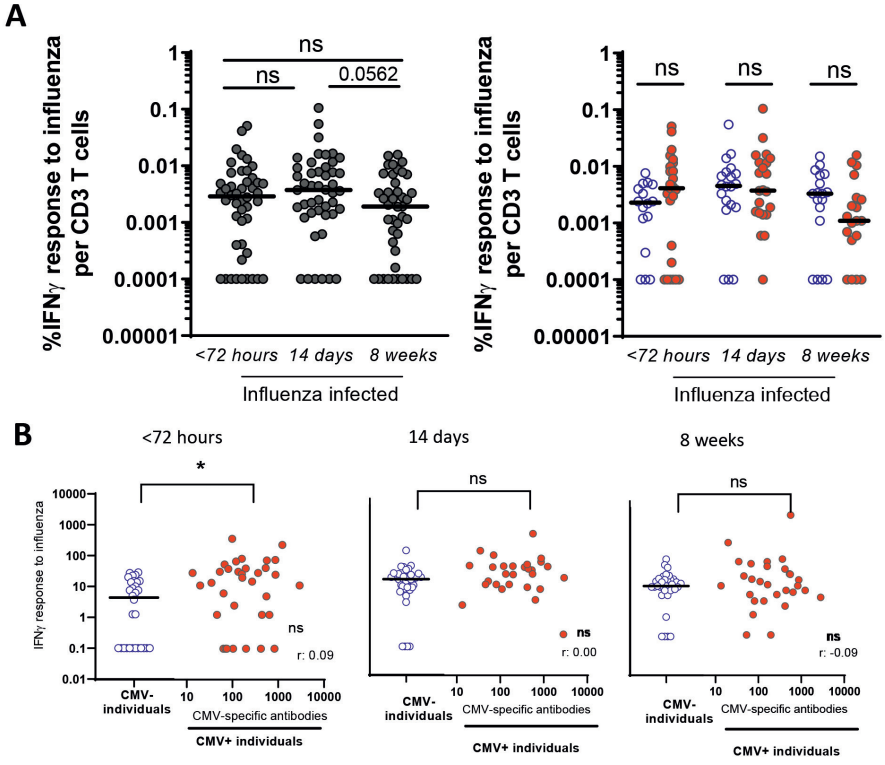
59. Mekker, A., et al., *Immune senescence: relative contributions of age and cytomegalovirus infection.* PLoS Pathog, 2012. 8(8): p. e1002850.

60. Cicin-Sain, L., et al., *Cytomegalovirus infection impairs immune responses and accentuates T-cell pool changes observed in mice with aging.* PLoS Pathog, 2012. 8(8): p. e1002849.

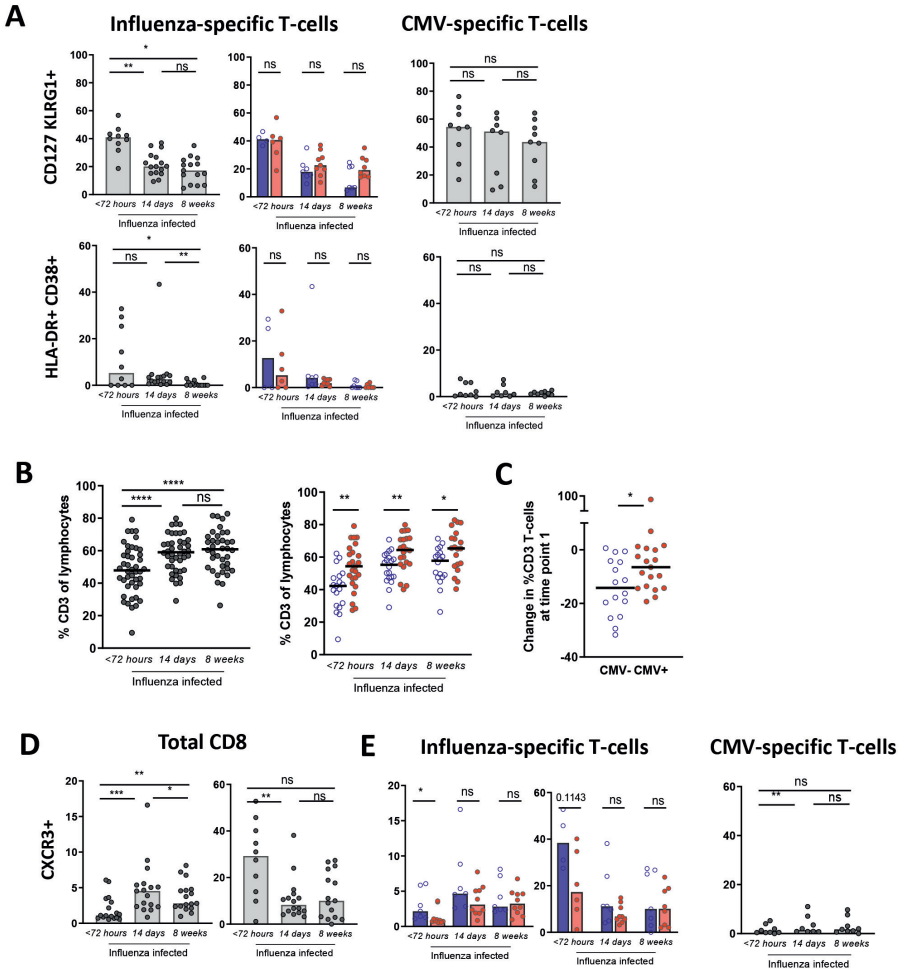
61. Alonso Arias, R., et al., *Intensity of the humoral response to cytomegalovirus is associated with the phenotypic and functional status of the immune system.* J Virol, 2013. 87(8): p. 4486-95.



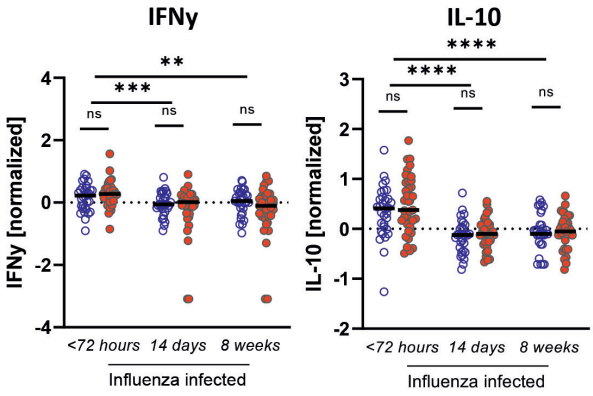
Supplementary Figure 1. Percentage of CD8+ T-cells per t-SNE cluster in CMV- and CMV+ individuals. Percentage of CD8⁺ T-cells per t-SNE cluster in CMV- (blue bars; N=40) and CMV+ (red bars; N=57) individuals based on MFI of CD57, KLRG-1, CXCR3, CD95, CD127, CD45RO, CD27 and CCR7. Data shown as median. Differences between groups were compared by Mann Whitney. Stars indicate statistical differences.



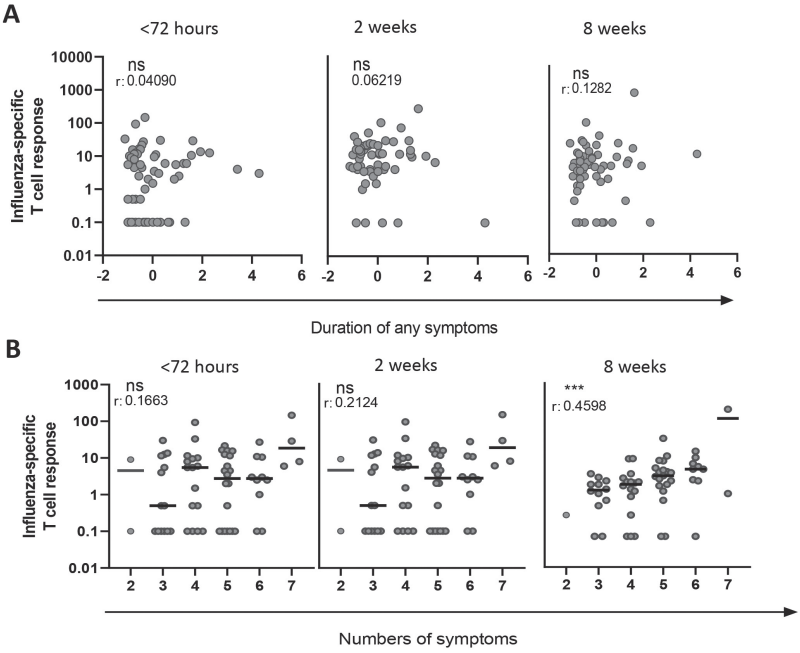
Supplementary Figure 2. Influenza virus-specific IFNγ T-cell response in CMV- and CMV+ individuals differentiated on the level of CMV-specific antibodies. A) All influenza virus-specific IFNγ response at <72 hours, 2 weeks and 8 weeks after fever onset, depicted as percentage of total CD3 T-cells (left panel), and compared between CMV- and CMV+ individuals (right panel). **B)** Correlation between CMV-specific antibody levels and influenza virus-specific T-cell responses in CMV+ individuals on the three time points after fever onset. Influenza-specific IFNγ T-cell responses are depicted in blue open circles in scatter plot for CMV- individuals and in red solid circles in correlation of CMV-specific IFNγ T-cell responses for CMV+ individuals. Correlation was assessed by Spearman correlation.



Supplementary Figure 3. Expression of activation and migration markers in T-cells during influenza virus infection. A) The percentage of CD127-KLRG-1+ and HLA-DR+CD38+ influenza virus-specific CD8⁺ T-cells upon influenza infection in all HLA-A2 positive individuals upon influenza infection (left panels), or comparing CMV-(blue bars) and CMV+ (red bars) (middle panels). Right panels shows CD127-KLRG-1+ and HLA-DR+CD38+ expression on CMV-specific CD8⁺ T-cells. Influenza virus-specific CD8+ T-cells were identified using a tetramer for matrix protein-1 GILG-epitope. CMV-specific CD8+ T-cells were identified using a tetramer for pp65 protein NLV-epitope. **B)** Frequencies of CD3⁺ T-cells in the blood of influenza virus-infected individuals at <72 hours, 2 weeks and 8 weeks after infection (left panel) and in CMV- and CMV+ individuals (right panel). **C)** The change in percentage of CD3⁺ T-cells at time point 1 compared to time point 2 in CMV- and CMV+ individuals. **D)** Percentage of CXCR3+ within total CD8⁺ T-cells upon infection (left panel) and in CMV- and CMV+ individuals (right panel). **E)** CXCR3+ influenza-virus specific CD8+ T-cells upon infection (left panel) and CMV- and CMV+ individuals (middle panel) and on CMV-specific CD8+ T-cells (right panel). Wilcoxon test was used to compare time points. Differences between CMV- and CMV+ individuals were tested using Mann-Whitney U test.



Supplementary Figure 4. Serum levels of IFNγ and IL-10 in CMV- and CMV+ individuals after influenza virus-infection. Serum levels of IFNγ and IL-10 upon influenza infection for CMV+ and CMV- individuals at <72hours after fever onset, and 2 and 8 weeks later. CMV- individuals are indicated with the blue open circles and the CMV+ individuals in red solid circles. Serum levels of the cytokines were measured by multiplex assays and batch effects were normalized based by subtracting the mean per plate. Differences between CMV- and CMV+ individuals were tested using unpaired T-test. Differences between time points were tested by Wilcoxon test.



Supplementary Figure 5. Duration of symptoms is not linked to T-cell responses to influenza virus-infection. A) Association was tested for influenza-specific T-cell responses upon influenza virus infection at <72 hours after fever onset, and 2 and 8 weeks later with the duration of symptoms of influenza infection. **B)** Association was tested for influenza-specific T-cell responses upon influenza virus infection at <72 hours after fever onset, and 2 and 8 weeks later with the number of symptoms. Due to study design, participants had a minimal of two symptoms; fever (≥37.8 °C) and at least 1 other symptoms, either cough, sore throat, runny nose, headache, pain while breathing or muscle pain. Associations was tested by Spearman correlation.

5

The hallmarks of CMV-specific CD8$^+$ T-cell differentiation

2019 Medical Microbiology and Immunology, doi: 10.1007/s00430-019-00608-7

S. P. H. van den Berg[1,2*], I. N. Pardieck[3*], J. Lanfermeijer[1,2], D. Sauce[4], P. Klenerman[5,6], D. van Baarle[1,2*], R. Arens[3*]

*These authors contributed equally to the work

1 Center for Infectious Disease Control, National Institute for Public Health and the Environment, Bilthoven, Netherlands
2 Laboratory of Translational Immunology, Department of Immunology, University Medical Center Utrecht, Utrecht University, Utrecht, Netherlands
3 Department of Immunohematology and Blood Transfusion, Leiden University Medical Center, Leiden, Netherlands
4 Sorbonne Université, INSERM, Centre d'Immunologie et des Maladies Infectieuses (CIMI-Paris), Paris, France
5 Nuffield Department of Medicine, Peter Medawar Building for Pathogen Research, University of Oxford, Oxford, United Kingdom
6 NIHR Biomedical Research Centre, Oxford, John Radcliffe Hospital, Oxford United Kingdom

ABSTRACT

Upon cytomegalovirus (CMV) infection large T-cell responses are elicited that remain high or even increase over time, a phenomenon named memory T-cell inflation. Besides, the maintained robust T-cell response, CMV-specific T-cells seem to have a distinctive phenotype, characterized by an advanced differentiation state. Here, we will review this "special" differentiation status by discussing the cellular phenotype based on the expression of CD45 isoforms, costimulatory, inhibitory and natural killer receptors, adhesion and lymphocyte homing molecules, transcription factors, cytokines and cytotoxic molecules. In addition, we focus on whether the differentiation state of CMV-specific CD8 T-cells is unique in comparison with other chronic viruses and we will discuss the possible impact of factors such as antigen exposure and aging on the advanced differentiation status of CMV-specific CD8 T-cells.

INTRODUCTION

Human cytomegalovirus (HCMV), a beta-herpesvirus family member, infects around 60% of the worldwide population [1]. In healthy individuals, HCMV establishes a persistent latent infection with episodes of reactivation. Although HCMV infection is usually asymptomatic, in immunocompromised (e.g. HCMV-seronegative recipients receiving organs of HCMV-positive donors) and immune immature individuals (neonates), HCMV can cause serious disease [2].

A remarkable feature of HCMV infection is the capacity to elicit large T-cell responses that do not follow the typical contraction pattern after primary infection. Instead, the percentages of CMV-specific T-cells remain high or even increase over time [3], a phenomenon named memory T-cell inflation [4, 5]. In the western world, frequencies around 10% of HCMV-specific T-cells of the total memory T-cell pool are commonly observed (with outliers >50%), and this is found in both healthy and immunocompromised individuals [6, 7]. In elderly, the frequency of circulating HCMV-specific T-cells is higher than in younger adults, and the reactivity of these cells can be restricted to a limited number of epitopes [8-11]. The increase in frequency of HCMV-specific CD8 T-cells with age is also observed in studies with immunocompromised individuals and is similar to frequencies found in healthy donors [12].

Besides the sustained large T-cell response, the phenotype of CMV-specific T-cells seems to be characteristic as well, typified by an advanced differentiation state. Here, we discuss the particulars of this "special" differentiation phenotype and asked the question whether the differentiation state of CMV-specific CD8 T-cells is unique. In addition, we discuss the potential impact of antigen exposure and aging on the differentiation status of CMV-specific CD8 T-cells.

THE DIFFERENTIATION PHENOTYPE OF CMV-SPECIFIC CD8 T-CELLS

CD45 isoforms

Isoforms of the protein tyrosine phosphatase CD45 are expressed at various levels on hematopoietic cell lineages. The high molecular weight isoform CD45RA is expressed by naïve T-cells while the low molecular weight isoform CD45RO is expressed on activated and memory T-cells and is implicated in increasing the sensitivity of TCR signalling [13]. Advanced differentiation of T-cells is however characterized by a lack of CD45RO while CD45RA is re-expressed. A large proportion of the HCMV-specific T-cells have the latter phenotype (in combination with downregulation of costimulatory molecules also called TEMRA), and this seems quite unique for HCMV [14]. For example, Epstein-Barr virus (EBV)-specific CD8 T-cells are predominantly CD45RO positive [15] and human immunodeficiency virus (HIV)-specific T-cells express lower levels of CD45RA [16].

Costimulatory and inhibitory receptors

The advanced differentiation state of CMV-specific T-cells is also marked by the lack of expression of the costimulatory receptors CD27 and CD28, which are otherwise constitutively expressed on naïve T-cells [17]. This is in contrast to other virus-specific CD8 T-cells. For example, EBV and hepatitis C virus (HCV)-specific T-cells more often display expression of CD27 and CD28, and HIV-specific CD8 T-cells, despite advanced loss of CD28, still express CD27 [17], although this may also depend on the disease state [18].

Acute HCMV infections frequently occur in CMV-negative transplant recipients receiving a CMV-positive organ. In these individuals, the CMV-specific T-cell response consists of mainly $CD27^{+}$ $CD28^{-}CD45RA^{-}CD45RO^{+}$ memory T-cells shortly after the peak of CMV infection [19]. In time, expression of CD27 is lost and CD45RA is re-expressed on the majority of the cells [20, 21]. The gradual loss of CD27 is also observed in mouse models, and is likely caused by chronic antigenic triggering [22].

In mouse models, the functional role of CD27 and CD28 has been studied in CMV infection and indicated that CD28 costimulation is especially important during primary infection to enhance CMV-specific T-cell expansions while CD27 and its ligand CD70 seem to play an activating role during both the primary and latent phase of infection [22-26]. The costimulatory receptor OX40 is transiently upregulated upon activation, and is important during the latent phase [27].

Programmed cell death 1 (PD-1), cytotoxic T-lymphocyte antigen 4 (CTLA-4), T-cell immunoglobulin domain and mucin domain protein 3 (TIM-3), lymphocyte activation gene 3 (LAG-3) and CD160 are inhibitory receptors associated with the exhaustion phenotype of T-cells [28]. PD-1 was identified to be abundant on chronic lymphocytic choriomeningitis virus (LCMV)-specific T-cells in mice models [29] and was next shown to be upregulated on T-cells in a number of chronic viral infections including HIV [30, 31], hepatitis B virus (HBV) [32] or HCV [33]. In addition, PD-1 and other inhibitory molecules are abundantly found on T-cells in the tumor-microenvironment and this aspect forms the basis for reinforcing exhausted T-cells by blocking these inhibitory molecules [34]. Indeed, as demonstrated by variants of LCMV eliciting either acute or chronic infection, the induction of the exhausted phenotype is caused by strong chronic antigenic triggering [35], and is elevated by the lack of CD4 T-cell help [29, 36].

Interestingly, during the latent phase, circulating CMV-specific T-cells express relatively low levels of inhibitory receptors [37, 38]. PD-1 expression on CMV-specific T-cells is lower compared to chronic virus-specific T-cells against HBV [32], HIV [30, 38-40] and EBV-specific T-cells [37, 38]. Likewise, also TIM-3, CD160 and 2B4 are expressed at lower levels in CMV-specific T-cells compared to HIV-specific T-cells [40]. Nonetheless, the inter-individual variation of PD-1 and 2B4 expression observed for CMV-specific T-cells can be substantial [33, 40]. This heterogeneity of PD-1 expression could reflect different differentiation phenotypes of

virus-specific memory T-cells [38], and this may be independent of their capacity to control viruses. Indeed, PD-1 expression is not associated with functional capacity (e.g. secretion of cytokines and degranulation). In addition, data showed that CD8 T-cells can further up-regulate PD-1 when they are activated [38]. Altogether this suggests that PD-1, expressed on CMV-specific T-cells, is independent from T-cell exhaustion.

Natural killer receptors

Although originally reported as natural killer cell receptors (NKRs), a number of these receptors such as immunoglobulin-like receptors (KIRs, LIRs such as CD85j) and lectin-like receptors (CD94/NKG2, KLRG1) are also expressed on CD8 T-cells [41]. These molecules are likely implicated in the fine-tuning of the anti-viral response. Indeed, primary CMV infection induces an increased expression of both inhibitory and activating NKRs, which remains high during the latent infection phase while viral load is undetectable [42]. Yet, the precise role of different NKRs remains to be determined.

Similarity between CMV-specific T-cells and other chronic viruses is found in the expression patterns for several inhibiting NKRs. CMV-specific T-cells, just like EBV- and HIV-specific T-cells, show substantial expression of CD85j (ILT2/LIR-1) compared to the overall T-cell pool [43-45]. This CD85j expression is most abundant in TEMRAs and $CD28^-$ CD8 T-cells [44], suggesting an advanced differentiation phenotype. In addition, the overwhelming majority of CMV, EBV and HIV-specific T-cells express KLRG1, often together with loss of expression of CD28 and CCR7, indicating that these cells have undergone multiple cell divisions but are still active in cytokine production [46, 47]. Also, expression of NKG2A is increased on CMV-specific CD8 T-cells [42, 48]. However, KIRs do not seem upregulated on CMV-specific T-cells and/or HIV-specific T-cells: only small fractions express CD158 variants or NKB-1 (KIR3DL1) [43, 49]. Although increased expression of NKG2C on CD8 T-cells is associated with CMV-seropositivity [42] and CMV-reactive T-cells upon restimulation show NKG2C expression [44], CMV-specific T-cells stained with MHC class I tetramers do not seem to express NKG2C [42, 50]. Overall, CD8 T-cells specific for CMV show low expression of KIRs and NKG2C and increased expression of CD85j, NKG2A and KLRG1 during the latent phase of the infection.

CMV-specific CD8 T-cells also express the NKRs CD56 and CD57. $CD56^+$ CD8 T-cells are known for their natural-killer like cytotoxity [51], and CD56 is shown on CMV-specific T-cells in renal transplant patients [52] and healthy individuals (unpublished observations, S. van den Berg and D. van Baarle). CD57 expression represents a cellular phenotype associated with poor proliferative capacity but high cytotoxic potential [53]. On CMV-specific T-cells, CD57 expression, often co-expressed with CD85j, increases with age, but a large variation in expression exist [44, 54]. CD57 expression on CMV, EBV, and HIV-specific CD8 T-cells was low to moderate in adults [32, 46], whereas others reported overall a high expression on these virus-specific T-cells of CD57 in older subjects [55]. In the latter, CMV-specific T-cells seem to express CD57 at higher levels than EBV and HIV-specific T-cells, albeit not substantially.

Adhesion molecules and lymphocyte homing

CMV-specific CD8 T-cells are largely negative for CCR7 and CD62L [16, 17, 49], which are homing receptors for lymphoid organs. This property, which is shared with T-cells specific for other chronic viruses, allows the cells to circulate throughout the body, and reside in peripheral tissue, spleen and blood.

CX3CR1, which recognizes fractalkine expressed by endothelial cells, is abundantly expressed by CMV-specific cells [10, 56] during the primary and latent infection, whereas CCR1 and CXCR6 are only present during the acute phase [37]. High and intermediate expression of CX3CR1 seems to be unique for CMV specific CD8 T-cells in both human and mice [37, 56], as the frequency of this chemokine receptor on EBV [57], HBV and HCV-specific T-cells is much lower [58]. CMV-specific CD8 T-cells with intermediate expression of CX3CR1 associates with self-renewal potential, but the role of CX3CR1 seems to be redundant since memory T-cell inflation is unaltered in case of CX3CR1 deficiency [56]. In addition, CXCR3 is commonly expressed on CMV-specific T-cells as well as EBV-specific T-cells [57]. The homing cell adhesion molecule CD44 is uniformly high expressed on all CMV-specific T-cells [48, 59].

Transcription factors, cytokines and cytotoxic molecules

Transcription factors (TFs) are crucial regulators of cellular differentiation and function including the cytotoxic potential and cytokine secretion. For CD8 T-cells, the TFs Eomes and T-bet are particularly useful to determine the functional profile. For example, T-bet^{dim} and Eomes^{high} expression profiles are associated with expression of exhaustion markers as observed in HIV-specific T-cells, whereas many CMV and EBV-specific T-cells exhibit intermediate levels of Eomes and high levels of T-bet [37, 40, 60]. Blimp-1 and the Homolog of Blimp-1 in T-cells (Hobit) are also clearly expressed by CMV-specific CD8 T-cells [61, 62].

Related to the above described TF profile is the high granzyme B and perforin expression in CMV-specific CD8 T-cells [39, 40, 49, 63]. These cells also abundantly produce IFN-γ and TNF after re-stimulation, while IL-2 is produced by only a subset of the inflationary CMV-specific CD8 T-cells [63]. The expression of the above described effector molecules is consistent with the functional non-exhaustion phenotype of CMV-specific T-cells, and underline their functional status and requirement for lifelong protection against viral dissemination [64]. Analysis of transcriptional networks in inflating cells reveals a module of genes strongly driven by T-bet, not seen in T-cell exhaustion [65].

The low IL-2 production may coincide with the reduced expression of IL-2Rβ (CD122/IL-15Rβ) on CMV-specific CD8 T-cells [37, 48, 63, 66]. In addition, virus-specific effector CD8 T-cells activated *in vivo* during primary EBV or CMV infection down-regulate IL-7Rα (CD127) and IL-15Rα (CD215) expression [67]. With time, CMV-specific CD8 T-cells maintain high level of IL-15Rα. This contrasts with the lower expression of IL7Rα on CMV-specific CD8+ T-cells compared to EBV-specific CD8 T-cells [32, 68-70]. Interestingly, IL-7Rα expression was tightly associated with population size in blood [70]. However, this correlation was not

sustained in tonsillar lymphoid tissue where CMV-specific T-cells were less abundant than EBV-specific T-cells despite higher IL-7Rα expression [70].

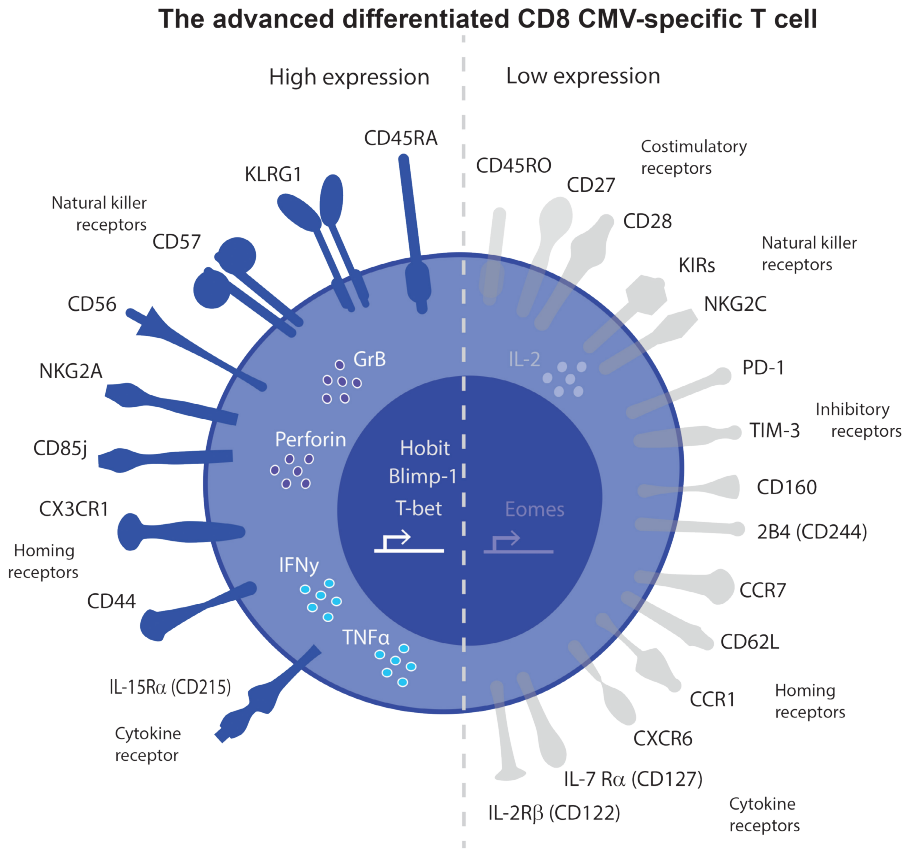


Figure 1. The advanced differentiation phenotype of CMV-specific CD8 T-cells. The advanced differentiated CMV-specific CD8 T-cell are typified by either expression or down-modulation of different surface receptors, cytokines and transcription factors. Surface receptors that are expressed are depicted in blue on the left side of the cell, whereas down-modulated or non-expressed surface receptors are depicted in gray on the right side of the cell. CMV-specific CD8 T-cells express the CD45 isoform CD45RA, different natural killer receptors (CD85j, CD56, CD57, NKG2A and KLRG1), IL-15Rα and the homing receptors CX3CR1 and CD44. These cells do not express or lowly express CD45RO, costimulatory receptors CD27 and CD28, natural killer receptors (KIRs and NKG2C), inhibitory receptors (PD-1, TIM-3, CD160 and 2B4), homing receptors (CXCR6, CCR1, CD62L and CCR7) and cytokine receptors IL-2Rβ (CD122) and IL-7Rα (CD127). CMV-specific CD8 T-cells have intermediate expression of the transcription factor Eomes and strong expression of transcription factors Hobit, Blimp-1 and T-bet. Related to this transcription profile is the high expression of cytokines IFN-γ and TNF-α and the cytotoxic molecules granzyme B (GrB) and perforin. In general, IL-2 production by CMV-specific CD8 T-cells is low.

IS THE ADVANCED DIFFERENTIATED T-CELL PHENOTYPE UNIQUE?

The above described advanced differentiated CD8 T-cell phenotype is clearly observed for CMV-specific T-cells, and could be considered as a distinct type of effector-memory (EM) T-cells. The phenotype involves expression of inhibitory molecules such as KLRG1, CD57

and CD56, yet the cells are nevertheless functional with respect to cytokine production and cytotoxicity (**Figure 1**). However, the advanced differentiated phenotype is not entirely exclusive as also other viruses can elicit CD8 T-cells with a similar differentiation status. Less attention is given to this since either only a small subset among the total memory pool has this phenotype (e.g. upon infection with EBV or HIV) or the frequencies of the late-stage differentiated CD8 T-cells is generally lower compared to these frequencies in CMV infection (e.g. infection with herpes simplex virus-1 (HSV-1) [71] and parvoviruses B19 and PARV4 [72]. One clear feature is that high doses of adenovirus-based vaccine vectors can actually induce a comparable phenotype (and transcriptome) to CMV [56], which is also accompanied with a high frequency of the cells, which makes this a vaccine platform with great potential. As variation exists in the differentiation state of CMV-specific T-cells between individuals, we will next discuss factors that influence the T-cell differentiation.

ESTABLISHMENT OF THE ADVANCED DIFFERENTIATION PHENOTYPE

The phenotype of the CMV-specific CD8 T-cells is strongly connected with the magnitude of the CMV-specific T-cell response. Cross-sectional human studies show that in both healthy and immunosuppressed individuals a high HCMV-specific T-cell response is associated with a high percentage of advanced differentiated T-cells within the total specific T-cell population [44, 73-75]. Nevertheless, the association between the differentiation state and level of CMV-specific T-cells is shown in experimental mouse models [74, 76]. Low dose inoculums elicit fewer circulating CMV-specific CD8 T-cells, and these cells have a less advanced differentiation phenotype. Accordingly, interference with an established mouse CMV infection by antiviral treatment reduces the frequency of the CMV-specific CD8 T-cell response, and also in this setting the CD8 T-cells acquired a lesser differentiated phenotype compared to CMV-infected mice that are untreated [77].

Differences in the infectious dose of primary CMV infection may be instrumental in causing the large variation of the advanced-stage differentiation status of CMV-specific T-cell that exists between individuals. CMV-specific CD8 T-cells may reach an advanced differentiation phenotype already early after infection, and then maintain this status stably over time. In young individuals and even in children an advanced differentiated CMV-specific T-cells can appear [78-80]. Thus, the (primary) infectious dose might determine the viral setpoint (the initial balance between virus and host after primary infection)[81] and thereby subsequently influencing the level and amount of viral reactivation episodes and consequent antigen triggering of CMV-specific T-cells.

Notably, within the inflationary epitope-specific memory T-cell population not all CMV-specific T-cells acquire the late-stage differentiation. Depending on the viral dose, a significant portion can attain a central-memory (CM) phenotype [76]. These CM-like CD8 T-cells produce more IL-2 and are probably dominantly contributing to T-cell expansion upon re-challenge [82]. Also, within the total pool of CMV-specific T-cells also non-inflationary

T-cells exist directed against a distinct subset of epitopes, which never acquire the EM-like differentiation during the latent phase of infection [63]. In line with this are the observations that the enhanced differentiation state of the HCMV-specific T-cell is observed for different epitopes [32]. A critical aspect for virus-specific T-cells undergoing memory inflation or not, does not depend on the intrinsic property of the peptide epitope but on the context of viral gene expression. CMV epitopes that normally induce non-inflationary CD8 T-cell responses from its native site can induce an inflationary response due to C-terminal localization allowing better peptide processing, also leading to a more advanced differentiated phenotype [83, 84].

Besides the infectious dose, aging impacts also the differentiation status of the CMV-reactive T-cells. In cross-sectional studies it was observed that the number of HCMV-specific T-cells increases over time [6, 47]. And this is accompanied by an increase of HCMV-specific cells that re-express CD45RA [11] and express KLRG1 [47]. Moreover, by using new computational tools it was recently shown that inflationary MCMV-specific T-cells are progressively differentiating in time (based on the markers KLRG1, CD44, CD27 and CD62L), long after the initial infection [74, 85]. In line with these studies is the observation that telomeres of HCMV-specific CD8 T-cells are significantly shorter compared to the corresponding phenotypic subsets of the total CD8 T-cell pool [86]. The shortest telomere lengths were found in old individuals compared to young individuals in all different memory subsets (based on CD27 and CD45RA distinction). Overall, this indicates that with aging CMV-specific cells undergo more proliferation and enhanced differentiation.

Important for the enhanced differentiation after CMV infection is the capacity of CMV to become latent. Essentially, latent genomes can sporadically desilence at certain genetic loci, which lead to gene expression of antigenic peptide-encoding genes without entering the productive cycle [87, 88] This allows intermittent re-exposure of antigen to the virus-specific T-cells, which keeps these cells "tickled" during a lifetime, but avoids continuous strong antigenic stimulation leading eventually to exhaustion as is the case for chronic infections with HIV or certain LCMV strains [89]. The large and gradual expansion of CMV-specific CD8 T-cells with an enhanced differentiation phenotype could be interpreted as a lack of complete control of the virus. The T-cells that show enhanced differentiation thus attempt to retain control over full reactivation of the virus. Accordingly, interference with an established MCMV infection by antiviral treatment reduces the frequency of the CMV-specific CD8 T-cell response, and also in this setting the CD8 T-cells reverted to a lesser differentiated phenotype compared to CMV-infected mice that are untreated [77]. It is generally assumed that the immune evasion strategies of CMV targeting the innate and adaptive immunity are critical for the long-term persistence of the virus [90, 91], but whether some of these strategies are capable of specifically modulating particular phenotypic characteristics of the CMV-specific T-cell is unknown. The need and purpose of the maintenance of high frequencies CMV specific CD8 T-cells that progressively differentiate is thus unclear and may be driven by an ongoing shift in the virus-host equilibrium.

Another important aspect might be the broad tropism of CMV and its systemic spread as localized CMV infection results in lesser inflation and less advanced differentiation [92, 93]. The distinctive tropism of CMV, including the wide variety of targeT-cells, innate immune cells such as myeloid cells as CMV vehicles, and the infrequent expression of immediate early genes leading to abortive reactivation, may thus co-determine the fate of the T-cell response, and such characteristics may be the key differences compared to other chronic viruses that frequently reactivate, like EBV. Finally, the size of the genome of CMV is relatively large (compared to most other viruses), which may contribute to elicit larger T-cell responses and to the likelihood to encompass epitopes inducing inflationary T-cell responses.

CONCLUDING REMARKS

The characteristics of CMV-specific T-cells, i.e. maintenance of high numbers and the late differentiated EM-like phenotype, has been a subject of interest. Although the CMV-specific memory T-cell populations are diverse (in magnitude and phenotype) between individuals, it is evident that a large proportion of these cells are advanced differentiated. This particular phenotype seems to be related to the nature of CMV infection because it is more abundantly found upon CMV infection compared to other chronic viruses. The CMV-specific T-cells are often late-stage differentiated T-cells, have shorter telomeres and express inhibitory molecules such as KLRG1, CD57 and CD85j, yet the cells are nevertheless functional with respect to cytokine production and cytotoxicity [94]. Further studies are needed to unravel this seemingly conflicting feature of CMV-specific T-cells. Large prospective studies in humans could provide further insight, but such studies may still be complicated given the possible impact of MHC heterogeneity in the human population compared to inbred mice [95]. Notably, the data discussed here reflects mainly the differentiation of the circulating CMV-specific T-cells, which represents a subgroup of the total CD8 T-cell pool in the body. Whether a late-differentiated phenotype “uniquely” related to CMV infection is also present in the tissue-resident memory T-cell population remains to be elucidated. Several papers reveal a dual impact of CMV infection and aging on immune subsets [96-100]. Prevalence of CMV infection increases with age [101, 102], suggesting that CMV may take advantage over a senescent immune system. How long term infection of CMV is able to change the virus-host balance leading to gradual higher levels of advanced differentiated T-cells is unknown. Due to aging, immune control may gradually wane leading to more frequent reactivation.

REFERENCES

1. Cannon MJ, Schmid DS, Hyde TB. Review of cytomegalovirus seroprevalence and demographic characteristics associated with infection. Rev Med Virol. 2010;20(4):202-13.
2. Sissons JG, Wills MR. How understanding immunology contributes to managing CMV disease in immunosuppressed patients: now and in future. Medical microbiology and immunology. 2015;204(3):307-16.
3. Holtappels R, Pahl-Seibert MF, Thomas D, Reddehase MJ. Enrichment of immediate-early 1 (m123/pp89) peptide-specific CD8 T-cells in a pulmonary CD62L(lo) memory-effector cell pool during latent murine cytomegalovirus infection of the lungs. J Virol. 2000;74(24):11495-503.
4. Karrer U, Sierro S, Wagner M, Oxenius A, Hengel H, Koszinowski UH, et al. Memory inflation: continuous accumulation of antiviral CD8+ T-cells over time. J Immunol. 2003;170(4):2022-9.
5. O'Hara GA, Welten SP, Klenerman P, Arens R. Memory T-cell inflation: understanding cause and effect. Trends Immunol. 2012;33(2):84-90.
6. Khan N, Hislop A, Gudgeon N, Cobbold M, Khanna R, Nayak L, et al. Herpesvirus-specific CD8 T-cell immunity in old age: cytomegalovirus impairs the response to a coresident EBV infection. J Immunol. 2004;173(12):7481-9.
7. Sylwester AW, Mitchell BL, Edgar JB, Taormina C, Pelte C, Ruchti F, et al. Broadly targeted human cytomegalovirus-specific CD4+ and CD8+ T-cells dominate the memory compartments of exposed subjects. J Exp Med. 2005;202(5):673-85.
8. Komatsu H, Sierro S, Cuero V, Klenerman P. Population analysis of antiviral T-cell responses using MHC class I-peptide tetramers. Clin Exp Immunol. 2003;134(1):9-12.
9. Vescovini R, Biasini C, Fagnoni FF, Telera AR, Zanlari L, Pedrazzoni M, et al. Massive load of functional effector CD4+ and CD8+ T-cells against cytomegalovirus in very old subjects. J Immunol. 2007;179(6):4283-91.
10. Remmerswaal EB, Havenith SH, Idu MM, van Leeuwen EM, van Donselaar KA, ten BA, et al. Human virus-specific effector-type T-cells accumulate in blood but not in lymph nodes. Blood. 2012;119(7):1702-12.
11. Griffiths SJ, Riddell NE, Masters J, Libri V, Henson SM, Wertheimer A, et al. Age-associated increase of low-avidity cytomegalovirus-specific CD8+ T-cells that re-express CD45RA. J Immunol. 2013;190(11):5363-72.
12. Engstrand M, Lidehall AK, Totterman TH, Herrman B, Eriksson BM, Korsgren O. Cellular responses to cytomegalovirus in immunosuppressed patients: circulating CD8+ T-cells recognizing CMVpp65 are present but display functional impairment. Clin Exp Immunol. 2003;132(1):96-104.
13. Leitenberg D, Boutin Y, Lu DD, Bottomly K. Biochemical association of CD45 with the T-cell receptor complex: regulation by CD45 isoform and during T-cell activation. Immunity. 1999;10(6):701-11.
14. Derhovanessian E, Maier AB, Hahnel K, Beck R, de Craen AJ, Slagboom EP, et al. Infection with cytomegalovirus but not herpes simplex virus induces the accumulation of late-differentiated CD4+ and CD8+ T-cells in humans. J Gen Virol. 2011;92(Pt 12):2746-56.
15. Kuijpers TW, Vossen MT, Gent MR, Davin JC, Roos MT, Wertheim-van Dillen PM, et al. Frequencies of circulating cytolytic, CD45RA+CD27-, CD8+ T lymphocytes depend on infection with CMV. J Immunol. 2003;170(8):4342-8.
16. Chen G, Shankar P, Lange C, Valdez H, Skolnik PR, Wu L, et al. CD8 T-cells specific for human immunodeficiency virus, Epstein-Barr virus, and cytomegalovirus lack molecules for homing to lymphoid sites of infection. Blood. 2001;98(1):156-64.

17. Appay V, Dunbar PR, Callan M, Klenerman P, Gillespie GM, Papagno L, et al. Memory CD8+ T-cells vary in differentiation phenotype in different persistent virus infections. Nat Med. 2002;8(4):379-85.

18. van Baarle D, Kostense S, van Oers MH, Hamann D, Miedema F. Failing immune control as a result of impaired CD8+ T-cell maturation: CD27 might provide a clue. Trends Immunol. 2002;23(12):586-91.

19. Wills MR, Okecha G, Weekes MP, Gandhi MK, Sissons PJ, Carmichael AJ. Identification of naive or antigen-experienced human CD8(+) T-cells by expression of costimulation and chemokine receptors: analysis of the human cytomegalovirus-specific CD8(+) T-cell response. J Immunol. 2002;168(11):5455-64.

20. van Leeuwen EM, Koning JJ, Remmerswaal EB, van BD, van Lier RA, ten Berge IJ. Differential usage of cellular niches by cytomegalovirus versus EBV- and influenza virus-specific CD8+ T-cells. J Immunol. 2006;177(8):4998-5005.

21. Pipeling MR, West EE, Osborne CM, Whitlock AB, Dropulic LK, Willett MH, et al. Differential CMV-specific CD8+ effector T-cell responses in the lung allograft predominate over the blood during human primary infection. J Immunol. 2008;181(1):546-56.

22. Welten SP, Redeker A, Franken KL, Benedict CA, Yagita H, Wensveen FM, et al. CD27-CD70 costimulation controls T-cell immunity during acute and persistent cytomegalovirus infection. J Virol. 2013;87(12):6851-65.

23. Arens R, Loewendorf A, Her MJ, Schneider-Ohrum K, Shellam GR, Janssen E, et al. B7-mediated costimulation of CD4 T-cells constrains cytomegalovirus persistence. J Virol. 2011;85(1):390-6.

24. Arens R, Loewendorf A, Redeker A, Sierro S, Boon L, Klenerman P, et al. Differential B7-CD28 costimulatory requirements for stable and inflationary mouse cytomegalovirus-specific memory CD8 T-cell populations. J Immunol. 2011;186(7):3874-81.

25. Welten SP, Melief CJ, Arens R. The distinct role of T-cell costimulation in antiviral immunity. Curr Opin Virol. 2013;3(4):475-82.

26. Welten SP, Redeker A, Franken KL, Oduro JD, Ossendorp F, Cicin-Sain L, et al. The viral context instructs the redundancy of costimulatory pathways in driving CD8(+) T-cell expansion. Elife. 2015;4:e07486.

27. Humphreys IR, Loewendorf A, De TC, Schneider K, Benedict CA, Munks MW, et al. OX40 costimulation promotes persistence of cytomegalovirus-specific CD8 T-cells: A CD4-dependent mechanism. J Immunol. 2007;179(4):2195-202.

28. Blackburn SD, Shin H, Haining WN, Zou T, Workman CJ, Polley A, et al. Coregulation of CD8+ T-cell exhaustion by multiple inhibitory receptors during chronic viral infection. Nat Immunol. 2009;10(1):29-37.

29. Barber DL, Wherry EJ, Masopust D, Zhu B, Allison JP, Sharpe AH, et al. Restoring function in exhausted CD8 T-cells during chronic viral infection. Nature. 2006;439(7077):682-7.

30. Trautmann L, Janbazian L, Chomont N, Said EA, Gimmig S, Bessette B, et al. Upregulation of PD-1 expression on HIV-specific CD8+ T-cells leads to reversible immune dysfunction. Nat Med. 2006;12(10):1198-202.

31. Day CL, Kaufmann DE, Kiepiela P, Brown JA, Moodley ES, Reddy S, et al. PD-1 expression on HIV-specific T-cells is associated with T-cell exhaustion and disease progression. Nature. 2006;443(7109):350-4.

