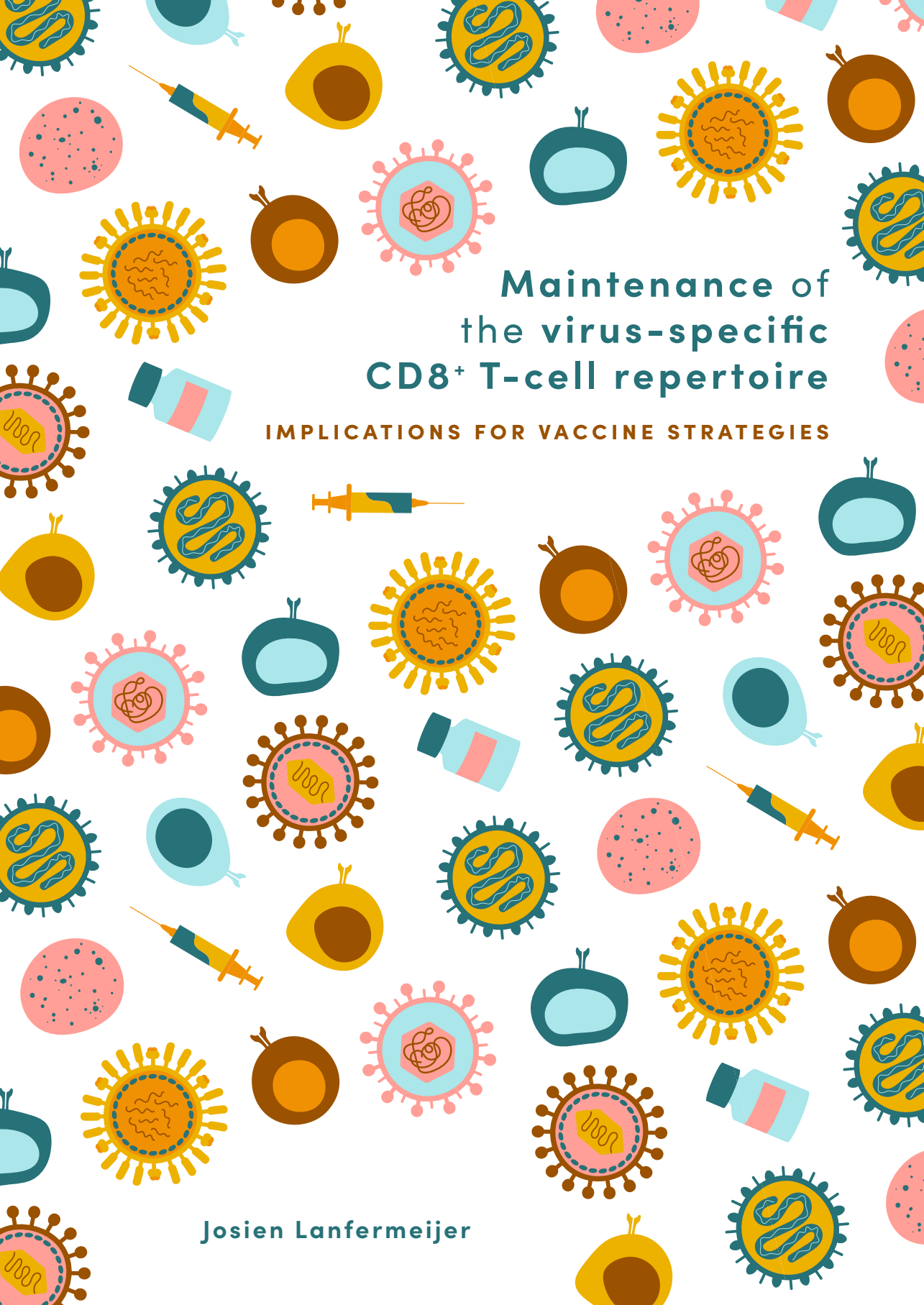
Maintenance of the virus-specific CD8+ T-cell repertoire

IMPLICATIONS FOR VACCINE STRATEGIES



Josien Lanfermeijer

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COLOFON

QR-code

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Josien Lanfermeijer

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Maintenance of the virus-specific CD8$^+$ T-cell repertoire

Implications for vaccine strategies

Behoud van het virusspecifieke CD8$^+$ T-cel repertoire
Implicaties voor vaccinatiestrategieën

(met een samenvatting in het Nederlands)

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Voor papa en mama

Mijn grote voorbeeld
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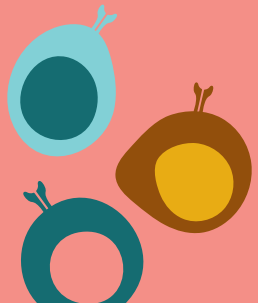