32. He XH, Jia QT, Li FY, Saltis M, Liu Y, Xu LH, et al. CD8(+) T-cells specific for both persistent and non-persistent viruses display distinct differentiation phenotypes but have similar level of PD-1 expression in healthy Chinese individuals. Clin Immunol. 2008;126(2):222-34.

33. Owusu Sekyere S, Suneetha PV, Kraft AR, Zhang S, Dietz J, Sarrazin C, et al. A heterogeneous hierarchy of co-regulatory receptors regulates exhaustion of HCV-specific CD8 T-cells in patients with chronic hepatitis C. J Hepatol. 2015;62(1):31-40.

34. Topalian SL, Drake CG, Pardoll DM. Immune checkpoint blockade: a common denominator approach to cancer therapy. Cancer Cell. 2015;27(4):450-61.

35. Shin H, Blackburn SD, Blattman JN, Wherry EJ. Viral antigen and extensive division maintain virus-specific CD8 T-cells during chronic infection. J Exp Med. 2007;204(4):941-9.

36. Matloubian M, Concepcion RJ, Ahmed R. CD4+ T-cells are required to sustain CD8+ cytotoxic T-cell responses during chronic viral infection. J Virol. 1994;68(12):8056-63.

37. Hertoghs KM, Moerland PD, van SA, Remmerswaal EB, Yong SL, van de Berg PJ, et al. Molecular profiling of cytomegalovirus-induced human CD8+ T-cell differentiation. J Clin Invest. 2010;120(11):4077-90.

38. Sauce D, Almeida JR, Larsen M, Haro L, Autran B, Freeman GJ, et al. PD-1 expression on human CD8 T-cells depends on both state of differentiation and activation status. Aids. 2007;21(15):2005-13.

39. Petrovas C, Casazza JP, Brenchley JM, Price DA, Gostick E, Adams WC, et al. PD-1 is a regulator of virus-specific CD8+ T-cell survival in HIV infection. J Exp Med. 2006;203(10):2281-92.

40. Buggert M, Tauriainen J, Yamamoto T, Frederiksen J, Ivarsson MA, Michaelsson J, et al. T-bet and Eomes are differentially linked to the exhausted phenotype of CD8+ T-cells in HIV infection. PLoS Pathog. 2014;10(7):e1004251.

41. Huard B, Karlsson L. KIR expression on self-reactive CD8+ T-cells is controlled by T-cell receptor engagement. Nature. 2000;403(6767):325-8.

42. van Stijn A, Rowshani AT, Yong SL, Baas F, Roosnek E, ten Berge IJ, et al. Human cytomegalovirus infection induces a rapid and sustained change in the expression of NK cell receptors on CD8+ T-cells. J Immunol. 2008;180(7):4550-60.

43. Anfossi N, Doisne JM, Peyrat MA, Ugolini S, Bonnaud O, Bossy D, et al. Coordinated expression of Ig-like inhibitory MHC class I receptors and acquisition of cytotoxic function in human CD8+ T-cells. J Immunol. 2004;173(12):7223-9.

44. Gustafson CE, Qi Q, Hutter-Saunders J, Gupta S, Jadhav R, Newell E, et al. Immune Checkpoint Function of CD85j in CD8 T-cell Differentiation and Aging. Front Immunol. 2017;8:692.

45. Ince MN, Harnisch B, Xu Z, Lee SK, Lange C, Moretta L, et al. Increased expression of the natural killer cell inhibitory receptor CD85j/ILT2 on antigen-specific effector CD8 T-cells and its impact on CD8 T-cell function. Immunology. 2004;112(4):531-42.

46. Ibegbu CC, Xu YX, Harris W, Maggio D, Miller JD, Kourtis AP. Expression of killer cell lectin-like receptor G1 on antigen-specific human CD8+ T lymphocytes during active, latent, and resolved infection and its relation with CD57. J Immunol. 2005;174(10):6088-94.

47. Ouyang Q, Wagner WM, Voehringer D, Wikby A, Klatt T, Walter S, et al. Age-associated accumulation of CMV-specific CD8+ T-cells expressing the inhibitory killer cell lectin-like receptor G1 (KLRG1). Exp Gerontol. 2003;38(8):911-20.

48. Snyder CM, Cho KS, Bonnett EL, van DS, Shellam GR, Hill AB. Memory inflation during chronic viral infection is maintained by continuous production of short-lived, functional T-cells. Immunity. 2008;29(4):650-9.

49. Gamadia LE, Rentenaar RJ, Baars PA, Remmerswaal EB, Surachno S, Weel JF, et al. Differentiation of cytomegalovirus-specific CD8(+) T-cells in healthy and immunosuppressed virus carriers. Blood. 2001;98(3):754-61.

50. Guma M, Angulo A, Vilches C, Gomez-Lozano N, Malats N, Lopez-Botet M. Imprint of human cytomegalovirus infection on the NK cell receptor repertoire. Blood. 2004;104(12):3664-71.

51. Pievani A, Borleri G, Pende D, Moretta L, Rambaldi A, Golay J, et al. Dual-functional capability of CD3+CD56+ CIK cells, a T-cell subset that acquires NK function and retains TCR-mediated specific cytotoxicity. Blood. 2011;118(12):3301-10.

52. Almehmadi M, Hammad A, Heyworth S, Moberly J, Middleton D, Hopkins MJ, et al. CD56+ T-cells are increased in kidney transplant patients following cytomegalovirus infection. Transpl Infect Dis. 2015;17(4):518-26.

53. Kared H, Martelli S, Ng TP, Pender SL, Larbi A. CD57 in human natural killer cells and T-lymphocytes. Cancer Immunol Immunother. 2016;65(4):441-52.

54. Northfield J, Lucas M, Jones H, Young NT, Klenerman P. Does memory improve with age? CD85j (ILT-2/LIR-1) expression on CD8 T-cells correlates with 'memory inflation' in human cytomegalovirus infection. Immunol Cell Biol. 2005;83(2):182-8.

55. Hoji A, Connolly NC, Buchanan WG, Rinaldo CR, Jr. CD27 and CD57 expression reveals atypical differentiation of human immunodeficiency virus type 1-specific memory CD8+ T-cells. Clin Vaccine Immunol. 2007;14(1):74-80.

56. Gordon CL, Lee LN, Swadling L, Hutchings C, Zinser M, Highton AJ, et al. Induction and Maintenance of CX3CR1-Intermediate Peripheral Memory CD8(+) T-cells by Persistent Viruses and Vaccines. Cell Rep. 2018;23(3):768-82.

57. van de Berg PJ, Yong SL, Remmerswaal EB, van Lier RA, ten Berge IJ. Cytomegalovirus-induced effector T-cells cause endothelial cell damage. Clin Vaccine Immunol. 2012;19(5):772-9.

58. Bottcher JP, Beyer M, Meissner F, Abdullah Z, Sander J, Hochst B, et al. Functional classification of memory CD8(+) T-cells by CX3CR1 expression. Nat Commun. 2015;6:8306.

59. Lang KS, Moris A, Gouttefangeas C, Walter S, Teichgraber V, Miller M, et al. High frequency of human cytomegalovirus (HCMV)-specific CD8+ T-cells detected in a healthy CMV-seropositive donor. Cell Mol Life Sci. 2002;59(6):1076-80.

60. van Aalderen MC, Remmerswaal EB, Verstegen NJ, Hombrink P, ten Brinke A, Pircher H, et al. Infection history determines the differentiation state of human CD8+ T-cells. J Virol. 2015;89(9):5110-23.

61. Vieira Braga FA, Hertoghs KM, Kragten NA, Doody GM, Barnes NA, Remmerswaal EB, et al. Blimp-1 homolog Hobit identifies effector-type lymphocytes in humans. Eur J Immunol. 2015;45(10):2945-58.

62. Kragten NAM, Behr FM, Vieira Braga FA, Remmerswaal EBM, Wesselink TH, Oja AE, et al. Blimp-1 induces and Hobit maintains the cytotoxic mediator granzyme B in CD8 T-cells. Eur J Immunol. 2018;48(10):1644-62.

63. Munks MW, Cho KS, Pinto AK, Sierro S, Klenerman P, Hill AB. Four distinct patterns of memory CD8 T-cell responses to chronic murine cytomegalovirus infection. J Immunol. 2006;177(1):450-8.

64. Jackson SE, Mason GM, Okecha G, Sissons JG, Wills MR. Diverse specificities, phenotypes, and antiviral activities of cytomegalovirus-specific CD8+ T-cells. J Virol. 2014;88(18):10894-908.

65. Marchi E, Lee LN, Klenerman P. Inflation vs. Exhaustion of Antiviral CD8+ T-Cell Populations in Persistent Infections: Two Sides of the Same Coin? Front Immunol. 2019;10(197).

66. Sierro S, Rothkopf R, Klenerman P. Evolution of diverse antiviral CD8+ T-cell populations after murine cytomegalovirus infection. Eur J Immunol. 2005;35(4):1113-23.

67. Sauce D, Larsen M, Curnow SJ, Leese AM, Moss PA, Hislop AD, et al. EBV-associated mononucleosis leads to long-term global deficit in T-cell responsiveness to IL-15. Blood. 2006;108(1):11-8.

68. van Leeuwen EM, de Bree GJ, Remmerswaal EB, Yong SL, Tesselaar K, ten Berge IJ, et al. IL-7 receptor alpha chain expression distinguishes functional subsets of virus-specific human CD8+ T-cells. Blood. 2005;106(6):2091-8.

69. Boutboul F, Puthier D, Appay V, Pelle O, Ait-Mohand H, Combadiere B, et al. Modulation of interleukin-7 receptor expression characterizes differentiation of CD8 T-cells specific for HIV, EBV and CMV. Aids. 2005;19(17):1981-6.

70. Sauce D, Larsen M, Leese AM, Millar D, Khan N, Hislop AD, et al. IL-7R alpha versus CCR7 and CD45 as markers of virus-specific CD8+ T-cell differentiation: contrasting pictures in blood and tonsillar lymphoid tissue. The Journal of infectious diseases. 2007;195(2):268-78.

71. Lang A, Brien JD, Nikolich-Zugich J. Inflation and long-term maintenance of CD8 T-cells responding to a latent herpesvirus depend upon establishment of latency and presence of viral antigens. J Immunol. 2009;183(12):8077-87.

72. Isa A, Kasprowicz V, Norbeck O, Loughry A, Jeffery K, Broliden K, et al. Prolonged activation of virus-specific CD8+T-cells after acute B19 infection. PLoS Med. 2005;2(12):e343.

73. Gamadia LE, van Leeuwen EM, Remmerswaal EB, Yong SL, Surachno S, Wertheim-van Dillen PM, et al. The size and phenotype of virus-specific T-cell populations is determined by repetitive antigenic stimulation and environmental cytokines. J Immunol. 2004;172(10):6107-14.

74. Redeker A, Remmerswaal EBM, van der Gracht ETI, Welten SPM, Hollt T, Koning F, et al. The Contribution of Cytomegalovirus Infection to Immune Senescence Is Set by the Infectious Dose. Front Immunol. 2017;8:1953.

75. Northfield J, Lucas M, Jones H, Young NT, Klenerman P. Does memory improve with age? CD85j (ILT-2/LIR-1) expression on CD8 T-cells correlates with 'memory inflation' in human cytomegalovirus infection. Immunol Cell Biol. 2005;83(2):182-8.

76. Redeker A, Welten SP, Arens R. Viral inoculum dose impacts memory T-cell inflation. Eur J Immunol. 2014;44(4):1046-57.

77. Beswick M, Pachnio A, Lauder SN, Sweet C, Moss PA. Antiviral therapy can reverse the development of immune senescence in elderly mice with latent cytomegalovirus infection. J Virol. 2013;87(2):779-89.

78. van den Heuvel D, Jansen MA, Dik WA, Bouallouch-Charif H, Zhao D, van Kester KA, et al. Cytomegalovirus- and Epstein-Barr Virus-Induced T-Cell Expansions in Young Children Do Not Impair Naive T-cell Populations or Vaccination Responses: The Generation R Study. J Infect Dis. 2016;213(2):233-42.

79. Gibson L, Piccinini G, Lilleri D, Revello MG, Wang Z, Markel S, et al. Human cytomegalovirus proteins pp65 and immediate early protein 1 are common targets for CD8+ T-cell responses in children with congenital or postnatal human cytomegalovirus infection. J Immunol. 2004;172(4):2256-64.

80. Komatsu H, Inui A, Sogo T, Fujisawa T, Nagasaka H, Nonoyama S, et al. Large scale analysis of pediatric antiviral CD8+ T-cell populations reveals sustained, functional and mature responses. Immun Ageing. 2006;3:11.

81. Reddehase MJ, Balthesen M, Rapp M, Jonjic S, Pavic I, Koszinowski UH. The conditions of primary infection define the load of latent viral genome in organs and the risk of recurrent cytomegalovirus disease. J Exp Med. 1994;179(1):185-93.

82. Redeker A, Welten SP, Baert MR, Vloemans SA, Tiemessen MM, Staal FJ, et al. The Quantity of Autocrine IL-2 Governs the Expansion Potential of CD8+ T-cells. J Immunol. 2015;195(10):4792-801.

83. Dekhtiarenko I, Jarvis MA, Ruzsics Z, Cicin-Sain L. The context of gene expression defines the immunodominance hierarchy of cytomegalovirus antigens. J Immunol. 2013;190(7):3399-409.

84. Dekhtiarenko I, Ratts RB, Blatnik R, Lee LN, Fischer S, Borkner L, et al. Peptide Processing Is Critical for T-Cell Memory Inflation and May Be Optimized to Improve Immune Protection by CMV-Based Vaccine Vectors. PLoS Pathog. 2016;12(12):e1006072.

85. Pardieck IN, Beyrend G, Redeker A, Arens R. Cytomegalovirus infection and progressive differentiation of effector-memory T-cells. F1000Research. 2018;7(F1000 Faculty Rev):1554

86. Riddell NE, Griffiths SJ, Rivino L, King DC, Teo GH, Henson SM, et al. Multifunctional cytomegalovirus (CMV)-specific CD8(+) T-cells are not restricted by telomere-related senescence in young or old adults. Immunology. 2015;144(4):549-60.

87. Simon CO, Holtappels R, Tervo HM, Bohm V, Daubner T, Oehrlein-Karpi SA, et al. CD8 T-cells control cytomegalovirus latency by epitope-specific sensing of transcriptional reactivation. J Virol. 2006;80(21):10436-56.

88. Seckert CK, Griessl M, Buttner JK, Scheller S, Simon CO, Kropp KA, et al. Viral latency drives 'memory inflation': a unifying hypothesis linking two hallmarks of cytomegalovirus infection. Medical microbiology and immunology. 2012;201(4):551-66.

89. Cicin-Sain L, Arens R. Exhaustion and Inflation at Antipodes of T-cell Responses to Chronic Virus Infection. Trends Microbiol. 2018;26(6):498-509.

90. Mocarski ES, Jr. Immunomodulation by cytomegaloviruses: manipulative strategies beyond evasion. Trends Microbiol. 2002;10(7):332-9.

91. Jackson SE, Redeker A, Arens R, van Baarle D, van den Berg SPH, Benedict CA, et al. CMV immune evasion and manipulation of the immune system with aging. GeroScience. 2017;39(3):273-91.

92. Beyranvand Nejad E, Ratts RB, Panagioti E, Meyer C, Oduro JD, Cicin-Sain L, et al. Demarcated thresholds of tumor-specific CD8 T-cells elicited by MCMV-based vaccine vectors provide robust correlates of protection. Journal for immunotherapy of cancer. 2019;7(1):25.

93. Oduro JD, Redeker A, Lemmermann NA, Ebermann L, Marandu TF, Dekhtiarenko I, et al. Murine cytomegalovirus infection via the intranasal route offers a robust model of immunity upon mucosal CMV infection. J Gen Virol. 2015.

94. Wallace DL, Masters JE, De Lara CM, Henson SM, Worth A, Zhang Y, et al. Human cytomegalovirus-specific CD8(+) T-cell expansions contain long-lived cells that retain functional capacity in both young and elderly subjects. Immunology. 2011;132(1):27-38.

95. Mansfield SA, Dwivedi V, Elgharably H, Griessl M, Zimmerman PD, Limaye AP, et al. Cytomegalovirus immunoglobulin G titers do not predict reactivation risk in immunocompetent hosts. J Med Virol. 2019.

96. Roux A, Mourin G, Larsen M, Fastenackels S, Urrutia A, Gorochov G, et al. Differential impact of age and cytomegalovirus infection on the gammadelta T-cell compartment. J Immunol. 2013;191(3):1300-6.

97. Souquette A, Frere J, Smithey M, Sauce D, Thomas PG. A constant companion: immune recognition and response to cytomegalovirus with aging and implications for immune fitness. GeroScience. 2017;39(3):293-303.

98. Bayard C, Lepetitcorps H, Roux A, Larsen M, Fastenackels S, Salle V, et al. Coordinated expansion of both memory T-cells and NK cells in response to CMV infection in humans. Eur J Immunol. 2016;46(5):1168-79.

99. Sauce D, Larsen M, Fastenackels S, Duperrier A, Keller M, Grubeck-Loebenstein B, et al. Evidence of premature immune aging in patients thymectomized during early childhood. J Clin Invest. 2009;119(10):3070-8.

100. Lopez-Sejas N, Campos C, Hassouneh F, Sanchez-Correa B, Tarazona R, Pera A, et al. Effect of CMV and Aging on the Differential Expression of CD300a, CD161, T-bet, and Eomes on NK Cell Subsets. Front Immunol. 2016;7:476.

101. Pawelec G, McElhaney JE, Aiello AE, Derhovanessian E. The impact of CMV infection on survival in older humans. Curr Opin Immunol. 2012;24(4):507-11.

102. Bate SL, Dollard SC, Cannon MJ. Cytomegalovirus seroprevalence in the United States: the national health and nutrition examination surveys, 1988-2004. Clin Infect Dis. 2010;50(11):1439-47.

6

Quantification of T-cell dynamics during latent human cytomegalovirus infection

Manuscript in preparation

S.P.H. van den Berg[1,2], L.Y. Derksen[2], J. Drylewicz[2], N. M. Nanlohy[1], L. Beckers[1], J. Lanfermeijer[1,2], S. N. Gessel[1], M. Vos[1], S. Otto[2], R. de Boer[3], K. Tesselaar[2], J.A.M. Borghans[2]* & D. van Baarle[1,2]*

* These authors contributed equally to this study.

1 Center for Infectious Disease Control, National Institute for Public Health and the Environment, Bilthoven, the Netherlands
2 Laboratory of Translational Immunology, Department Immunology, University Medical Center Utrecht, Utrecht University, the Netherlands
3 Theoretical Biology, Utrecht University, the Netherlands

ABSTRACT

Background: Cytomegalovirus (CMV) infection is known to have a major impact on the T-cell pool, which is typically ascribed to the presence of large numbers of CMV-specific memory $CD8^+$ T-cells. It has been suggested that these CMV-specific $CD8^+$ T-cell populations are established through gradual accumulation of long-lived cells. It remains unknown whether the impact of CMV on the T-cell pool stretches beyond the presence of large numbers of CMV-specific $CD8^+$ T-cells, and whether CMV infection leads to changes in the dynamics of the memory T-cell pool as a whole.

Methods: In this study, we aimed to investigate the effect of CMV infection on $CD4^+$ and $CD8^+$ T-cell dynamics in healthy older adults, and to unravel the mechanisms of maintenance of large numbers of CMV-specific $CD8^+$ T-cells. We studied the expression of senescence, proliferation, and apoptosis markers (among others CD57, KLRG-1, and Ki-67) in 22 healthy CMV-seronegative (CMV-) and 32 CMV-seropositive (CMV+) individuals. Additionally, we quantified the *in vivo* dynamics of different $CD4^+$ and $CD8^+$ memory T-cell populations and specifically of CMV-specific $CD8^+$ T-cells in 5 CMV+ and 5 CMV- individuals by deuterium labelling.

Results: The increased expression of late-stage differentiation markers by $CD8^+$ T-cells of CMV+ versus CMV- individuals was not solely explained by the presence of CMV-specific $CD8^+$ T-cells. The average production rates of CMV-specific $CD8^+$ T-cells and of different $CD8^+$ memory T-cell populations in CMV- and CMV+ individuals were not significantly different. $CD4^+$ memory T-cells of CMV+ individuals showed an increased expression of late-stage differentiation markers, decreased Ki-67 expression and a trend towards lower T-cell production rates as assessed by *in vivo* deuterium labelling compared to CMV- individuals. Overall, the expression of senescence markers correlated negatively with *in vivo* production rates.

Conclusion: Together, this work suggests that i) CMV-specific $CD8^+$ T-cell inflation is not due to a gradual accumulation of long-lived cells, ii) the impact of CMV infection stretches beyond the presence of large numbers of CMV-specific $CD8^+$ T-cells, but iii) CMV infection hardly affects the dynamics of the $CD8^+$ T-cell pool, while it is associated with reduced $CD4^+$ T-cell production rates.

INTRODUCTION

In an ongoing fight against new and emerging viruses and bacteria, the adaptive immune system provides a unique line of defence for its host. Adaptive immunity is not only highly specific against pathogens, but also enables protection for many years. This immunological memory requires long-term maintenance of memory T-cells. After an infection is cleared, the pool of generated effector memory (T_{EM}) and central memory (T_{CM}) T-cells typically contracts, and a small population of antigen-specific T-cells is maintained over time. Under steady state conditions, cell numbers are maintained by balancing the influx and efflux of cells; the production of new cells, either from a source or from proliferation, matches the death and differentiation of cells. Although it is challenging to measure the production rates of cell populations *in vivo* in humans, let alone to subsequently put this in context of the expression of proliferation, apoptosis, or senescence markers, it is important to obtain such quantitative insights in order to understand how T-cell memory is maintained. Moreover, features of the T-cell compartment can be severely impacted by chronic viral infections, specifically by human cytomegalovirus (CMV).

Cytomegaloviruses are archaic double-stranded DNA viruses that have infected vertebrates for millennia [1]. CMV can cause pathology in severely immunocompromised states (e.g. after organ transplantation, or in advanced HIV infection), but in healthy individuals the primary infection typically remains asymptomatic. Nevertheless, CMV-seropositivity greatly influences the T-cell phenotype of healthy individuals — $CD8^+$ T-cells in particular. It leads to a marked increase in the absolute number of $CD8^+$ T_{EM} [2] and effector memory re-expressing CD45RA (T_{EMRA}) T-cells [3, 4]. These cells also seem functionally different as they show a decreased expression of the costimulatory molecules CD27 and CD28 [3, 4], and upregulation of the late-differentiation markers CD57 and KLRG-1 [5]. Currently, it is still unclear to what extent the presence of CMV-specific T-cells themselves is responsible for these changes in the T-cell pool, and to what extent other antigen-specific memory T-cells are affected. The fact that T-cell changes in CMV infection resemble those observed in healthy ageing [2] has prompted the hypothesis that CMV infection contributes to the age-related decay of immune function, including diminished responses to infectious diseases and vaccination [6, 7].

The factor that is thought to be key in the unique T-cell response to CMV, is the 'dynamic' latency CMV establishes within its host [8] with frequent episodes of viral reactivation from latency [9, 10]. These reactivations are believed to play a significant role in the establishment of high numbers of CMV-specific T-cells. Especially in older adults, CMV-specific $CD8^+$ T-cells can take up as much as 30%-90% of the total $CD8^+$ T-cell pool [11, 12]. In mice, it has been shown that the number of CMV-specific memory T-cells gradually increases over time, a process termed 'memory inflation' [13-17]. There is also some evidence for memory inflation in humans [14, 18-20]. However, *how* a handful of T-cell clones can become and remain so numerous, or even increase over time, is not well understood. The underlying dynamics, i.e. the production and loss rates, of both CMV-specific T-cells and other cells in the CMV-reshaped T-cell pool, remain largely unknown. Based on *in vivo* deuterated glucose labelling

of two older CMV-seropositive (CMV+) individuals, it has previously been proposed that CMV-specific $CD8^+$ T-cells are relatively long-lived and thereby accumulate over time [21]. In contrast, the one aged individual that was CMV+ in a deuterated water labelling study [22] had much higher $CD8^+$ memory T-cell production rates than the four CMV- individuals, which could possibly be explained by chronic, age-related immune activation [23] as was suggested in mice [24]. As CMV has such a broad impact on the appearance of the T-cell pool, understanding the functional implications and mechanisms underlying CMV-induced changes is important.

In this study, we aimed to investigate the effect of CMV infection on $CD4^+$ and $CD8^+$ T-cell dynamics in healthy older adults, and to unravel the mechanisms of maintenance of large numbers of CMV-specific $CD8^+$ T-cells. Hereto, we extensively characterized the lifespan-associated phenotype of different T-cell subsets, based on flow cytometric markers for senescence, proliferation, and apoptosis, in a cohort of 32 CMV+ and 22 CMV- healthy older adults. In five CMV- and five CMV+ healthy older adults, we additionally performed a long-term, *in vivo* deuterated water (^2H_2O) labelling study to assess the *in vivo* production rate of cells. We show that the changes in the T-cell pool phenotype of CMV+ individuals cannot solely be explained by the presence of large numbers of CMV-specific $CD8^+$ T-cells. $CD4^+$ T_{EM}, $CD4^+$ T_{EMRA}, and $CD8^+$ T_{EM} cells differ most between CMV+ and CMV- individuals in terms of lifespan-associated phenotype, e.g. by increased expression of senescence markers and decreased Ki-67 expression in CMV+ individuals. A trend towards lower $CD4^+$ $T_{EM/EMRA}$ production rates in CMV+ individuals compared to CMV- individuals was found, but we found no significant difference between the $CD8^+$ T-cell dynamics of CMV+ versus CMV- individuals, nor for CMV-specific $CD8^+$ T-cells versus bulk memory $CD8^+$ T-cells. We found a significant correlation between *in vivo* production rates and the expression of senescence markers.

MATERIALS AND METHODS

Study design

Fifty-four healthy older adults were included based on very strict health criteria to ensure that we were investigating a steady-state situation. Individuals were excluded from participation if they had a condition that influenced the immune system (e.g. infection with human immunodeficiency virus, hepatitis B or C virus, Lyme disease, malaria, or chronic diseases such as asthma or diabetes mellitus, or (a history of) cancer) or drug use (with the exception of occasional use of paracetamol or ibuprofen)). For the heavy water labelling study, we sub-selected five CMV+ individuals based on high T-cell responses against CMV, as well as five age-matched CMV- individuals. All participants gave written informed consent. This study was approved by the local ethical committee of the University Medical Center Utrecht (UMCU) (METC 15/745), The Netherlands, and was conducted in accordance with the Helsinki Declaration, last amended in 2013. We obtained heparinised blood by venepuncture from all fifty-four participants at one time point. The ten participants that were included in the deuterium labelling study donated blood an additional eight times.

Cytomegalovirus (CMV)-specific antibodies

CMV-specific antibody (Ab) levels were measured in serum by a sensitive multiplex immunoassay (Tcheriaeva 2018, Journal Immunological Methods). A cut-off value of 5 (RU) mL^{-1} was used to define CMV-seropositivity. To decrease the chance of false-positive or false-negative results, we only considered samples to be CMV-seronegative if the Ab level was ≤ 4 (RU) mL^{-1} and CMV-seropositive if the Ab level was >7.5 (RU) mL^{-1}. All of our samples were clearly CMV-seropositive or CMV-seronegative according to these definitions.

Cell numbers and HLA typing by flow cytometry

Whole blood was used to calculate absolute leukocyte counts by Trucount™ analysis (BD Biosciences) according to manufacturer's protocol. The following antibodies were used: CD45-PerCP (BioLegend), and CD3-APC-R700, CD4-BV711, CD8-BV786, all purchased from BD Biosciences. Using the bead count of the Trucount™ tube and the $CD3^+$ cell count, absolute cell numbers of the proliferation/apoptosis and senescence panel were also calculated. In addition, for all individuals, HLA typing on whole blood by HLA-antibodies was performed for the most relevant HLA-types describing dominant CMV epitopes by flow cytometry at the first visit. Antibodies used were HLA-A1/36-biotin/strep-PE-CF594, HLA-A2-V450, HLA-A3-APC, HLA-A24-PE, HLA-B7-FITC, and HLA-B8-PE-Cy7. Cells were measured on a Fortessa™ flow cytometer (BD Biosciences) and analysed with FlowJo V10 software.

PBMC, neutrophilic granulocytes, and serum isolation and storage

Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Paque PLUS (GE) density centrifugation according to manufacturer's protocol. After isolation of the PBMCs, cells were washed with phosphate buffered saline (PBS) supplemented with 0.2% FCS, and then frozen in a solution with 90% fetal calf serum and 10% dimethyl sulfoxide in liquid nitrogen or -135°C until further use. Neutrophilic granulocytes were obtained from the Ficoll-Paque pellet after erythrolysis with shock buffer (8.3 g NH_4Cl, 1 g $KHCO_3$, and 37 mg EDTA in 1 L Aqua Millipore). Serum was collected separately and stored at -80°C.

Proliferation/apoptosis and senescence markers by flow cytometry

Thawed PBMCs of all 54 individuals were stained with a proliferation/apoptosis and a senescence panel for flow cytometry. The apoptosis panel consisted of an extracellular antibody mix (CD3-PerCP and CD45RO-PE-Cy7 (both from Biolegend), CD27-APC-R700, Live/dead-APC-Cy7, CD28-BV510, CD56-BV711, CD8-BV786, , CCR7-BUV395, CD4-BUV737 (all from BD Biosciences)), and, after fixing and permeabilising (Cytofix/Cytoperm; BD Biosciences), an additional intracellular antibody mix (Ki-67-FITC and Bcl-2-PE/Dazzle594 (both from BD Biosciences)). Washing steps were performed using Perm/Wash buffer (BD Biosciences). The senescence panel contained CD57-FITC (eBioscience), CD3-AF700, Live/dead-APC-Cy7, CD95-BV421, CD8-BV510, CD27-BV786, CCR7-BUV395, and CD4-BUV737 (all from BD Biosciences), CD127-BV650, CD45RO-BV711, and KLRG1-PE-Cy7 (all from Biolegend). In all flow cytometric panels, CMV+ donors were first stained with the specific

tetramer, either with HLA-B*0702/TPRVTGGGAM-PE, or HLA-A*0201/NLVPMVATV-PE or -APC, or both HLA-A*0101/VTEHDTLLY-APC and HLA-B*0801/ELRRKMMYM-PE (all from Immudex), for 30 minutes at room temperature. An overview of CMV-tetramers used per donor can be found in **Supplementary Table 1**. Cells were measured on a Fortessa™ flow cytometer (BD Biosciences) and data was analysed with FlowJo V10 software. T-SNE analysis was performed with of every donor 10.000 $CD8^+$ T-cells with the senescence panel for $CD8^+$ T-cells (cytobank.org).

In vivo deuterated water labelling protocol

Ten participants (five CMV+ and five CMV-) were included in a longitudinal heavy water labelling study to quantify the underlying dynamics, i.e. production and loss rates, of different sorted T-cell subsets. In short, ^2H is incorporated via *de novo* DNA synthesis by cells undergoing cell division and lost when cells die, differentiate, or migrate to a different body compartment [25]. Participants received a ramp-up dose of ^2H_2O on the first day, after which they drank a daily dose of ^2H_2O for five weeks (except for participant E30 who drank ^2H_2O for only three and a half weeks for logistical reasons). The procedures on the first day and the follow-up schedule were largely as described before [22], though we decreased the length of labelling from nine to five weeks. Urine was collected at thirteen time points during the up- and down-labelling phases to correct for ^2H_2O-availability in the body. Blood was collected to sort T-cell subsets at eight time points, of which four during up-labelling and four during down-labelling, with an average follow-up time of 448 days (range 378 to 511 days).

Sorting of T-cell subpopulations by flow cytometry

Directly after Ficoll-Paque isolation, between $5{\cdot}10^7$ and $20{\cdot}10^7$ PBMCs were stained with CD95-FITC, CD4-APC-eF780 (both eBioscience), CD3-PerCP, CCR7-BV421, CD8a-BV510, and CD45RO-PE-Cy7 (all from BioLegend), and either CD56-APC, CD56-PE, or CD56-PE/Dazzle-594 (the first purchased from BD Biosciences and the latter two from BioLegend). T-cell subpopulations were defined as follows: truly naive, T_{TN} ($CCR7^+CD45RO^-CD27^+CD95^+$), central memory, T_{CM} ($CD45RO^+CD27^+$), effector memory, T_{EM} ($CD27^-CD45RO^+$) and effector memory re-expressing RA, T_{EMRA} ($CD27^-CD45RO^-$). The full gating strategy can be found in **Supplementary Figure 1**. Cells were sorted on a FACSAria™ II or FACSAria™ III sorter (BD Biosciences), and data was analysed with the BD FACSDiva™ v8.0.1 and FlowJo V10 software.

DNA isolation and measurement of deuterium enrichment by GC/MS

After sorting of the different T-cell populations, DNA was isolated after overnight storage of the cell pellets at 4^oC using the ReliaPrep™ DNA isolation kit (Promega). DNA samples were stored in 200 µL DNAse free water at -20^oC until further processing. Next, we followed the protocols described previously [22, 25], with minor modifications, to obtain the percentage deuterium enrichment in body water and immune cell populations. Briefly, DNA samples from sorted lymphocytes were enzymatically hydrolysed into deoxyribonucleotides and conjugated to pentafluorotriacetate (PFTA). The PFTA derivative of deoxyadenosine was analysed by

measuring the ions m/z 435 u (M+0) and m/z 436 u (M+1) in the gas chromatograph-mass spectrometer (GC/MS) (7890A GC System, 5975C inert XL EI/CI MSD with Triple-Axis Detector; Agilent Technologies). The tracer-to-tracee ratio (TTR) was then calculated, by dividing the enriched, deuterated ion (M+1) by the unenriched, naturally occurring ion (M+0).

Correction for abundance sensitivity of the GC/MS

The measured TTR of each sample was corrected for abundance sensitivity, that is the positive correlation observed between sample input and *measured* TTR at fixed *theoretical* TTR [25, 26]. In short, eight standards with different known enrichments ([M+1]/[M+0] = 0, 0.0016, 0.0031, 0.0063, 0.0126, 0.0255, 0.0523, and 0.01097) were measured on the GC/MS at different sample input (M+0), within the same time period as the samples (<3 months). A second-order polynomial was fitted to each standard (M0 versus M1), and the corrected TTR of each sample was calculated by linear interpolation between these polynomials [26]. All data were arcsin(sqrt) transformed before fitting different models to the data (see below). To allow for this transformation, any negative values had to be set to zero. Finally, the atom percent excess (APE) of each sample was calculated from the corrected TTR values (as APE = TTR/(1-TTR)) and presented in the paper. Data points that were out of range of the standards (N = 317/1059) were excluded from the analysis and indicated by in red in **Supplementary Figure 5,6**.

Mathematical modelling to estimate T-cell production and loss rates

We first fitted a simple label enrichment/decay curve to the urine enrichment data of each individual:

during label intake ($t \leq \tau$): $U(t) = f\,(1 - e^{-\delta t}) + \beta\, e^{-\delta t}$

after label intake ($t > \tau$): $U(t) = \left[f(1 - e^{-\delta \tau}) + \beta\, e^{-\delta}\right] e^{-\delta(t-\tau)}$

as described previously [22], where $U(t)$ represents the fraction of ^2H_2O in urine at time t (in days), f is the fraction of ^2H_2O in the drinking water, labelling was stopped at $t = \tau$ days, δ represents the turnover rate of body water per day, and β is the plasma enrichment attained after the boost of label by the end of day 0. We used these best fits when analyzing the enrichment in the different T-cell populations. The estimated maximum level of ^2H enrichment in the granulocyte population of each individual was considered to be the maximum level of label incorporation that cells could possibly attain, and was used to scale the enrichment data of the other cell subsets. Because the granulocyte enrichment curves had not yet reached plateau after 5 weeks of labelling, we constrained the fits to the granulocyte data by imposing that all individuals have the same granulocyte turnover rate d and a fixed delay of bone marrow maturation (Δ) of 5 days [27] in the following differential equation, describing the granulocyte dynamics for all individuals:

$$\frac{dL}{dt} = dcU(t-\Delta) - dL$$

where *L* represents the fraction of labelled DNA in the cell population, here the granulocyte population. All T-cell deuterium labelling curves were fitted using the kinetic heterogeneity model introduced by Asquith et al. [28].

$$\frac{dL}{dt} = pcU(t) - d^{*}L$$

where *L* again represents the fraction of labelled DNA, here in the T-cell population of interest, yielding the average production rate *p* of the T-cell population, and the average loss rate of the labelled T-cells *d** for each cell subset. As CMV-specific cell numbers may not be constant over time, we did not make a steady-state assumption and therefore interpreted *p* as the average *production rate* of a cell population. We then calculated the average *loss rate d* of each cell subset using the cell number data, by fitting an exponential function to the number or frequency of CMV-specific T-cells for each individual:

$$X(t) = X_0 e^{(p-d)t}$$

where X_0 is the CMV-specific T-cell number or frequency at the time of inclusion in the study and p was fixed to the estimated values from the deuterium analyses. In this paper, we present death rates *d* based on the fits to the frequencies of CMV-specific T-cells, because T-cell numbers including CMV-specific T-cell numbers fluctuated considerably between visits, as a direct consequence of fluctuations in total leukocyte counts. Although the kinetic heterogeneity model has the disadvantage that the average production rate *p* is somewhat dependent on the length of the labelling period, we nevertheless used it because the multi-exponential model that we previously proposed [29], frequently led to overfitting of the data. The estimated production rates p are presented in this paper either as rates per day (in tables and figures), or as percentages per year (where percentage production per year = production rate per day*365*100). For cell populations in steady state, the estimated production rate *p* can be translated into the average lifespan of the cells in the population by taking the inverse of the production rate.

Statistical analysis

Differences between groups were assessed using Mann-Whitney *U* test, and comparisons within the same individuals with the Wilcoxon singed-rank test. Correlations were tested with Spearman's rank correlation coefficient. Principal component analysis (PCA) was performed in SPSS, factors were included of an eigenvalue >1, iteration was set at a maximum of 25. For all analyses *p*-values <0.05 were considered significant. Data were analyzed using GraphPad Prism 8.3 and SPSS statistics 22 for Windows (SPSS Inc., Chicago, IL, USA). Deuterium-enrichment data were fitted using R (version 3.6.1, R Core Team (2019). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria (https://www.R-project.org/)), parameters were estimated using a maximum

likelihood approach. Using a bootstrap method, the 95% confidence limits of the parameter were determined by resampling 500 times the residuals to the optimal fit.

RESULTS

Characterization of the study cohort

We included 54 healthy older adults of on average 68.8 years of age (SD 4.6 years, range 61.0 to 79.8 years) who were free of systemic diseases or immunocompromising conditions. Individuals with any (history of) immune-mediated diseases or any current medicine use (with the exception of occasional use of paracetamol or ibuprofen) were excluded. Using a multiplex immunoassay to test for CMV antibodies, we identified 32 of the 54 individuals (59%) as CMV+ and 22 as CMV- (**Supplementary Table 1**). No significant differences in sex or age were observed between CMV+ and CMV- individuals (data not shown).

We investigated the influence of CMV-seropositivity on the T-cell pool by measuring absolute T-cell numbers, and their memory or senescence-associated phenotype in CMV- and CMV+ individuals. In line with previous reports [2, 3, 30, 31], we found significantly increased cell numbers of $CD4^+$ and $CD8^+$ T-cells with a T_{EM} or T_{EMRA} phenotype in CMV+ compared to CMV- individuals (**Figure 1A**). Moreover, CMV-seropositivity was associated with a significantly increased expression of the senescence markers CD57 and KLRG-1 and a loss of expression of the co-stimulatory receptor CD28 on $CD4^+$ T_{EM}, $CD4^+$ T_{EMRA} and $CD8^+$ T_{EM} cells, identifying a late-stage differentiation state (**Figure 1B**). For $CD8^+$ T_{EMRA} cells, the differences in CD57, KLRG-1, and CD28 did not reach statistical significance, but similar trends were observed.

In addition, 38 CMV-specific $CD8^+$ T-cell populations were characterized in 32 CMV+ participants using HLA-class I tetramers for immunodominant CMV epitopes, based on the HLA-type of each participant (see **Supplementary Table 1**). The frequencies of CMV-specific $CD8^+$ T-cells specific for one epitope reached on average 3.7% of total $CD8^+$ T-cells and could reach up to 15-20% for some epitopes (**Figure 1C upper panel**). The dominant phenotype of CMV-specific $CD8^+$ T-cells varied between individuals, although mosT-cells had a T_{EM}/T_{EMRA} phenotype (**Figure 1C middle panel**). Interestingly, we observed a significant positive correlation between the frequency of CMV-specific $CD8^+$ T-cells and the percentage of CMV-specific $CD8^+$ T-cells with a $T_{EM/EMRA}$ phenotype ($p = 0.035$, $r = 0.37$) (**Figure 1C lower panel**). Furthermore, CMV-specific $CD8^+$ T-cells showed a significantly higher expression of the markers CD57 and KLRG-1 and a lower expression of the receptor CD28 than bulk memory T-cells of CMV+ individuals (**Supplementary Figure 2A**). Even within the different memory subsets, CMV-specific $CD8^+$ T-cells showed a more pronounced late-stage differentiation state compared to the total memory subset, especially within the T_{CM} and T_{EM} compartment (**Figure 1D**). Thus, CMV-specific $CD8^+$ T-cells were the most differentiated, followed by bulk memory $CD8^+$ T-cells in CMV+ individuals and then bulk memory $CD8^+$ T-cells in CMV- individuals.

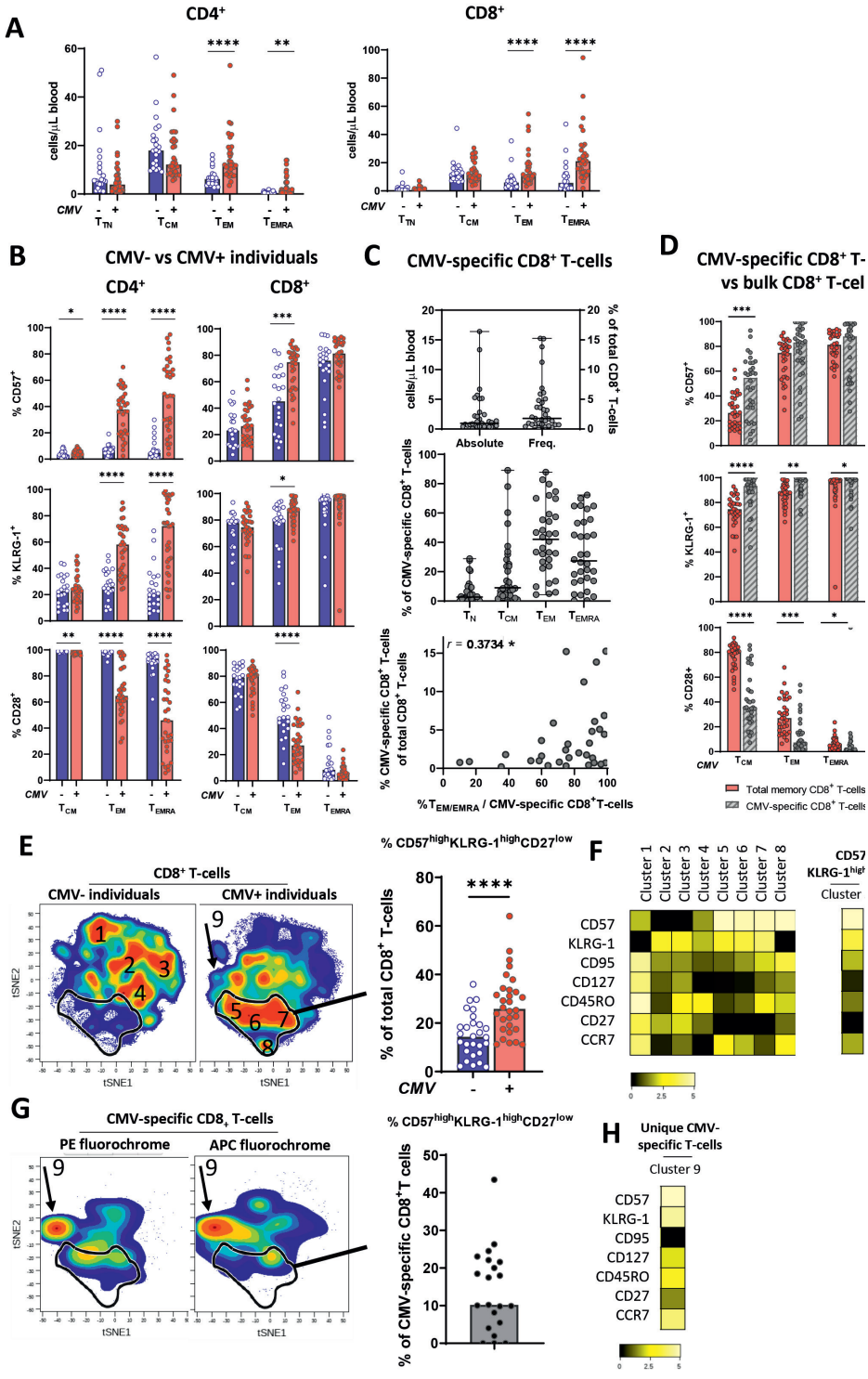

A
CD4+
CD8+
cells/µL blood
CMV
TTN
TCM
TEM
TEMRA
B
CMV- vs CMV+ individuals
CD4+
CD8+
% CD57+
% KLRG-1+
% CD28+
C
CMV-specific CD8+ T-cells
Absolute
Freq.
% of total CD8+ T-cells
% of CMV-specific CD8+ T-cells
% CMV-specific CD8+ T-cells of total CD8+ T-cells
r = 0.3734 *
%TEM/EMRA / CMV-specific CD8+T-cells
D
CMV-specific CD8+ T- vs bulk CD8+ T-cel
Total memory CD8+ T-cells
CMV-specific CD8+ T-cells
E
CD8+ T-cells
CMV- individuals
CMV+ individuals
tSNE1
tSNE2
% CD57highKLRG-1highCD27low
% of total CD8+ T-cells
F
Cluster 1
Cluster 2
Cluster 3
Cluster 4
Cluster 5
Cluster 6
Cluster 7
Cluster 8
CD57
KLRG-1
CD95
CD127
CD45RO
CD27
CCR7
G
CMV-specific CD8+ T-cells
PE fluorochrome
APC fluorochrome
% of CMV-specific CD8+ T cells
H
Unique CMV-specific T-cells
Cluster 9

Figure 1. Alterations in T-cell pool in CMV infection are not solely explained by the presence of CMV-specific CD8⁺ T-cells. A. Absolute T-cell numbers in CMV- and CMV+ individuals for CD4⁺ (left panel) and CD8⁺ (right panel) T_{TN}, T_{CM}, T_{EM}, and T_{EMRA} cells. **B.** Expression of CD57, KLRG-1, and CD28 by CD4⁺ and CD8⁺ T-cells, measured per memory subpopulation (T_{CM}, T_{EM}, and T_{EMRA}) and compared between CMV+ and CMV- individuals. **C.** CMV-specific CD8⁺ T-cells analyzed using 7 different HLA-class I tetramers for 8 immunodominant CMV epitopes (yielding *N* = 38 CMV-specific CD8⁺ T-cell populations in *N* = 32 CMV+ individuals – see **Supplementary Table 1** for epitopes used). Upper panel: CMV-specific CD8⁺ T-cells in absolute numbers and frequency of total CD8⁺ T-cells. Middle panel: phenotype of CMV-specific CD8⁺ T-cells based on division in T_N (naïve, $CD27^+CD45RO^-$), T_{CM}, T_{EM}, and T_{EMRA} cells. Lower panel: association of percentage $T_{EM/EMRA}$ phenotype of CMV-specific CD8⁺ T-cells and the frequency of CMV-specific cells within the CD8⁺ T-cell pool. Error bars in upper and middle panel represent the range. **D.** Comparison of expression of CD57, KLRG-1, and CD28 by CMV-specific CD8⁺ T-cells and total memory CD8⁺ T-cells of CMV+ individuals, analyzed per memory subpopulation (T_{CM}, T_{EM}, and T_{EMRA}). **E.** Left panels: CD8⁺ T-cells in CMV- and CMV+ individuals, clustered by t-SNE analysis based on the expression of CD57, KLRG-1, CD127, CD27, CD45RO, CCR7, and CD95. Numbers 1-9 represent identified clusters of CD8⁺ T-cells based on cell density (with red representing high density of cells, and blue low density of cells). Cluster 5-8 ($CD57^{high}$, KLRG-1^{high}, $CD27^{low}$) are outlined in black in all t-SNE graphs. Cluster 9 represents a cluster that was only observed in CMV+ individuals. Right panels: Percentage of cluster 5-8 within the total CD8⁺ T-cell pool of CMV- and CMV+ individuals**. F.** Phenotype of clyster 5-8 is presented in a heatmap by the mean fluorescence intensity of CD57, KLRG-1, CD127, CD27, CD45RO, CCR7, and CD95 as used in the t-SNE analysis. **G.** Left panel: CMV-specific CD8⁺ T-cells clustered by the same t-SNE analysis as total CD8⁺ T-cells. Right panel: Percentage of clusters 5-8 among CMV-specific T-cells. **H.** Phenotype of cluster 9 is presented in a heatmap by the mean fluorescence intensity of CD57, KLRG-1, CD127, CD27, CD45RO, CCR7, and CD95 as used in the t-SNE analysis. Expression levels are given in arbitrary units ranging from zero (black) to high (white). Bars and horizontal lines in all figures represent medians. Differences between CMV- and CMV+ individuals were assessed by Mann-Whitney *U* test. Correlation was tested by Spearman's correlation. Stars indicate significant differences as follows: * *P*-value <0.05, ** *P*-value <0.01, *** *P*-value <0.001, *** *P*-value <0.0001.

Alterations in the CD8+ T-cell pool in CMV infection are not solely explained by the presence of CMV-specific T-cells

Although the impact of latent CMV infection on the T-cell pool is well known [2, 4, 5], the underlying mechanism for these changes remains unclear. Given that the above described changes in the phenotype of the CD8⁺ T-cell pool of CMV+ individuals resembles the phenotype of CMV-specific T-cells, we investigated whether these changes could be explained by the sheer presence of large numbers of CMV-specific CD8⁺ T-cells. We used an MFI-based t-SNE cluster analysis, including the markers CD57, KLRG-1, CD127, CD27, CD45RO, CCR7, and CD95. T-SNE analysis of CMV- and CMV+ individuals (*N* = 22 and *N* = 32, respectively) again revealed large differences in T-cell phenotype (**Figure 1E**, **F**). CMV+ individuals had significantly more cells in clusters 5-8 (**Figure 1E**, outlined in black, $p < 0.0001$) (**Supplementary Figure 2**). These clusters represent $CD57^{high}$KLRG-$1^{high}$$CD27^{low}$ T-cells (**Figure 1F**), whereas clusters 1-4 in CMV- individuals is characterised by either low KLRG-1 (cluster 1) or low CD57 (clusters 2-4) expression. Subsequently, CMV-specific CD8⁺ T-cells specific for different CMV-epitopes were plotted on the same t-SNE cluster-axes (**Figure 1G**). Only 10.0% (median) of CMV-specific CD8⁺ T-cells were part of the $CD57^{high}$KLRG-$1^{high}$$CD27^{low}$ cluster (**Figure 1E right panel**). A proportion of CMV-specific CD8⁺ T-cells (15% on average per donor) contributed to a relatively small cluster (cluster 9) of T-cells in CMV+ individuals (**Figure 1G right panel**). This cluster was hardly observed in CMV- individuals. The unique combination of T-cell markers in cluster 9 seems to be exclusive for CMV-specific CD8⁺ T-cells. It can be described as a T_{CM} or T_{EM} like population ($CD27^{medium}CD45RO^{high}$) with high

expression of the senescence markers CD57 and KLRG-1, but low expression of the death receptor CD95 (**Figure 1H**). Collectively, these results show (1) that the increased frequency of advanced differentiation state CD8⁺ T-cells in CMV+ individuals is not simply explained by the sheer presence of large numbers of CD8⁺ CMV-specific T-cells, and (2) that part of the CMV-specific CD8⁺ T-cells cluster separately.

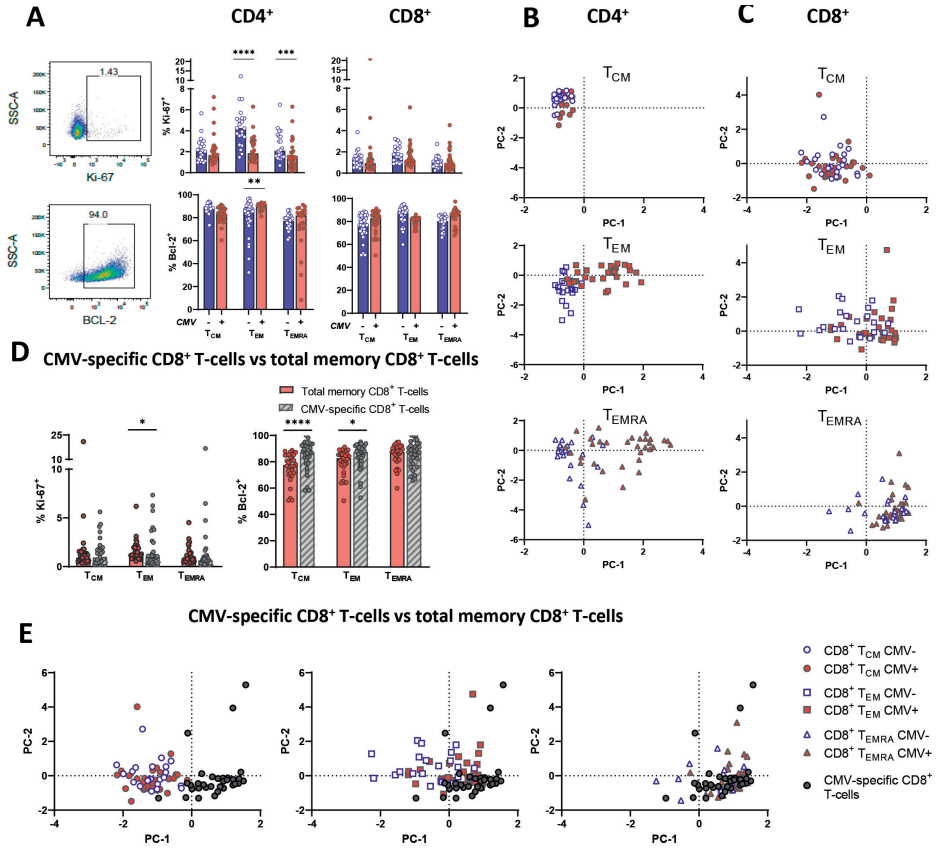


Figure 2. Altered expression of lifespan-associated markers by CD4⁺ $T_{EM/EMRA}$ cells in CMV infection. A. Representative flow cytometric plots for gating of Ki-67 and Bcl-2 expression. Graphs represent percentage of positive cells for Ki-67 and Bcl-2 in CMV- and CMV+ individuals by CD4⁺ and CD8⁺ T-cells, measured per memory subpopulation. **B, C.** Principal component (PC) analysis of CD4⁺ (**B**) and CD8⁺ (**C**) T-cells based on lifespan-associated markers KLRG-1, CD57, CD28, Ki-67 and Bcl-2 per memory subpopulation. Blue dots represent CMV- individuals, red dots represent CMV+ individuals. **D.** Comparison of expression of Ki-67 and Bcl-2 by the total memory CD8⁺ T-cell pool of CMV+ individuals and by CMV-specific CD8⁺ T-cells, measured per memory subpopulation. **E.** PC analysis of CD8⁺ T-cells based on lifespan-associated markers KLRG-1, CD57, CD28, Ki-67 and Bcl-2 per memory subpopulation. Bars in **A** and **D** represent medians. Differences between CMV- and CMV+ individuals were tested by Mann-Whitney *U* test. Stars indicate significant differences as follows: * *P*-value <0.05, ** *P*-value <0.01, *** *P*-value <0.001, *** *P*-value <0.0001.

CMV infection affects expression of lifespan-associated markers in the CD4+ T-cell pool

CD57 and KLRG-1 expression is often associated with proliferative capacity [32, 33] and CD95 is known as a death receptor. Therefore, we assessed if, apart from the changes in T-cell numbers and phenotype, CMV infection also affected other lifespan-associated markers. T-cell proliferation was assessed by measuring the expression of Ki-67, and apoptosis sensitivity by Bcl-2 (**Figure 2A**). Both CD4$^+$ T_{EM} and T_{EMRA} cells expressed significantly lower levels of Ki-67 in CMV+ compared to CMV- individuals (**Figure 2A**), suggesting that a lower percentage of cells were undergoing cell division in CMV+ individuals at the moment of blood withdrawal. Expression of the anti-apoptotic protein Bcl-2 was significantly higher in CD4$^+$ T_{EM} cells of CMV+ individuals (**Figure 2A**). For CD8$^+$ T-cells, no significant differences in expression of these markers were observed between CMV+ and CMV- individuals (**Figure 2A**). As a proxy for lifespan, we combined the information of both previous flow cytometry panels (CD28, KLRG-1, CD57, Ki-67 and Bcl-2) in a principal component (PC) analysis (explaining 73.3% of the variance with two PCs). We found that CD4$^+$ T_{EM} and T_{EMRA} cells in CMV+ individuals clustered separately from CD4$^+$ T_{EM} and T_{EMRA} in CMV- individuals (**Figure 2B**). This was also the case for CD8$^+$ T_{EM} cells albeit to a lesser extent, but not for CD8$^+$ T_{EMRA} cells (**Figure 2C**). Thus, the effect of CMV infection on the expression of senescence and lifespan-associated markers was most pronounced for CD4$^+$ T_{EM} and CD4$^+$ T_{EMRA} cells.

				HLA type						
	Sex	Age	CMV-specific IgG level	A1/ A36	A2	A3	A24	B7	B8	CMV-specific epitope
Total CMV+		***69.14***	***781.58***	***2***	***3***	***2***	***2***	***2***	***1***	
E04	Male	68.8	185.9	+	-	-	-	-	+	A1-VTE, B8-ELR
E08	Male	66.6	191.1	+	+	-	+	-	-	A2-NLV
E34	Male	67.7	145.1	-	+	-	-	-	-	A2-NLV
E39	Male	66.6	123.1	-	-	+	-	+	-	B7-TPR
E59	Female	76	3262.7	-	+	+	+	+	-	B7-TPR
Total CMV-		***69.22***	***1.58***	***1***	***3***	***3***	***2***	***2***	***1***	
E35	Female	69.8	1.8	-	+	-	-	-	-	N/A
E30	Male	66	1.4	+	-	+	-	-	+	N/A
E23	Male	71.3	1.6	-	-	+	+	+	-	N/A
E21	Male	69.7	1.5	-	+	-	-	+	-	N/A
E10	Female	69.3	1.6	-	+	+	+	-	-	N/A

Table 1. Selected individuals for heavy water labelling study

CMV-specific CD8+ T-cells do not differ from bulk memory CD8+ T-cells in terms of lifespan-associated markers

We next investigated if CMV-specific CD8$^+$ T-cells differ in their expression of lifespan-associated markers compared to bulk memory CD8$^+$ T-cells. CMV-specific CD8$^+$ T-cells showed significantly lower expression of Ki-67 compared to bulk memory CD8$^+$ T-cells of CMV+ individuals (**Supplementary Figure 2D**). Also within the memory T_{CM} subset, CMV-specific CD8$^+$ T-cells expressed significantly less Ki-67 compared to bulk T_{CM} cells (**Figure**

2D). The expression of Bcl-2 was significantly higher for CMV-specific CD8$^+$ T_{CM} and T_{EM} cells compared to bulk CD8$^+$ T_{CM} and T_{EM} cells of CMV+ individuals (**Figure 2D**). Finally, we performed a PC analysis on these markers for CMV-specific CD8$^+$ T-cells and the different memory CD8$^+$ T-cell subsets (explaining 72.5% of variance with two PCs). We confirmed that CMV-specific CD8$^+$ T-cells mostly resemble bulk CD8$^+$ T_{EMRA} cells and, to a lesser extent, bulk CD8$^+$ T_{EM} cells (**Figure 2E**).

Ten individuals selected for quantification of T-cell dynamics using heavy water labelling

The previously described markers gave a snapshot of ongoing proliferation and apoptosis resistance of different T-cell subsets. However, to address the question whether T-cells of CMV- and CMV+ individuals differ in their production and loss rates, and if CMV-specific CD8$^+$ T-cells accumulate due to accumulation of long-lived cells, we quantified the *in vivo* production and loss rates of different T-cell populations using heavy water (^2H_2O) labelling. The principle of ^2H_2O labelling is that the ^{2}H-atoms present in the body are incorporated via *de novo* DNA synthesis by cells undergoing cell division and lost when cells die, differentiate, or migrate to another body compartment [25]. Ten individuals were selected to participate in such a ^2H_2O labelling study (**Figure 3A**). We hypothesized that any potential differences in T-cell production and loss rates between CMV-specific CD8$^+$ T-cells and bulk memory CD8$^+$ T-cells in CMV+ and CMV- individuals would be most evident in individuals with the highest CMV-specific CD8$^+$ T-cell responses. We therefore selected the five CMV+ participants with the highest absolute numbers of CMV-specific CD8$^+$ T-cells (**Supplementary Figure 3**). Next, five CMV- participants were selected based on matching for age and sex to the CMV+ group (**Table 1**). Percentages of memory and naive T-cells in these selected ten individuals were similar to those in the total group of participants (**Supplementary Figure 4A,B**). Based on the longitudinal data of each sorted T-cell subset per individual during the 5 week up- and on average 59 week (range 49-70) down-labelling phase of the ^2H_2O labelling study, the average production rate of each T-cell subset was estimated by fitting a mathematical model to the deuterium enrichment data (**Supplementary Figure 5,6**). T_{EM} and T_{EMRA} cells were sorted together as $T_{EM/EMRA}$ for the assessment of production rate, to enable a fair comparison with CMV-specific T-cells.

In earlier deuterium labelling studies, we used quite broad definitions to separate memory (CD45RO$^+$) and naïve (CD45RO$^-$CD27$^+$) T-cell subsets. Here, we refined our sorting strategy to determine the production rates of different CD4$^+$ and CD8$^+$ T-cell subsets in all older adults. We sorted truly naive (T_{TN}) cells (CD45RO$^-$CD27$^+$CCR7^{hi}CD95$^-$) to exclude contamination with CD95$^+$ T-cells, which are known to be highly proliferating [22] and include stem cell memory T-cells (T_{SCM}) [34]. For total memory and naïve T-cell populations, we assumed that the number of cells in the population remained constant over time, and thus that production rates equal loss rates. The median T_{TN} production rate was 0.00065 per day (23.9% per year) for CD4$^+$ T_{TN} cells and 0.00067 per day (24.3% per year) for CD8$^+$ T_{TN} cells (**Figure 3C and Table 2**), indicating that naive T-cells have a life expectancy of approximately 4 years. The

average production rates of T_{CM} and $T_{EM/EMRA}$ cells were, respectively 0.00740 and 0.00722 per day for $CD4^+$ T-cells (270.1% and 263.5% per year) and 0.00351 and 0.00279 per day for $CD8^+$ T-cells (128.1% and 101.8% per year), and thereby both for $CD4^+$ and $CD8^+$ T-cells higher than for their T_{TN} counterparts (**Supplementary Figure 7A, Figure 3C**).

$CD4^+$ $T_{EM/EMRA}$ cells had a higher production rate than $CD8^+$ $T_{EM/EMRA}$ cells (**Supplementary Figure 7A, Figure 3C**). Confidence intervals of production rate estimations are presented in **Supplementary Figure 7B,C**.

	Median (range) of production rates per day		
	All individuals	*CMV- individuals*	*CMV+ individuals*
$CD4^+$ T_{TN} cells	0.00065 (0.00011 – 0.00249)	0.00137 (0.00063 – 0.00179)	0.00030 (0.00011 – 0.00249)
$CD8^+$ T_{TN} cells	0.00067 (0.00003 – 0.00370)	0.00097 (0.00014 – 0.00137)	0.00049 (0.00003 – 0.00370)
$CD4^+$ T_{CM} cells	0.00740 (0.00322 – 0.00967)	0.00755 (0.00578 – 0.00875)	0.007098 (0.00322 – 0.00967)
$CD8^+$ T_{CM} cells	0.00351 (0.00168 – 0.02625)	0.00332 (0.00220 – 0.02625)	0.00460 (0.00168 – 0.00813)
$CD4^+$ $T_{EM/EMRA}$ cells	0.00722 (0.00353 – 0.01359)	0.00739 (0.00708 – 0.01359)	0.00678 (0.00353 – 0.00746)
$CD8^+$ $T_{EM/EMRA}$ cells	0.00279 (0.00198 – 0.00793)	0.00279 (0.00234 – 0.00793)	0.00263 (0.00198 – 0.00536)
CMV-specific $CD8^+$ T-cells	0.00228 (0.00148 – 0.00745)	N/A	0.00228 (0.00148 – 0.00745)

Table 2. Median (range) of production rates of the different T-cell subsets

The average production rate of CD4+ $T_{EM/EMRA}$ cells tends to be lower in CMV+ versus CMV- individuals

Next, we investigated whether the increased number of memory T-cells and the upregulation of senescence markers seen in CMV infection were associated with altered T-cell dynamics. Therefore, the production rates of T_{CM} and $T_{EM/EMRA}$ $CD4^+$ and $CD8^+$ cells were compared between CMV- and CMV+ individuals. The deuterium enrichment curves of CMV+ and CMV- individuals were largely overlapping for $CD4^+$ T_{TN} and T_{CM} cells and for all $CD8^+$ T-cell subsets (**Figure 3B**), but $CD4^+$ $T_{EM/EMRA}$ cells seemed to reach lower enrichment levels in CMV+ compared to CMV- individuals (**Figure 3B left lower panel**). Mathematical modelling confirmed a trend towards a lower production rate of $CD4^+$ $T_{EM/EMRA}$ cells in CMV+ versus CMV- individuals ($p = 0.0952$, average production rate of 0.005762 (210.3% per year) and 0.008985 per day (327.85% per year), respectively) (**Figure 3C**). In conclusion, the dynamics of T-cell populations are hardly influenced by CMV infection, apart from a slight decrease in the production rate of $CD4^+$ $T_{EM/EMRA}$ cells in CMV+ individuals.

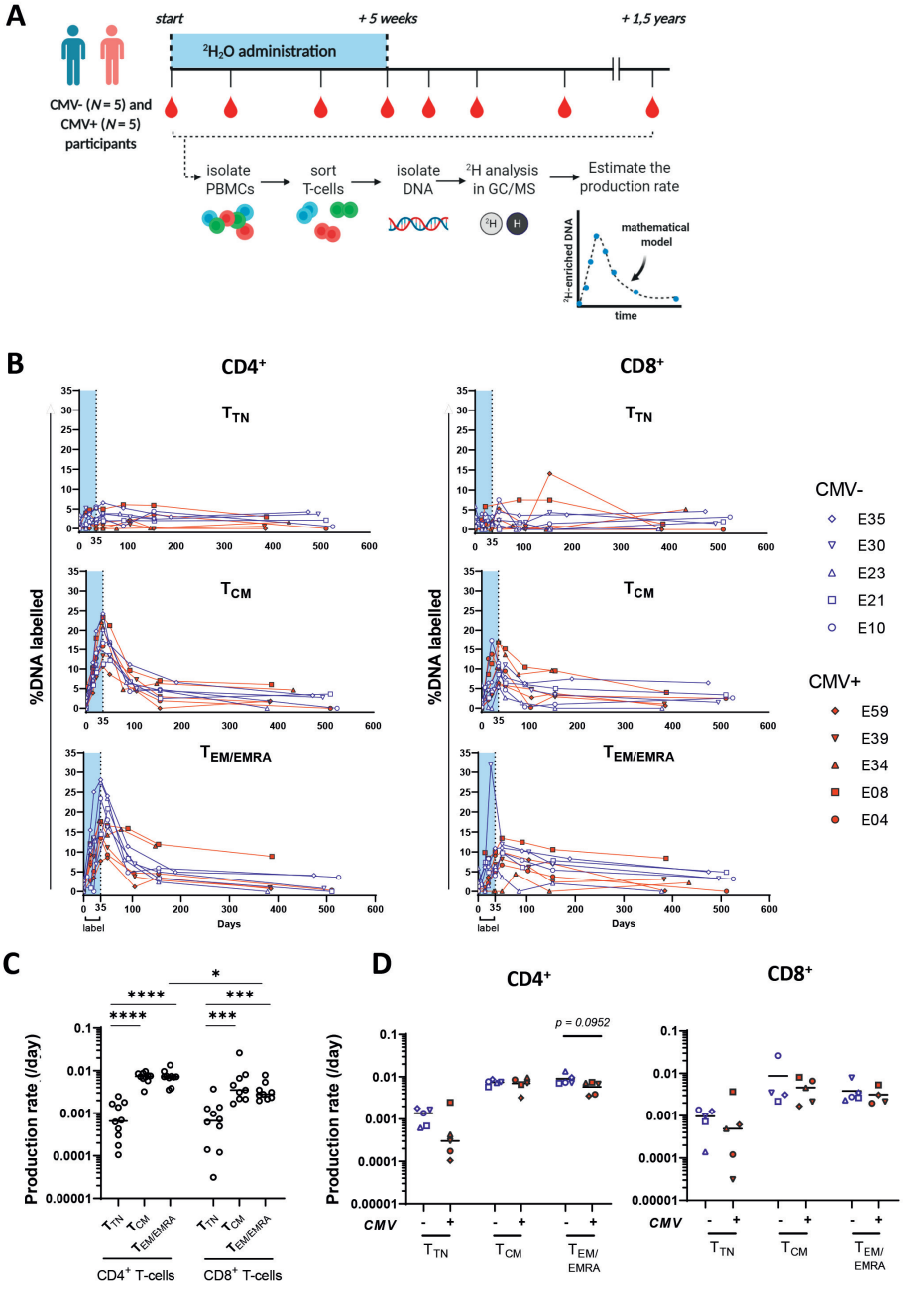


Figure 3. Dynamics of CD4⁺ and CD8⁺ T-cells in CMV- and CMV+ individuals. A. Graphical representation of the study design for the deuterated water labelling protocol. **B.** Deuterium labelling enrichment (%DNA labelled) of different T-cell subpopulations in CMV- and CMV+ individuals. Label enrichment was scaled between 0 and 100% by normalizing for the estimated maximum enrichment of granulocytes (see **Materials and methods**). Lines go through the mean of duplicate measurements. **C.** Summary of estimated production rates of T_{TN}, T_{CM}, and $T_{EM/EMRA}$ CD4⁺ and CD8⁺ T-cells in CMV- (blue symbols) and CMV+ (red symbols) individuals. All estimates

were obtained by fitting a single-exponential model to the data sets per individual (see **Materials and methods** and **Supplementary Figure 5**. Note that steady state is assumed in these cell populations and loss rates are expected to equal the production rate. Horizontal lines represent median values. *P*-values <0.1 are presented in the figure. Data from each individual are represented by unique symbols.

Kinetics of CMV-specific CD8+ memory T-cells are similar to those of bulk memory T-cells

We investigated if the high numbers of CMV-specific CD8$^+$ T-cells can be explained by accumulation of long-lived cells. Therefore, we compared the dynamics of CMV-specific CD8$^+$ T-cells to those of $T_{EM/EMRA}$ CD8$^+$ T-cells. The frequency of tetramer-positive cells in the total CD8$^+$ T-cell population remained fairly constant during the follow-up time of ~1,5 years (**Figure 4A upper panel**). Although there were relatively large fluctuations in absolute numbers of CMV-specific CD8$^+$ T-cells (**Figure 4A lower panel**) this could be explained by fluctuations in total T-cell counts (data not shown). For each individual, the incorporation of deuterium by CMV-specific CD8$^+$ T-cells was largely overlapping with that of total CD8$^+$ $T_{EM/EMRA}$ cells (**Figure 4B upper panel**). There is a risk that potential kinetic differences between CMV-specific CD8$^+$ T-cells and non-CMV-specific CD8$^+$ T-cells may go unnoticed in this comparison as bulk $T_{EM/EMRA}$ CD8$^+$ T-cells from CMV+ individuals in fact *include* lots of CMV-specific CD8$^+$ T-cells. We therefore also compared the dynamics of CMV-specific CD8$^+$ T-cells to those of bulk $T_{EM/EMRA}$ CD8$^+$ T-cells of CMV- individuals, that contain simply no CMV-specific CD8$^+$ T-cells. Again, no obvious differences in deuterium incorporation between CMV-specific CD8$^+$ T-cells and non-CMV-specific $T_{EM/EMRA}$ CD8$^+$ T-cells were observed (**Figure 4B lower panel**). Indeed, the estimated average production rates of CMV-specific CD8$^+$ T-cells were not significantly different from those of total CD8$^+$ $T_{EM/EMRA}$ cells in CMV+ nor CMV- individuals (**Figure 4C, Table 2**). Because CMV-specific CD8$^+$ T-cells are thought to increase over time [14], in the analyses of CMV-specific CD8$^+$ T-cells we did not assume thaT-cell numbers were in steady state. Thus, we deduced the average loss rates of CMV-specific CD8$^+$ T-cells from the changes in cell numbers over time and using the average production rates as estimated by deuterium labelling (see **Materials and methods**). We found that the average loss rates of CMV-specific CD8$^+$ T-cells were nearly identical to their average production rates (**Figure 4D**), suggesting that CMV-specific CD8$^+$ T-cells are not substantially longer-lived than other memory T-cells. Although the sizes of the CMV-specific CD8$^+$ T-cell expansions differed more than threefold between individuals, we found no significant correlation between absolute numbers (or frequencies) of CMV-specific CD8$^+$ T-cells and their average production or loss rates (**Figure 4E**). This suggests that the large size of clonally expanded CMV-specific CD8$^+$ T-cell subsets is neither due to increased T-cell production nor to a clearly increased lifespan of these cells.

Expression of senescence markers and Ki-67 associate with production, while Bcl-2 does not

Finally, we wondered if the estimated production rates of memory T-cell populations that we quantified using deuterium labelling was in part reflected by the expression of senescence, proliferation, and apoptosis markers.

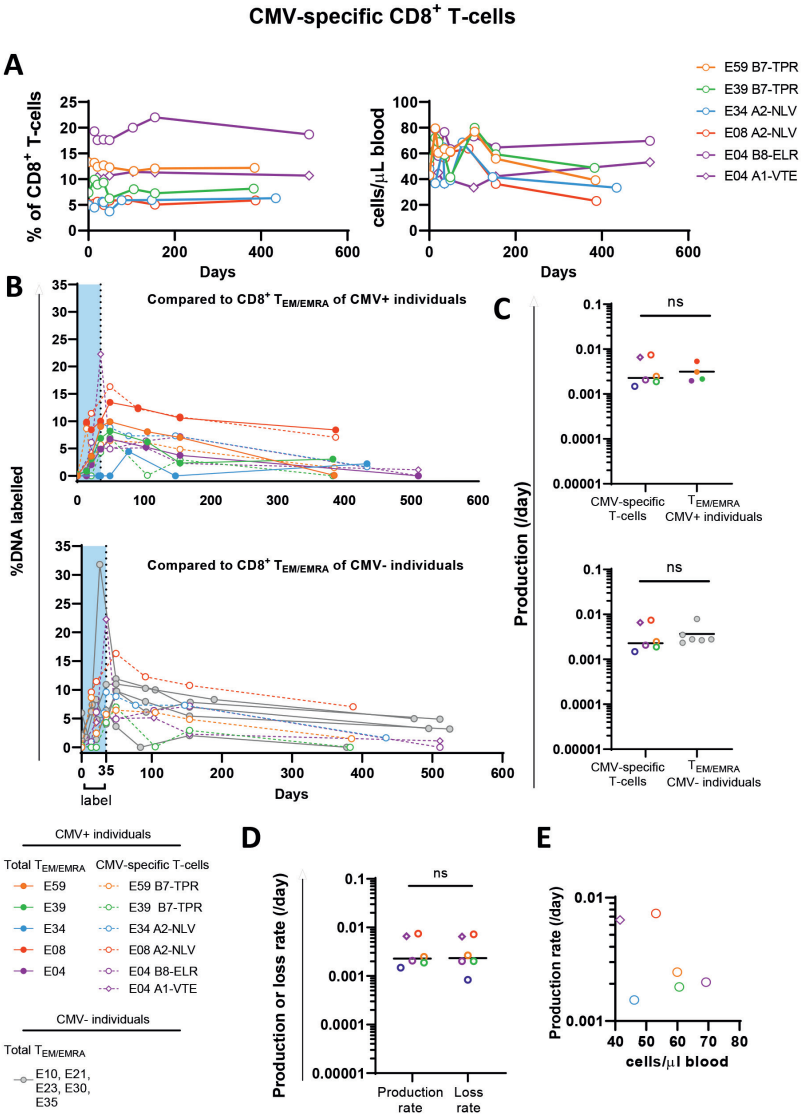


Figure 4. Dynamics of CMV-specific CD8⁺ T-cells and CD8⁺ $T_{EM/EMRA}$ cells in CMV+ and CMV- individuals. **A.** Frequency within the total CD8⁺ T-cell pool (upper panel) and absolute count (lower panel) of CMV-specific CD8⁺ T-cells over time in the CMV+ individuals selected for the deuterium labelling study. **B.** Deuterium labelling enrichment (%DNA labelled) of CMV-specific CD8⁺ T-cells compared to CD8⁺ $T_{EM/EMRA}$ cells of CMV+ (upper panel) or CMV- (lower panel) individuals. Label enrichment was scaled between 0 and 100% by normalizing for the estimated maximum enrichment of granulocytes (see **Materials and methods**). Lines go through the mean of duplicate measurements. It should be noted that for one individual (E34) it was not possible to determine the production rate of CD8⁺ $T_{EM/EMRA}$ cells because too many samples were out of range of the standard lines for deuterium enrichment measurement. **C.** Estimated average production rates of CMV-specific CD8⁺ T-cells compared to CD8⁺ $T_{EM/EMRA}$ cells of CMV+ (upper panel) or CMV- (lower panel) individuals. All estimates were obtained by fitting a single-exponential model to the data sets per individual (see **Materials and methods** and **Supplementary Figure 5**). **D.** Comparison of production and loss rate of CMV-specific T-cells. **E.** Association between the absolute cell number and the average production rate of CMV-specific CD8⁺ T-cells. Horizontal lines represent median values.

Differences between groups were assessed by Mann-Whitney *U* test, or Wilcoxon matched-pairs signed rank test for comparisons within the same individuals. Correlation was tested by Spearman's correlation. *P*-values < 0.1 are presented in the figure, ns = not significant. Data from each individual are represented by unique symbols.

Interestingly, the expression of CD57, KLRG-1, and CD28 show moderate, but highly significant correlations with the estimated production rates (r = -0.6196, r = -0.6075, r = 0.6243, respectively; for all markers $p < 0.0001$) (**Figure 5**). Thus, late T-cell differentiation states are associated with reduced production rates *in vivo*. Expression of the proliferation marker Ki-67 correlated only weakly with the *in vivo* production rate (r = 0.3304, p = 0.0285). This correlation was partly obscured due to the data points of one individual (E04), whose Ki-67 expression levels were relatively high given the corresponding production rate. Excluding this individual from the analysis yielded a moderate significant correlation between Ki-67 expression and the estimated production rate (r = 0.5115, p = 0.0010).

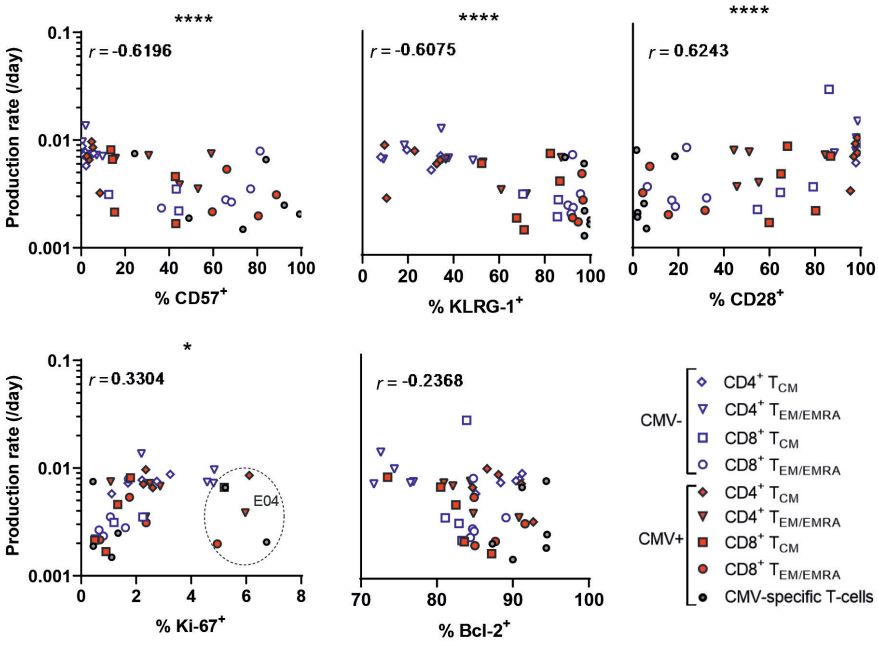


Figure 5. Association between T-cell production rates and expression of senescence, proliferation, and apoptosis markers. The estimated average production rates of different CD4+ and CD8+ T-cell subsets as estimated by heavy water labelling were plotted against their expression (% positive) of CD57, KLRG-1, CD28, Ki-67, and Bcl-2. Horizontal lines represent median values. Data points of donor E04 are circled in the Ki-67 graph. Correlation was tested by Spearman's correlation, *r*-values are indicated in the graphs. Stars indicate significant differences as follows: * *P*-value <0.05, ** *P*-value <0.01, *** *P*-value <0.001, *** *P*-value <0.0001. Different symbols represent different memory subpopulations.

DISCUSSION

Immunological memory is indispensable for healthy ageing and therefore it is essential to understand the underlying biology of lymphocyte maintenance. CMV exerts a major effect on the composition of the T-cell pool. The abundancy of antigen-specific CD8+ T-cells

that are generated by CMV infection is unparalleled by any other antigenic stimulus, and CMV-specific T-cells display a unique late differentiated phenotype [35]. In this study, we investigated the effect of CMV infection on the dynamics of the T-cell pool in healthy older adults. We show that the CMV-induced late-stage differentiated state of the $CD8^+$ T-cell memory pool is not explained by the sheer presence of large numbers of CMV-specific $CD8^+$ T-cells. In the $CD4^+$ memory T-cell pool, latent CMV infection is associated with increased expression of late-stage differentiation markers and decreased proliferation, supported by a marked decrease in Ki-67 expression and a trend towards lower T-cell production rates assessed by *in vivo* deuterium labelling. Despite clear differences in the expression of late-stage differentiation markers, we find no significant differences in the expression of proliferation and apoptosis markers or in the *in vivo* production rates of different $CD8^+$ memory T-cell subsets between CMV- and CMV+ individuals. CMV-specific $CD8^+$ T-cells also express a late-stage differentiated phenotype, but do not differ significantly in *in vivo* production or loss rates when compared to $T_{EM/EMRA}$ or T_{CM} cells in CMV- or CMV+ individuals. Finally, we find that the expression of late-stage differentiation markers correlated negatively with T-cell production rates, coupling high expression of senescence markers to an increased lifespan *in vivo*.

Originally, the typical features of the T-cell pool in CMV+ individuals were thought to reflect an effect of CMV on the whole T-cell pool [30]. With the invention of MHC class I tetramers, however, it became clear that CMV-specific $CD8^+$ T-cells are present in large numbers, and *themselves* possess a phenotype that resembles the changes observed in the total T-cell pool of CMV+ individuals. We therefore investigated whether the phenotypical changes of the T-cell pool in CMV+ individuals are the direct consequence of the presence of large expansions of CMV-specific late-differentiated $CD8^+$ T-cells. By performing a t-SNE analysis, we found that CMV-specific $CD8^+$ T-cells only partially explain the differences in the $CD8^+$ T-cell pool between CMV- and CMV+ individuals. CMV infection thus seems to affect non-CMV-specific memory T-cells as well. Two different fluorochromes (PE and APC) for different epitopes of CMV-specific $CD8^+$ T-cells yielded comparable results in the t-SNE analysis, indicating that the mean fluorescence intensity (MFI)-based clustering of the $CD8^+$ T-cells was not biased by compensation for spectral overlap. The investigated epitopes in our analysis captured at most 18% of CMV-specific $CD8^+$ T-cells out of the total $CD8^+$ T-cell pool, while some studies suggest that in CMV+ individuals as much as 30-90% of $CD8^+$ T-cells is CMV-specific [11, 12]. We cannot exclude the possibility that $CD8^+$ T-cells specific for other (non-tested) CMV-epitopes may have been responsible for the observed differences between CMV+ and CMV- individuals. We regard this unlikely, however, because the 7 epitopes that were tested are the most immunodominant ones known for CMV. Ideally, a method that would pick up all CMV-specific $CD8^+$ T-cells would need to be used to reveal the precise contribution of CMV-specific T-cells to the changes in the T-cell pool observed in CMV+ individuals.

Even though most research has focused on the large numerical expansions of CMV-specific $CD8^+$ T-cells, also the $CD4^+$ T-cell pool is significantly altered in CMV+ compared to CMV- individuals. We found that the expression of late-stage differentiation markers, as well as proliferation and anti-apoptotic markers was affected by CMV. Also, a trend towards a decreased $CD4^+$ $T_{EM/EMRA}$ cell production rate was observed in CMV+ versus CMV- individuals *in vivo* by deuterium analysis. As we did not use peptide-MHC class II tetramers, we could not quantify to what extent CMV-specific $CD4^+$ T-cells were responsible for these differences and to what extent CMV affected non-CMV-specific memory $CD4^+$ T-cells. We speculate that the $CD4^+$ $T_{EM/EMRA}$ cell population in CMV+ individuals mainly consists of CMV-specific cytotoxic T-cells. Cytotoxic $CD4^+$ T-cells express a $T_{EM/EMRA}$ phenotype and are typically only present in small numbers in CMV- individuals [36, 37]. As CMV harnesses multiple mechanisms to escape neutralizing antibodies as well as the $CD8^+$ cytotoxic T-cell response, $CD4^+$ cytotoxic T-cells may be essential in the control of CMV [38]. $CD8^+$ T-cells, which are known to have larger cytotoxic potential than $CD4^+$ memory T-cells, have a longer expected lifespan than $CD4^+$ T-cells [22], suggesting that cytotoxic function may be related to longevity.

We also assessed if maintenance of CMV-specific $CD8^+$ T-cells occurs due to the accumulation of long-lived cells, as was suggested by Wallace *et* al. [21]. In this deuterated glucose labelling study, tetramer+ CMV-specific T-cells were compared to kinetically heterogeneous tetramer- T-cell subsets ($CD45RO^+$ (i.e. T_{EM} and T_{CM}) and $CD45RO^-$ (i.e. T_N and T_{EMRA}) T-cells) *within* the same individual and were found to be longer-lived (lower in production and loss rate). In theory, our approach should be able to pick up even smaller differences by comparing to more strictly defined memory T-cell populations and also comparing to non-CMV-specific memory T-cell populations of CMV- individuals. Nevertheless, we did not find a significantly decreased production or loss rate of CMV-specific $CD8^+$ T-cells in comparison with $CD8^+$ $T_{EM/EMRA}$ or T_{CM} cells from CMV+ or CMV- individuals. In line with this, in a recent study of murine CMV, we found that the *in vivo* production rates of MCMV-specific T-cells were very similar to those of bulk memory T-cells (Baliu-Piqué *et al.* in preparation). We therefore believe that the maintenance of high numbers of CMV-specific $CD8^+$ T-cells is not linked to substantially altered production or loss rates compared to other memory $CD8^+$ T-cells, and that the large size of the CMV-specific T-cell pool is set shortly after CMV infection (Samson, Van den Berg *et al.* in preparation). The underlying mechanisms explaining these large CMV-specific expansions remain to be investigated.

Despite the increased $CD8^+$ T-cell numbers and their late stage differentiation phenotype, no significant differences in $CD8^+$ T-cell proliferation and apoptosis markers or production and loss rates (as measured by *in vivo* deuterium labelling) were observed between CMV+ and CMV- individuals. It was previously proposed that chronic infection may lead to increased T-cell production rates due to the continuous recruitment of new antigen-specific T-cells from the naïve or central memory pool into the effector memory compartment [39]. Alternatively, it has been proposed that CMV-specific $CD8^+$ T-cells might outcompete other pre-existing antigen-specific T-cells [40-42], and thereby affect their lifespan. In our

selected CMV+ individuals with high $CD8^+$ CMV-specific T-cell numbers, we did not find any kinetic differences between CMV-specific and bulk memory T-cells. We do not know whether any CMV reactivations occurred during follow-up in our participants, and cannot exclude the possibility that in these extremely healthy individuals CMV reactivation may occur less frequently than in individuals with an impaired health, which may in turn influence the dynamics of the $CD8^+$ T-cell pool. Thanks to the rather strict selection criteria, we were able to study the influence of CMV infection on the T-cell pool in the absence of other underlying (chronic) conditions.

To understand how T-cell memory is maintained, we investigated the production and loss rates of cell populations *in vivo* in humans and put this in context of the expression of proliferation, apoptosis, and senescence markers. Generally speaking, expression of senescence markers as well as Ki-67 correlated with T-cell production rates *in vivo*. CMV-specific $CD8^+$ T-cells had on average a higher expression of senescence markers than their bulk memory counterparts, though this was not reflected in differences in *in vivo* T-cell production rates. This might be partly explained by the fact that we compared the *in vivo* production rates of total $CD8^+$ CMV-specific T-cells to that of bulk $T_{EM/EMRA}$ $CD8^+$ T-cells, a comparison for which the expression of Ki-67 also did not differ significantly. Deuterium labeling studies tend to be limited to relatively small numbers of participants, which makes it more difficult to draw firm conclusions on kinetic comparisons between groups with a lot of interindividual variation. Nevertheless, deuterium labelling provides a powerful tool to quantify cellular dynamics, not in the least because kinetic estimates are based on multiple data points per individual, making the estimates per individual less sensitive to fluctuations over time. As a result, deuterium labelling studies, even when based on relatively small numbers of participants, can yield very reliable and reproducible results. We and others have for example reproducibly shown that $CD4^+$ T-cells have higher production rates than $CD8^+$ T-cells [43]. Furthermore, we used the kinetic heterogeneity model as presented here [28] and the multi-compartment model (data not shown) [29]. When using the latter, we also did not find differences in production rates between $CD8^+$ CMV-specific T-cells and bulk $CD8^+$ $T_{EM/EMRA}$ cells. Even when estimating production rates by simple linear regression on the data during the uplabeling period, the conclusion on the effect of CMV did not change (**Supplementary Figure 9A,B**). However, our data might suggest a need for new mathematical models that can account for smaller differences in label uptake without overfitting the data. Since the estimations of cellular loss rates relied on a combination of deuterium labelling data and measurements of cell frequencies or numbers, which are notoriously fluctuating, these estimates are probably less precise than those of the production rates. Differences therein could in theory still bridge the discrepancy between an increased late-stage differentiation state and a similar production rate. We thus cannot completely rule out that CMV-specific T-cells might be slightly longer lived. A downside of deuterium labelling is that it cannot be used to study single-cell kinetics and needs to be performed on sorted populations based on predefined cell surface markers. It could be that kinetically different subpopulations cannot be subdivided along the same lines as the well-established memory T-cell populations (e.g. T_{CM}, T_{EM}, T_{EMRA}), and that other

markers or functionalities govern cellular production and lifespan. In conclusion, although we cannot rule out the possibility that small differences in production rates have been missed in our *in vivo* deuterium labelling study, our data suggest that no substantial differences in underlying dynamics are required to maintain high $CD8^+$ CMV-specific T-cell numbers.

Finally, we found that the estimated *in vivo* production rates of memory T-cells based on deuterium labelling correlated with the expression of late-stage differentiation markers and the proliferation marker Ki-67. Expression of Ki-67 correlated weakly with the *in vivo* production rate, possibly due to the 'snapshot' nature of this marker, which makes it more sensitive to day to day variations and ongoing immune activations. The expression of the late-stage differentiation markers CD57 and KLRG-1 correlated negatively with the *in vivo* production rates. This is in line with *in vitro* results, which showed that $CD57^+$ and KLRG-1^+ T-cells are less able to proliferate upon *in vitro* T-cell receptor (TCR) stimulation [32, 33]. However, in mice, it was shown that $CD57^+$ as well as $CD57^-$ antigen-specific T-cells are able to divide upon IL-7 stimulation, a measure for homeostatic proliferation, and are therefore not hampered in their maintenance [44]. To give more clarity into this matter, a follow-up study would be needed where the *in vivo* production rate of sorted $CD57^+$ and $CD57^-$ cells would be directly compared. Altogether, this suggests that a combination of senescence markers could potentially be used as a proxy marker for cellular lifespan.

In the context of ageing of the immune system, or immuno-senescence, potential differences in T-cell maintenance between CMV- and CMV+ individuals are of special interest. CMV has been suggested to be a driving force behind accelerated ageing of the immune system [45-47]. Previously, we did not find a difference in memory T-cell maintenance between aged and young individuals [22]. Here we expand on this finding by showing that infection with CMV has only minor effects on T-cell dynamics. Together, this suggests that during healthy ageing, with or without CMV infection, no substantial changes in memory T-cell dynamics arise. Secondly, our work shows that maintenance of CMV-specific memory T-cells, despite their high abundance, does not necessarily require altered cellular dynamics. This suggests that the characteristics of large memory T-cell responses may be set at the start of the T-cell response. This work thereby also offers important implications for vaccination strategies that aim to induce long-term and high immunological memory responses, for example using CMV-vector based vaccines.

ACKNOWLEDGEMENTS:

We thank René van Boxtel and Inge Pronk for help in designing the study and Jeroen van Velzen, Pien van der Burght and Sebastian van Burgh for help with the flow cytometry sorting.

REFERENCES

1. McGeoch, D.J., F.J. Rixon, and A.J. Davison, *Topics in herpesvirus genomics and evolution.* Virus Res, 2006. 117(1): p. 90-104.
2. Wertheimer, A.M., et al., *Aging and cytomegalovirus infection differentially and jointly affect distinct circulating T-cell subsets in humans.* J Immunol, 2014. 192(5): p. 2143-55.
3. van der Heiden, M., et al., *Differential effects of Cytomegalovirus carriage on the immune phenotype of middle-aged males and females.* Sci Rep, 2016. 6: p. 26892.
4. Jackson, S.E., et al., *Latent Cytomegalovirus (CMV) Infection Does Not Detrimentally Alter T-cell Responses in the Healthy Old, But Increased Latent CMV Carriage Is Related to Expanded CMV-Specific T-cells.* Front Immunol, 2017. 8: p. 733.
5. Derhovanessian, E., et al., *Infection with cytomegalovirus but not herpes simplex virus induces the accumulation of late-differentiated CD4+ and CD8+ T-cells in humans.* J Gen Virol, 2011. 92(Pt 12): p. 2746-56.
6. Solana, R., et al., *CMV and Immunosenescence: from basics to clinics.* Immun Ageing, 2012. 9(1): p. 23.
7. Wills, M., et al., *Report from the second cytomegalovirus and immunosenescence workshop.* Immun Ageing, 2011. 8(1): p. 10.
8. Dupont, L. and M.B. Reeves, *Cytomegalovirus latency and reactivation: recent insights into an age old problem.* Rev Med Virol, 2016. 26(2): p. 75-89.
9. van Boven, M., et al., *Infectious reactivation of cytomegalovirus explaining age- and sex-specific patterns of seroprevalence.* PLoS Comput Biol, 2017. 13(9): p. e1005719.
10. Stowe, R.P., et al., *Chronic herpesvirus reactivation occurs in aging.* Exp Gerontol, 2007. 42(6): p. 563-70.
11. Sylwester, A.W., et al., *Broadly targeted human cytomegalovirus-specific CD4+ and CD8+ T-cells dominate the memory compartments of exposed subjects.* J Exp Med, 2005. 202(5): p. 673-85.
12. van de Berg, P.J., et al., *A fingerprint left by cytomegalovirus infection in the human T-cell compartment.* J Clin Virol, 2008. 41(3): p. 213-7.
13. Cicin-Sain, L., *Cytomegalovirus memory inflation and immune protection.* Med Microbiol Immunol, 2019. 208(3-4): p. 339-347.
14. Klenerman, P., *The (gradual) rise of memory inflation.* Immunol Rev, 2018. 283(1): p. 99-112.
15. Welten, S.P.M., N.S. Baumann, and A. Oxenius, *Fuel and brake of memory T-cell inflation.* Med Microbiol Immunol, 2019. 208(3-4): p. 329-338.
16. Karrer, U., et al., *Memory inflation: continuous accumulation of antiviral CD8+ T-cells over time.* J Immunol, 2003. 170(4): p. 2022-9.
17. Holtappels, R., et al., *Enrichment of immediate-early 1 (m123/pp89) peptide-specific CD8 T-cells in a pulmonary CD62L(lo) memory-effector cell pool during latent murine cytomegalovirus infection of the lungs.* J Virol, 2000. 74(24): p. 11495-503.
18. Vescovini, R., et al., *Impact of Persistent Cytomegalovirus Infection on Dynamic Changes in Human Immune System Profile.* PLoS One, 2016. 11(3): p. e0151965.
19. Hosie, L., et al., *Cytomegalovirus-Specific T-cells Restricted by HLA-Cw*0702 Increase Markedly with Age and Dominate the CD8(+) T-Cell Repertoire in Older People.* Front Immunol, 2017. 8: p. 1776.
20. Klenerman, P. and A. Oxenius, *T-cell responses to cytomegalovirus.* Nat Rev Immunol, 2016.

21. Wallace, D.L., et al., *Human cytomegalovirus-specific CD8(+) T-cell expansions contain long-lived cells that retain functional capacity in both young and elderly subjects.* Immunology, 2011. 132(1): p. 27-38.

22. Westera, L., et al., *Lymphocyte maintenance during healthy aging requires no substantial alterations in cellular turnover.* Aging Cell, 2015. 14(2): p. 219-27.

23. Macaulay, R., A.N. Akbar, and S.M. Henson, *The role of the T-cell in age-related inflammation.* Age (Dordr), 2013. 35(3): p. 563-72.

24. Snyder, C.M., et al., *Memory inflation during chronic viral infection is maintained by continuous production of short-lived, functional T-cells.* Immunity, 2008. 29(4): p. 650-9.

25. Busch, R., et al., *Measurement of cell proliferation by heavy water labeling.* Nat Protoc, 2007. 2(12): p. 3045-57.

26. Patterson, B.W., G. Zhao, and S. Klein, *Improved accuracy and precision of gas chromatography/mass spectrometry measurements for metabolic tracers.* Metabolism, 1998. 47(6): p. 706-12.

27. Ahmed, R., et al., *Reconciling Estimates of Cell Proliferation from Stable Isotope Labeling Experiments.* PLoS Comput Biol, 2015. 11(10): p. e1004355.

28. Asquith, B., et al., *Lymphocyte kinetics: the interpretation of labelling data.* Trends Immunol, 2002. 23(12): p. 596-601.

29. Westera, L., et al., *Closing the gap between T-cell life span estimates from stable isotope-labeling studies in mice and humans.* Blood, 2013. 122(13): p. 2205-12.

30. Looney, R.J., et al., *Role of cytomegalovirus in the T-cell changes seen in elderly individuals.* Clin Immunol, 1999. 90(2): p. 213-9.

31. Di Benedetto, S., et al., *Impact of age, sex and CMV infection on peripheral T-cell phenotypes: results from the Berlin BASE-II Study.* Biogerontology, 2015.

32. Ruiz-Mateos, E., et al., *High levels of CD57+CD28- T-cells, low T-cell proliferation and preferential expansion of terminally differentiated CD4+ T-cells in HIV-elite controllers.* Curr HIV Res, 2010. 8(6): p. 471-81.

33. Strioga, M., V. Pasukoniene, and D. Characiejus, *CD8+ CD28- and CD8+ CD57+ T-cells and their role in health and disease.* Immunology, 2011. 134(1): p. 17-32.

34. Ahmed, R., et al., *Human Stem Cell-like Memory T-cells Are Maintained in a State of Dynamic Flux.* Cell Rep, 2016. 17(11): p. 2811-2818.

35. van den Berg, S.P.H., et al., *The hallmarks of CMV-specific CD8 T-cell differentiation.* Med Microbiol Immunol, 2019. 208(3-4): p. 365-373.

36. Pera, A., et al., *CD28(null) pro-atherogenic CD4 T-cells explain the link between CMV infection and an increased risk of cardiovascular death.* Theranostics, 2018. 8(16): p. 4509-4519.

37. Juno, J.A., et al., *Cytotoxic CD4 T-cells-Friend or Foe during Viral Infection?* Front Immunol, 2017. 8: p. 19.

38. Phetsouphanh, C., S. Pillai, and J.J. Zaunders, *Editorial: Cytotoxic CD4+ T-cells in Viral Infections.* Front Immunol, 2017. 8: p. 1729.

39. Akondy, R.S., et al., *Origin and differentiation of human memory CD8 T-cells after vaccination.* Nature, 2017. 552(7685): p. 362-367.

40. Derhovanessian, E., A. Larbi, and G. Pawelec, *Biomarkers of human immunosenescence: impact of Cytomegalovirus infection.* Curr Opin Immunol, 2009. 21(4): p. 440-5.

41. Pawelec, G.A., A. Caruso, C. Grubeck-Loebenstein, B. Solana, R. Wikby, A. , *Human immunosenescence: is it infectious?* Immunological Reviews, 2005. 205: p. 257–268.

42. Tu, W. and S. Rao, *Mechanisms Underlying T-cell Immunosenescence: Aging and Cytomegalovirus Infection.* Front Microbiol, 2016. 7: p. 2111.

43. Macallan, D.C., J.A. Borghans, and B. Asquith, *Human T-cell Memory: A Dynamic View.* Vaccines (Basel), 2017. 5(1).

44. Lang, A. and J. Nikolich-Zugich, *Functional CD8 T-cell memory responding to persistent latent infection is maintained for life.* J Immunol, 2011. 187(7): p. 3759-68.

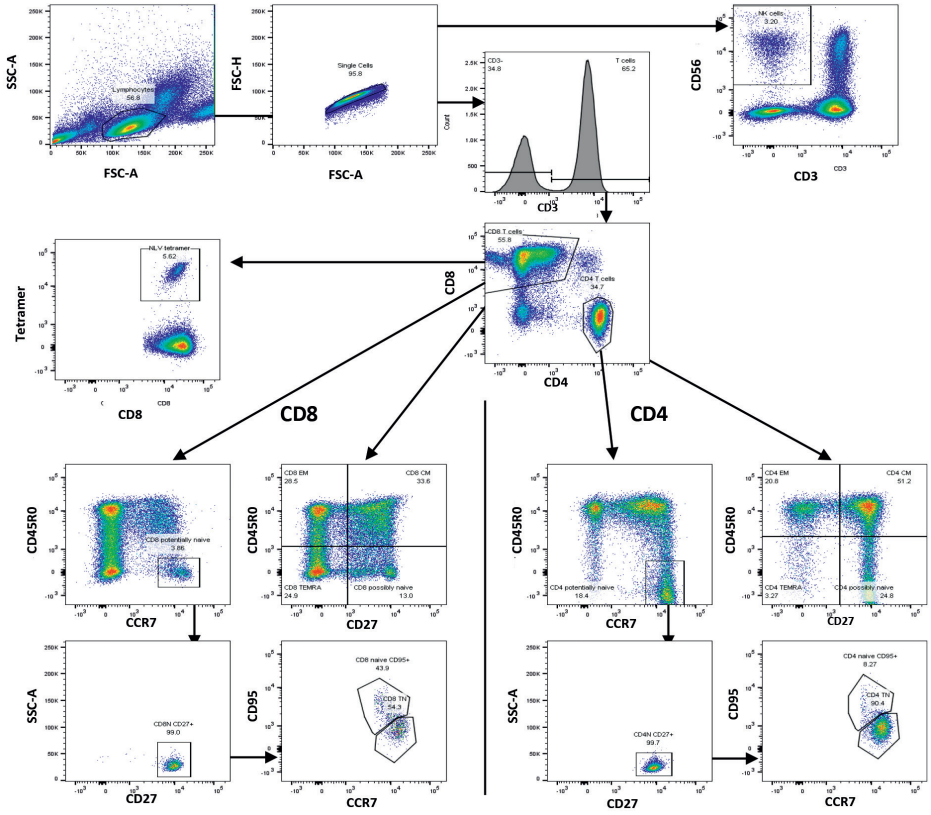
45. Pawelec, G., *Immunosenenescence: role of cytomegalovirus.* Exp Gerontol, 2014. 54: p. 1-5.

46. Olsson, J., et al., *Age-related change in peripheral blood T-lymphocyte subpopulations and cytomegalovirus infection in the very old: the Swedish longitudinal OCTO immune study.* Mech Ageing Dev, 2000. 121(1-3): p. 187-201.

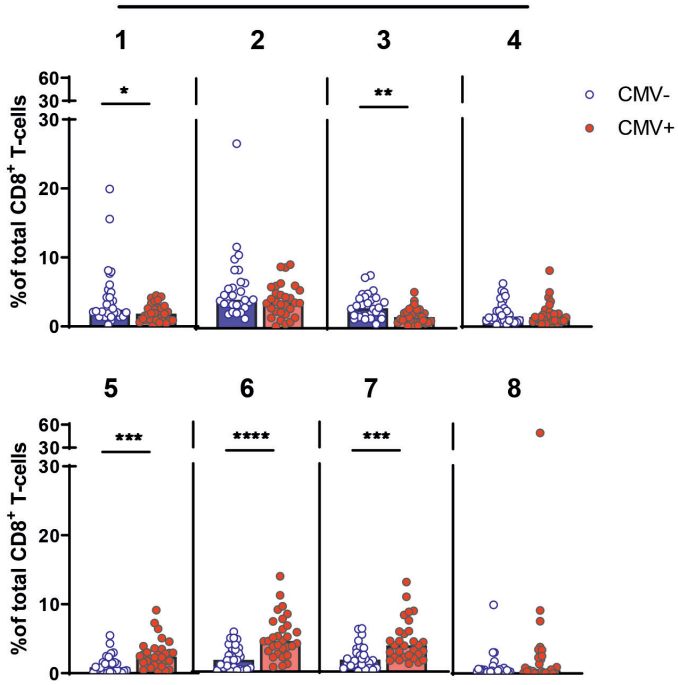
47. Wikby, A., et al., *Expansions of peripheral blood CD8 T-lymphocyte subpopulations and an association with cytomegalovirus seropositivity in the elderly: the Swedish NONA immune study.* Exp Gerontol, 2002. 37(2-3): p. 445-53.

Donor	Sex	Age	Log CMV IgG (MIA)	A1-VTE	A2-NLV	A3-KLG	A24-QYD	B7-RPH	B7-TPR	B8-ELR	B35-PE	Number of tetramer+ populations
				(Frequency of tetramer+ cells out of total CD8⁺ T-cells.)								
E02	Male	65.5	2.05	1.59	1.78	0	0	0	0	0	0	2
E04	*Male*	*68.8*	*2.27*	*13.83*	0	0	0	0	0	*15.25*	0	2
E07	Male	62.1	2.44	0	0.98	0	0	0	3.68	0	0	2
E08	*Male*	*66.6*	*2.28*	0	*4.43*	0	0	0	0	0.3	0	2
E09	Female	69.6	2.46	0	0	0	0	0	0	0	0	0
E17	Female	69.8	1.23	0	0	0	0	0	0	0	0	0
E20	Male	61.3	1.9	0	0	0.72	0	0	0	0	0	1
E22	Female	68.1	2.31	0	1.74	0	0	0	0	0	0	1
E24	Female	67.6	1.79	0	0	0	0	0.87	0.76	0	0	2
E26	Female	73.3	2.15	0	0	0	0	0	0	0	0	0
E27	Male	63.7	1.11	4.81	0	0	0	0	0	1.34	0	2
E28	Male	69.2	2.26	0	0	6.87	0	0	0	0	0	1
E31	Male	77.5	2.24	0	0	0	0	0	0	0	0	0
E32	Male	76.9	1.34	0	0	0	0	0	0	0	0	0
E34	*Male*	*67.7*	*2.16*	0	*5.9*	0	0	0	0	0	0	1
E38	Female	64.2	2.04	0	0.42	0	0	0	0	0	0	1
E39	*Male*	*66.6*	*2.09*	0	0	0	0	0.36	*11.25*	0	0	2
E42	Female	79.8	2.9	0.72	0	0	0	0	0	5.07	0	2
E43	Female	65.3	2.02	0	0.54	0	0	0	0	0	0	1
E44	Female	73.2	1.57	0	0	0	0	0	0	0	0	0
E45	Male	67.4	1.07	0	0	0	0	0	0	0	0	0
E48	Female	68.0	2.19	3.61	6.1	0	0	0	0	0	0	2
E49	Male	75.7	2.31	0	2	0	0	0	0	0	0	1
E51	Female	68.3	2.12	0	0	3.15	0	0	0	0	0	1
E53	Male	69.3	1.98	0	0	0	0	0	0	0	0	0
E54	Female	74.9	2.06	0	2.39	0	0	0	0	0	0	1
E55	Female	72.7	1.95	3.28	0	0	0	0	0	0	0	1
E56	Male	69.1	1.5	0	0.19	0	0	0	0	0	0	1
E57	Male	67.8	2.39	0	0.99	0	0	0	0	0	0	1
E58	Male	63.2	2.19	1.37	0	0	0	0	0	0	0	1
E59	*Female*	*76.0*	*3.51*	0	0	0	0	0.81	*15.2*	0	0	2
E60	Male	61.0	2.05	0	0	0	0	0	0	0	0	0
											Total tetramer+ CMV-specific CD8+ T-cell populations	33
											Total number of CMV+ participants	32

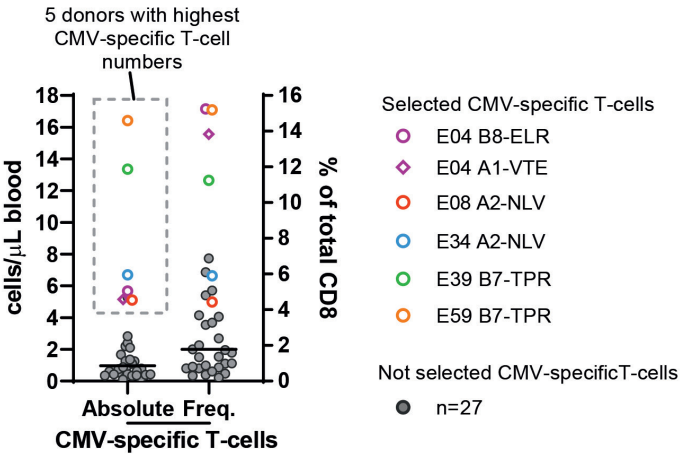
Supplementary Table 1. Cases of CMV-specific CD8+ T-cell tetramer stainings
In italics the selected individuals and the selected tetramer+ CMV-specific CD8⁺ T-cell populations for the deuterated water labelling study.



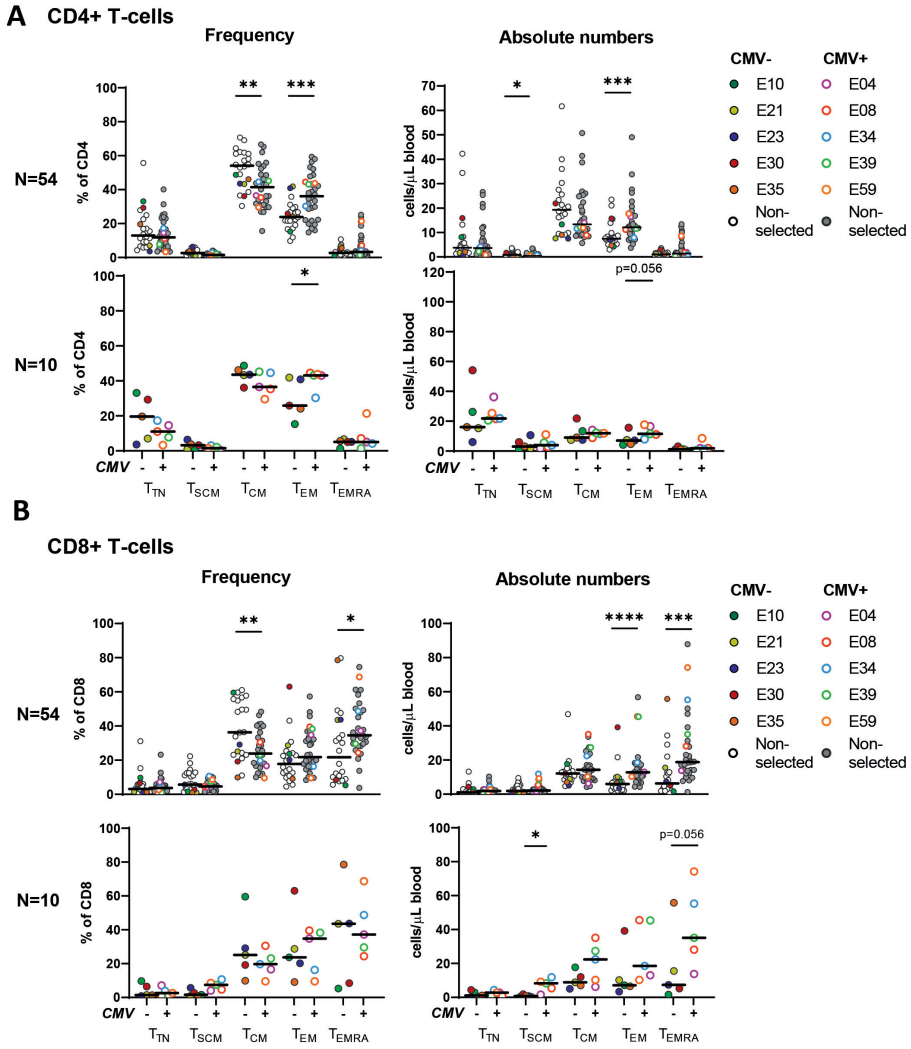
Supplementary Figure 1. Gating strategy for sorting of different T-cell populations. Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Paque PLUS density centrifugation and stained for CD3, CD56, CD4, CD8, CD45RO, CCR7, CD27, and CD95 (see **Materials and methods** for antibody specificities). Live lymphocytes were gated based on SSC-A/FSC-A and FSC-H/FSC-A plots. T-cells were subsequently defined as $CD3^+CD56^-$. T-cell subpopulations were defined as follows: truly naive, T_{TN} ($CCR7^+CD45RO^-CD27^+CD95^+$), central memory, T_{CM} ($CD45RO^+CD27^+$), effector memory, T_{EM} ($CD27^-CD45RO^+$) and effector memory re-expressing RA, T_{EMRA} ($CD27^-CD45RO^-$).



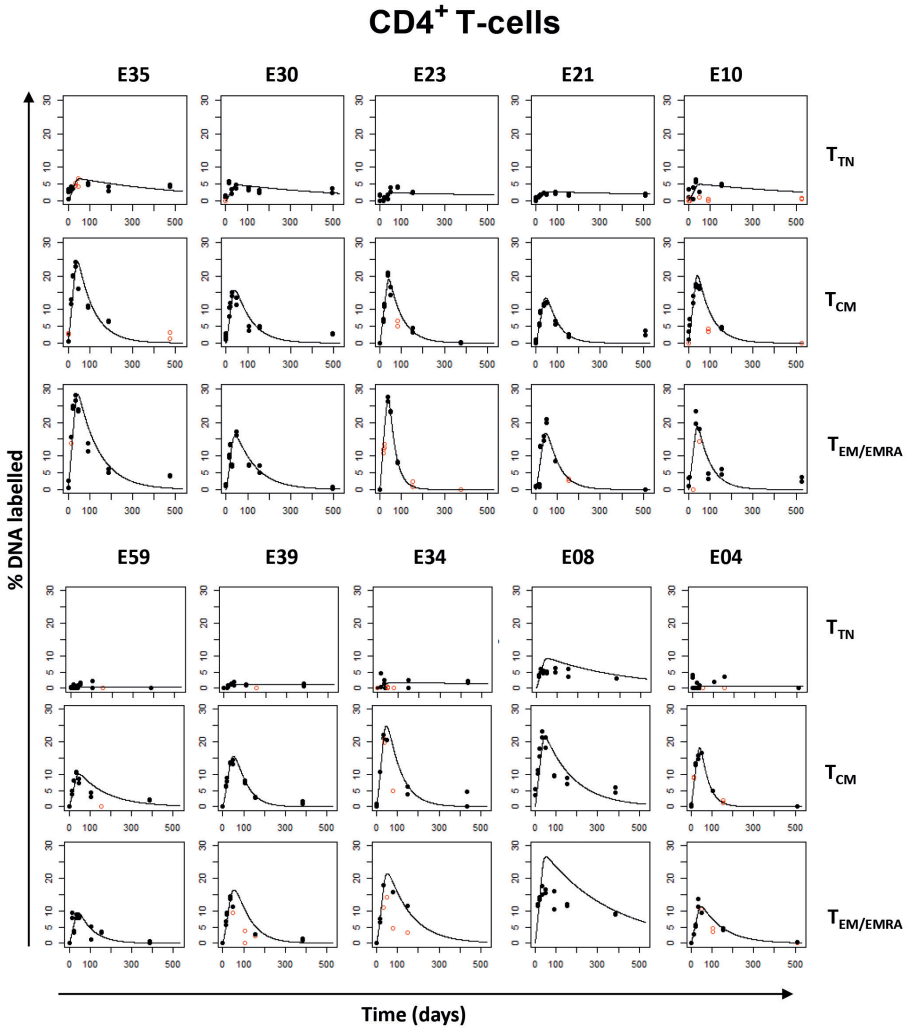
Supplementary Figure 2. Percentage of CD8⁺ T-cells per cluster in the t-SNE analysis. CD8⁺ T-cells of CMV- and CMV+ individuals were clustered by t-SNE analysis based on the expression of CD57, KLRG-1, CD127, CD27, CD45RO, CCR7, and CD95 (see **Figure 1E**). Nine clusters were identified within the t-SNE plot were identified based on cell density. Here the percentage of CD8⁺ T-cells that fall into each of these clusters out of total CD8⁺ T-cells is shown for CMV- and CMV+ individuals. Differences between CMV- and CMV+ individuals were tested by Mann-Whitney *U* test. Stars indicate significant differences as follows: * *P*-value <0.05, ** *P*-value <0.01, *** *P*-value <0.001, *** *P*-value <0.0001.



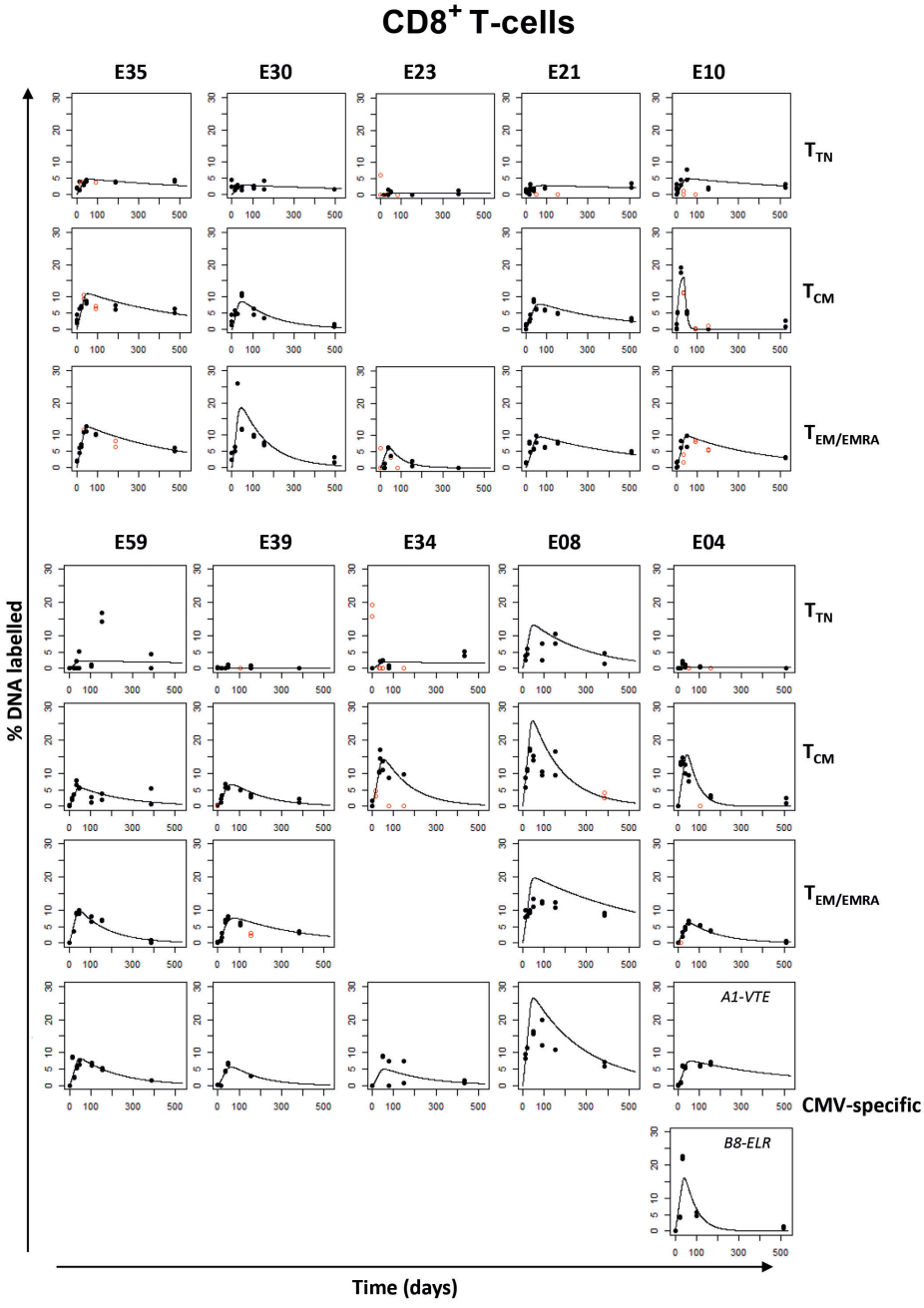
Supplementary Figure 3. Number and frequency of CMV-specific T-cells. Absolute numbers (in cells/µL) and frequency (in % of total CD8⁺ T-cells) of CMV-specific CD8⁺ T-cells are shown for all 32 CMV+ individuals. For the heavy water labelling study, five CMV+ participants were selected based on highest absolute CMV-specific T-cell numbers (shown with individual symbol). CMV+ participants that were not selected are shown in grey.



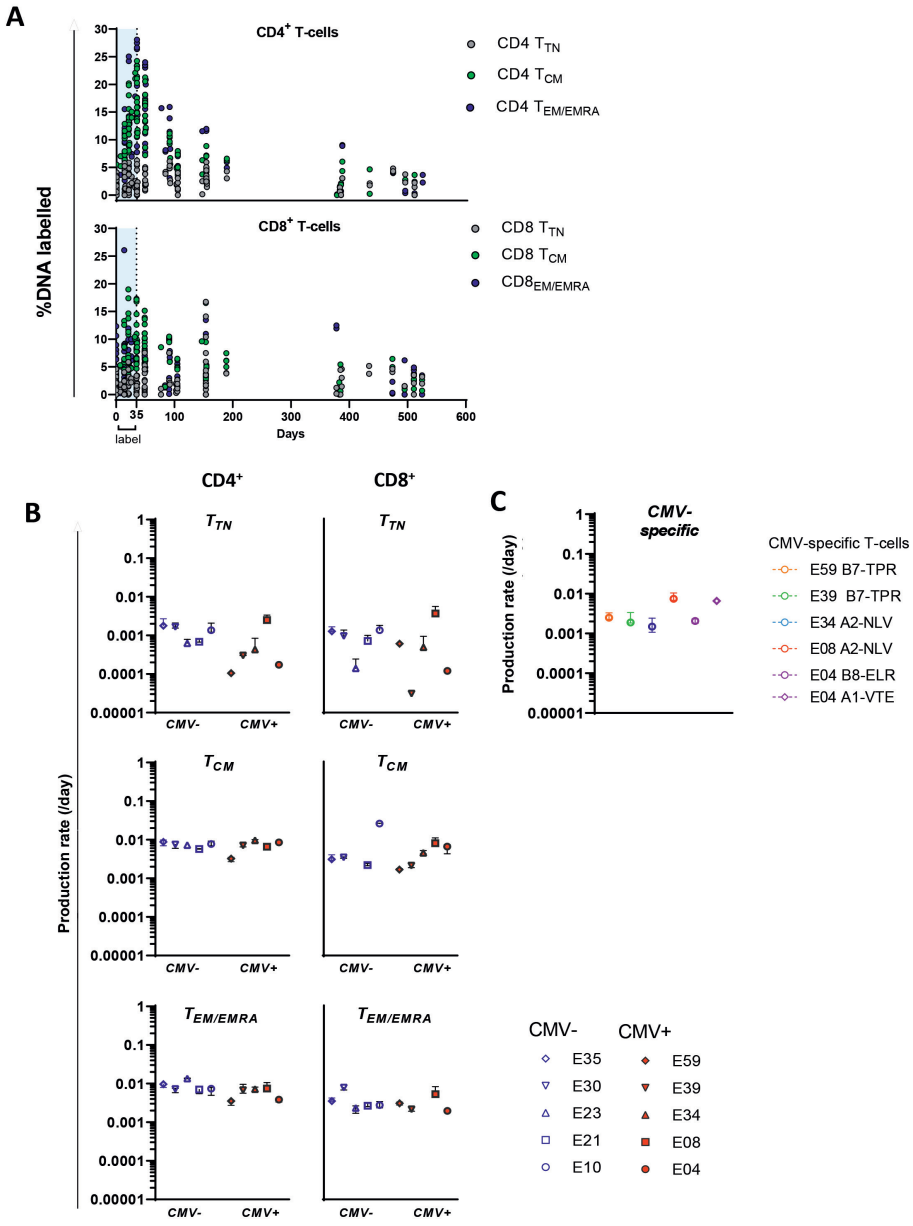
Supplementary Figure 4. Percentages of T-cells in all participants and in the ten selected for the heavy water labelling study. A&B. The frequency (left panels) and absolute numbers (right panels) of different CD4⁺ (**A**) or CD8⁺ (**B**) T-cell populations. In the top panels of both **A** and **B** all participants are shown, and in the lower panels only those selected for the heavy water labelling study, highlighted with a unique color. Differences between CMV- and CMV+ individuals were tested by Mann-Whitney *U* test. Stars indicate significant differences as follows: * *P*-value <0.05, ** *P*-value <0.01, *** *P*-value <0.001, *** *P*-value <0.0001.



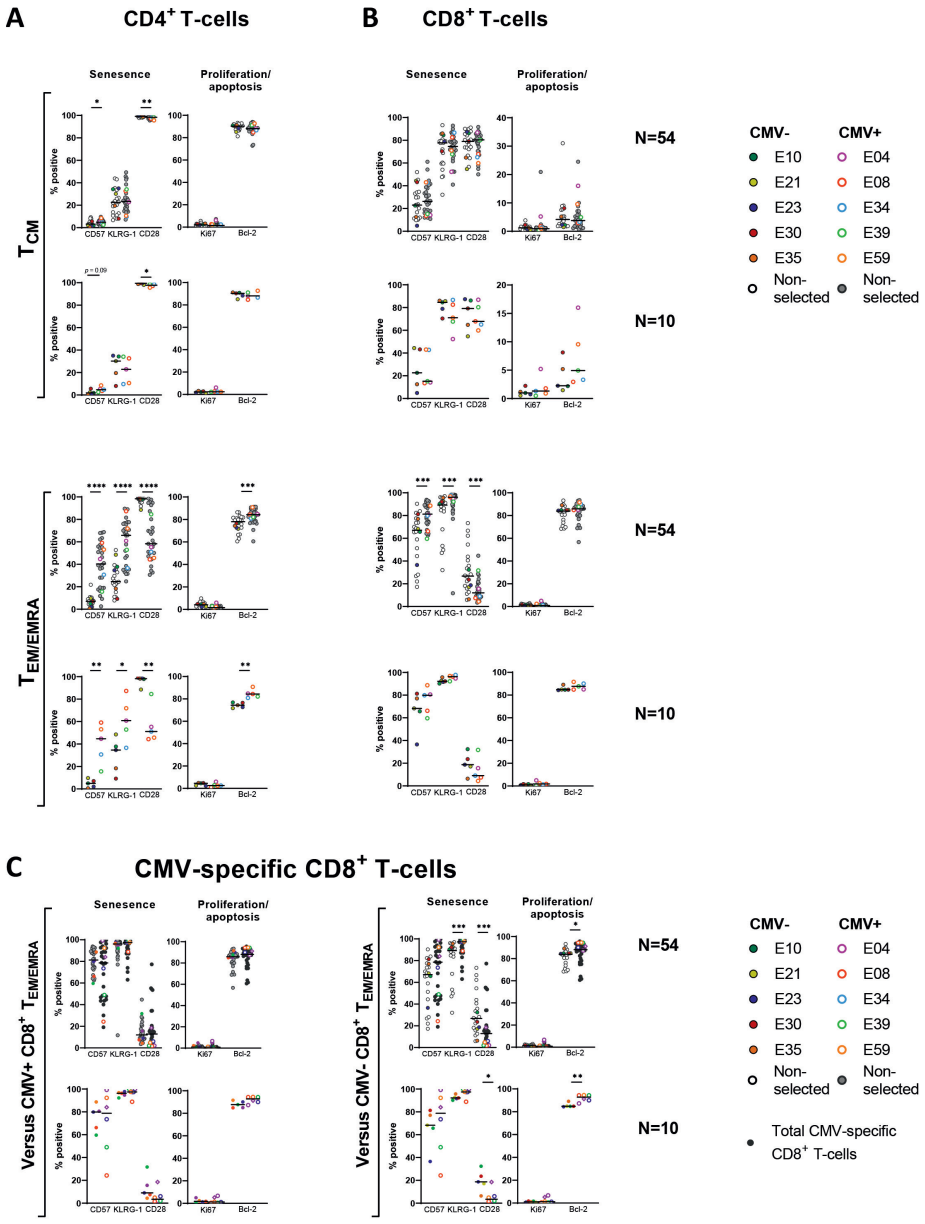
Supplementary Figure 5. Fits of mathematical model to the deuterium enrichment data of CD4⁺ T-cells. Best fits of the kinetic heterogeneity model to the enrichment in true naïve (T_{TN}), central memory (T_{CM}), and effector memory ($T_{EM/EMRA}$) CD4⁺ T-cells. Label enrichment in the DNA was scaled between 0 and 100% by normalizing for the maximum enrichment in granulocytes (see **Materials and methods**). The measurements that were out of range of the standards are indicated in red open symbols, those in range are indicated in black closed symbols.



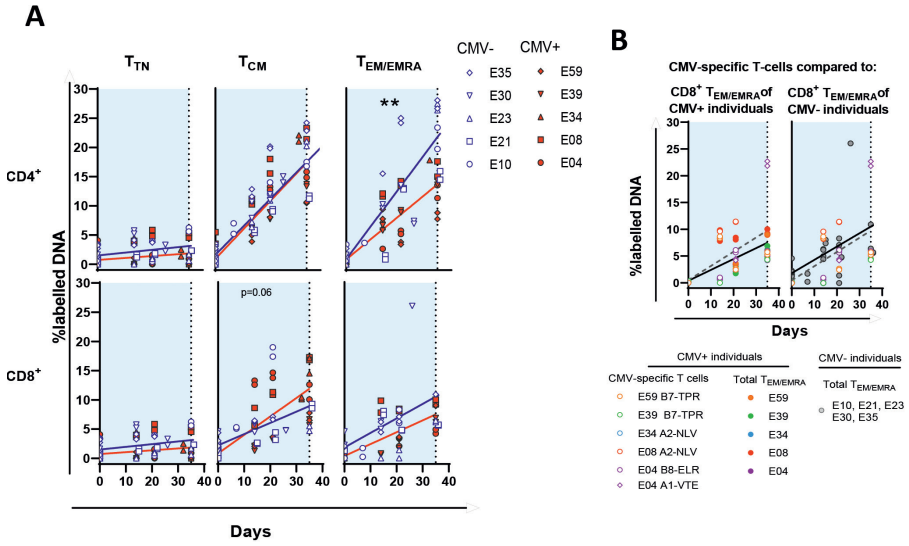
Supplementary Figure 6. Fits of mathematical model to the deuterium enrichment data of CD8⁺ T-cells. Best fits of the kinetic heterogeneity model to the enrichment in true naïve (T_{TN}), central memory (T_{CM}), effector memory ($T_{EM/EMRA}$), and CMV-specific CD8⁺ T-cells. Label enrichment in the DNA was scaled between 0 and 100% by normalizing for the maximum enrichment in granulocytes (see **Materials and methods**). The measurements that were out of range of the standards are indicated in red open symbols, those in range are indicated in black closed symbols.



Supplementary Figure 7. Dynamics of different T-cell subpopulations and estimated production rates including confidence intervals. A. Combined deuterium labelling enrichment (%DNA labelled) of different T-cell subpopulations for all individuals, both CMV+ and CMV-. Label enrichment was scaled between 0 and 100% by normalizing for the estimated maximum enrichment of granulocytes (see **Materials and methods**). **B.** Summary of estimated production rates with confidence intervals of T_{TN}, T_{CM}, and $T_{EM/EMRA}$ $CD4^+$ and $CD8^+$ T-cells in CMV- (blue symbols) and CMV+ (red symbols) individuals. Data from each individual are represented by unique symbols. **C.** Summary of estimated production rates with confidence intervals of CMV-specific $CD8^+$ T-cells. All estimates (both from **B** and **C**) were obtained by fitting a single-exponential model to the data sets per individual (see **Materials and methods** and **Supplementary Figure 5**.



Supplementary Figure 8. Percentages of senescence, proliferation, and apoptosis markers in all participants and the ten selected for the heavy water labelling study. A,B. The percentage of T_{CM} and $T_{EM/EMRA}$ positive for CD57, KLRG-1, CD28, Ki-67, and Bcl-2 are shown for $CD4^+$ (**A**) or $CD8^+$ (**B**) T-cell populations. In the top panels of both **A** and **B** all participants are shown, and in the lower panels only those selected for the heavy water labelling study, highlighted with a unique color. **C.** The expression of CD57, KLRG-1, CD28, Ki-67, and Bcl-2 on CMV-specific $CD8^+$ T-cells is compared to the expression on bulk $T_{EM/EMRA}$ $CD8^+$ T-cells from CMV+ individuals. Differences between CMV- and CMV+ individuals were tested by Mann-Whitney *U* test. Stars indicate significant differences as follows: * *P*-value <0.05, ** *P*-value <0.01, *** *P*-value <0.001, *** *P*-value <0.0001.



Supplementary Figure 9. Simple linear regression on the uplabelling data from the heavy water labelling study. A,B. The combined deuterium labelling enrichment datapoints (%DNA labelled) from all CMV+ and from all CMV- participants during the uplabelling phase were fitted to a simple linear regression line for T_{TN}, T_{CM}, and $T_{EM/EMRA}$ CD4+ and CD8+ T-cells (**A**) and CMV-specific CD8+ T-cells (**B**). Label enrichment was scaled between 0 and 100% by normalizing for the estimated maximum enrichment of granulocytes (see **Materials and methods**).

7

Limited effect of duration of CMV infection on adaptive immunity and frailty: insights from a 27-year long longitudinal study

2020 Clinical and Translational Immunology, Accepted for publication

L.D. Samson[1,2,3]*, S.P.H. van den Berg[1,4]*, P. Engelfriet[2], A.M.H. Boots[3], M. Hendriks, L.G.H. de Rond , M. de Zeeuw-Brouwer, M. Verschuren[2], J.A.M. Borghans[4], A. Buisman[1#], D. van Baarle[1,4#]

* first authors contributed equally

# last authors contributed equally

1 Center for Infectious Disease Control, National Institute for Public Health and the Environment, The Netherlands

2 Center for Nutrition, Prevention and Health Services, National Institute for Public Health and the Environment

3 Department of Rheumatology and Clinical Immunology, University of Groningen, University Medical Center Groningen, The Netherlands

4 Center for Translational Immunology, University Medical Center Utrecht, The Netherlands.

ABSTRACT

CMV infection is thought to affect the immune system and to impact general health and specifically the prevalence of cardiovascular disease. These effects are believed to accumulate over time. The increased CMV-specific antibody levels observed in elderly are generally assumed to reflect experienced viral reactivation during life. Furthermore, high levels of terminally-differentiated T-cells and large expansions of CMV-specific T-cells are hallmarks of CMV infection, which are thought to increase over time, a process also referred to as memory inflation. We had the unique opportunity to study CMV-specific antibody levels over ~27 years in 268 individuals (aged 60-87 years at endpoint), and to link CMV-duration to T-cell numbers, CMV-specific T-cell functions, frailty and cardiovascular disease at endpoint. In our study, 136/268 individuals were long-term CMV-seropositive and 19 persons seroconverted during follow-up (seroconversion rate: 0.56%/year). Although CMV-specific antibody levels increased slightly over time, differences in CMV-specific antibody levels at endpoint were hardly explained by CMV-duration. CMV-duration was not related to the size and function of the memory T-cell pool at endpoint. Elevated levels of CMV-specific antibodies were associated with cardiovascular disease, but not with frailty. Age at CMV-seroconversion was positively associated with CMV-specific antibody levels, memory $CD4^+$ T-cell numbers and frailty. In conclusion, CMV-specific memory T-cells develop shortly after CMV infection but do not seem to further increase over time. Age-related effects other than duration of CMV infection seem to contribute to CMV-induced changes in the immune system. Although CMV-specific immunity is not linked to frailty, it might be related to increased prevalence of cardiovascular disease.

INTRODUCTION

Primary infection with CMV results in life-long latency, but only leads to severe disease and viral dissemination in severely immunocompromised individuals. For example as individuals use immunosuppressive medication after organ transplantation or individuals with advanced and uncontrolled human immunodeficiency virus (HIV) infection. In immunocompetent individuals, CMV infection usually remains asymptomatic due responses by the humoral and cellular immune system [1-3]. In immunocompetent individuals, the virus is thought to reactivate and to activate the immune system regularly [1-3] which may explain the changes in the T-cell compartment observed with CMV infection [4].

CMV-specific antibody levels have frequently been reported to be positively associated with age [5-7]. CMV latent viral load as measured by CMV-infected monocytes [7] or whole cells [8] as well as CMV viral load in plasma [8] and urine [2] have also been shown to be higher in older adults, and are associated with CMV-specific antibody levels. CMV-specific antibody levels are often used as a measure for experienced CMV reactivation and to identify CMV-seropositive elderly with poor control of the virus, who are at increased risk of adverse clinical outcomes [9-15]. Since most studies rely on cross-sectional data, the underlying factors influencing CMV-specific antibody levels, and in particular the role of duration of CMV infection, in fact remain unknown.

Many parallels can be drawn between the well-described changes in the T-cell pool caused by CMV infection and those observed during ageing [16, 17]. Effects of CMV on the T-cell compartment [18] are in large part thought to be due to the presence of large CMV-specific T-cell expansions, which can mount up to 30% to 90% [19, 20] of the CD8+ T-cell pool in many elderly [21]. CMV-specific CD8+ memory T-cell numbers are assumed to increase over time, a process that is often referred to as 'memory inflation.' Memory inflation is unique for CMV [22, 23] and is believed to be most prominent for CD8+ T-cells [21, 23, 24] but also observed for CD4+ T-cells [22]. However, how prolonged exposure to a chronic virus-infection like CMV enhances immune reactivity, is not well understood. Further insight in CMV-induced memory inflation is valuable in understanding the potential harmful effect of CMV upon ageing.

The accumulating changes in the immune system due to prolonged exposure to CMV infection may eventually influence general health. CMV-infected individuals have been reported to have a higher prevalence of age-related conditions such as rheumatoid arthritis [25] and cardiovascular diseases [14]. Also mortality rates were higher in CMV infected individuals [26, 27] and in individuals with higher CMV-specific antibody levels [11, 15, 27]. The mechanisms underlying the relationships between CMV infection and general health outcomes are still unknown. Although several studies show a relation between CMV infection or CMV-specific antibody levels and frailty [11, 28, 29], others do not support this [30] [31]. These reported conflicting results might be explained by differences in duration of CMV infection, which is generally unknown.

Here we used a unique long-term longitudinal design to study the effect of duration of CMV infection on the immune system and on general health. We assessed how CMV-specific antibody levels developed within a cohort of older individuals (60-89 years at endpoint) over a follow-up time of 25-30 years. We relate duration of CMV infection to CMV-specific antibody levels, numbers of various T-cell subsets, function of CMV-specific T-cells, frailty and prevalence of cardiovascular disease.

EXPERIMENTAL PROCEDURES

Study design

This study was performed with a selected group of participants from the longitudinal Doetinchem Cohort Study (DCS) [32, 33], which we refer to as the DCS subcohort (n=289). Individuals have been followed as part of the DCS since 1987, with assessments every five years, resulting in six measurement rounds (1987-1992, 1993-1997, 1998-2002, 2003-2007, 2008-2012, 2013-2017). Every DCS measurement round, blood plasma samples were taken and stored for later use. The selection of the DCS subcohort from the DCS was described in more detail elsewhere [34]. Briefly, all active DCS participants 60-87 years of age (*n=289*) were randomly stratified by sex, age, and a frailty index score (see below), leading to a selection of equal numbers of men and women, distributed evenly over the included age range and over three frailty groups (healthy, intermediate, frail). The DCS subcohort participants were invited for an additional blood sample between October 2016 and March 2017, which was not only used to retrieve plasma, but also to perform immune cell phenotyping on fresh whole blood and to store PBMCs for later use (**Figure 1A**). Furthermore, at round 5 and round 6, the participants' general health was determined by a frailty index, which has been validated based on 36 deficits [34]. For most individuals the additional blood sample (round 7) taken for the DCS subcohort was the end point of study, for some (n=55) end point sampling was some months later (round 6).This study was approved by the Medical Ethics Committee of the University Medical Center Utrecht. All participants gave written informed consent for every DCS round and for this DCS subcohort study separately.

Exclusion criteria

From the original 289 participants, individuals were excluded from the longitudinal analyses if their follow-up time was less than 25 years (n=17) (**Supplementary Figure 1**), or because of an inconclusive CMV-serostatus (n=4). Thus, the results are based on 268 individuals with an average follow up time of 27.7 years (minimum of 25, maximum of 30 years).

Frailty index

A frailty index as previously explained (Samson et al 2019) is based on 36 "health deficits". The concept of this index was based on previous studies [30, 35-37] and was adapted for and validated in the DCS. The index is a variable with values between zero and one, zero representing the 'best' and one representing the 'worst' health status. The frailty index was calculated for each individual based on the data collected during the DCS measurements of

round 5 and round 6 (missing n=1 for round 5 and n=12 for round 6). Increase in frailty index was defined as the difference between the frailty indices assessed in rounds 5 and 6.

Cytomegalovirus (CMV)-specific antibodies

CMV-specific IgG antibody levels were measured in plasma by a multiplex immunoassay developed in-house [38]. A cutoff of 5 arbitrary units mL^{-1} was shown to discriminate best between CMV- and CMV+ study groups [39]. To decrease the chance of false-positive or false-negative results, samples with antibody levels <= 4 mL^{-1} were defined as CMV-seronegative (CMV-) and those with antibody levels >7.5) mL^{-1} CMV-seropositive (CMV+), while all levels in between were considered inconclusive. To reduce intra-assay variation, all samples from the same individual were measured on the same plate

Cell numbers by flow cytometry

Fresh whole blood samples collected in 2016-2017 were used to quantify cell numbers of T-cell subpopulations by flow cytometry, as previously described in more detail [39]. Briefly, absolute cell numbers were determined using TruCOUNT tubes (BD Biosciences, San Jose, CA, USA), in which whole blood was stained with CD3(UCHT1)-BV711 (BD Biosciences). After erythrocyte lysis with FACS Lysing Solution (BD Biosciences), tubes were measured directly on a flow cytometer (FortessaX20). Another tube was used with the following antibodies: CD3(UCHT1)-BV711, CD8(SK1)-APC-H7, CCR7(150503)-PECF594, CD27(M-T271)-BV421, CD28(CD28.2)-PerCPCy5.5 (all BD Biosciences), CD4(RPA-T4)-BV510, CD45RA(HI100)-BV650 (all Biolegend). Using the bead count of the TruCOUNT tube and the CD3 cell count of both tubes absolute cell numbers were calculated. T-cell subsets of naive (T_N), central memory (T_{CM}), effector memory (T_{EM}) and effector memory re-expressing CD45RA (T_{EMRA}) were defined based on the expression of CD45RA and CCR7, after which T_{EM} and T_{EMRA} cells were categorized as early, intermediate and late differentiated based on the expression of CD27 and CD28 [40].

PBMC isolation

Peripheral blood mononuclear cells from the DCS subcohort were obtained by Lymphoprep (Progen) density gradient centrifugation from heparinized blood, according to the manufacturer's instructions. After isolation of the PBMCs, the cells were washed with PBS medium containing 0.2% FCS (Sigma), and then frozen in a solution with 90% FCS and 10% dimethyl sulfoxide at -135°C until further use.

CMV-specific functional T-cell analysis

CMV-specific T-cell responses were analyzed for a selected subpopulation (n=30): first, ten CMV+ individuals were selected with the shortest duration of CMV infection for which PBMCs were available. Ten CMV- individuals were subsequently selected based on matched age and sex only, and ten CMV+ individuals with long term CMV infection also based on CMV-specific antibody levels. Cryopreserved PBMCs were rapidly thawed at 37 °C and washed once in AIM-V (Gibco) 2% human AB serum medium (AIM-V 2% hAB). The cells

were then resuspended in AIM-V 2% hAB and rested at 37 °C at 5% CO2 for 30 min before they were dispended in 10^6 PBMCs/150ul per well in a 96 wells plate with stimulation, anti-CD107a PerCP Cy5.5 and Monensin (1:1500) (BD bioscience, GolgiStop) and Brefaldin A (1:1000) (BD bioscience, GolgPlug). Stimulation for CMV-specific responses was done using one of the CMV overlapping peptide pools (15-mers with 11 overlap) of UL55 (1μg/ml) (JPT peptides), IE-1 (1μg/ml) (JPT peptides), or pp65 (1μg/ml) (JPT peptides). Medium was used as negative control. Following 6-hour incubation, cells were washed once and stained with the extracellular markers Fixable Viability Staining-780 (Thermofisher), CD3(UCHT1)-FITC, CD8+(RPA-T8)-BV510, CD4+(SK3)-BUV737 (BD bioscience), CD27(O323)-BV785 (Biolegend), CD45RO(UCHL1)-BUV395 (BD bioscience) and CD107a(H4A3)-PerCP Cy5.5. Next, cells were washed once in FACS buffer and twice in perm/wash buffer (fix/perm kit BD, diluted 10x in MilliQ H_2O). Intracellular staining was performed with IFN-γ(4S.B3)-PE-Cy7 (Thermofisher), IL-2(MQ1-17H12)-BV650 (BD bioscience), TNF-a(MAb11)-BV711 (Biolegend), Perforin(B-D48)-BV421, MIP-1β(D21-1351)-AlexaFluor700 (BD bioscience)and Granzyme B(GB11)-PE-CF594 (BD bioscience). Cells were subsequently washed three times and analyzed by flow cytometry (on an LSRII Fortessa). Sum of the three CMV peptides pools minus the negative control medium is calculated.

Quantification of CMV-specific T-cells

CMV-specific T-cells were stained using HLA-class A2 tetramers specific for the NLV epitope of the CMV-pp65 protein in the HLA-A2+, CMV+ individuals of the selected sub-population for CMV-specific functional T-cell analysis (n=30). First, HLA-A2 staining by HLA-A2(BB7.2)-V450 (BD Bioscience) was performed on the CMV+ individuals (n=20) to select all HLA-A2 + individuals. For the HLA-A2+ individuals (12/20) , tetramer staining was performed for 15 minutes at room temperature with CMV-(A*0201/NLVPMVATV)-APC (Immudex) and next extracellular staining was performed for 20 minutes at 4 °C with Fixable Viability Staining-780 (APC-Cy7) (Thermofisher), CD3 APC-R700(SK7)-AF700(BD bioscience), CD8+(RPA-T8)-BrilliantViolet510 (Biolegend), CD45RO+(UCHL1)-BrilliantViolet711 (Biolegend), CD27(O323)-BrilliantViolet786 (Biolegend), CCR7(150503)-BrilliantUV395(BD bioscience), KLRG-1(13F12F2)-PE-Cy7 (eBioscience), PD-1(EH12.2H7)-PerCP Cy5.5 (Biolegend). Cells were measured by flow cytometry (on an LSRII Fortessa).

Statistical analysis

Statistics on CMV-specific antibody levels were performed by parametric testing on log-transformed data. Comparison within individuals between two time points was done by paired testing and comparison among different groups by non-paired testing (paired t-test and independent Student's t-test or Mann Whitney test for non-normally distributed data). Associations between continuous variables were tested by Pearson's or Spearman's correlation depending on the distribution of the data. Seroconversion rate was calculated by dividing the number of seroconversion cases by the sum of the total timespan until seroconversion or until end of follow up of all people who were seronegative at baseline. Sex differences in seroconversion rate were tested using a chi-square test. To estimate the

change in CMV-specific antibody levels over a longer period of time within individuals, we used a median based linear model, the Theil-Sen estimator [41-43] with the R package 'mblm' (https://CRAN.R-project.org/package=mblm}). The slopes of the log-transformed antibody levels with age between all repeated measurements (at least 3 CMV+ time points) were calculated per individual for calculating the median of the slopes (representing the Theil-Sen estimator). This model is less affected by outliers or by skewed distributions than ordinary linear regression estimators. To estimate which variables are important predictors of CMV-specific antibody levels at endpoint, we performed a random forest prediction analysis with CMV-specific antibody levels at endpoint as dependent variable, using the random Forest R package [44]. The proportion of explained variance was calculated to estimate the prediction accuracy. The importance of the variables to predict CMV-specific antibody levels at endpoint, was shown a variable importance plot. Differences in T-cell subsets between the groups (CMV-, ST CMV+ and LT CMV+) were tested by one-way ANOVA and two groups comparisons were made using Tukey's multiple comparison test. Frailty index differences were compared by non-parametric testing, Spearman correlation for comparing continuous variables, a Kruskal-Wallis test for comparison between groups, and post-hoc analysis for comparison between multiple groups by Tukey correction. Data were analyzed using SPSS statistics 22 for Windows (SPSS Inc., Chicago, IL, USA) and R 3.6.0 (https://www.rproject.org/).

		Serostatus			Duration of CMV infection		
	Total (n = 268)	**CMV$^-$ (n = 113)**	**CMV$^+$ (n = 155)**	***P*-value***	**ST CMV$^+$ (n = 19)**	**LT CMV$^+$ (n = 136)**	***P*-value***
Age							
Age at baseline (years)	43.3 (6.6)	43.9 (7.1)	42.9 (6.2)	0.38	42.2 (7.5)	43 (6)	0.510
Age at endpoint (years)	70.9 (6.7)	71.7 (7.2)	70.6 (6.2)	0.32	70.4 (7.5)	70.6 (6.1)	0.780
Follow-up duration							
Total follow-up timespan (years)	27.7 (1.2)	27.7 (1.2)	27.7 (1.1)	0.61	28.2 (1.3)	27.6 (1.1)	0.030
Sex							
Women, % (n)	49.6 (133)	43.4 (49)	54.2 (84)	0.08	73.7 (14)	51.5 (70)	0.069
Education							
Low, % (n)	44 (118)	41.6 (47)	45.8 (71)	0.78	47.4 (9)	45.6 (62)	0.410
Middle, % (n)	27.6 (74)	28.3 (32)	27.1 (42)		36.8 (7)	25.7 (35)	
High, % (n)	28.4 (76)	30.1 (34)	27.1 (42)		15.8 (3)	28.7 (39)	
CMV serostatus							
CMV at baseline, % (n)	50.7 (136)						
CMV at endpoint, % (n)	57.8 (155)						

Note: Age and follow-up duration are summarized by mean (SD).

* Tested between serostatus groups

Table 1: Summary statistics of the study population

RESULTS

Characteristics of the study population

In total, 268 participants were included in this study (n=135 men, n=133 women), with an average age of 70.6 (60.3 – 88.6) years at end point (**Table 1**). Follow-up time was 27.7 years on

average (range 25-30 years). All individuals were analyzed for the presence of CMV-specific antibodies. At endpoint, 58% of the participants (n=155) were CMV-seropositive (**Table 1 and Supplementary Figure 1**). No significant differences in age, educational level and sex were observed between CMV-seronegative (CMV-) and CMV-seropositive (CMV+) individuals (**Table 1**). Follow-up time was similar for CMV- and CMV+ participants.

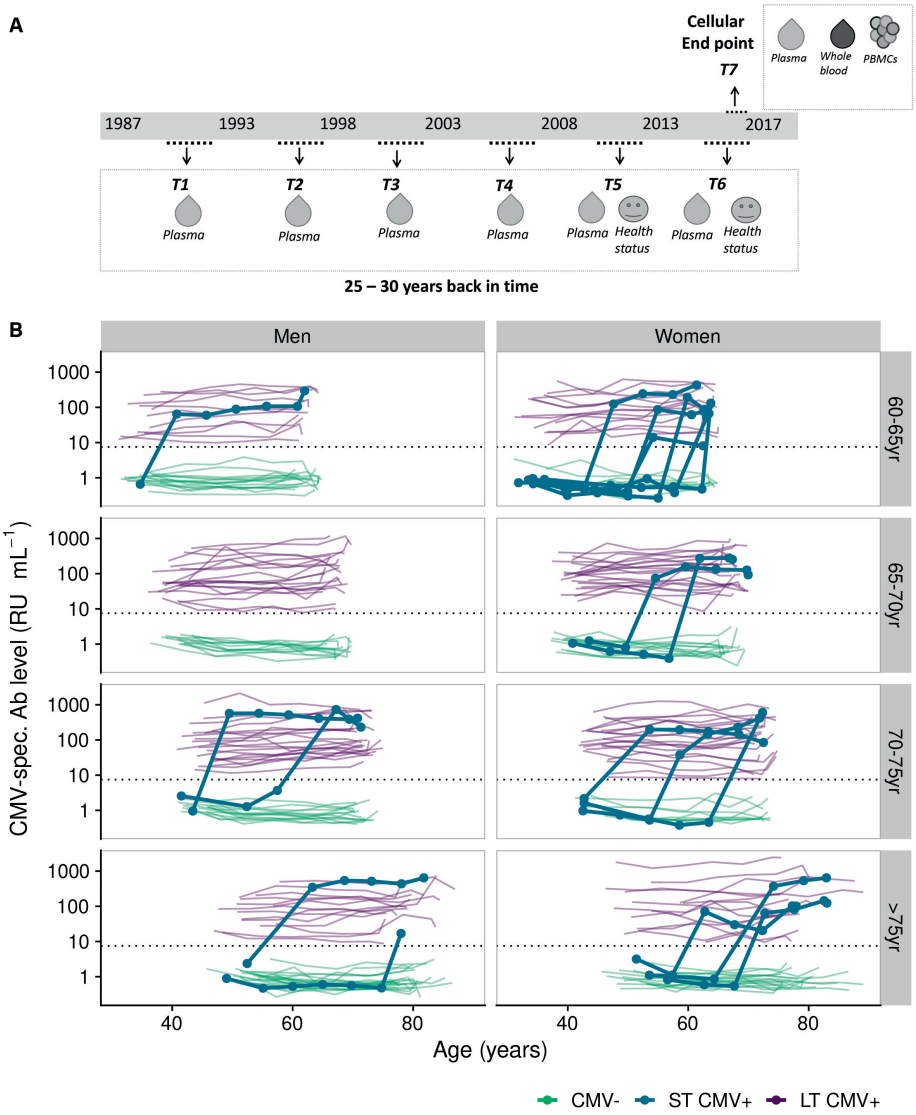


Figure 1. Overview of the CMV antibody levels of all participants during time. **A.** Study design. Participants donated blood 6 times in ~27 years. In 2016-2017, an extra sample was taken for extensive phenotyping of leukocytes subsets. **B.** CMV antibody levels were measured every 5-years and presented per age category at endpoint separately for men and woman. Green lines show the antibody trajectories of CMV- participants, purple lines those of LT CMV+ participants and the bold blue lines those the participants that seroconverted during follow-up (ST CMV+ participants). Dotted horizontal line shows the cutoff value for seropositivity.

The majority of CMV+ individuals were CMV+ during entire follow-up

The duration of CMV infection in the 155 individuals who were CMV+ at endpoint was determined by measuring CMV-specific antibody levels in the preceding 25 years. The vast majority (85.9%, n=136) of these participants had been CMV+ during the entire follow-up, and are therefore referred to as long-term CMV+ (LT CMV+). The other CMV+ participants (n=19, of which 14 were women and 5 men) seroconverted during follow-up and are referred to as short-term CMV+ (ST CMV+) (**Figure 1B**, **Supplementary Figure 2A**). An overview of the CMV-specific antibody levels over time of all CMV+ participants per 5 years age category, for men and women separately, is shown in **Figure 1B**. CMV-seroconverters were seen in all age categories and showed a sharp increase in antibody levels from undetectable to positive (**Figure 1B**). The average CMV-seroconversion rate was 0.56% per year in this adult population, and was higher for women than for men (0.88% versus 0.27% per year, p=0.03) (**Supplementary Figure 2B**). ST CMV+ individuals did not differ from LT CMV+ individuals in terms of age at endpoint or educational level (**Table 1**).

CMV-specific antibody levels within CMV+ individuals increase over time

We investigated whether CMV-specific antibody levels increased with age. To this end, we selected samples at all time points of all CMV+ participants. We confirmed data from previous cross-sectional studies by showing a significant, positive correlation between geomean CMV-specific antibody levels and age (P<0.0001, r=0.15) (**Figure 2A, Supplementary Figure 3A**). We then used a median-based linear model to estimate the fold-change in CMV-specific antibody levels over time per individual using all available time points. This showed a significant increase within individuals over time (**Figure 2B**). On average, the increase was 1.01 (95%CI: 1.01-1.02) fold per year, or 1.42 (95%CI: 1.26-1.60) fold in 25 years (P<0.001), although in some individuals CMV-specific antibody levels decreased (**Figure 2B**)**.** Together, these data demonstrate a slight increase in CMV-specific antibody levels within CMV+ individuals over time.

Variation in CMV-specific antibody levels at endpoint is largely explained by baseline CMV-specific antibody levels

Antibody levels at endpoint showed considerable variation between individuals (**Figure 2G**). We studied which factors explained these differences using a random forest prediction algorithm. Explained variance in the algorithm to predict CMV-specific antibody levels at endpoint was 66%. The most important variable to predict turned out to be the "baseline" CMV-specific antibody level, where baseline is defined as the time of the first CMV+ observation, which was on average 27 years ago (**Figure 2H**). The increase in antibody levels over time was second in ranking of variable importance.

Other factors, such as age at endpoint, age at seroconversion, duration of CMV infection, sex (**Figure 2H**) and educational level (data not shown) were much less predictive for CMV-specific antibody levels at endpoint.

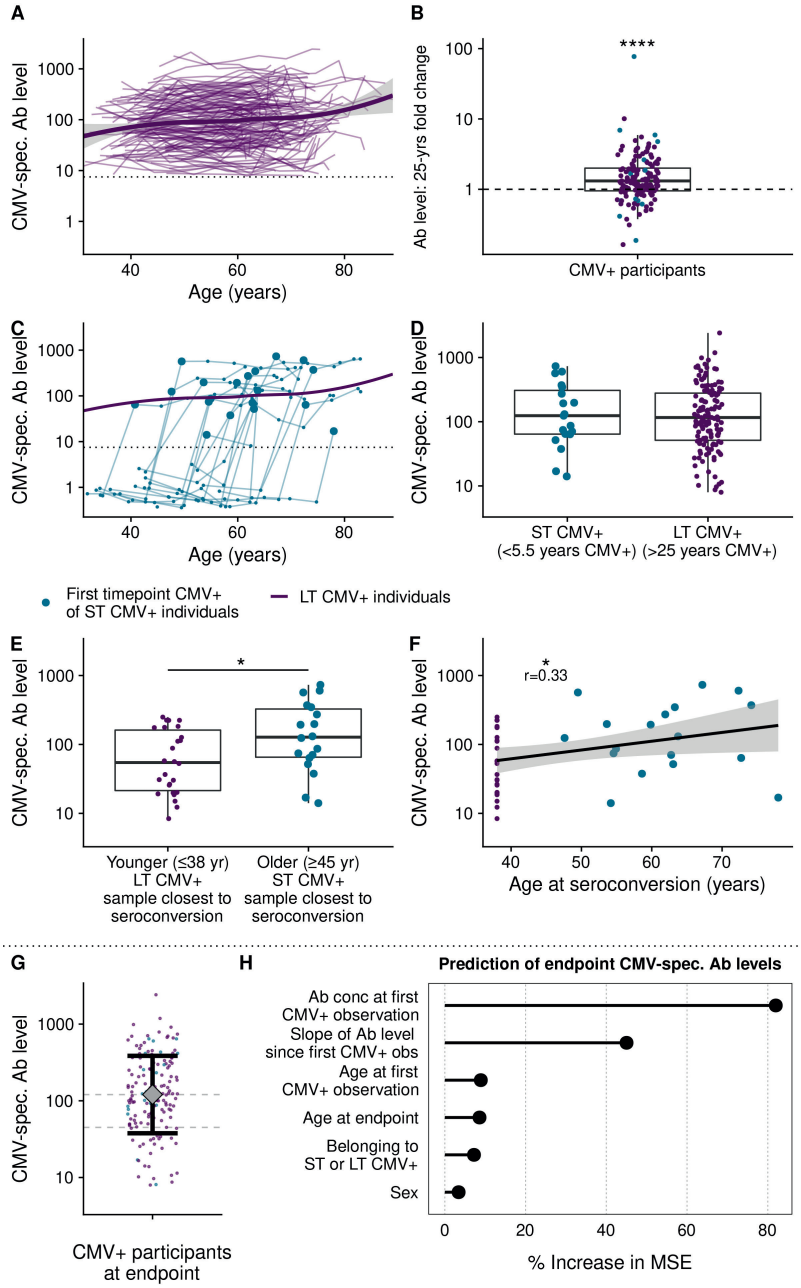


Figure 2. CMV-specific antibody levels followed over time. A. Antibody levels of all CMV+ individuals. The trend line shows local polynomial regression through the data of long-term (LT) CMV+ samples. **B.** Fold increase in CMV-specific antibody levels over 25 years for each individual. Dashed line shows no increase or decrease. **C.** Individual CMV-specific antibody levels during time of the CMV seroconverters (n=19) (blue lines) compared to the average trend line of the long-term CMV+ individuals. The first CMV+ time point of CMV seroconverters after CMV-seroconversion (<5.5 year after CMV conversion) is highlighted by a larger blue dot. **D.** Duration of CMV infection: CMV-specific antibody levels of recently seroconverted individuals (max <5.5 year after CMV

conversion) compared with those of long-term CMV+ individuals (> 25 years CMV+). **E.** Age at seroconversion: Antibody levels of individuals that seroconverted at younger age (≤38yr of age) or older age (≥45yr of age, mean age 58.5±8.1, shortly after CMV-seroconversion (<max 5.5 years)). **F.** Antibody levels associated with age at CMV seroconversion. For the selection of long-term CMV+ individuals, CMV+ individuals of round 1 were included that were ≤38yr of age and age of seroconversion was set at 38 years. **G.** Antibody levels of all CMV+ individuals at end point. Interval represents geometric mean level ±geometric standard deviation. **H.** Variable importance when predicting CMV antibody levels at endpoint with a random forest algorithm. % increase in MSE: proportion increase in mean squared error when the variable is removed from the model. Slope of Ab level: log-linear variation in CMV-specific antibody levels after first CMV+ measurement, until timepoint 6. *=P<0.05 **=P<0.01 Dotted line in A) and C) is the upper boundary of the cutoff for CMV seropositivity.

Changes in the CD8+ T-cell pool are established early after CMV infection

In addition to effects on antibody levels, we investigated the effect of CMV-seropositivity and duration of CMV infection on the CD8+ T-cell pool at endpoint. Both factors did not affect the total number of naive (T_N) or central memory (T_{CM}) CD8+ T-cells (**Figure 3A**). As expected, numbers of effector memory (T_{EM}) and terminally differentiated effector (T_{EMRA}) T-cells were significantly higher in CMV+ compared to CMV- individuals (**Figure 3A**). This was mainly explained by an increase in the number of late-stage differentiated (CCR7-CD45RA+CD27-CD28-) T-cells (data not shown) in CMV+ individuals. No significant association was observed between duration of CMV infection and numbers of T_{EMRA} cells, even within ST CMV+ individuals (**Figure 3B**). Also no significant difference was found in CD8+ T_{EMRA} cell numbers at end point between individuals who seroconverted at a young age (≤38yrs of age, exact date unknown) and those who seroconverted at older age (≥45yrs of age, range 47-88 years) (**Figure 3C**).

Next, we investigated whether CMV-specific CD8+ T-cell responses differed between ST CMV+ and LT CMV+ individuals (n=27 in total, matched for age and sex). Based on tetramer staining (using HLA-class I pp65 NLV-epitope) in all HLA-A2 positive individuals (n=11) , we found no significant differences in CMV-specific CD8+ T-cell numbers (**Figure 3D**)**,** percentages of CMV-specific CD8+ T-cells in the total CD8+ T-cell pool (**Figure 3E**)**,** percentages of T_{EMRA} cells in the CMV-specific T-cell pool (**Figure 3F**) and expression of the senescence marker KLRG-1 of the CMV-specific CD8 T-cells between ST CMV+ and LT CMV+ individuals (**Figure 3G**). Also, after CMV-specific stimulation using overlapping CMV peptide pools, no significant differences were observed in IFNγ production between ST CMV+ and LT CMV+ participants (**Figure 3H**). We also studied the polyfunctionality of the CMV-specific T-cell response and identified that CMV-specific CD8+ T-cell responses were mainly IFNγ+TNFa+MIP-1Bβ +CD107a+ but lacked IL-2 (**Supplementary Figure 4A,B**)**,** suggestive of an end-stage highly functional T-cell phenotype. The height of these functional CMV-specific CD8+T-cells did not differ between ST CMV+ and LT CMV+ individuals (**Figure 3I**). This suggests that high CD8+ T_{EM} and T_{EMRA} cell numbers are induced shortly after primary CMV infection and that CMV-specific T-cell numbers, phenotype and polyfunctionality are not much dependent on duration of CMV infection.

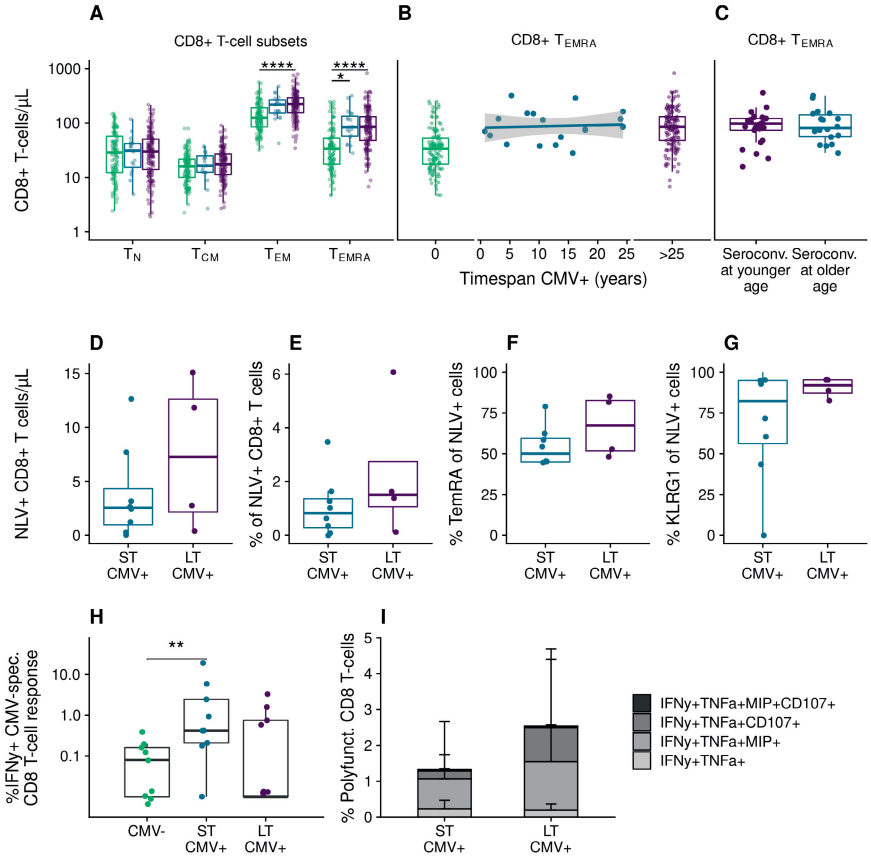


Figure 3. CMV-induced changes in the CD8+ T-cell pool are established early after CMV infection. A. Absolute numbers of CD8+ T-cell subsets compared between CMV-, ST CMV+ and LT CMV+ individuals indicated by boxplots. **B.** Relationship of duration of CMV infection with CD8+ T_{EMRA} cells numbers. **C.** CD8+ T_{EMRA} cells numbers at endpoint in individuals that seroconverted at younger age (≤38yr of age) compared to those individuals who converted at older age (≥45yr of age, mean age 58.5±8.1). **D-G.** CMV-specific T-cells in HLA-A2 positive individuals for pp65-epitope NLVPMVATV are compared between ST CMV+ and LT CMV+ individuals in numbers of cells (**D**), percentage of total CD8 (**E**), percentage of T_{EMRA} cells (**F**) and expression of senescence marker KLRG-1 (**G**). **H.** IFNγ production of CD8+ T-cells after CMV-specific peptide stimulation in CMV-, ST CMV+ and LT CMV+ individuals (n=27) **I.** Percentage of CD8+ T-cells responding polyfunctionally to CMV with production of IFNγ, TNFa, MIP-1β and/orCD107 in ST CMV+ and LT CMV+ individuals. *=P<0.05 ****P<0.0001. Boxplots show median with interquartile range. Colors of symbols represent CMV-serostatus. Green: CMV-, blue: ST CMV+, purple: LT CMV+.

Changes in the CD4+ T-cell pool are established early after CMV infection, and are most pronounced in older CMV-seroconverters

Next, we studied the impact of duration of CMV infection on the CD4+ T-cell pool by comparing CD4+ T-cells at end point between ST CMV+ individuals and LT CMV+ individuals. CMV seropositivity was associated with higher numbers of T_{EM} and T_{EMRA} CD4+ T-cells (**Figure 4A**), which was mainly due to the presence of relatively high numbers of intermediate and late-stage differentiated T_{EM} and T_{EMRA} cells (data not shown).

The higher CD4+ T_{EM} and T_{EMRA} numbers in ST CMV+ individuals were not related to sex or age at end point (data not shown). Since ST CMV+ individuals were significantly older at CMV seroconversion than LT CMV+ individuals (average of 57 years and a maximum of 43 years respectively, p<0.0001), the association may be driven by differences in age at seroconversion. Indeed, individuals who seroconverted at older age (≥45 years old, n=18/19) had significantly higher CD4+ T_{EM} and T_{EMRA} cell numbers at end point than individuals who seroconverted when they were younger than 38 years of age (n=26/116 LT CMV+ individuals, p=0.018 and p=0.006 respectively, **Figure 4C**). However, within the group of ST CMV+ individuals, age of seroconversion was not significantly related to CD4+ T_{EM} or T_{EMRA} cell numbers (data not shown), possibly due to the smaller age range. Within the group of ST CMV+ individuals, we found a positive correlation between duration of CMV infection and CD4+ T_{EM} and T_{EMRA} cell numbers at endpoint (**Figure 4B**). Similar results were observed for the differentiation states of T_{EM} and T_{EMRA} cells (data not shown). Together, these results suggest that duration of CMV infection has only a minor effect on CMV-induced CD4+ T_{EM} and T_{EMRA} cell numbers, and that becoming CMV+ at older age leads to higher CD4+ T_{EM} and T_{EMRA} numbers at end point.

We further investigated the functionality of the CMV-specific CD4+ T-cells of ST CMV+ and LT CMV+ individuals. CD4+ T_{EMRA} cell numbers at endpoint correlated positively with the percentage of granzyme B and perforin positive T_{EMRA} T-cells (**Figure 4D**) and with the CMV-specific IFNγ response after stimulation with overlapping CMV-peptide pools (UL55, pp65 and IE-1) (**Figure 4E**), suggesting that CD4+ T_{EMRA} cells have cytotoxic potential and are responding to CMV by cytokine production. After stimulation of CD4+ T-cells with overlapping CMV peptide pools, ST CMV+ individuals showed higher IFNγ-production than LT CMV+ participants (**Figure 4F**). CD4+ CMV-specific T-cell responses were polyfunctional, producing IFNγ, TNFα, MIP-1β and CD107 but not IL-2, suggestive of an end-stage highly functional T-cell phenotype. Higher percentages of these cells were found in ST CMV+ compared to LT CMV+ individuals (**Figure 4G, Supplementary Figure 4C)**. This was mainly due to the fact that ST CMV+ individuals had significantly higher polyfunctional responses to peptide pool UL55 than LT CMV+ individuals (data not shown). These results suggest that many of the CD4+ T_{EM} and T_{EMRA} cells in CMV-infected individuals are CMV-specific T-cells with a polyfunctional late-stage memory phenotype, and that these cells are induced early after primary CMV infection. In addition, these cells are induced in higher numbers in individuals who became CMV infected at older age.

The median frailty index score of all participants was 0.069 at round 5 (Inter Quartile Range (IQR) 0.027– 0.167) and 0.081 (IQR 0.029 – 0.186) at round 6, indicating that on average frailty increased with age (p<0.001). Median increase in frailty index was 0.013 (IQR -0.017– 0.049) (p<0.001) (**Supplementary Figure 5**). We found no significant differences in frailty between CMV-, ST CMV+, or LT CMV+ individuals (**Figure 5A**). While there seemed to be a negative trend between frailty and duration of CMV infection within ST CMV+ participants (n=19), this correlation was not statistically significant (p=0.098, ρ=-0.27 **Figure 5B**).

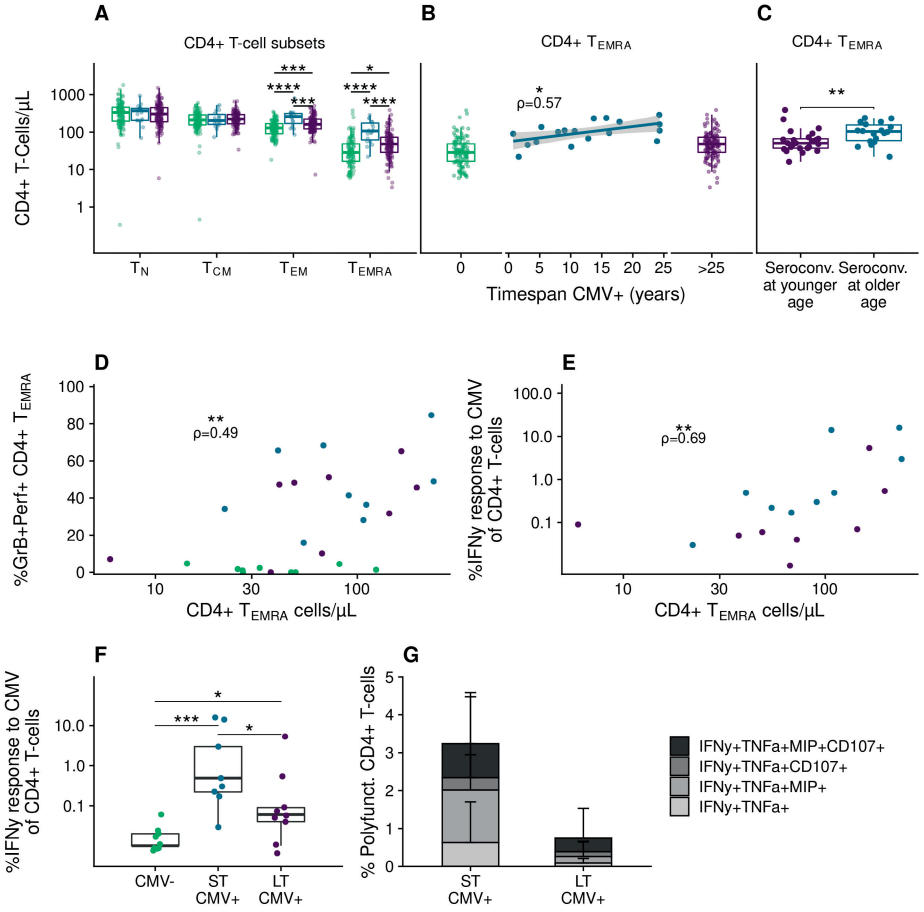


Figure 4. CMV-induced changes in the CD4+ T-cell pool are established early after CMV infection, and are most pronounced in older CMV-seroconverters. A. Absolute numbers of CD4+ T-cell subsets compared between CMV-, ST CMV+, and LT CMV+ individuals indicated by geometric mean with geometric standard deviation. **B.** Relationship of duration of CMV infection with CD4+ T_{EMRA} cells numbers. ρ and p value are from correlation tested in ST CMV+ individuals. **C.** CD4+ T_{EMRA} cells numbers at endpoint are compared between individuals that seroconverted at younger age (≤38yr) or older age (seroconverted ≥45yr, mean age 58.5±8.1). **D,E.** Correlation of CD4 T_{EMRA} cells numbers with their percentage producing granzyme B and perforin (**D**) and IFNγ production after CMV-peptide stimulation (n=27) (**E**). **F.** Percentages of CD4 T-cells producing IFNγ after CMV-specific stimulation in CMV-, ST CMV+, and LT CMV+ individuals. **H.** Percentage of CD4+ T-cells responding polyfunctionally to CMV with production of IFNγ, TNFa, MIP-1β, and/or CD107 in ST CMV+ and LT CMV+ individuals. *=P<0.05, **=P<0.01, ***=P<0.001, ****=P<0.0001. Colors of symbols represent CMV-serostatus. Green: CMV-, blue: ST CMV+, purple: LT CMV+.

Interestingly, within the group of ST CMV+ individuals, older age at CMV seroconversion was associated with a higher frailty index (p=0.03, ρ= 0.48) (**Figure 5C**), which reduced to a non-significant trend after adjusting for age at frailty index assessment (p=0.11, ρ=0.39). We further studied whether CMV-specific antibody levels at endpoint or the increase of CMV-specific antibodies per year were associated with frailty. None of these two indicators were significantly associated with frailty (**Figure 5D,E**). The median increase in frailty

index tended to be higher in recent CMV seroconverters (i.e. converted after DCS round 5), although the effect was not significant (p=0.11, **Figure 5F**; n=4 participants recently seroconverted). When restricting this analysis to women aged 60-65 years, because 3 out of 4 recent CMV seroconverters were women in this age category, we observed that women who seroconverted recently had a higher increase in frailty index than their CMV- (p=0.006) (**Figure 5G**). Although the sample size of ST CMV+ individuals was very small, these unique human data suggest a relationship between becoming infected with CMV at older age and frailty. Together, these results suggest that there is no significant association between CMV-seropositivity, duration of CMV infection or CMV-specific antibody levels and frailty, but age of seroconversion might be associated with frailty, and recent CMV seroconversion with an increase in frailty, in older individuals.

CMV-	CMV+
9.2% (n = 11)	12.7% (n = 21)
3.4% (n = 4)	9.1% (n = 15)
1.7% (n = 2)	1.8% (n = 3)
Total	
14.3% (n = 17)	23.6% (n = 39)

Serostatus is serostatus at endpoint. Cardiovascular intervention: participant undergone either bypass surgery, Cardiac valve dilation surgery, Cardiac catheterization, Pacemaker placement, or peripheral vascular surgery.

Table 2: Cardiovascular disease prevalence

CMV-specific antibody levels are increased in CMV+ individuals with cardiovascular disease

A more specific health outcome that has been associated with CMV infection is cardiovascular disease [45]. Of all participants in this cohort, 20.9% (n=56) had any form of cardiovascular disease (**Table 2**), most of them men (n=38, $\chi 2$=8.9, p=0.003). The percentage of individuals with any form of cardiovascular disease tended to be higher among CMV+ compared to CMV- individuals (23.6% versus 14.3%, Table 2) (χ^2 =5.3, p=0.02, analysis stratified by sex as confounder). Furthermore, the occurrence of cardiovascular disease was positively associated with CMV-specific antibody levels at endpoint (p=0.04 **Figure 5H**). Although in the group with CVD the median increase in CMV-specific antibodies seemed higher, no significant difference was observed (p=0.12 **Figure 5I**). Within the ST CMV+ group, 3 out of 19 individuals had any form of cardiovascular disease, a number too low to investigate a possible association between cardiovascular disease and CMV-duration. In conclusion, CMV-specific antibody levels in CMV+ individuals might be related to the risk of cardiovascular disease.

DISCUSSION

We investigated how duration of CMV infection was related to CMV-specific antibody levels, T-cell numbers, CMV-specific T-cell responses, frailty and cardiovascular disease prevalence. We demonstrated that within individuals, CMV-specific antibody levels increased over time, albeit only slightly. Nevertheless, duration of CMV infection was not the major determinant

of CMV-specific antibody levels at endpoint and age at CMV-seroconversion turned out to play a major role. Duration of CMV infection was not related to the size and function of the memory CD8+ T-cell pool, suggesting that CD8+ T-cell memory responses do not further inflate over time. In contrast, CD4+ T-cell numbers, similar to CMV-specific antibody levels, were higher in individuals who seroconverted at older age. Furthermore, we did not find an association of frailty with CMV-serostatus or with duration of CMV infection in elderly individuals, although increased CMV-specific antibody levels were associated with higher prevalence of cardiovascular disease.

We are the first showing the CMV seroconversion rate in an observational longitudinal cohort of healthy older adults. The average seroconversion rate we found in (0.56% per year) was similar to a previous estimate (0.55% per year) based on a large cross-sectional study in adults [46]. This thus provides a valuable estimate, although it is lower than that previously reported for individuals at higher risk like pregnant women, day-care providers and parents with young children (respectively 2.3%, 8.5% and 2.1%) that can be explained by the lower number of contacts with young children [47, 48].

Higher CMV-specific antibody levels in older adults are generally thought to reflect multiple experienced CMV reactivations during life [2, 3, 9, 11, 23]. However, little is known about how CMV-specific antibody levels are established and maintained during a lifetime in healthy individuals. We observed a small increase in CMV-specific antibodies over a substantial period of time (~27 years). Two other studies reported a small [13] or even no increase [49] in CMV-specific antibody levels over time, but these covered a much shorter follow-up time (5 years and 13 years, respectively). The slight increase in CMV-specific antibody levels we observed over time suggests that CMV reactivation, and probably to a lesser extent reinfection [3], indeed occurs in CMV-infected individuals. Antibody levels can be stable over prolonged periods of time as has been seen for other viruses [50], although CMV reactivation will play a role in maintaining CMV-specific antibody levels. The persistence of CMV antigen may contribute to activation of memory B-cells and the continuous replenishment of long-lived plasma cells and antibody production [50-52].

Furthermore, we show that variation of CMV antibody levels at end point is just for a minor part explained by changes in these levels over the preceding 27 years. Therefore, our results argue against the use of CMV-specific antibody levels as a surrogate marker for experienced CMV reactivation. Interestingly, while we found a positive relationship between age and CMV-specific antibody levels, short-term and long-term CMV+ individuals did not differ in their CMV-specific antibody levels at end point. In contrast, age, regardless of duration of CMV infection, was associated with increased CMV-specific antibody levels. Thus age-related effects might play a prominent role in the variation in antibody levels.

Memory inflation, characterized by an expansion of the memory T-cell pool over time, is a hallmark for CMV infection, especially shown in CMV mouse models [24, 53, 54]. Memory

inflation of CMV-specific T-cells in humans was questioned recently [55]. Longitudinal studies in humans are very limited, and some report evidence for memory inflation [56, 57], while others do not [58, 59]. Our study allowed us to investigate how duration of CMV infection influences the T-cell memory pool in humans. We show that large polyfunctional CMV-specific T-cell responses and memory/effector CD4+ and CD8+ T-cell populations are already high shortly after CMV seroconversion. Thus, both the humoral immune response and the CD4+ and CD8+ T-cell pools do not require a long duration of CMV infection to develop and expand. We found weak evidence for memory inflation in the CD4+ T-cell pool, as duration of CMV infection correlated positively with CD4+ T_{EM} and T_{EMRA} numbers in short-term converters, but not in LT CMV+ individuals. Regardless of duration of CMV infection, we found a positive correlation between CMV seroconversion at older age and CD4+ T_{EM} and T_{EMRA} cell numbers, similar to what we observed for CMV-specific antibody levels. This may be explained either by the aged immune system requiring after delayed primary control a high number of CD4+ T_{EM} and T_{EMRA} cells to control latent CMV infection, or by enhanced stimulation of cells due to limited control of the virus. Taken together, our data contribute to the view that there is no evidence for a time dependent memory inflation during CMV infection in humans. Further longitudinal studies, possibly covering even longer periods of time might strengthen our results.

We also assessed whether prolonged CMV infection might impair clinically relevant health outcomes. We did not find an association between frailty and CMV seroprevalence, or between frailty and duration of CMV infection, with frailty expressed by a frailty index score [34]. This is in line with some other papers [30], although several studies show a relation between CMV infection or CMV-antibody levels and frailty [11, 28, 29] or even the opposite relationship with a higher CMV seroprevalence in healthy people [31]. The lack of a relationship between frailty and CMV-seroprevalence or CMV-specific antibody levels in our study could be due to the heterogeneity between individuals with a high frailty index score, since various conditions and deficits (n=36) are included in the frailty score we used based on Rockwood criteria. Importantly, another study that used a Rockwood-based frailty index score also did not observe an effect of CMV-seropositivity on frailty [30]. Moreover, our study was performed in a relative younger population compared to the two other studies not showing a relation between CMV and frailty in people above 80 years of age that might be obscured by a survival bias. Also, the frail population in our study was overrepresented due to the stratified selection, which generally increases the chance of finding an association with frailty, emphasizing the negative relation we found. In sum, our study does not support the hypothesis that CMV causes 'accelerated aging' influencing general health. However, we found an association between frailty and age at first seropositive time point during follow up within short-term CMV+ individuals. In addition, a small sub-group of women who seroconverted recently (after round 5, n=3) had a steeper increase in frailty index than those who did not seroconvert that might suggest that people who become frail are more susceptible to CMV infection.

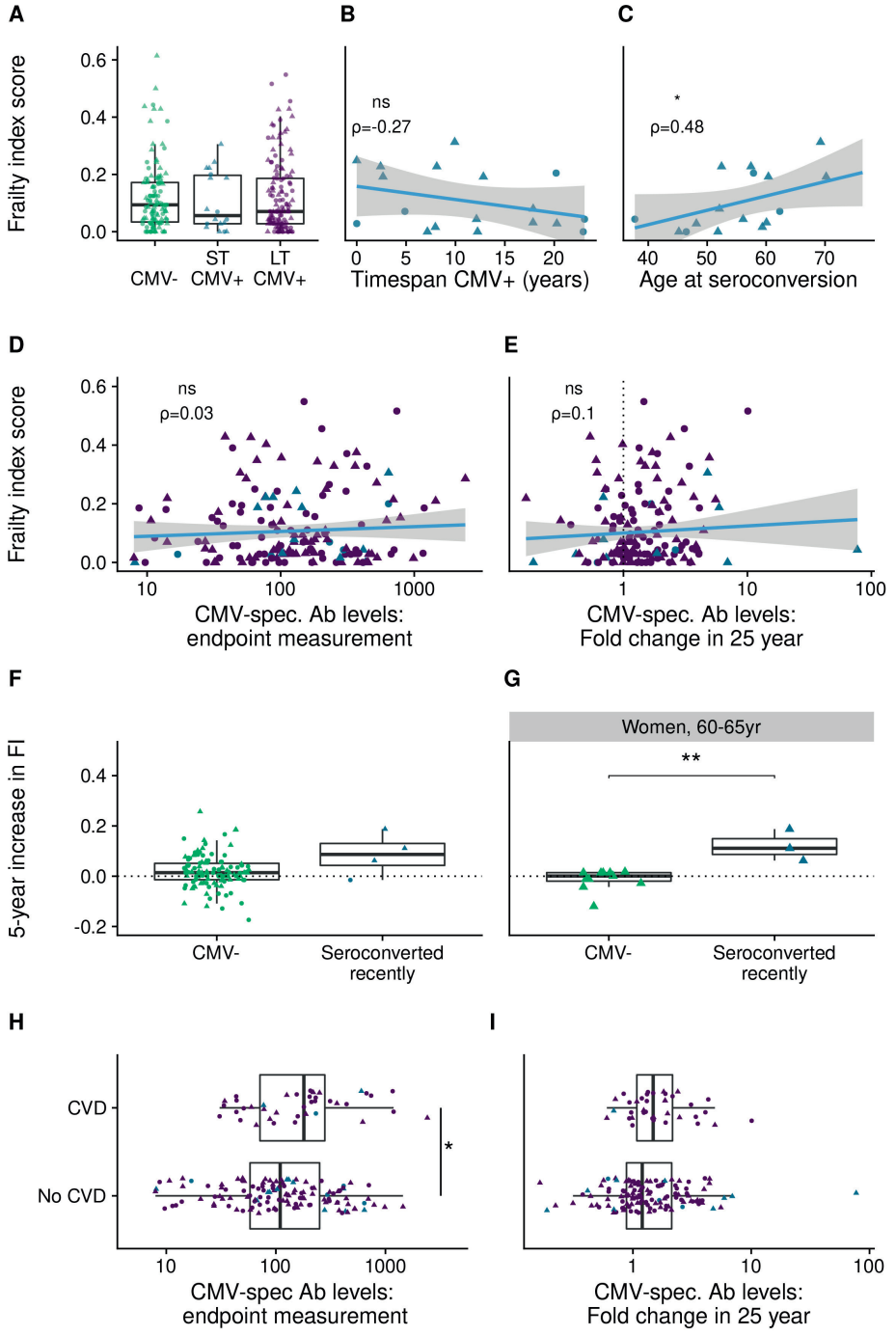


Figure 5. CMV infection is not associated with frailty, but is related to prevalence of cardiovascular disease.
A. Comparison of frailty index score between CMV-, short-term (ST) CMV+ and long-term (LT) CMV+ participants. **B,C.** The relation of frailty with (**B**) the duration of CMV infection and (**C**) age of seroconversion in ST CMV+

individuals. **D,E**. Relationships are shown respectively between frailty and CMV-specific antibody levels at endpoint (**D**) and between frailty and fold change in CMV-specific antibody levels over 26 years (**E**). **F, G.** Difference in increase in frailty index of CMV- participants and those who seroconverted recently after measurement point T5 (**F**) and in recently converted women aged 60-65 years (**G**). Increase in frailty index: difference between frailty index as measured between T5 and T6. **H, I.** Comparison of cardiovascular disease (CVD) prevalence with CMV-specific antibody levels at endpoint (**H**) and fold change in 25 years (**I**) in these antibody levels (Prevalence of CVD is indicated in table 2). Symbols: Triangles represent women, circles represent men. Colors of symbols represent CMV-serostatus. Green: CMV-, blue: ST CMV+, purple: LT CMV+.

As the effects of CMV might be related to specific chronic conditions and not to a general health state such as frailty we investigated the association with cardiovascular disease [45, 60]. Indeed, the prevalence of CVD was significantly higher in CMV-seropositive individuals. Furthermore, within CMV+ individuals CVD was associated with higher CMV-specific antibody levels at end point. A study in an "unhealthy" population above 90 years of age also reported a relationship between high CMV-specific antibody levels and the prevalence of CVD [13]. One of the mechanisms that could explain this association is that the lytic viral CMV lifecycle in endothelial cells induces vascular damage and contributes to CVD [61, 62] and in particular to atherosclerosis [63]. Alternatively, progressive endothelial damage in individuals with CVD and a pro-inflammatory environment could initiate inflammation leading to CMV reactivation [64].

We show that, age-related effects other than duration of CMV infection seem to contribute to the variation in CMV-specific immune responses at endpoint between individuals. We hypothesize that this variation between individuals is due to individual differences in the balance between virus and host factors and that these immune responses may in part be determined by viral reactivation, but for the larger part by the establishment of the latent CMV reservoir after primary infection (**Figure 6**)**.** Due to immunosenescence at higher age, older individuals may be less able to control primary infection and viral spread. This may lead to enhanced activation of the immune system to enable control of (latent) CMV infection, regardless of duration of CMV infection. Other factors that could influence this balance include the route of viral transmission [65] and the viral inoculum during primary infection [24] as well as the state of the immune system at primary infection [53, 66].

A comparable model was previously proposed based on data from children, which suggested that the relatively immature state of the immune system in children leads to the establishment of a larger viral reservoir [53, 66], which may subsequently increase the chance of viral reactivation. The state of the immune system at primary CMV infection might thus affect the levels of life-long immunity to CMV (**Figure 6**). Whether general health status, i.e. frailty, also enhances the establishment of the latent CMV reservoir and subsequent viral reactivation, remains to be investigated.

In conclusion, our results indicate that age, regardless of duration of CMV infection, has a larger influence on the CMV-specific response than previously anticipated. High CMV-specific antibody levels should therefore not be interpreted as a measure of experienced viral reactivation or duration of CMV infection.

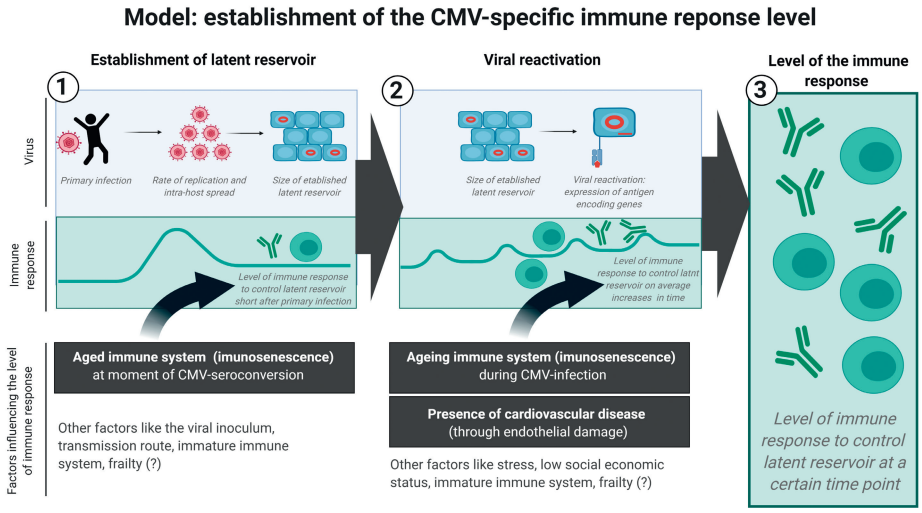


Figure 6. Model of establishment of the size of the immune response to control latent CMV infection. Both establishment of the viral latent reservoir after primary infection (1) and the subsequent viral reactivation in time (2) will affect the CMV-specific immune response. Different factors may influence both phases; those based on our data are presented in black rectangles. Figure created with Biorender.com.

In fact, elderly individuals with high CMV-specific antibody levels and high numbers of CMV-specific T_{EMRA} cells could also be the ones who were more recently infected with CMV. While we confirmed that CMV infection is related to cardiovascular disease, we found limited evidence for a relationship with general health i.e. frailty. We therefore propose an alternative hypothesis that high CMV-specific immune responses in elderly may not be a *cause* of poor health outcomes, but may instead be a sign of impaired health status.

ACKNOWLEDGEMENTS

We thank Irina Tcherniaeva, Marjan Bogaard-van Maurik, Ronald Jacobi and Gerco den Hartog for help with the CMV serology and Petra Vissink for the longitudinal samples of the DCS.

REFERENCES

1. Dupont, L. and M.B. Reeves, *Cytomegalovirus latency and reactivation: recent insights into an age old problem.* Rev Med Virol, 2016. 26(2): p. 75-89.
2. Stowe, R.P., et al., *Chronic herpesvirus reactivation occurs in aging.* Exp Gerontol, 2007. 42(6): p. 563-70.
3. van Boven, M., et al., *Infectious reactivation of cytomegalovirus explaining age- and sex-specific patterns of seroprevalence.* PLoS Comput Biol, 2017. 13(9): p. e1005719.
4. Wertheimer, A.M., et al., *Aging and Cytomegalovirus Infection Differentially and Jointly Affect Distinct Circulating T-cell Subsets in Humans.* The Journal of Immunology, 2014. 192(5): p. 2143-2155.
5. Korndewal, M.J., et al., *Cytomegalovirus infection in the Netherlands: seroprevalence, risk factors, and implications.* J Clin Virol, 2015. 63: p. 53-8.
6. Stowe, R.P., et al., *Reactivation of herpes simplex virus type 1 is associated with cytomegalovirus and age.* J Med Virol, 2012. 84(11): p. 1797-802.
7. Parry, H.M., et al., *Cytomegalovirus viral load within blood increases markedly in healthy people over the age of 70 years.* Immun Ageing, 2016. 13: p. 1.
8. Furui, Y., et al., *Cytomegalovirus (CMV) seroprevalence in Japanese blood donors and high detection frequency of CMV DNA in elderly donors.* Transfusion, 2013. 53(10): p. 2190-7.
9. Alonso Arias, R., et al., *Intensity of the humoral response to cytomegalovirus is associated with the phenotypic and functional status of the immune system.* J Virol, 2013. 87(8): p. 4486-95.
10. Turner, J.E., et al., *Rudimentary signs of immunosenescence in Cytomegalovirus-seropositive healthy young adults.* Age (Dordr), 2014. 36(1): p. 287-97.
11. Wang, G.C., et al., *Cytomegalovirus infection and the risk of mortality and frailty in older women: a prospective observational cohort study.* Am J Epidemiol, 2010. 171(10): p. 1144-52.
12. Araujo Carvalho, A.C., et al., *Association between human herpes virus seropositivity and frailty in the elderly: A systematic review and meta-analysis.* Ageing Res Rev, 2018. 48: p. 145-152.
13. Vescovini, R., et al., *Intense antiextracellular adaptive immune response to human cytomegalovirus in very old subjects with impaired health and cognitive and functional status.* J Immunol, 2010. 184(6): p. 3242-9.
14. Gkrania-Klotsas, E., et al., *Higher immunoglobulin G antibody levels against cytomegalovirus are associated with incident ischemic heart disease in the population-based EPIC-Norfolk cohort.* J Infect Dis, 2012. 206(12): p. 1897-903.
15. Roberts, E.T., et al., *Cytomegalovirus antibody levels, inflammation, and mortality among elderly Latinos over 9 years of follow-up.* Am J Epidemiol, 2010. 172(4): p. 363-71.
16. Kaczorowski, K.J., et al., *Continuous immunotypes describe human immune variation and predict diverse responses.* Proceedings of the National Academy of Sciences, 2017. 114(30): p. E6097-E6106.
17. Wertheimer, A.M., et al., *Aging and cytomegalovirus infection differentially and jointly affect distinct circulating T-cell subsets in humans.* J Immunol, 2014. 192(5): p. 2143-55.
18. Weltevrede, M., et al., *Cytomegalovirus persistence and T-cell immunosenescence in people aged fifty and older: A systematic review.* Exp Gerontol, 2016.
19. Sylwester, A.W., et al., *Broadly targeted human cytomegalovirus-specific CD4+ and CD8+ T-cells dominate the memory compartments of exposed subjects.* J Exp Med, 2005. 202(5): p. 673-85.
20. van de Berg, P.J., et al., *A fingerprint left by cytomegalovirus infection in the human T-cell compartment.* J Clin Virol, 2008. 41(3): p. 213-7.
21. Klenerman, P. and A. Oxenius, *T-cell responses to cytomegalovirus.* Nat Rev Immunol, 2016.

22. Pourgheysari, B., et al., *The cytomegalovirus-specific CD4+ T-cell response expands with age and markedly alters the CD4+ T-cell repertoire.* J Virol, 2007. 81(14): p. 7759-65.

23. Klenerman, P., *The (gradual) rise of memory inflation.* Immunol Rev, 2018. 283(1): p. 99-112.

24. Redeker, A., S.P. Welten, and R. Arens, *Viral inoculum dose impacts memory T-cell inflation.* Eur J Immunol, 2014. 44(4): p. 1046-57.

25. Aiello, A.E., Y.-L. Chiu, and D. Frasca, *How does cytomegalovirus factor into diseases of aging and vaccine responses, and by what mechanisms?* GeroScience, 2017. 39(3): p. 261-271.

26. Savva, G.M., et al., *Cytomegalovirus infection is associated with increased mortality in the older population.* Aging Cell, 2013. 12(3): p. 381-7.

27. Gkrania-Klotsas, E., et al., *Seropositivity and Higher Immunoglobulin G Antibody Levels Against Cytomegalovirus Are Associated With Mortality in the Population-Based European Prospective Investigation of Cancer–Norfolk Cohort.* Clinical Infectious Diseases, 2013. 56(10): p. 1421-1427.

28. Thomasini, R.L., et al., *Aged-associated cytomegalovirus and Epstein-Barr virus reactivation and cytomegalovirus relationship with the frailty syndrome in older women.* PLoS One, 2017. 12(7): p. e0180841.

29. Schmaltz, H.N., et al., *Chronic Cytomegalovirus Infection and Inflammation Are Associated with Prevalent Frailty in Community-Dwelling Older Women.* Journal of the American Geriatrics Society, 2005. 53(5): p. 747-754.

30. Collerton, J., et al., *Frailty and the role of inflammation, immunosenescence and cellular ageing in the very old: cross-sectional findings from the Newcastle 85+ Study.* Mech Ageing Dev, 2012. 133(6): p. 456-66.

31. Matheï, C., et al., *Associations Between Cytomegalovirus Infection and Functional Impairment and Frailty in the BELFRAIL Cohort.* Journal of the American Geriatrics Society, 2011. 59(12): p. 2201-2208.

32. Picavet, H.S.J., et al., *Cohort Profile Update: The Doetinchem Cohort Study 1987-2017: lifestyle, health and chronic diseases in a life course and ageing perspective.* Int J Epidemiol, 2017. 46(6): p. 1751-1751g.

33. Verschuren, W.M., et al., *Cohort profile: the Doetinchem Cohort Study.* Int J Epidemiol, 2008. 37(6): p. 1236-41.

34. Samson, L.D., et al., *Frailty is associated with elevated CRP trajectories and higher numbers of neutrophils and monocytes.* Experimental Gerontology, 2019. 125: p. 110674.

35. Rockwood, K., X. Song, and A. Mitnitski, *Changes in relative fitness and frailty across the adult lifespan: evidence from the Canadian National Population Health Survey.* CMAJ, 2011. 183(8): p. E487-94.

36. Schoufour, J.D., et al., *Design of a frailty index among community living middle-aged and older people: The Rotterdam study.* Maturitas, 2017. 97: p. 14-20.

37. Searle, S.D., et al., *A standard procedure for creating a frailty index.* BMC Geriatr, 2008. 8: p. 24.

38. Tcherniaeva, I., et al., *The development of a bead-based multiplex immunoassay for the detection of IgG antibodies to CMV and EBV.* J Immunol Methods, 2018. 462: p. 1-8.

39. Samson, L.D., et al., *In-depth immune cellular profiling reveals sex-specific associations with frailty.* Immunity & Ageing, 2020. 17(1): p. 20.

40. Appay, V., et al., *Memory CD8+ T-cells vary in differentiation phenotype in different persistent virus infections.* Nat Med, 2002. 8(4): p. 379-85.

41. Theil, H., *A rank-invariant method of linear and polynomial regression analysis.* Indigationes Mathematicae, 1950. 12: p. 85-91.

42. Sen, P.K., *Estimates of the Regression Coefficient Based on Kendall's Tau.* Journal of the American Statistical Association, 1968. 63(324): p. 1379-1389.

43. Wilcox, R., *A Note on the Theil-Sen Regression Estimator When the Regressor Is Random and the Error Term Is Heteroscedastic.* Biometrical Journal, 1998. 40(3): p. 261-268.

44. Liaw, A. and M. Wiener, *Classification and regression by randomForest.* R news, 2002. 2(3): p. 18-22.

45. Wang, H., et al., *Cytomegalovirus Infection and Relative Risk of Cardiovascular Disease (Ischemic Heart Disease, Stroke, and Cardiovascular Death): A Meta‐Analysis of Prospective Studies Up to 2016.* Journal of the American Heart Association, 2017. 6(7): p. e005025.

46. Hecker, M., et al., *Continuous cytomegalovirus seroconversion in a large group of healthy blood donors.* Vox Sang, 2004. 86(1): p. 41-4.

47. Hyde, T.B., D.S. Schmid, and M.J. Cannon, *Cytomegalovirus seroconversion rates and risk factors: implications for congenital CMV.* Rev Med Virol, 2010. 20(5): p. 311-26.

48. Cannon, M.J., T.B. Hyde, and D.S. Schmid, *Review of cytomegalovirus shedding in bodily fluids and relevance to congenital cytomegalovirus infection.* Rev Med Virol, 2011. 21(4): p. 240-55.

49. Lustig, A., et al., *Telomere Shortening, Inflammatory Cytokines, and Anti-Cytomegalovirus Antibody Follow Distinct Age-Associated Trajectories in Humans.* Front Immunol, 2017. 8: p. 1027.

50. Amanna, I.J., N.E. Carlson, and M.K. Slifka, *Duration of humoral immunity to common viral and vaccine antigens.* N Engl J Med, 2007. 357(19): p. 1903-15.

51. Wrammert, J. and R. Ahmed, *Maintenance of serological memory.* Biol Chem, 2008. 389(5): p. 537-9.

52. Siegrist, C.A. and R. Aspinall, *B-cell responses to vaccination at the extremes of age.* Nat Rev Immunol, 2009. 9(3): p. 185-94.

53. Reddehase, M.J., *'Checks and balances' in cytomegalovirus-host cohabitation.* Med Microbiol Immunol, 2019. 208(3-4): p. 259-261.

54. Cicin-Sain, L., *Cytomegalovirus memory inflation and immune protection.* Med Microbiol Immunol, 2019. 208(3-4): p. 339-347.

55. Jackson, S.E., et al., *Generation, maintenance and tissue distribution of T-cell responses to human cytomegalovirus in lytic and latent infection.* Med Microbiol Immunol, 2019.

56. Vescovini, R., et al., *Impact of Persistent Cytomegalovirus Infection on Dynamic Changes in Human Immune System Profile.* PLoS One, 2016. 11(3): p. e0151965.

57. Hosie, L., et al., *Cytomegalovirus-Specific T-cells Restricted by HLA-Cw*0702 Increase Markedly with Age and Dominate the CD8(+) T-Cell Repertoire in Older People.* Front Immunol, 2017. 8: p. 1776.

58. Jackson, S.E., et al., *Diverse specificities, phenotypes, and antiviral activities of cytomegalovirus-specific CD8+ T-cells.* J Virol, 2014. 88(18): p. 10894-908.

59. Abana, C.O., et al., *Cytomegalovirus (CMV) Epitope-Specific CD4(+) T-cells Are Inflated in HIV(+) CMV(+) Subjects.* J Immunol, 2017. 199(9): p. 3187-3201.

60. Lebedeva, A.M., et al., *Cytomegalovirus Infection in Cardiovascular Diseases.* Biochemistry (Mosc), 2018. 83(12): p. 1437-1447.

61. van de Berg, P.J., et al., *Cytomegalovirus-induced effector T-cells cause endothelial cell damage.* Clin Vaccine Immunol, 2012. 19(5): p. 772-9.

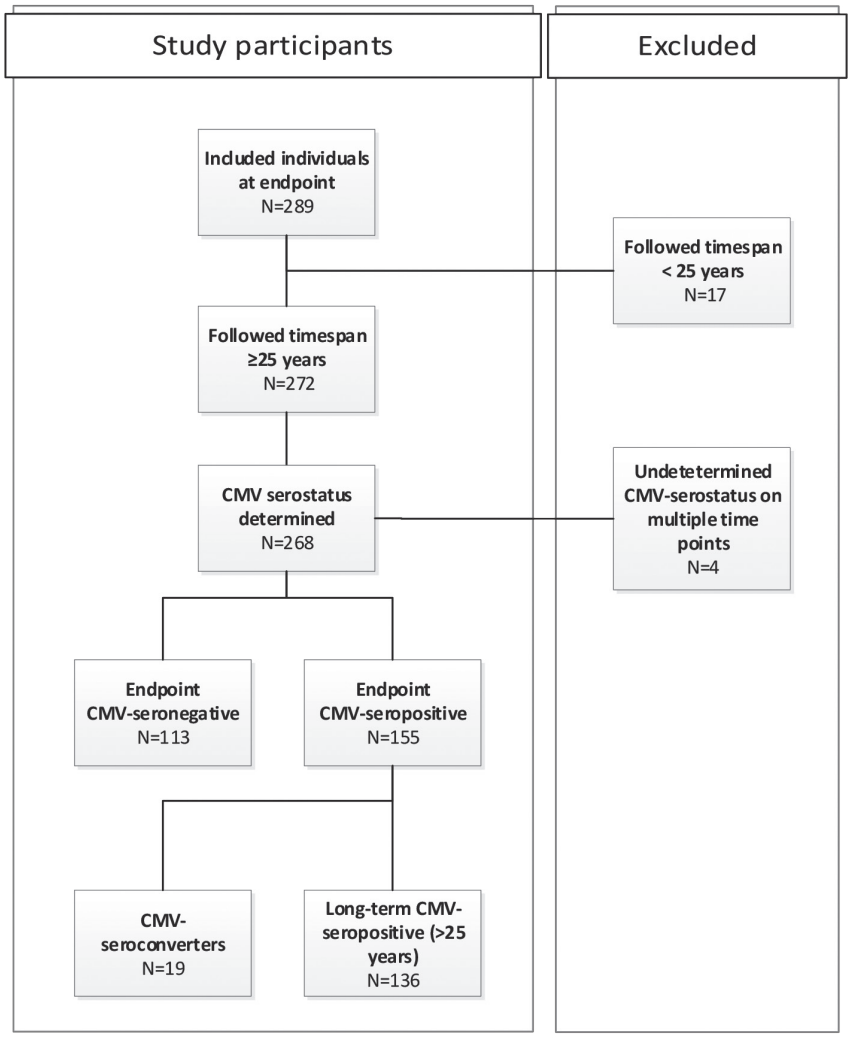
62. Pachnio, A., et al., *Cytomegalovirus Infection Leads to Development of High Frequencies of Cytotoxic Virus-Specific CD4+ T-cells Targeted to Vascular Endothelium.* PLOS Pathogens, 2016. 12(9): p. e1005832.

63. Yaiw, K.-C., et al., *High prevalence of human cytomegalovirus in carotid atherosclerotic plaques obtained from Russian patients undergoing carotid endarterectomy.* Herpesviridae, 2013. 4(1): p. 3.

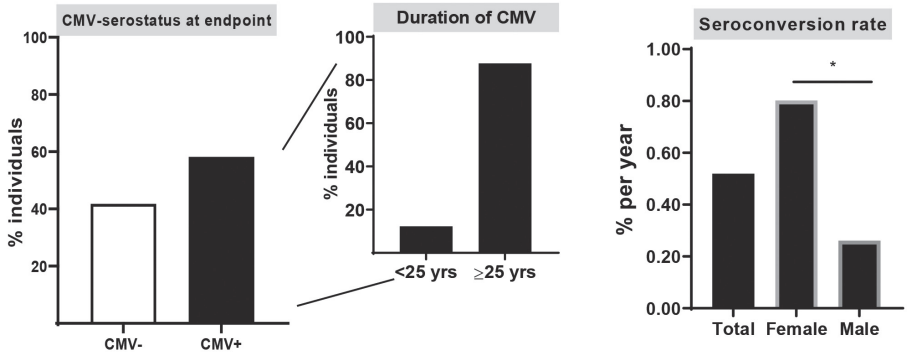
64. Dupont, L. and M.B. Reeves, *Cytomegalovirus latency and reactivation: recent insights into an age old problem.* Reviews in Medical Virology, 2016. 26(2): p. 75-89.

65. Farrell, H.E., et al., *Murine Cytomegalovirus Exploits Olfaction To Enter New Hosts.* mBio, 2016. 7(2): p. e00251-16.

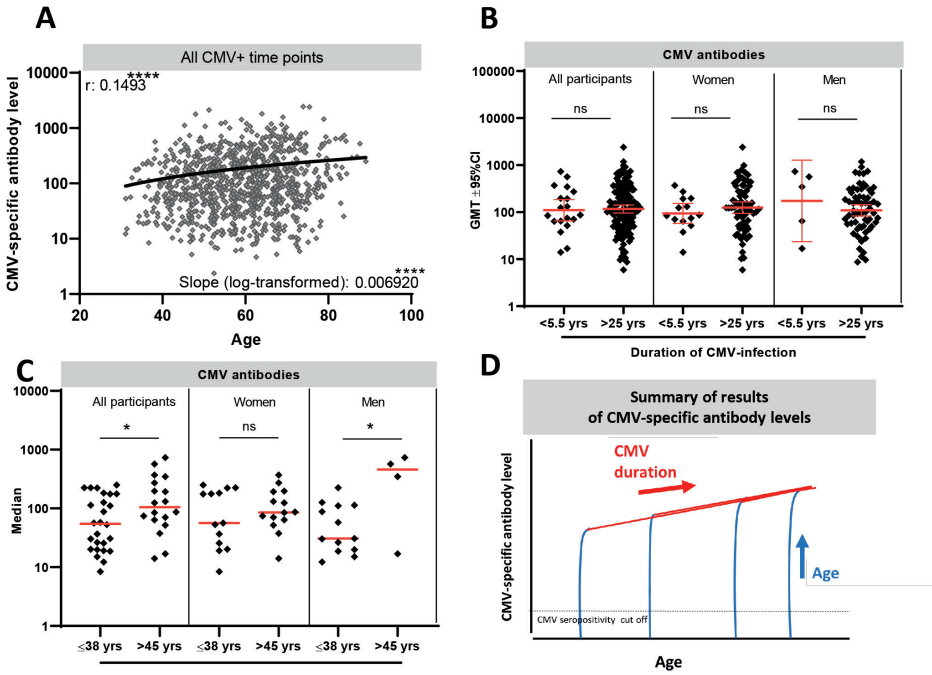
66. Adler, S.P. and M.J. Reddehase, *Pediatric roots of cytomegalovirus recurrence and memory inflation in the elderly.* Medical Microbiology and Immunology, 2019. 208(3): p. 323-328.



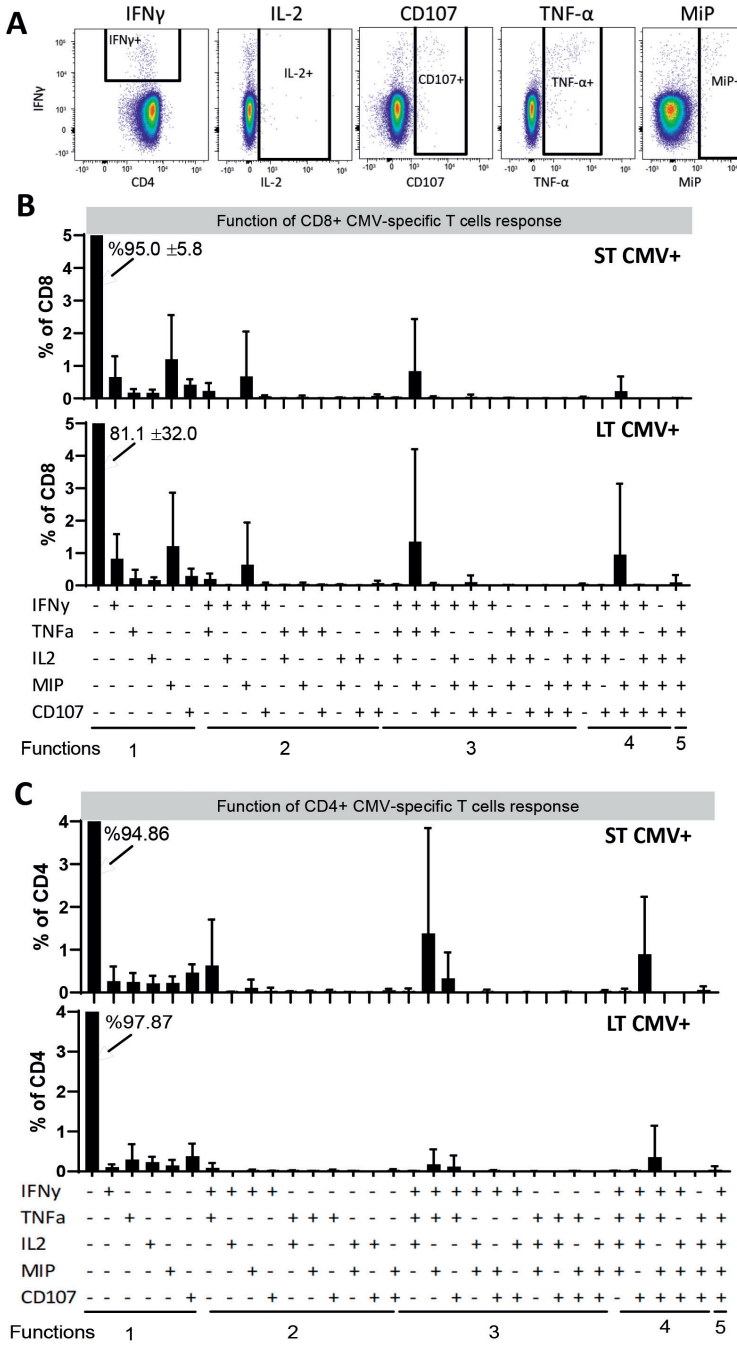
Supplementary Figure 1. Flow diagram of selection of study subjects.



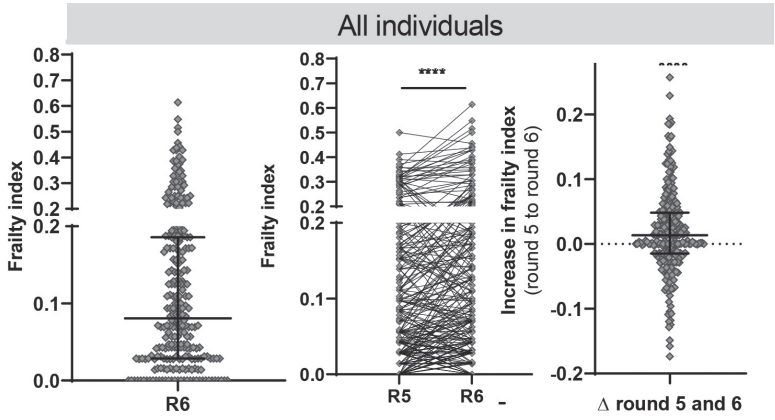
Supplementary Figure 2. CMV-serostatus at endpoint and seroconversion rate in the past 27 years. A. Left panel: CMV serostatus at end point of all individuals. Right panel: duration of CMV infection in CMV+ individuals. **B.** Seroconversion rate for total individuals and separated for women and men. Statistics was performed by chi-square test. Star indicates a *p*-vale <0.05.



Supplementary Figure 3. CMV-specific antibody levels. A. Results of the data of CMV-specific antibodies in both long-term CMV+ individuals and CMV seroconverters is summarized. **B**. Duration of CMV infection: CMV-specific antibody levels of recently seroconverted individuals (max <5.5 year after CMV conversion) compared with those of long-term CMV+ individuals (> 25 years CMV+) per sex. **C.** Age at seroconversion: CMV-specific antibody levels of individuals that seroconverted at younger age (≤38yr of age) or older age (≥45yr of age, mean age 58.5±8.1, shortly after CMV-seroconversion (<max 5.5 years)).Analysis of age at seroconversion was performed on a sub selection 18/19 ST CMV+ individuals and 26/116 LT CMV+ individuals. **D.** Summary of results of CMV-speciifc antibody levels.



Supplementary Figure 4. Cytokine profiles after stimulation with CMV-specific peptide pools. A. Representative flow cytometry plots in the CMV-specific T-cell stimulation assay after stimulation with a CMV-specific peptides. **A,B**. Figures show percentage of cells that produce IFNγ, TNFα, MIP-1β, CD107, IL-2, or a combination of these cytokines after in-vitro stimulation of PBMCs for CD8 T-cells (**A**) and (**B**) CD4 T-cells.



Supplementary Figure 5. Frailty index score at round 5 and 6 and the difference between these rounds.

8

General discussion

CMV-ENHANCED AGEING OF THE IMMUNE SYSTEM

In the early 2000s, it was first suggested that CMV may be a driving force of immunological ageing, as many hallmarks of the T-cell pool observed in older individuals were also observed in individuals with CMV infection. The life-long control of infection with CMV drives exceptionally large T-cell responses, which may even increase with age. This is thought to lead to overcrowding or exhaustion of resources of the immune system. Mechanistically, this was suggested to be due to out-competition of non-CMV-specific T-cells. Alternatively, or additionally, CMV was suggested to induce a chronic sub-clinical systemic inflammatory state, which gets even more pronounced in older adults and is associated with impaired health outcomes. In line with this, CMV was identified as part of the 'immune risk profile' for mortality [1, 2]. Either way, it is thought that duration of CMV infection and the number of experienced viral reactivations of CMV define the size of the CMV effect on the immune system. In mouse models, long-term infection with mouse CMV (MCMV) was indeed shown to hamper the T-cell response to heterologous infections [3, 4]. Cross-sectional studies in humans also supported the view that the immune response to CMV increases with age [5-7], and the first studies into the clinical consequences of CMV infection suggested a decreased immune response to influenza vaccination in CMV-infected individuals [8, 9]. Lastly, large cohorts showed an overall increased mortality risk in CMV-infected individuals [10, 11] and in individuals with higher CMV-specific antibody levels [11-13]. CMV, a previously seemingly harmless virus for healthy individuals, suddenly did not seem so harmless anymore, and might actually have major consequences for health and even reduce people's life expectancy. The intuitively logical theory of CMV-enhanced ageing of the immune system quickly became firmly established. However, "if it quacks like a duck, it is not always a duck", and several key questions remained to be investigated about the CMV-enhanced ageing theory.

First, in contrast to the observations in mouse models that CMV leads to impaired immune responses to heterologous infections [3, 4, 14], evidence in humans turned out to be much less solid. Some recent studies even revealed a *positive* association between CMV infection and the immune response to influenza [15]. This raises the following questions: *does CMV really impair immune responses to heterologous infections in humans and is this clinically relevant? Could CMV infection even be beneficial for the immune response to heterologous infections?* In addition, one may wonder what would be the mechanism of such effects of CMV. To answer these questions we need better insights into the generation and maintenance of the CMV-specific immune response and the way CMV infection impacts the total T-cell pool. *Are high CMV-specific T-cell numbers established over time? How are CMV-specific T-cell responses maintained at such high numbers? Are the large effects of CMV on the T-cell pool detrimental?* Answers to these key questions are crucial to understand the effects of life-long CMV infection on the immune system. They are needed to identify the potential threat of CMV on public health. This thesis attempts to contribute to these questions and reports 4 main findings. First, the immune response to influenza vaccination (**chapters 2,3**) and influenza infection (**chapter 4**) is not impaired by CMV infection, and we found no evidence that CMV infection leads to increased competition between T-cells or increased inflammation levels

(**chapter 4**). Secondly, the dynamics of memory $CD8^+$ T-cells are not substantially affected by CMV infection, while the production and loss rate of memory $CD4^+$ T-cells is lower in CMV+ individuals than CMV- individuals (**chapter 6**). Third, CMV-specific T-cells express a late-stage differentiation state, and these markers correlate with a decreased production and death rates *in vivo*, but CMV-specific T-cells do not substantially differ in their in vivo dynamics compared to bulk memory T-cells, as assessed by heavy water labelling (**chapters 5, 6**). Lastly, we found no evidence that the CMV-induced changes in the T-cell pool and the size of the CMV-specific immune response accumulate to a large extent during long-term CMV infection (**chapter 7**). Interestingly, older age, regardless of duration of CMV infection, turned out to lead to more prominent CMV-induced changes in the T-cell pool and to larger CMV-specific immune responses (**chapter 7**), which suggests that high CMV immune responses may be merely a consequence of immunological ageing.

DOES CMV IMPAIR IMMUNE RESPONSES TO HETEROLOGOUS INFECTIONS?

CMV does not substantially hamper the immune response to influenza

The role of CMV infection on heterologous immune challenges *in vivo* in humans has mainly been investigated for the clinically relevant immune response to influenza. Predominantly negative effects of CMV on the influenza antibody response have been reported [16, 17], but conflicting studies [15, 18] have led to controversy [19]. We observed no significant effect of CMV on the antibody response to influenza vaccination, despite a significant effect of age, when the possible confounder of pre-existing immunity was reduced by studying the response to a pandemic novel vaccine strain (**chapter 2**). This is in line with a study in a large group of subjects, which reported no effect of CMV infection on the antibody response to influenza vaccination [20]. Our systematic review and meta-analysis of all studies on the association between CMV infection and the antibody response to influenza vaccination in younger and older adults showed that there is no unequivocal evidence for a negative effect of CMV infection (**chapter 3**).

Importantly, CMV mainly affects the T-cell pool, but cellular responses to influenza are investigated less frequently than antibody responses. This is probably due to logistic reasons as influenza vaccines, at least in Europe, primarily induce antibody and not T-cell responses. T-cell responses are however induced by influenza virus infection and play an important role in elimination of the virus and reduction of severity of disease. Therefore, we also studied the effect of CMV infection on the T-cell response to influenza virus infection in older adults. Despite the fact that CMV infection reduced the frequency of memory influenza virus-specific T-cells, it did not affect the T-cell response to acute influenza virus infection. Also, severity of symptoms of influenza virus infection was not increased by CMV infection, suggesting no substantial clinical impact of CMV on influenza virus infection. While others showed a lower activation level of $CD8^+$ T-cells after influenza vaccination in CMV+ compared to CMV- older adults [21], we did not observe this after influenza virus infection (**chapter 4**). Two other papers investigated the association between CMV and the cellular immune response

to influenza virus *in vitro* in healthy individuals, and revealed either a negative association [22] or a positive association [23]. Taken together, we conclude that there is no unequivocal evidence that CMV infection leads to an impaired immune response to influenza, one of the most relevant and most frequently used models for heterologous infections in humans.

As the CMV-enhanced ageing theory seems to persevere and influenza in older adults remains an important matter, more studies into CMV and the immune response to influenza will probably be performed. It remains important to interpret small-scale studies, especially with reports of only one influenza strain or one antibody outcome, such as the response rate, with caution. For future studies, we recommend to at least always report the geometric mean titer (GMT) pre- and post-vaccination (or the ratio, GM ratio) and confidence intervals, in line with European Medicines Agency-guidelines. This enables objective comparison between studies, and prevents to some extent influences of pre-existing immunity that may act as confounders. Furthermore, statistically adjusted analysis for the confounders age and pre-existing immunity are always needed.

Beyond influenza

The impact of CMV-enhanced ageing of the immune system on public health, and on the economy, may be most profound for the immune response to influenza, and influenza remains a leading cause of infectious morbidity, hospitalization and death in older adults. Also, the influenza vaccine is the number one vaccine recommended in Europe and the United States, next to the pertussis and varicella zoster vaccines, for older adults. Thus, clarifying the role of CMV on influenza infection and vaccination may help to identify possible vaccine optimization strategies that could have large impact. On the other hand, antibody responses to influenza vaccination are quite complicated to investigate. They constitute mostly a recall response, established by relatively frequent, natural exposure to the influenza virus and by possible previous seasonal vaccinations. Due to relatively large cross-reactivity among strains, also pre-existing immunity of other influenza virus types may influence the subsequent seasonal influenza vaccine response. Therefore, other models might be more suitable to test the principle of CMV-enhanced ageing. Beyond influenza, only a handful of studies have been performed in humans, with contradicting results. In a study in adult patients with antineutrophil cytoplasmic antibody–associated vasculitis, it was shown that expansion of $CD4^{+}CD28^{null}$ T-cells, most likely induced by CMV reactivation, was associated with a reduced antibody response to pneumococcal vaccination [24]. In contrast, in healthy young children CMV infection was associated with an increased differentiation state of T-cells without hampering the $CD8^{+}$ T-cell response to staphylococcal enterotoxin B (SEB) and the antibody response to measles[25]. In addition, in adults [26] and in our cohort of healthy to frail older adults (**chapter 7**, data not shown), the response to SEB was not negatively associated with CMV infection. Thus, results on heterologous challenges other than influenza, also indicate no clear evidence for a negative association of CMV infection and the immune response to a heterologous infection.

MAJOR HYPOTHESIS ON THE MECHANISM OF CMV-ENHANCED AGEING HAS LOST SUPPORT

Competition for 'limited immunological space' between the large numbers of CMV-specific T-cells and other antigen-specific T-cells has been proposed as a main explanation for the negative impact of CMV by several researchers [27-29]. In fact only a handful of studies report data supporting this view. In support of this view, Epstein-Barr virus-specific T-cells were found to be decreased in frequency and absolute number in CMV+ compared to CMV- older adults [30]. Influenza T-cell frequencies (**chapter 4**) or responses [22]were found to be decreased in CMV-infected individuals in some studies, but not in others [23]. It is important to realize that decreased *frequencies* of influenza virus specific T-cells or T-cell responses in CMV-infected individuals do not necessarily imply that influenza-specific T-cell *numbers* are also reduced. CMV-specific T-cell numbers can become very large and may thereby reduce the frequency of other T-cell specificities (**chapter 4**) [22]). In fact, there is no convincing evidence for loss of memory T-cells in CMV+ individuals due to competition for homeostatic growth or survival factors, also referred to as passive memory attrition, in humans. We also found no evidence for reduced T-cell production rates or increased T-cell death rates *in vivo in* CMV+ individuals (**chapter 6**), thereby contradicting the theory of continuous recruitment of new CMV-specific T-cells in to the memory pool thereby actively pushing out pre-existing memory T-cells specific for influenza (a.o.), also referred to as 'active memory attrition' [31, 32]. Furthermore, the presence of high CMV-specific T-cells numbers is typically assessed in the blood, reflecting only about 2% of the total T-cell population in the body, which might not be representative for the total number of T-cells. Large numbers of CMV-specific T-cells were shown to be present outside the blood as well, e.g. in bone marrow, lymph nodes as well as in other organs [33], although others reported only high numbers of CMV-specific T-cells in blood and not in lymph nodes [34]. We believe that the immune system might, especially during acute infections, be flexible in 'space' in order to host clonal expansions of T-cells to combat infections. Indeed, we found that large CMV-specific T-cell responses in CMV+ older individuals did not hamper the T-cell response to acute influenza virus infection (**chapter 4**).

The other major hypothesis on how CMV could impact the function of the immune system, is through the induction of pro-inflammatory cytokines. These pro-inflammatory cytokines may in turn influence other immune challenges as a bystander effect, for example through the induction of TNFα, IL-6 or high granzyme B levels. In mice, CMV-induced IL-6 production was shown to reduce the number of bystander memory T-cells and affects the subsequent immune response to a heterologous challenge [35]. However, we observed no association between CMV and pro-inflammatory cytokine levels in humans (**chapter 4**). In line with this, a longitudinal study over 10 years also showed that CMV infection was not associated with a steeper increase in pro-inflammatory cytokine levels[36]. In theory, influenza antibody responses may also be hampered by CMV infection through reduced $CD4^+$ T-helper cell responses, thereby hampering the antibody production of B-cells. In mice, it was recently shown that CMV infection led to delayed B-cell class switching, which impaired the peak

antibody response to VSV [37]. In line with this, in humans it was shown that a lower percentage of class-switched B-cells pre-vaccination in CMV+ versus CMV- individuals, correlated with a reduced vaccine response to influenza [38]. It remains unclear *how* CMV leads to reduced B-cell class-switching [39]. Thus, no clear evidence on how CMV would impact the function of the immune system is present.

COULD CMV BE BENEFICIAL FOR IMMUNE RESPONSES AGAINST HETEROLOGOUS INFECTIONS?

Increasing evidence for a positive association between CMV and immune responses to heterologous infections

Despite the many reported negative associations between CMV infection and the function of the immune system, there is currently increasing evidence for a *positive* association between CMV infection and immune responses to heterologous infections, especially in younger adults. A model in which CMV might be beneficial during young adulthood, and become detrimental over time during CMV infection has been proposed [40]. A positive association has been observed between CMV and the antibody response to influenza in humans in CMV+ mice within 3 months after CMV infection compared to CMV– mice [15]. This association was explained by IFNγ-dependent bystander activation, as increased IFNγ levels in human were associated with increased influenza antibody responses and IFNγ knock-out mice showed increased virus titers and decreased antibody responses [15]. In older adults, we found increased T-cell responses to influenza virus infection early after infection in CMV+ as compared to CMV- individuals, and a positive association between influenza virus-specific and CMV-specific T-cell responses was observed later on. However, this was not associated with levels of IFNγ in serum (**chapter 4**). Positive associations were previously observed between CMV-specific T-cell responses and influenza virus T-cell responses [23], and SEB responses [26]. We observed similar associations between CMV-specific T-cell responses and SEB T-cells responses and T-cell responses after CD3/28 stimulation in CMV+ individuals (**chapter 7**, data not shown). Whether these associations are merely explained by inter-individual differences in size of T-cell responses, are due to cross-reactivity of T-cells, to bystander effects on other antigen-specific T-cells or non-antigen specific memory T-cells (virtual memory cells), remains unclear. Alternatively, NK cells might play a role, which have been shown to be affected by CMV as well [41], are not specific to a specific peptide, and might also respond in Elispot assays.

The effect of publication bias and chance

A beneficial effect of CMV on the immune system might be more likely than previously anticipated. We showed that a publication bias towards a negative association of CMV and the response rate to influenza antibody vaccination is present (**chapter 3**). Currently, publications concluding a beneficial effect of CMV on immune response to a heterologous infection seem to increase in number. This indicates that the publications of a negative association between CMV infection and the immune response to influenza, might be merely variation around the possible truth: CMV does not affect the function of the immune system

to a heterologous infection. Whether the claim of CMV-enhanced ageing on the influenza antibody response represents outliers based on chance, or instead a true, small, negative effect of CMV in certain populations or individuals remains to be investigated.

LARGE EFFECTS OF CMV ON THE T-CELL POOL: DETRIMENTAL CHANGES, OR THE REQUIRED CMV IMMUNE RESPONSE?

CMV-induced changes in the T-cell pool are no direct proof of CMV-enhanced ageing of the immune system

The many parallels between age-induced and CMV-induced changes in the T-cell pool have laid the basis for the CMV-enhanced ageing theory. In CMV-enhanced ageing research, hallmarks of an aged T-cell pool, for example increased expression of senescence markers, are sometimes used as a read-out of a CMV-impaired immune system. However, CMV induces large numbers of CMV-specific T-cells that express high levels of senescence markers [42]. Consequently, there is an increased expression of senescence markers, which resemble the changes that occur with age in the T-cell pool. Investigating the effect of CMV on the T-cell pool changes that resemble the hallmarks of CMV-specific T-cells is like investigating the effect on the alcohol level when adding vodka to wine. The fact that CMV-specific memory T-cells are present in high frequencies ('lots of vodka') and themselves express high levels of senescence markers ('high alcohol') is often not taken into account when interpreting the overall level of immunosenescence ('the alcohol level in the mixed drink'). Since the *specificity* of the T-cells is not taken into account, the increased level of senescence in the T-cell pool is taken as a 'bad thing', regardless of the specificity of the cells. However, possible associations between the frequency of for example senescent T-cells and an immunological outcome should really be based on senescent T-cells *specific* for the immunological challenge. It is therefore crucial to investigate not only general immunosenescence levels in the T-cell pool, but specifically investigate non-CMV-specific memory T-cells as well.

Does CMV affect the phenotype of non-CMV specific memory cells?

It has long remained unknown to what extent CMV-specific T-cells themselves are responsible for the CMV-induced changes in the T-cell pool. Studies have suggested a change by CMV infection in phenotype of other antigen-specific T-cells like EBV-specific T-cells [30]. Unfortunately, influenza-virus specific T-cell numbers (**chapter 4**) were too low to reliably measure their expression of senescence markers. However, other work from our group on EBV-specific T-cells, showed no changes in the expression of senescence markers by CMV infection (*Lanfermeijer et al*, manuscript in preparation). Our t-SNE analysis did suggest that the CMV-induced alterations in the T-cell pool are not fully explained by the presence of CMV-specific T-cells (**chapter 6**). The increased T-cell numbers with high senescence marker expression in CMV+ individuals were only to some extent overlapping with the phenotype of CMV-specific T-cells. Note that a t-SNE analysis is based on the expression level of markers (continuous outcome: mean fluorescence intensity), while most papers merely investigate presence of or absence of a marker (dichotomic outcome: negative or positive) [30] (Lanfermeijer et al, manuscript in preparation). So differences in non-CMV

specific memory T-cells by CMV infection may be small and restricted to a increased expression level of senescence markers. When expression levels were assessed in our group, no difference in senescence markers for EBV-specific T-cells, but differences in other markers (increased CD45RO and decreased PD-1) were observed in CMV+ individuals compared to CMV- individuals (Lanfermeijer et al, manuscript in preparation). Further research, possibly investigating the expression level of senescence- and memory markers of other antigen-specific T-cells in CMV- and CMV+ individuals, is needed to confirm these CMV-induced changes. Alternatively, non-CMV-specific memory T-cells as a whole can be investigated. Ideally, all CMV-specific T-cells would have to be deleted from the T-cell pool of CMV-infected individuals, to compare the characteristics of the non-CMV specific T-cell pool of CMV-infected individuals with the T-cell pool of CMV- individuals. Taken together, our work (**chapter 6**) indicates that a part of the CMV-induced changes in the T-cell pool might not be explained by CMV-specific T-cells, but to what extent this is reflected in other-antigen specific T-cells needs further investigation.

MEMORY INFLATION: HOW ARE HIGH CMV-SPECIFIC T-CELL NUMBERS ESTABLISHED AND MAINTAINED OVER TIME?

Viral reactivation is thought to be key in memory inflation of CMV-specific T-cells

In order to understand the potential effect of CMV on the immune system, we need to know whether memory CMV-specific T-cell responses increase over time in humans ('memory inflation'). A lot of the knowledge about memory inflation was gained from mouse studies, which reported memory inflation predominantly for $CD8^+$ CMV-specific T-cells, and sometimes also for $CD4^+$ CMV-specific T-cells. In humans, high CMV-specific T-cell responses have been shown to be associated with older age in cross sectional studies [30]. It is generally assumed that continuous TCR triggering by antigen presentation is key in the maintenance of high numbers of CMV-specific T-cells. CMV expresses latency-associated genes during latent infection, but the large T-cell responses against CMV are mainly directed against lytic-expressed genes. Viral reactivation is expected to lead to antigen presentation to CMV-specific T-cells, regardless of production of full virus particles and viral dissemination. This way, the number of CMV-specific T-cells is thought to increase in time. An increased number of CMV-specific T-cell numbers might depend on the recruitment of more CMV-specific memory T-cells after TCR-triggering. Production of CMV-specific memory T-cells may probably be mostly due to proliferation of CMV-specific memory T-cells by TCR-triggering of existing memory CMV-specific T-cells, clonal expansion and limited contraction to form memory T-cells. Thereby, this would lead to an increase of CMV-specific T-cell number over time. In this case, increased proliferation in CMV-specific T-cells would be the underlying mechanism of the high CMV-specific T-cells numbers. Alternatively, the increase of CMV-specific T-cell number might be explained by accumulation of cells having an extended lifespan, possibly by an increased resistance for apoptosis. This effect can be especially large when not only homeostatic proliferation, but also antigen stimulation results in production of memory CMV-specific T-cells. If these are long-lived (having a lower loss rate than production

rate), CMV-specific T-cells will accumulate over time. Previously, a study in 3 individuals reported a lower turnover of CMV-specific T-cells *in vivo* compared to bulk memory T-cells, arguing for a model of accumulation of long-lived CMV-specific T-cells over time [43].

Our work investigated the phenomenon of memory inflation in two different studies; (1) by investigating the effect of duration of CMV infection on the T-cell response and (2) by investigating the underlying dynamics of T-cells (**Figure 1, Model A**).

The role of duration of CMV infection and underlying dynamics of CMV-specific T-cells

The association between age and the size of the CMV-specific T-cell response in human was previously assed in some longitudinal studies. These were often limited to a few years of follow-up and have reported conflicting results [44-47]. We followed CMV-specific T-cell numbers for up to 1.5 years, and found no increase over time over this timespan (**chapter 6**). In a unique longitudinal study with 27 years of follow-up, we were able to assess time since CMV-seroconversion based on consecutive antibody analysis in healthy individuals. We found that large CMV-specific T-cell responses were already present shortly after CMV seroconversion (**chapter 7**), which does not support a role for memory inflation in $CD8^+$ T-cells. Furthermore, even in young children, i.e. short duration of CMV infection, high CMV-specific T-cell numbers have been observed [25], suggesting that already within a short period of time large numbers of CMV-specific T-cells are established. Thus, long-term infection with CMV seems to play a smaller role in the establishment of high CMV-specific T-cell numbers than previously anticipated based on mouse studies. Interestingly, we found that high $CD4^+$ CMV-specific T-cell responses were present in elderly that seroconverted at older age, than in elderly that seroconverted at younger age. We expect that this may be explained by an impaired primary immune response in an aged immune system and subsequent a larger viral reservoir that is controlled by high CMV-specific immune responses (**Chapter 7**). Thus, the reported associations between age and the magnitude of the $CD4^+$ CMV-specific T-cell response will most likely not be explained by inflation of $CD4^+$ CMV-specific T-cell numbers over time, but (at least partly) by increased $CD4^+$ CMV-specific T-cell response when CMV-seroconverting older age. For $CD8^+$ T-cells, no proof for an association between age at seroconversion and the magnitude of the CMV-specific T-cell response was found.

We also investigated the dynamics of CMV-specific T-cells compared to bulk memory. We found lower expression of proliferation markers and reduced sensitivity for apoptosis of CMV-specific T-cells compared to bulk memory T-cells (**chapter 6**). Also, CMV-specific T-cells expressed a late-stage differentiation state, which in turn was shown to be associated with a reduced turnover rate *in vivo*, probably reflecting homeostatic proliferation (**chapter 6**). Despite this association and despite the observed indications based on snapshot markers like Ki-67 and Bcl-2, no significant differences in the *in vivo* turnover rate were observed between CMV-specific T-cells and bulk memory T-cells from CMV- and CMV+ donors (**chapter 6**).

Our study suggests that the maintenance of high numbers of CMV-specific $CD8^+$ T-cells does not require a substantially altered production or death rate compared to other memory $CD8^+$ T-cells. Thus, this would suggest that maintenance of CMV-specific T-cells numbers is accomplished by similar dynamics than other antigen-specific T-cells of lower numbers.

Thus, our work entails three new insights, namely (1) the effect of duration of CMV infection on the magnitude of the CMV-specific T-cells response is limited, (2) older age, regardless of duration of CMV infection, is associated with increased CMV-specific T-cell $CD4^+$ responses, and (3) CMV-specific T-cells have similar underlying dynamics as bulk memory. These insights argue against both an increase of CMV-specific T-cell numbers over time as well as an effect of viral reactivation on the CMV-specific T-cell dynamics. If we incorporate these three findings in the model of memory inflation, no large role for viral reactivation and TCR triggering in the maintenance of high CMV-specific T-cell numbers is to be expected (**Figure 1, model B**).

Alternative model and further research

Our results challenge the mainstream of 'T-cell memory inflation by viral reactivation' and highlight that the underlying mechanism of the large CMV-specific T-cell responses in humans needs further attention. Further research into the association between age at the moment of CMV-seroconversion and both $CD4^+$ and $CD8^+$ CMV-specific T-cell responses shortly after seroconversion ask for further attention, starting with mouse studies. To further understand the phenomenon of memory inflation, two other questions would be particularly of interest in further research.

The first question is if memory inflation of CMV-specific T-cells occurs in the first years after primary CMV infection. Obviously, in humans the moment of primary CMV infection is typically unknown, making it very complicated to draw firm conclusions on memory inflation, as this might already have occurred short-term after CMV-seroconversion. In fact, the initial contraction phase after the T-cell peak during primary infection or the set point within 1 or 2 years after primary infection might play a more crucial role in the establishment of these large T-cell numbers. To address this, others have investigated the CMV-specific T-cell response in recipients of a kidney transplant when a CMV+ donor kidney was transplanted into a CMV- recipient. These studies also revealed no direct evidence for memory inflation [48-50], although the percentage effector memory of total CMV-specific T-cells did seem to increase over time [48]. However, these patients were undergoing various degrees of immunosuppression. Further research, although very challenging, would of course ideally be performed in recently CMV-seroconverted healthy individuals to investigate the T-cell response in the first years after primary CMV infection. It might be that, maybe especially when primary CMV infection occurs in older adults affected by an aged immune system, memory inflation of CMV-specific T-cells will occur (**Figure 1, Model C**).

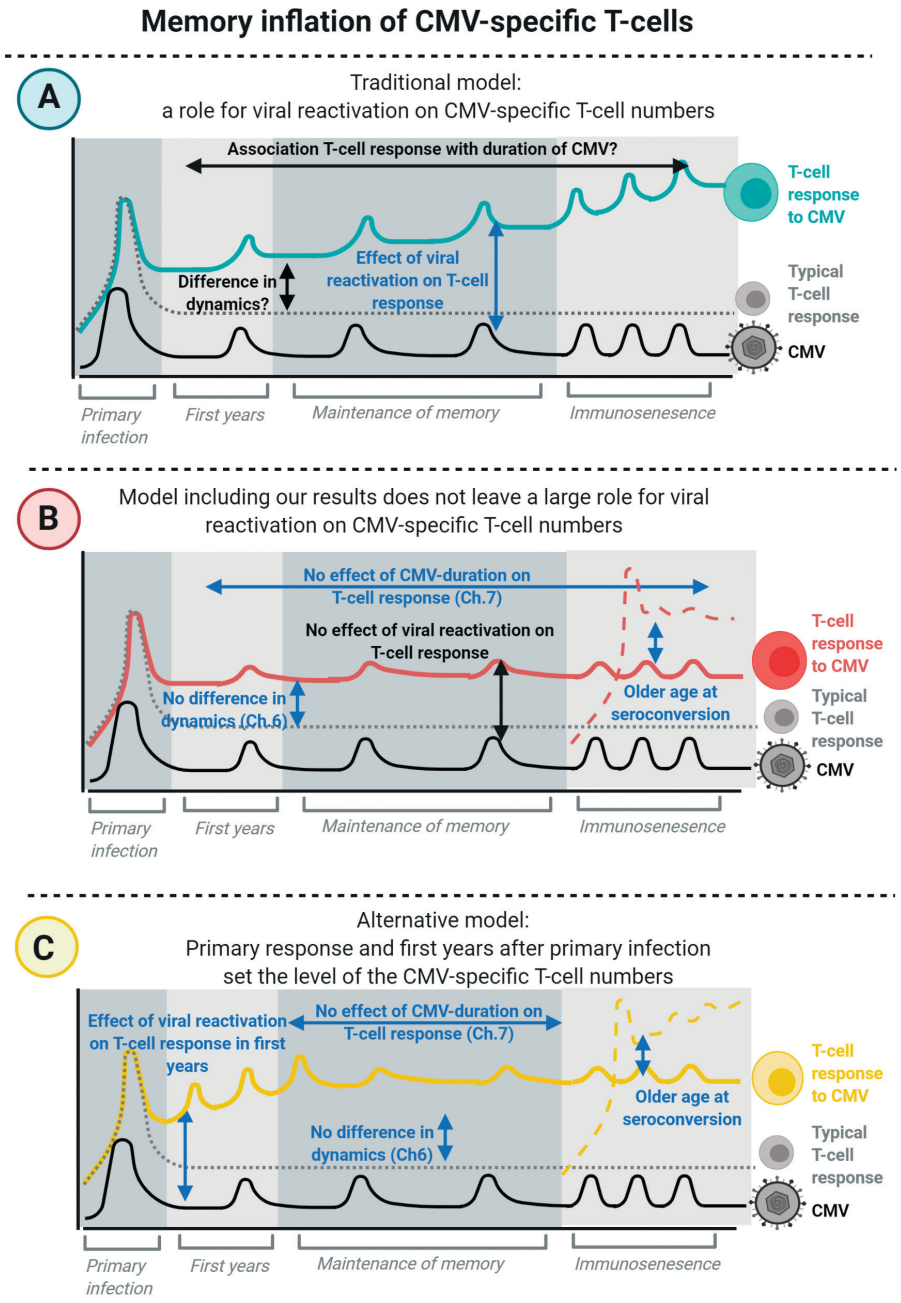


Figure 1. Models of memory inflation of CMV-specific T-cells in healthy individuals.

Secondly, recent insights from adoptive transfer studies in mice (David Masopust, conference) might have identified a key process in the establishment of large numbers of high quality T-cell responses. Generally, excessive and sustained stimulation by antigen

drives exhaustions of T-cells [51]. In mice, it was shown that frequent *in vivo* boosting of T-cells by sequential antigenic challenge can result in non-exhausted high quality T-cells in cases where the immune system gets enough time to "rest" in between the challenges ('boosting-with-breaks') (adoptive transfer mice experiments, David Masopust, conference). Interestingly, multiple boosts with breaks led to a restricted contraction phase after the peak of the T-cell response, thereby yielding high numbers of memory T-cells. We speculate that a process like this may also occur in CMV infection: CMV may just give the immune system the required rest in between reactivation episodes to achieve high numbers of memory T-cells while maintaining their high quality. When the rest period in between sequential challenges was shortened in mice, an exhausted T-cell phenotype was established (David Masopust, conference), a phenomenon that is also seen in humans during chronic infections (**chapter 5**). Limited contraction after numerous T-cell challenges with in-between-breaks seems applicable to the unique situation of 'dynamic latency' in CMV. However, this idea of limited contraction after *numerous* T-cell challenges including in-between rests, would imply an effect of time on memory CMV-specific T-cell numbers. As discussed above, our results indicate duration of CMV infection does not seem to play a large role in humans. Whether a system of "boosting-with-breaks" is indeed responsible for reduced contraction of T-cell responses to CMV in humans, and might be key in the unique T-cell phenotype and magnitude of the T-cell response, would also be of great interest for further research and will help to further understand the phenomenon of memory inflation in human.

A CURRENT VIEW ON CMV AND AGEING OF THE IMMUNE SYSTEM: HIGH CMV-SPECIFIC IMMUNE RESPONSES AS CAUSE OR CONSEQUENCE OF IMMUNOLOGICAL AGEING?

A changing mainstream view

The mainstream view on the impact of CMV on ageing of the immune system has changed quite a lot over the last couple of years [52, 53]. Associations between CMV infection and impaired immunological health outcomes turned out to be less strong than initially thought, and even positive associations between CMV and health outcomes have been reported. We observed no link between CMV infection and frailty. In fact, the link between CMV infection and increased risk for mortality might to a large extent be due to increased cardiovascular-related death and not due to a direct effect on the immune system. The evidence for CMV-enhanced prevalence of cardiovascular disease and severity of disease seems rather solid [54]. Mechanistically, CMV in endothelial cells is thought to reactivate quite often, causing endothelial dysfunction, which may in turn contribute to the development of atherosclerosis and ischemic heart disease [55]. For immunological clinical outcomes such as the immune response to influenza, no clear evidence pointing into specific mechanisms of CMV as a direct cause is currently available, e.g. for increased pro-inflammatory levels [36] and out competition of T-cells (**Chapter 4**) . Importantly, more recent longitudinal studies into the role of CMV in the 'immune risk profile' could not always reproduce the findings of the original Swedish longitudinal immune risk study [56], which may be partly explained by genetic differences. Taken together, a current model of the association of CMV infection and ageing

of the immune system, would include a moderate, modulatory role of CMV [40]. CMV may only marginally influence the immune system by inducing bystander effects, which may be negligible or even beneficial during young adulthood and which may become detrimental only at older age [40]. However, it goes without saying that correlations and associations between CMV and hallmarks of immunological ageing or health outcomes cannot distinguish between cause and consequence. Therefore, an alternative model that we would like to propose is that a high immune response to CMV may not be the cause, but the result of an aged immune system.

Model 1: CMV is only detrimental in individuals with impaired control of the virus

Several studies identified individuals with high CMV-specific antibody levels as at higher risk for impaired immune responses to heterologous viruses [8, 57] or poor general health status [12, 13, 54, 58, 59]. However, we suggest that the link between experienced viral reactivation and the CMV-specific antibody level is probably more complicated than simply reflecting experienced viral reactivation (**chapter 7**). We expect that the size of the established latent reservoir after primary infection is essential in the level of the CMV-specific immune response. We speculate that after a delayed or diminished primary immune response to CMV, a high level of CMV-specific immunity is necessary to maintain control of latent infection. Therefore, we think it is crucial to measure the viral reservoir and reactivation itself, instead of the immune response as a surrogate marker. Some groups have managed to measure the, relatively low, latent reservoir of CMV in monocytes using digital droplet PCR [60] or even nested PCR [61]. We were not able to reproduce such measurements of CMV DNA by nested PCR in the healthy older adults in our studies (**chapters 4 and 6**), even though 1-10 virus copies could be detected in control samples (data not shown). Whether the latent reservoir of CMV in monocytes is representative of the total CMV reservoir, being aware of the broad tropism of CMV, and whether the size of the latent reservoir indeed reflects the chance for the virus to reactivate, remains to be investigated. Nevertheless, there are some first indications for a positive correlation between the height of the CMV-specific immune response and the size of the latent reservoir[60], highlighting the importance of the host-virus balance.

Next to genetics, environmental experiences determine our immune status to a great extent, which could obscure relatively small modulatory effects of CMV. In humans, CMV infection might be just one of many important environmental influences defining the immune status. In the last decade, studies have uncovered a major role for the microbiome in general health, including regulation of the immune system. In mice [62] and monkeys [63], CMV was shown to affect the microbiome composition of the gut, and thereby increase inflammatory bowel disease [62] and decrease the influenza antibody immune response [63]. Almost all the animal models of CMV until now have been performed in clean, specific-pathogen-free mice. New mouse models, in which clean inbred mice are cohoused with pet shop mice [64] or born from wild mice [65], and thereby exposed to large numbers of different antigens, have received a lot of attention in the last couple of years. The immune systems of these so-called 'dirty mice'

are more representative of the human immune system, which also encounters numerous pathogens and antigens during life. Whether CMV also plays a dominant role in dirty mice would be interesting to investigate. Another important difference between studies in mice and humans might be the order in which different infections occur. Humans are thought to be exposed to influenza virus approximately every 5 years. In the first five years of life, most children have already encountered their first influenza infection, while only about 25% of them are CMV-seropositive [66]. Thus, in humans it is more likely that influenza exposure precedes CMV exposure. It has been suggested that, at least for antibody responses, the first encountered influenza strain in childhood confers lifelong protection against severe disease for this group of influenza strains (the HA antigen group) – also referred to as 'first flu forever' [67, 68]. Thus, the first influenza virus infection might affect sequential influenza infections for life, thereby possibly reducing the effect of CMV on the immune response to this type of influenza. In mice, CMV infection always given before the immune response to a heterologous infection is investigated. Also the number of influenza infections might be crucial. In mice, most studies look into the effect of CMV on one heterologous infection. However, humans are likely to encounter multiple influenza infections after CMV infection, inducing a recall response instead of a *de novo* immune response. Mice experiments looking into the impact of the number and sequence of infections, and different environmental influences, could help to understand the difference between mouse and human in the CMV-induced effects on the function of the immune system.

Model 2: High CMV-specific immune responses may be a hallmark rather than a cause of immunological ageing

Prolonged exposure to CMV infection has been suggested to be harmful because in older adults the CMV-specific immune response was found to be higher than in younger adults. However, as ageing is inevitable, it is impossible to pull apart the factors age and time in CMV+ individuals. In order to distinguish between age and time of CMV infection comparative studies, separately assessing ageing in CMV- individuals and CMV+ individuals, are required [69]. We found that higher age at the moment of CMV seroconversion was associated with more profound changes in the T-cell pool and with higher CMV-immune responses (**chapter 7**), highlighting that multiple reactivation events are not necessary to establish a high CMV-specific immune response. As CMV leads to full viral reactivation in severely immunocompromised individuals, such as transplantation patients on immunosuppressive drugs and HIV-infected individuals, it is not surprising that CMV partially reactivates in moderately immune-affected situations, such as in aged individuals or immune immature children. Indeed, detection of viral load in urine and saliva occur to a greater extent in older adults than in younger adults. In conclusion, high CMV-specific immune responses may identify individuals in which the immune system is impaired by ageing. Even more, receiving primary infection with CMV at older age was associated with a worse overall health status as assessed by a multiple deficit frailty index (**chapter 7**). This indicates that receiving a primary CMV infection may in fact be a consequence of immunological ageing and poor health. Sensitivity for CMV infection indeed seems to play a role in general, as only 2/3rd of

individuals living with a CMV+ partner is CMV-infected themselves [70]. Thus, it might even be that receiving a primary CMV infection in older adults is a reflection of the ageing status of individuals, or their so-called biological age.

CONCLUDING REMARKS

The research presented in this thesis has generated some important insights into the CMV-enhanced ageing theory. On the one hand it questions the negative effect of CMV on the function of the immune system. Our work suggests that when trying to tackle the aged-impaired immune response to influenza, not much will be gained by interfering with CMV e.g. by vaccination. On the other hand, this thesis again stresses the uniqueness of CMV-specific T-cells and the large impact of CMV infection on the T-cell pool. To what extent non-CMV specific T-cells are also affected by CMV asks for further research. A next step could be to measure expression of senescence markers in multiple non-CMV antigen-specific T-cells in large cohorts, thereby focusing on the known CMV-induced changes in the T-cell pool. Further knowledge on how CMV-specific T-cells in humans become so abundant, while retaining their high quality, would be of great value. This will contribute to our current understanding of fundamental T-cell concepts, including maintaining T-cell quality. Also, this will offer important implications for vaccination strategies that aim to induce long-term and high immunological memory responses, for example using CMV-vector based vaccines to combat infections like HIV [71]. Before potentially extrapolating these high quality T-cell responses into CMV-vector based vaccination strategies, the role of antigen stimulation during CMV infection should be investigated in more detail. A combination of measuring the viral reservoir and viral reactivation itself, and the CMV-specific immune responses is needed. This will help to further understand the rather paradoxical increase of CMV-specific immune responses with age and the complex viral-host balance. In all this, the $CD4^+$ T-cell pool should not be disregarded. As CMV mainly affected $CD4^+$ T-cells in terms of T-cell dynamics, there might be a broader role for cytotoxic CD4+ T-cells in control of CMV infection. Finally, the possibility of high CMV-specific immune responses not as a cause, but merely as a hallmark of ageing, deserves further attention. Rather than solely looking for biomarkers to identify individuals that are most prone to severe CMV-related changes in the immune system and to clinical consequences, we may have to explore the possibility to use the immune response to CMV or the size of the CMV latent reservoir as biomarkers of immunological ageing. Considering this view might shine a whole new light on the association between CMV and ageing of the immune system.

REFERENCES

1. Olsson, J., et al., *Age-related change in peripheral blood T-lymphocyte subpopulations and cytomegalovirus infection in the very old: the Swedish longitudinal OCTO immune study.* Mech Ageing Dev, 2000. 121(1-3): p. 187-201.
2. Wikby, A., et al., *Expansions of peripheral blood CD8 T-lymphocyte subpopulations and an association with cytomegalovirus seropositivity in the elderly: the Swedish NONA immune study.* Exp Gerontol, 2002. 37(2-3): p. 445-53.
3. Redeker, A., et al., *The Contribution of Cytomegalovirus Infection to Immune Senescence Is Set by the Infectious Dose.* Front Immunol, 2017. 8: p. 1953.
4. Mekker, A., et al., *Immune senescence: relative contributions of age and cytomegalovirus infection.* PLoS Pathog, 2012. 8(8): p. e1002850.
5. Komatsu, H., et al., *Population analysis of antiviral T-cell responses using MHC class I-peptide tetramers.* Clin Exp Immunol, 2003. 134(1): p. 9-12.
6. Karrer, U., et al., *Memory inflation: continuous accumulation of antiviral CD8+ T-cells over time.* J Immunol, 2003. 170(4): p. 2022-9.
7. Klenerman, P., *The (gradual) rise of memory inflation.* Immunol Rev, 2018. 283(1): p. 99-112.
8. Trzonkowski, P., et al., *Association between cytomegalovirus infection, enhanced proinflammatory response and low level of anti-hemagglutinins during the anti-influenza vaccination--an impact of immunosenescence.* Vaccine, 2003. 21(25-26): p. 3826-36.
9. Wald, A., et al., *Impact of human cytomegalovirus (CMV) infection on immune response to pandemic 2009 H1N1 influenza vaccine in healthy adults.* J Med Virol, 2013. 85(9): p. 1557-60.
10. Savva, G.M., et al., *Cytomegalovirus infection is associated with increased mortality in the older population.* Aging Cell, 2013. 12(3): p. 381-7.
11. Gkrania-Klotsas, E., et al., *Seropositivity and Higher Immunoglobulin G Antibody Levels Against Cytomegalovirus Are Associated With Mortality in the Population-Based European Prospective Investigation of Cancer–Norfolk Cohort.* Clinical Infectious Diseases, 2013. 56(10): p. 1421-1427.
12. Roberts, E.T., et al., *Cytomegalovirus antibody levels, inflammation, and mortality among elderly Latinos over 9 years of follow-up.* Am J Epidemiol, 2010. 172(4): p. 363-71.
13. Wang, G.C., et al., *Cytomegalovirus infection and the risk of mortality and frailty in older women: a prospective observational cohort study.* Am J Epidemiol, 2010. 171(10): p. 1144-52.
14. Cicin-Sain, L., et al., *Cytomegalovirus infection impairs immune responses and accentuates T-cell pool changes observed in mice with aging.* PLoS Pathog, 2012. 8(8): p. e1002849.
15. Furman, D., et al., *Cytomegalovirus infection enhances the immune response to influenza.* Science Translational Medicine, 2015. 7(281).
16. Trzonkowski, P., et al., *From bench to bedside and back: The SENIEUR Protocol and the efficacy of influenza vaccination in the elderly.* Biogerontology, 2009. 10(1): p. 83-94.
17. Wald, A., et al., *Impact of human cytomegalovirus (CMV) infection on immune response to pandemic 2009 H1N1 influenza vaccine in healthy adults.* Journal of Medical Virology, 2013. 85(9): p. 1557-1560.
18. Strindhall, J., et al., *Humoral response to influenza vaccination in relation to pre-vaccination antibody titres, vaccination history, cytomegalovirus serostatus and CD4/CD8 ratio.* Infectious Diseases, 2016. 48(6): p. 436-442.
19. Merani, S., et al., *Impact of Aging and Cytomegalovirus on Immunological Response to Influenza Vaccination and Infection.* Front Immunol, 2017. 8: p. 784.

20. den Elzen, W.P., et al., *Cytomegalovirus infection and responsiveness to influenza vaccination in elderly residents of long-term care facilities.* Vaccine, 2011. 29(29-30): p. 4869-74.

21. Enani, S., et al., *Impact of ageing and a synbiotic on the immune response to seasonal influenza vaccination; a randomised controlled trial.* Clin Nutr, 2017.

22. Derhovanessian, E., et al., *Latent infection with cytomegalovirus is associated with poor memory CD4 responses to influenza A core proteins in the elderly.* J Immunol, 2014. 193(7): p. 3624-31.

23. Theeten, H., et al., *Cellular Interferon Gamma and Granzyme B Responses to Cytomegalovirus-pp65 and Influenza N1 Are Positively Associated in Elderly.* Viral Immunol, 2016. 29(3): p. 169-75.

24. Chanouzas, D., et al., *Subclinical Reactivation of Cytomegalovirus Drives CD4+CD28null T-Cell Expansion and Impaired Immune Response to Pneumococcal Vaccination in Antineutrophil Cytoplasmic Antibody-Associated Vasculitis.* J Infect Dis, 2019. 219(2): p. 234-244.

25. Miles, D.J., et al., *Cytomegalovirus infection induces T-cell differentiation without impairing antigen-specific responses in Gambian infants.* Immunology, 2008. 124(3): p. 388-400.

26. Pera, A., et al., *CMV latent infection improves CD8+ T response to SEB due to expansion of polyfunctional CD57+ cells in young individuals.* PLoS One, 2014. 9(2): p. e88538.

27. Derhovanessian, E., A. Larbi, and G. Pawelec, *Biomarkers of human immunosenescence: impact of Cytomegalovirus infection.* Curr Opin Immunol, 2009. 21(4): p. 440-5.

28. Pawelec, G.A., A. Caruso, C. Grubeck-Loebenstein, B. Solana, R. Wikby, A. , *Human immunosenescence: is it infectious?* Immunological Reviews, 2005. 205: p. 257–268.

29. Tu, W. and S. Rao, *Mechanisms Underlying T-cell Immunosenescence: Aging and Cytomegalovirus Infection.* Front Microbiol, 2016. 7: p. 2111.

30. Khan, N., et al., *Herpesvirus-specific CD8 T-cell immunity in old age: cytomegalovirus impairs the response to a coresident EBV infection.* J Immunol, 2004. 173(12): p. 7481-9.

31. Selin, L.K., et al., *Attrition of T-cell memory: selective loss of LCMV epitope-specific memory CD8 T-cells following infections with heterologous viruses.* Immunity, 1999. 11(6): p. 733-42.

32. Welsh, R.M. and L.K. Selin, *Attrition of memory CD8 T-cells.* Nature, 2009. 459(7247): p. E3-4; discussion E4.

33. Gordon, C.L., et al., *Tissue reservoirs of antiviral T-cell immunity in persistent human CMV infection.* J Exp Med, 2017. 214(3): p. 651-667.

34. Remmerswaal, E.B., et al., *Human virus-specific effector-type T-cells accumulate in blood but not in lymph nodes.* Blood, 2012. 119(7): p. 1702-12.

35. Barnstorf, I., et al., *Chronic virus infection compromises memory bystander T-cell function in an IL-6/STAT1-dependent manner.* J Exp Med, 2019. 216(3): p. 571-586.

36. Bartlett, D.B., et al., *The age-related increase in low-grade systemic inflammation (Inflammaging) is not driven by cytomegalovirus infection.* Aging Cell, 2012. 11(5): p. 912-5.

37. Marandu, T.F., et al., *Mouse CMV infection delays antibody class switch upon an unrelated virus challenge.* Exp Gerontol, 2014. 54: p. 101-8.

38. Frasca, D., et al., *Cytomegalovirus (CMV) seropositivity decreases B cell responses to the influenza vaccine.* Vaccine, 2015. 33(12): p. 1433-9.

39. Frasca, D. and B.B. Blomberg, *Aging, cytomegalovirus (CMV) and influenza vaccine responses.* Human Vaccines and Immunotherapeutics, 2016. 12(3): p. 682-690.

40. Moss, P., *'From immunosenescence to immune modulation': a re-appraisal of the role of cytomegalovirus as major regulator of human immune function.* Med Microbiol Immunol, 2019. 208(3-4): p. 271-280.

41. Goodier, M.R., et al., *CMV and natural killer cells: shaping the response to vaccination.* Eur J Immunol, 2018. 48(1): p. 50-65.

42. Derhovanessian, E., et al., *Infection with cytomegalovirus but not herpes simplex virus induces the accumulation of late-differentiated CD4+ and CD8+ T-cells in humans.* J Gen Virol, 2011. 92(Pt 12): p. 2746-56.

43. Wallace, D.L., et al., *Human cytomegalovirus-specific CD8(+) T-cell expansions contain long-lived cells that retain functional capacity in both young and elderly subjects.* Immunology, 2011. 132(1): p. 27-38.

44. Vescovini, R., et al., *Impact of Persistent Cytomegalovirus Infection on Dynamic Changes in Human Immune System Profile.* PLoS One, 2016. 11(3): p. e0151965.

45. Abana, C.O., et al., *Cytomegalovirus (CMV) Epitope-Specific CD4(+) T-cells Are Inflated in HIV(+) CMV(+) Subjects.* J Immunol, 2017. 199(9): p. 3187-3201.

46. Jackson, S.E., et al., *Diverse specificities, phenotypes, and antiviral activities of cytomegalovirus-specific CD8+ T-cells.* J Virol, 2014. 88(18): p. 10894-908.

47. Hosie, L., et al., *Cytomegalovirus-Specific T-cells Restricted by HLA-Cw*0702 Increase Markedly with Age and Dominate the CD8(+) T-Cell Repertoire in Older People.* Front Immunol, 2017. 8: p. 1776.

48. Gamadia, L.E., et al., *Primary immune responses to human CMV: a critical role for IFN-gamma-producing CD4+ T-cells in protection against CMV disease.* Blood, 2003. 101(7): p. 2686-92.

49. Hertoghs, K.M., et al., *Molecular profiling of cytomegalovirus-induced human CD8+ T-cell differentiation.* J Clin Invest, 2010. 120(11): p. 4077-90.

50. Jackson, S.E., et al., *Generation, maintenance and tissue distribution of T-cell responses to human cytomegalovirus in lytic and latent infection.* Med Microbiol Immunol, 2019.

51. Kahan, S.M., E.J. Wherry, and A.J. Zajac, *T-cell exhaustion during persistent viral infections.* Virology, 2015. 479-480: p. 180-93.

52. Nikolich-Zugich, J., et al., *Advances in cytomegalovirus (CMV) biology and its relationship to health, diseases, and aging.* Geroscience, 2020. 42(2): p. 495-504.

53. Nikolich-Zugich, J. and R.A.W. van Lier, *Cytomegalovirus (CMV) research in immune senescence comes of age: overview of the 6th International Workshop on CMV and Immunosenescence.* Geroscience, 2017.

54. Gkrania-Klotsas, E., et al., *Higher immunoglobulin G antibody levels against cytomegalovirus are associated with incident ischemic heart disease in the population-based EPIC-Norfolk cohort.* J Infect Dis, 2012. 206(12): p. 1897-903.

55. Lebedeva, A.M., et al., *Cytomegalovirus Infection in Cardiovascular Diseases.* Biochemistry (Mosc), 2018. 83(12): p. 1437-1447.

56. Mathei, C., et al., *Associations between cytomegalovirus infection and functional impairment and frailty in the BELFRAIL Cohort.* J Am Geriatr Soc, 2011. 59(12): p. 2201-8.

57. Alonso Arias, R., et al., *Intensity of the humoral response to cytomegalovirus is associated with the phenotypic and functional status of the immune system.* J Virol, 2013. 87(8): p. 4486-95.

58. Araujo Carvalho, A.C., et al., *Association between human herpes virus seropositivity and frailty in the elderly: A systematic review and meta-analysis.* Ageing Res Rev, 2018. 48: p. 145-152.

59. Vescovini, R., et al., *Intense antiextracellular adaptive immune response to human cytomegalovirus in very old subjects with impaired health and cognitive and functional status.* J Immunol, 2010. 184(6): p. 3242-9.

60. Jackson, S.E., et al., *Latent Cytomegalovirus (CMV) Infection Does Not Detrimentally Alter T-cell Responses in the Healthy Old, But Increased Latent CMV Carriage Is Related to Expanded CMV-Specific T-cells.* Front Immunol, 2017. 8: p. 733.

61. Parry, H.M., et al., *Cytomegalovirus viral load within blood increases markedly in healthy people over the age of 70 years.* Immun Ageing, 2016. 13: p. 1.

62. Onyeagocha, C., et al., *Latent cytomegalovirus infection exacerbates experimental colitis.* Am J Pathol, 2009. 175(5): p. 2034-42.

63. Santos Rocha, C., et al., *Subclinical cytomegalovirus infection associates with altered host immunity, gut microbiota and vaccine responses.* J Virol, 2018.

64. Beura, L.K., et al., *Normalizing the environment recapitulates adult human immune traits in laboratory mice.* Nature, 2016. 532(7600): p. 512-6.

65. Rosshart, S.P., et al., *Laboratory mice born to wild mice have natural microbiota and model human immune responses.* Science, 2019. 365(6452).

66. Korndewal, M.J., et al., *Cytomegalovirus infection in the Netherlands: seroprevalence, risk factors, and implications.* J Clin Virol, 2015. 63: p. 53-8.

67. Gostic, K.M., et al., *Potent protection against H5N1 and H7N9 influenza via childhood hemagglutinin imprinting.* Science, 2016. 354(6313): p. 722-726.

68. Viboud, C. and S.L. Epstein, *First flu is forever.* Science, 2016. 354(6313): p. 706-707.

69. Wertheimer, A.M., et al., *Aging and cytomegalovirus infection differentially and jointly affect distinct circulating T-cell subsets in humans.* J Immunol, 2014. 192(5): p. 2143-55.

70. Mortensen, L.H., et al., *Early-life environment influencing susceptibility to cytomegalovirus infection: evidence from the Leiden Longevity Study and the Longitudinal Study of Aging Danish Twins.* Epidemiol Infect, 2012. 140(5): p. 835-41.

71. Liu, J., et al., *Promising Cytomegalovirus-Based Vaccine Vector Induces Robust CD8(+) T-Cell Response.* Int J Mol Sci, 2019. 20(18).

A

APPENDIX

Nederlandse samenvatting
Curriculum vitae
List of publications
Dankwoord

NEDERLANDSE SAMENVATTING

De wereldbevolking is snel aan het vergrijzen. We worden allemaal steeds ouder. Ouder worden gaat vaak samen met het ontstaan van gebreken en ziekten. Op hogere leeftijd nemen voornamelijk de vatbaarheid voor infectieziekten, kanker en auto-immuunziekten toe. Een belangrijk voorbeeld van een infectieziekte is de griep, dat wordt veroorzaakt door het griepvirus (influenza). De griep komt niet alleen vaker voor bij ouderen, maar is ook veel schadelijker voor ouderen dan voor jongeren. De toename van verschillende ziekten op hogere leeftijd zorgt voor stijgende kosten in de gezondheidszorg. Gezond ouder worden is daarom belangrijk voor onze toekomstige gezondheidszorg. De belangrijkste manier om mensen te beschermen tegen infectieziekten is door middel van vaccinatie. Maar juist deze vaccinaties zijn minder werkzaam bij ouderen dan bij jongeren. Op deze manier blijven de ouderen kwetsbaar, terwijl zij de bescherming juist het hardst nodig hebben. De onderliggende reden voor deze problemen ligt bij de veroudering van het immuunsysteem. Daarom is het belangrijk om onderzoek te doen naar de veroudering van het immuunsysteem.

Het immuunsysteem is de belangrijkste afweer van het lichaam tegen infecties met ziektekiemen, zoals bacteriën en virussen. Het is een complex systeem, dat als taak heeft deze ongewenste ziektekiemen op te ruimen, maar ook – heel belangrijk hierover informatie op te slaan. De opgeslagen informatie zorgt bij een tweede besmetting voor een snellere en krachtigere reactie tegen de ziektekiem. Het systeem om specifiek aangeleerde informatie op te slaan wordt het *adaptieve immuunsysteem* genoemd. En juist dit systeem vertoont de meeste ouderdomsverschijnselen op hoge leeftijd. Belangrijke spelers in het adaptieve immuunsysteem zijn de witte bloedcellen, waaronder de zogenoemde *T-cellen* en *B-cellen*. T-cellen kunnen een cel die met een virus is geïnfecteerd direct herkennen en deze vernietigen. B-cellen kunnen *antistoffen* uitscheiden, kleine eiwitten die een ziekteverwekker onschadelijk kunnen maken. B-cellen worden hierbij weer geholpen door T-cellen. Al deze componenten van het immuunsysteem zijn belangrijk om weerstand te bieden en te behouden tegen ziekteverwekkers.

Het immuunsysteem van ouderen ziet er gemiddeld anders uit dan het immuunsysteem van jongeren. Met het ouder worden, worden er steeds minder nieuwe T-cellen aangemaakt, ook wel *naïeve T-cellen* genoemd. Deze naïeve T-cellen zijn nodig voor het aanleren van nieuwe immuunreacties. Het gevolg is dat het immuunsysteem bij ouderen minder uit naïeve T-cellen bestaat dan bij jongeren. Bij ouderen zijn juist de *geheugen T-cellen* in grote hoeveelheden aanwezig. Geheugen T-cellen onthouden de ziektekiemen die eerder in het leven tegengekomen zijn, zodat er de volgende keer sneller en sterker gereageerd kan worden tegen dezelfde ziekteverwekker. Bovendien zijn geheugen T-cellen bij ouderen anders wat betreft hun uiterlijk en functie vergeleken met geheugencellen van jongeren. Door het verouderingsproces zal een deel van de geheugen T-cellen vermoeid raken en zich anders gaan gedragen. Ze krijgen bijvoorbeeld moeite met *delen*. Celdeling is een belangrijke eigenschap in de strijd tegen een ziekteverwekker. Ook is vaak de hoeveelheid aanwezige antistoffen om ziekteverwekkers te vernietigen verminderd bij ouderen. Door al deze

verschijnselen van het ouder worden van het immuunsysteem, komen er meer infectieziekte gevallen voor op hogere leeftijd en wordt de werking van vaccins vaak minder.

Maar niet alle mensen zijn hetzelfde wat betreft hun immuunsysteem. De ene 70-jarige zal meer ouderdom gerelateerde verschijnselen vertonen in zijn immuunsysteem dan de andere 70-jarige. Of zelfs meer dan een 90-jarige die simpelweg nog weinig ouderdomsverschijnselen vertoont in zijn immuunsysteem. Dit kan door allerlei factoren komen. Begin jaren 2000 werd een nieuwe belangrijke factor ontdekt die een grote invloed heeft op deze immunologische verouderingsverschijnselen. Er werd ontdekt dat dragers van het cytomegalovirus, CMV, veel meer immunologische verouderingsverschijnselen vertonen dan mensen die geen drager van dit virus zijn. Dit was het begin van een nieuwe theorie: CMV versnelt het verouderingsproces van het immuunsysteem. Over deze theorie gaat dit proefschrift.

CMV is een veelvoorkomend virus. Ongeveer 50 tot 90 procent van de wereldbevolking is drager van CMV. Als mensen het virus eenmaal hebben, zal het altijd aanwezig blijven in het lichaam. CMV verstopt zich namelijk voor het immuunsysteem, in een soort slapende vorm, en kan zo het immuunsysteem van de mens ontwijken. Maar CMV probeert wel constant weer actief te worden en dan is het immuunsysteem nodig om CMV weer de kop in te drukken. Als het immuunsysteem ernstig verzwakt is, bijvoorbeeld na een stamceltransplantatie of door een HIV-infectie, lukt het niet om CMV onder controle te houden. Deze mensen worden zeer ziek van CMV en kunnen daaraan overlijden. Bij gezonde mensen levert CMV gewoonlijk geen directe ziekteverschijnselen op. Een gezond immuunsysteem heeft dus het slapende virus en de pogingen van het virus om weer actief te worden onder controle. Dit gaat echter niet zomaar: een zeer groot gedeelte van het immuunsysteem is bezig om CMV onder controle te houden. Tot wel 50%, of soms in unieke gevallen zelfs 90%, van de geheugen T-cellen is bijvoorbeeld specifiek gericht tegen CMV. Er zijn miljoenen ziekteverwekkers in de wereld, maar geen enkele andere ziekteverwekker die we kennen vraagt zo veel middelen van het immuunsysteem. Het kost dus erg veel om CMV onder controle te houden. CMV zou op deze manier het immuunsysteem gaandeweg kunnen uitputten, waardoor er steeds minder middelen, ruimte en energie overblijft voor de strijd tegen andere ziekteverwekkers. Het immuunsysteem van mensen die CMV bij zich dragen zou daardoor sneller verouderen. Als gevolg daarvan zouden deze mensen op hogere leeftijd meer last hebben van bijvoorbeeld de griep dan mensen zonder CMV.

Er zijn verschillende tekenen die ondersteunen dat CMV inderdaad de veroudering van het immuunsysteem versnelt. Op de eerste plaats is duidelijk dat CMV dezelfde verschijnselen introduceert in het immuunsysteem als ouderdom. CMV-dragerschap gaat ook samen met minder naïeve T-cellen, meer geheugen T-cellen en wijzigingen in het uiterlijk en de functie van geheugen T-cellen. Ten tweede hebben een aantal onderzoeken laten zien dat CMV-dragers een minder goede immuunreactie hebben op het griepvaccin dan niet-CMV-dragers. Bij CMV-dragers was er een lagere aanmaak van antistoffen tegen de griep dan bij niet-CMV-dragers. Ten derde laten onderzoeken waaraan heel veel mensen deelnamen een link zien

tussen het CMV-dragerschap en een verkorte levensduur. CMV-dragers leven gemiddeld 2 of 3 jaar korter dan niet-CMV-dragers. Ten vierde laten onderzoeken in muizen met CMV daadwerkelijk zien dat na langdurig dragerschap van CMV, het immuunsysteem van de muizen minder krachtig reageert op de griep. Al deze bewijzen ondersteunen de theorie dat CMV inderdaad de veroudering van het immuunsysteem kan versnellen. Toch zitten hier nog belangrijke haken en ogen aan. Die worden in **deel 1** van dit proefschrift onderzocht.

We vermoeden dat de versnelde veroudering van het immuunsysteem door het CMV-dragerschap komt doordat er zoveel middelen van het immuunsysteem nodig zijn om CMV onder controle te houden. Maar over deze middelen, de CMV-specifieke geheugen T-cellen en CMV-specifieke antistoffen, is nog veel onbekend. De meeste immuunreacties nemen af met de leeftijd, juist door veroudering. Maar verrassend genoeg neemt de immuunreactie tegen CMV juist toe met de leeftijd. Zowel het aantal CMV-specifieke geheugen T-cellen, als het aantal CMV-specifieke antistoffen zijn hoger in ouderen dan in jongeren. Het lijkt er dus op dat er steeds meer middelen en meer energie nodig is om CMV onder controle te houden. Elke keer als het virus actief probeert te worden, schakelt het immuunsysteem een tandje bij om het weer te kunnen controleren. Bij onderzoek met muizen kunnen we heel duidelijk zien dat het aantal CMV-specifieke geheugen T-cellen toeneemt met de tijd. Maar van de immuunreactie tegen CMV in de mens gedurende het leven is nog veel onbekend. Ook begrijpen we nog niet goed hoe zulke hoge aantallen CMV-specifieke geheugen T-cellen ontstaan en in stand gehouden worden in de mens. In **deel 2** van dit proefschrift wordt de immuunreactie tegen CMV in mensen verder onderzocht.

In elk hoofdstuk in dit proefschrift staat een andere bevinding en boodschap centraal. In de volgende paragrafen wordt kort toegelicht waar elk hoofdstuk over gaat.

Na een algemene inleiding over veroudering van het immuunsysteem en CMV in **hoofdstuk 1**, onderzoeken we in **hoofdstuk 2** op een nieuwe manier het effect van CMV op de griepvaccinatie. Eerdere studies van andere onderzoekers bleken tegenstrijdig te zijn. Alhoewel een aantal onderzoeken een negatief effect aantoonden van CMV op het griepvaccin, was dat toch niet in alle onderzoeken het geval. In al deze onderzoeken werd de reactie tegen standaard griepvaccinaties bestudeerd, die elk winterseizoen gegeven worden en in opeenvolgende jaren erg op elkaar kunnen lijken. De hoogte en kwaliteit van de immuunreactie op deze griepvaccinaties hangt erg af van eerder doorgemaakte griepvaccinaties en -infecties. Dit kan een effect van CMV vertroebelen. Wij vermoedden dat dat misschien kon verklaren waarom niet elk onderzoek een negatief effect van CMV kon vinden. Daarom baseerden wij ons onderzoek in **hoofdstuk 2** op een griepvaccinatie met een nieuw griepvirus, namelijk het Mexicaanse griepvirus. De mensen die dit vaccin kregen in 2009 waren nog niet eerder in aanraking geweest met dit virus. Ons onderzoek toonde aan dat er op dit nieuwe griepvaccin geen effect van het CMV-dragerschap was. CMV-dragers en niet-CMV-dragers maakten evenveel antistoffen aan na de griepvaccinatie. We vonden wel dat de leeftijd van de mensen invloed had op de hoeveelheid antistoffen tegen de griep, zelfs

al vanaf de leeftijd van circa 40 jaar. Ondanks deze tekenen van een verzwakte immuunreactie door de leeftijd deed het CMV-virus dus verder geen kwaad.

Onze resultaten in **hoofdstuk 2** waren in tegenspraak met de algemeen heersende gedachte naar het effect van CMV. Daarom hebben we in **hoofdstuk 3** een grondige en systematische analyse gedaan van alle bestaande onderzoeken naar CMV en de antistofreactie tegen het griepvaccin. Na uitgebreide analyse bleek dat onderzoekers inderdaad vaak concluderen dat er een negatieve link bestaat tussen CMV en de werkzaamheid van het griepvaccin. Maar dat in feite het daadwerkelijke bewijs beide kanten op wees. Er waren evenveel onderzoeken die geen effect, of zelfs een *positief* effect van CMV-dragerschap op het griepvaccin rapporteerden als dat er onderzoeken waren die een negatief effect van CMV op het griepvaccin vonden. Wij laten zien dat de verschillende onderzoeken de antistofreactie op het griepvaccin op veel verschillende manieren analyseerden. Dit leidde tot verschillende conclusies. Toen wij alle gegevens van alle onderzoeken op één manier analyseerden, vonden wij geen effect van CMV-dragerschap op de werking van het griepvaccin. Ook laten we zien in hoofdstuk 3 dat er een neiging is om de resultaten van onderzoeken met een negatief effect van CMV eerder de wereld in te brengen dan de resultaten van onderzoeken met een positief effect van CMV. Onderzoekers of tijdschriften leken dus een beetje bevooroordeeld te zijn over dit onderwerp. Samenvattend tonen hoofdstuk 2 en 3 aan dat het CMV-dragerschap niet per definitie nadelig hoeft te zijn voor een goede werking van het griepvaccin.

Maar er miste nog wat in onze kennis omdat tot nu toe alleen de antistoffen tegen de griep onderzocht waren. Een hoge leeftijd en CMV-dragerschap heeft juist met name effecten op geheugen T-cellen. Ook zijn de infectiegevallen van de griep nog niet onderzocht in de context van CMV. Daarom onderzochten wij in **hoofdstuk 4** het effect van CMV-dragerschap op T-cellen en hoe deze reageren op een griepinfectie in ouderen in plaats van een griep vaccinatie. We onderzochten ook de meest voorkomende theorieën over *hoe* CMV het immuunsysteem kan hinderen in de strijd tegen een ziekteverwekker, te weten: 1) door competitie tussen CMV-specifieke en griep-specifieke geheugen T-cellen, en 2) door een algemene belemmerende ontsteking bij ouderen door dragerschap van CMV. We vonden geen bewijs voor een verminderde werking van T-cellen tegen de griep-infectie door CMV-dragerschap bij ouderen. CMV-dragers reageerden even snel en krachtig op een griepinfectie als niet-CMV-dragers. Voor beide theorieën over *hoe* CMV dit zou doen vonden wij geen bewijzen. Dit onderzoek, samen met hoofdstuk 2 en 3, heeft bijgedragen aan een kerend beeld in de onderzoekswereld. In het algemeen wordt nu niet meer zo sterk gedacht dat CMV-dragerschap de immuunreactie tegen de griep in ouderen verzwakt. Omdat de griep hét meest gebruikte en beste voorbeeld is van infectieziekten bij ouderen staat de theorie van CMV-versnelde immuunveroudering ter discussie. We denken nu - en gelukkig maar - dat CMV niet of nauwelijks de immuunreactie tegen griep verzwakt. Of dit ook het geval is bij nieuwe ziekteverwekkers die mensen voor het eerst tegenkomen en nog geen geheugen voor hebben opgebouwd, zal nog onderzocht moeten worden.

Er lijkt dus sprake te zijn geweest van voorbarige conclusies over het nadelige effect van het CMV-dragerschap op de werking van het immuunsysteem op hogere leeftijd. Maar de verschijnselen in het uiterlijk van het immuunsysteem die samengaan met het CMV-dragerschap zijn wel heel duidelijk bewezen. Over de achterliggende mechanismen van deze verschijnselen is nog veel onduidelijk. Bijvoorbeeld *hoe* zulke grote aantallen CMV-specifieke geheugen T-cellen aangemaakt worden en hoe ze behouden blijven gedurende een mensenleven. Niet alleen voor onderzoek naar CMV-veroudering, maar ook om in het algemeen meer te begrijpen over de werking van geheugen T-cellen, is het van belang om hier meer over te weten te komen. Daarom is het belangrijk om de unieke grote immuunreactie tegen CMV in mensen beter te begrijpen.

In **hoofdstuk 5** onderzoeken we door middel van een literatuurstudie het uiterlijk van CMV-specifieke geheugen T-cellen. We vragen ons af of deze T-cel, naast het feit dat ze uniek zijn omdat ze met hele grote hoeveelheden aanwezig zijn, ook uniek zin in uiterlijk. We beschrijven het uiterlijk van de CMV-specifieke geheugen T-cel en vergelijken dit met andere geheugen T-cellen tegen chronische infecties. We tonen aan dat de CMV-specifieke T-cel inderdaad een uniek uiterlijk heeft. De cel lijkt ouder, lijkt minder te kunnen delen, maar lijkt dan wel weer bijzonder krachtig te zijn in het doden van cellen die geïnfecteerd zijn met CMV. Dit unieke uiterlijk van de CMV-specifieke geheugen T-cel lijkt afhankelijk te zijn van de leeftijd van mensen. Hoe ouder de persoon, hoe unieker het uiterlijk van de CMV-specifiek geheugen T-cel is.

Nu we weten dat de CMV-specifieke geheugen T-cel uniek is in zowel aantal als in het uiterlijk van de cellen, hebben we in **hoofdstuk 6** onderzocht *hoe* deze CMV-specifieke T-cellen in zulke hoge aantallen aanwezig kunnen blijven in het lichaam. Normaal gesproken worden geheugen T-cellen in lagere aantallen opgeslagen. In tijden van rust, delen de geheugen T-cellen maar af en toe. Zij doen dit enkel om de natuurlijk doodgaande cellen te compenseren en een stabiel aantal van geheugen T-cellen te behouden voor het geval deze weer nodig zijn. Zodra het nodig is, zullen de aanwezige geheugen T-cellen weer heel erg veel gaan delen, om op hele hoge aantallen te komen en de teruggekomen ziekteverwekker te bestrijden. Maar het bijzondere aan CMV-specifieke geheugen T-cellen is dat ze ook in tijden van rust in grote aantallen aanwezig zijn. In ons onderzoek in **hoofdstuk 6** vroegen we ons af of dit komt door een hogere aanmaaksnelheid, of een lagere sterftesnelheid van de CMV-specifieke geheugen T-cellen. We onderzochten dit door middel van een unieke methode, waarin we delende geheugen T-cellen in oudere mensen konden markeren. We tonen aan dat ondanks de hoge aantallen en het unieke uiterlijk van de CMV-specifieke geheugen T-cellen, deze cellen geen andere aanmaak- of sterftesnelheid hebben. Het lijkt erop dat er in het begin van de rustperiode een bepaald niveau van CMV-specifieke geheugen T-cellen neergezet is. En dat dit niveau van hoge aantallen CMV-specifieke geheugen T-cellen op dezelfde wijze wordt onderhouden als dat van andere geheugen T-cellen. Daarnaast tonen we aan dat in het algemeen een bepaalde groep T-cellen, de *helper T-cellen*, minder snel lijken te delen in CMV-dragers dan in niet-CMV-dragers. Dit betekent dat CMV-specifieke T-cellen zich

niet per se anders gedragen, maar dat de algemene *helper* T-cel zich misschien wel anders gedraagt door het CMV-dragerschap. Of dit inderdaad zo is, waardoor dit precies komt en of dit nadelige gevolgen heeft voor het immuunsysteem, heeft verder onderzoek nodig.

Tot slot onderzochten we in **hoofdstuk 7** het effect van CMV-dragerschap over de tijd in mensen. Er zijn tot nu toe bijna alleen maar muizenonderzoeken gedaan die een CMV-dragerschap over de tijd volgen. Voor mensen is dit natuurlijk logistiek heel uitdagend. Het is nou eenmaal niet eenvoudig om mensen over tientallen jaren te volgen. Ook is het lastig dat het moment van een CMV-infectie bijna altijd verloopt zonder ziekteverschijnselen. Daardoor weten we niet bij ouderen *wanneer* ze CMV bij zich zijn gaan dragen en of ze het virus al bijna hun hele leven, of pas enkele jaren bij zich dragen. In **hoofdstuk 7** hadden wij de unieke kans om het verloop van de immuunreactie tegen CMV te onderzoeken over een lange tijd in mensen. Dit kon in een studie waarin mensen 27 jaar lang gevolgd zijn en waarvan bloedmonsters zijn opgeslagen. In deze studie hebben we in deze bloedmonsters ook mensen gevonden die in de loop van die 27 jaar geïnfecteerd raakten met CMV. Zo konden we kortdurend CMV-dragerschap vergelijken met 27 jaar-durend CMV-dragerschap. Ook wisten we van deze oudere mensen de algemene gezondheidsstatus, op basis van een zogenoemde 'kwetsbaarheidsindex', en wisten we of ze last hadden van hart- en vaatziekten. In ons onderzoek tonen wij aan dat de immuunreactie tegen CMV inderdaad hoger is bij oudere mensen. Maar dat dit nauwelijks lijkt te komen door een toename in de tijd gedurende het leven van deze mensen. Een oudere die pas net CMV heeft, heeft al een hogere immuunreactie dan een jonger persoon. De leeftijd waarop je CMV krijgt doet er dus toe. Dit is een nieuwe en belangrijke bevinding. Dit zou betekenen dat het continu actief worden van CMV gedurende het CMV-dragerschap niet een oorzaak is van de verschijnselen in het immuunsysteem die gepaard gaan met CMV. Ook tonen we aan dat CMV, zowel bij lang als bij kort dragerschap, geen nadelige invloed lijkt te hebben op de algemene gezondheidsstatus van de dragers. Tot slot vinden we dat de hoge CMV-specifieke immuunreactie samen lijkt te gaan met het aantal gevallen van hart- en vaatziekten. Er lijkt dus een verband te zijn tussen hart- en vaatziekten en CMV.

Een samenvatting van alle bevindingen van dit proefschrift en nieuwe inzichten over de invloed van CMV op de veroudering van het immuunsysteem worden in **hoofdstuk 8 v**an dit proefschrift beschreven. Samenvattend zijn er drie hoofdbevindingen gedaan. Ten eerste, de immuun reactie tegen griepvaccinatie en griepinfectie wordt niet gehinderd door CMV. Ten tweede, hoge aantallen CMV-specifieke geheugen T-cellen kunnen behouden worden zonder grote wijzigingen in aanmaak- en sterftesnelheid. Ten derde, het aantal CMV-specifieke geheugen T-cellen neemt niet of nauwelijks toe in de tijd, maar de leeftijd op het moment van het krijgen van CMV heeft invloed op het aantal CMV-specifieke geheugen cellen. Nu we dit weten, zien we CMV-dragerschap niet meer als iets dat perse slecht is voor ons immuunsysteem op hogere leeftijd. Daarom is het belangrijk om verder onderzoek te doen naar andere redenen voor de verzwakking van de immuunreactie tegen de griep en het griepvaccin op hogere leeftijd. Ook weten we nu dat de link tussen de grote CMV-specifieke

immuunreactie en leeftijd nog wat ingewikkelder is dan we dachten. Misschien is het zelfs zo dat een hoge CMV immuunreactie juist *een gevolg is* van een verzwakt immuunsysteem, in plaats van dat het een *oorzaak* is van een verzwakt immuunsysteem. Om dit de gaan begrijpen, is verder onderzoek nodig waarin de ontwikkeling van de CMV-specifieke geheugen T-cellen en de ouderdomsverschijnselen van het immuunsysteem in mensen gevolgd wordt.

CURRICULUM VITAE

Sara van den Berg was born on March 21st 1990 in Velp, gemeente Rheden, the Netherlands. She finished her secondary school at the Stedelijk Gymnasium Arnhem in 2008. Subsequently, she started her bachelor studies Biomedical Sciences in Utrecht University. After 3 years, she took a gap year to board the study association M.B.V. Mebiose. After obtaining her degree Bachelor of Science in 2013, she enrolled in the master program Infection and Immunity at Utrecht University. During her master studies, she performed her first internship at the Center for Translational Immunology of the University Medical Centre in Utrecht (UMCU) under supervision of Dr. Kiki Tesselaar and Dr. José Borghans, where she focussed on T-cell proliferation and the stability of T-cell Receptor Excision Circles (TRECs). For her second internship, she went to Cambridge University in Cambridge, United Kingdom. Here, she worked in the lab of Dr. Mark Wills, where she assessed the control of cytomegalovirus by immune cells in an *in vitro* viral dissemination assay. During her master studies, she also completed the minor Fundamentals of Business and Economics.

In May 2015, she started as a PhD candidate at the Center for Immunology of Infectious Diseases and Vaccines of the National Institute for Public Health and the Environment (RIVM) in collaboration with the Center for Translational Immunology of the University Medical Centre in Utrecht (UMCU). Her supervisors were Prof. Dr. Debbie van Baarle, Dr. José Borghans and Dr. Jelle de Wit. The research of her PhD project focussed on the effect of cytomegalovirus (CMV) infection on ageing of the immune system. The results obtained during her PhD are described in this thesis.

Sara her life was not only focussed on her studies or her work, as she was also busy with activities concerning student representatives, her volunteer work giving LGBTI+ guest lectures at high schools, playing soccer in the best friends team imaginable, enjoying life with her family and friends and – most importantly – fell in love with Julia.

LIST OF PUBLICATIONS

S.P.H. van den Berg., A. Wong, M. Hendriks, R. H. J. Jacobi, D. van Baarle and J. van Beek (2018). *Negative Effect of Age, but Not of Latent Cytomegalovirus Infection on the Antibody Response to a Novel Influenza Vaccine Strain in Healthy Adults.* Front Immunol.

S.P.H. van den Berg, K. Warmink, J. A. M. Borghans, M. J. Knol and D. van Baarle (2019). *Effect of latent cytomegalovirus infection on the antibody response to influenza vaccination: a systematic review and meta-analysis.* Med Microbiol Immunol.

S.P.H. van den Berg, I. N. Pardieck, J. Lanfermeijer, D. Sauce, P. Klenerman, D. van Baarle and R. Arens (2019). *The hallmarks of CMV-specific CD8 T-cell differentiation.* Med Microbiol Immunol.

L.D. Samson*, **S.P.H. van den Berg***, P. Engelfriet, A. M. H. Boots, M. Hendriks, L.G.H. de Rond , M. de Zeeuw-Brouwer, M.Verschuren, J. A.M. Borghans, A. Buisman#, and D. van Baarle# (2020). *Limited effect of duration of CMV infection on adaptive immunity and frailty: insights from a 27-year long longitudinal study.* Accepted for publication. Clin Trans Immunol

* first authors contributed equally # last authors contributed equally.

S.P.H. van den Berg, L.Y. Derksen, J. Drylewicz, N. M. Nanlohy, L. Beckers, J. Lanfermeijer, S. N. Gessel, M. Vos, S. Otto, R. de Boer, K. Tesselaar, J.A.M.Borghans* and D. van Baarle*. *Quantification of T-cell dynamics during latent human cytomegalovirus infection.* Submitted for publication.

S.P.H. van den Berg, J. Lanfermeijer, R.H.J Jacobi, M. Hendriks, M. Vos, R. van Schuijlenburg, N.M. Nanlohy, J.A.M. Borghans, J. van Beek, D. van Baarle and J. de Wit. *Lower influenza virus-specific memory T-cell frequencies, but no impaired T-cell response to acute influenza virus infection by latent CMV infection.* Submitted for publication.

Jackson, S.E., A. Redeker, R. Arens, D. van Baarle, **S.P.H. van den Berg**, C.A. Benedict, L. Cicin-Sain, A.B. Hill and M.R. Wills (2017). *CMV immune evasion and manipulation of the immune system with aging.* Geroscience.

Shan, L., S. Li, J. Meeldijk, B. Blijenberg, A. Hendriks, K. van Boxtel, **S.P.H. van den Berg**, I. J. Groves, M. Potts, A. Svrlanska, T. Stamminger, M.R. Wills and N. Bovenschen (2020). *Killer cell proteases can target viral immediate-early proteins to control human cytomegalovirus infection in a noncytotoxic manner.* PLoS Pathog.

DANKWOORD

Ik wil graag iedereen bedanken die heeft bijgedragen aan de totstandkoming van dit proefschrift. Zonder het harde werken, de begeleiding en de liefdevolle steun van velen was er zeker weten geen boekje: dank jullie wel! Graag wil ik enkele mensen in het bijzonder bedanken.

Ten eerste gaat mijn dank uit naar mijn begeleiders: mijn promotor Debbie en copromotoren José en Jelle. Van begin af aan vormden Debbie en José een ijzersterk duo. Misschien juist door jullie complementaire eigenschappen. Zo'n begeleiding kan menig promovenda zich alleen maar wensen. Jelle, jij kwam gedurende mijn PhD op het RIVM werken en ik had het geluk dat jij ons team versterkte als een van mijn dagelijks begeleiders.

Debbie, je bent een enorm snelle denker en schakelaar, met een passie voor nieuwe ideeën. Verbanden leggen en 'muziek in de data zien' gaat jou natuurlijk af. We begrepen elkaars immunologische redenaties altijd direct, wat sparren met jou tot een feestje maakte. Door deze fijne samenwerking hebben we samen zes papers geschreven. Van jou heb ik geleerd om het verhaal in de data te zien en te verkopen. Ik heb veel gehad aan je resultaatgerichtheid hierin. Je persoonlijke begeleiding kenmerkte zich door het geven van ruimte, maar je was altijd dichtbij als ik het nodig had. Een mooie leerweg voor mij. Ontzettend bedankt!

José, ik heb intens genoten van onze inhoudelijke discussies. Ik bewonder jouw kritische blik, strakke logica en integriteit. Je bent een voorbeeld voor mij in de wetenschap. Hiermee vormde je een leerschool voor mijn eigen analytisch vermogen te ontwikkelen. Verder ken ik weinig mensen die zo strak en toegankelijk over hele complexe onderwerpen kunnen communiceren en deze kunnen uitleggen, waardoor je veel bereikt. Minstens zo bijzonder, is jouw aandacht voor de persoon achter de promovenda. Je coaching en toewijding heb ik als bijzonder waardevol ervaren en heeft mij enorm geholpen. Dankjewel!

Jelle, je persoonlijke touch en creatieve immunologische kennis in de vele werkbesprekingen, waren precies wat mij nog verder hielp. Ik heb het samenwerken met jou altijd als heel prettig ervaren. Je dacht out-of-the box en was toegankelijk en attent. Je hebt mij uit meerdere moeilijke momenten getrokken. Meer dan je zelf wellicht zag. Dankjewel daarvoor!

Grote dank gaat ook uit naar mijn twee paranimfen, tevens twee van mijn dichtstbijzijnde collega's en vrienden: Josien en Lyanne. Ik ben ontzettend blij dat jullie naast mij, of in elk geval ergens binnen 1.5 meter afstand, staan tijdens de verdediging. Ik kan me geen twee fijnere paranimfen indenken!

Lieve Josien, mijn sparringpartner en vrolijke metgezel. Wij hadden al een band voorafgaand aan mijn PhD en die is de afgelopen jaren alleen maar verder gegroeid. We waren beiden enthousiaste deelnemers aan dezelfde groep en deelden onze focus op ontwikkeling en streven naar kwaliteit. Je was, net als vroeger, vaak genoeg mijn reddende engel als ik vast

zat. Jouw ruime blik, je positiviteit en pragmatische tips hielpen mij verder relativeren en doorpakken. Daarnaast investeerden we bewust tijd in elkaars projecten en ik waardeer je passievolle meedenken zeer. Het maakte de wetenschap een stuk minder eenzaam. Ik kan me niet indenken hoe veel zwaarder en simpelweg minder leuk dit traject zou zijn geweest zonder jou en onze vriendschap. Bedankt voor alles!

Lyanne, in een moeilijke periode wat betreft mijn gezondheid kwam jij op mijn pad. Je werd de redder van het project, maar bovenal de persoonlijke kracht en vriend voor mij op het UMCU. Door onze moeiteloze diepgaande gesprekken over het leven en nog heel veel meer, werden onze pipetteersessies in de zuurkast – verrassend genoeg – ware lichtpuntjes voor mij in de werkweek. Nieuwe stof neem je gemakkelijk tot je, je argumentaties kloppen volgens mij altijd en ik ken niemand die de GCMS zo snel grondig begreep (en dat wil wat zeggen). Ik ben super dankbaar voor onze samenwerking, maar bovenal voor onze vriendschap!

Een speciaal plekje in mijn PhD werd ingenomen door mijn AIO kamergenoten: Daan, Elise, Leon en Liz. Samen hebben we een vertrouwd, open en positief plekje gecreëerd op onze kamer. Bedankt voor al jullie interesse en steun! Daan, met een vergelijkbaar PhD onderwerp was je van begin af aan mijn maatje. Ik heb veel gehad aan onze persoonlijke gesprekken, misschien wel juist door onze verschillende karakters. Liz, ik heb genoten van onze gezamenlijke initiatieven en je was een voorbeeld in je pragmatische aanpak van de PhD-afrondingsfase. Daarnaast was je ook nog mijn professionele coach tijdens mijn langdurige hersenschudding, ik ben je intens dankbaar hiervoor!

Het hebben van twee werkplekken betekent ook dat je altijd ergens níet bent. Hoewel minder aanwezig, voelde ik mij toch altijd op mijn gemak binnen mijn UMCU labgroep. Kiki, bedankt voor de leuke helpende gesprekken, je onuitputtelijke immunologisch kennis en je directheid. Julia en Rob, dankjulliewel voor jullie expertise en de samenwerking. Sigrid, dank voor al je hulp. Mariona, Elena en Carina; dankjulliewel voor de groep maken tot wat het was. Ik heb veel gehad aan onze kritische wekelijkse werkbesprekingen, zowel qua inhoudelijke ontwikkeling als qua energiegever. De persoonlijke sfeer en het teamverband binnen de groep zijn inspirerend!

Dit proefschrift is een resultaat van academische samenwerkingen met veel verschillende mensen, verschillend in kennis en persoonlijkheid. Juist daarin heb ik het meest mogen leren. Josine, we hebben samen een weg gevonden naar een goede samenwerking en resultaat. Bedankt voor je vertrouwen. Ronald, Marion en Roos, dank voor jullie labexpertise, inzet en welkome sfeer in de originele influenza groep. Ronald en Marion, ik vond het ontzettend leuk om jullie later in mijn PhD ook buiten influenza projecten weer terug te mogen zien in onze samenwerkingen. Nening en Lisa, het 'DICE team' in den beginne, ik ben dankbaar voor jullie tomeloze inzet en ondersteuning in dit logistiek uitdagende project. Samen hebben we de schouders eronder gezet. Bedankt voor de gezellige labdagen en het teamgevoel. Leon, Annemarie en Peter, bedankt voor onze aangegane samenwerking waarin we in een

uitdagende tocht verschillende disciplines bij elkaar brachten. Ik heb veel geleerd van jullie en ben erg trots op het resultaat! Albert en Mirjam, dank voor jullie expertrol en kundige statistiek respectievelijk epidemiologie bijdrage. Studenten Lisa, Kelly, Stephanie en Martijn, bedankt voor jullie enthousiasme en inzet. Ik vond het leerzaam en leuk jullie te mogen begeleiden. Martijn, tevens bedankt voor je technische noot in de verdere projecten en voor je humor. Lotte, Marlot en Rianne, bedankt dat jullie van het UMCU een nog meer welkom plekje maakten. Hella, thanks voor de vrolijke noot en onze fijne band. Iris en Ramon, bedankt voor jullie gedrevenheid, kennis en fijne manier van werken. In korte tijd hebben we samen een sterke review eruit weten te stampen!

Tevens mijn dank aan Ann Vossen, Emmanuel Wiertz, Janneke van de Wijgert, Paul Moss en René van Lier die als manuscriptcommissie dit proefschrift kritisch hebben gelezen en beoordeeld.

I would also like to take this chance to thank Mark Wills, my supervisor of my internship at Cambridge University during my master studies. Mark, you welcomed me in the fascinating world of viruses. And even welcomed me in your home when I had no place to live in Cambridge for some weeks. Because of this internship, I became again thrilled with science and started a PhD. It was lovely to see you in the last couple of years at international CMV conferences, and hope that we keep in touch. I wish you all the best in science and life!

Tot slot wil ik graag wat woorden richten en de mensen bedanken die indirect aan de totstandkoming van dit proefschrift hebben bijgedragen door ontspanning, steun en meedenken.

Een bijzonder bedankje gaat uit naar m'n groep LHBTI+ voorlichters. Ik heb de afgelopen jaren enorm genoten om deel uit te mogen maken van zo'n diverse en motiverende groep mensen. Jullie stellen je kwetsbaar op in onveilige situaties om de wereld steeds een klein stukje mooier maken. Het mezelf verder mogen ontwikkelen hierin en het geven van de interactieve sociaal dynamische voorlichtingen vormden voor mij een welkome – en soms broodnodige – afwisseling van de wetenschap. Florien, vriendin, ik ben erg blij met onze vriendschap, bedankt voor al onze mooie analytische – zeer snelle – gesprekken. Aukje, bedankt voor al onze gezellige borrels die voor de nodige afleiding van werkstress zorgden, en voor je interesse en onze hechte vriendschap.

Ook gaat mijn dank uit naar mijn studie vrienden, of studievereniging vrienden, bij onszelf beter bekend als de 'Utrecht for life'-groep. Bart, Brenda, Celine, Dieke, Ellen, Eric, Eva, Fabian, Jeroen, Johan, Joost, Joppe, Joske, Juan Luis, Kim, Lars, Lennart, Leonie, Loes, Lotte, Marina, Mark, Marleen, Mathieu, Roos en Ruud. Bedankt voor alle borrels, festiviteiten, gezelligheid, voor de gesprekken over 'een PhD doen of niet' en de humor. Ik ben intens blij om deel te mogen uit maken van zo'n inspirerende groep mensen! Lieve Marina, bedankt voor onze waardevolle diepe band en je loyaliteit. In de afgelopen jaren hebben wij ontelbare

mooie avonden analyseren – vaak onder het genot van een speciaal biertje – samen erop zitten. Altijd raak ik geïnspireerd of heb ik een nieuw inzicht. Onze gesprekken over mijn verschillende PhD projecten en alle randzaken en interacties die daarbij horen, zijn van onschatbare waarde geweest voor dit proefschrift.

Mijn voetbalteam, Dieke, Edith, Eefje, Elise, Ine, Irene, Iris, Jeanine, Judith, Judith, Karlijn, Linda, Lonneke, Marije, Marily, Marjolein, Marleen, Merel, Sari, Simone, Solveig, Susan, Vera en trainers Bas, Joakim, Martin en Stephan. Jullie zijn het beste team wat ik me kan wensen. Ik kom altijd enthousiast terug van een voetbaltraining of wedstrijd. Nog belangrijker voor mij, is het gevoel van thuiskomen. We zijn allemaal erg uiteenlopend als persoon en in karakter, maar juist zo hetzelfde in loyaliteit, interesse en ieder zichzelf laten zijn. Bedankt voor de fantastische sfeer, alle derde helften, festivals, vunzige deuntjes concerten, spelletjesavonden en vele andere activiteiten. In het bijzonder Jeanine, en natuurlijk Glenn en Tess. Dank voor onze hechte vriendschap en de relativerende insteek in een PhD doen. Impactfactors zijn toch wat overgewaardeerd..

Tot slot een woord tot mijn familie. Op de momenten van mijn PhD dat ik door de bomen de … struik… niet meer zag, kon ik altijd op jullie rekenen. Mama, je bent er altijd voor mij geweest en steunt me als ik ergens mee zit. Bedankt voor je onvoorwaardelijke liefde en je onophoudelijke interesse in mijn studie en Phd. Papa, bedankt voor al je analyses, eindeloze hulp en relativerende humor. Denise, bedankt voor je scherpe redenaties. Samen met Pap is geen vraagstuk voor jullie te complex en kan ik altijd bij jullie terecht. Mijn lieve zus Eva, Jelle en neefje Gijs. Wat vormen jullie een prachtig gezin en een fijne plek voor mij. Eef, onze band en de vele gezellige telefoontjes na een lange dag werken zijn de bron geweest voor praktische oplossingen. Ik ben gezegend met zo'n grote zus! Maes, lief broertje, dichtbij jouw ontwikkeling mogen staan is een bijzonder groot goed. De momenten met jou, van blokken bouwen, eindeloze potjes voetbal tot heuse wetenschapsproeven en Housepartyen, zijn de momenten waar ik het allermeest van geniet, ontspan en oplaad. Jij zorgt altijd voor nieuwe positieve energie!

Lieve Julia, toen ik jou 3 jaar geleden leerde kennen was ik inmiddels vrij steady met mijn PhD bezig, en zette je m'n wereld in positieve zin op z'n kop. Vervolgens kregen we het samen keihard voor onze kiezen doordat we geconfronteerd werden met verschillende gezondheidsissues over een langere periode. Hierin was je er voor me, ook als het niet makkelijk was. Jouw positieve noot, gekkigheid, interesse, analyses en liefde, hebben mij heel erg gesteund bij het avontuur dat een PhD doen heet. Je gaf me de ruimte voor de vele uren schrijven en het bespreken van mijn PhD gedachtes en perikelen. Ook probeerde je je kostbare energie over te houden voor al onze andere leuke analytische gesprekken en voor quality time. Bedankt voor je liefde, geduld en steun! Bovenal, ben ik gewoon heel erg blij en dankbaar dat jij mijn vriendin bent. Ik hou zielsveel van je!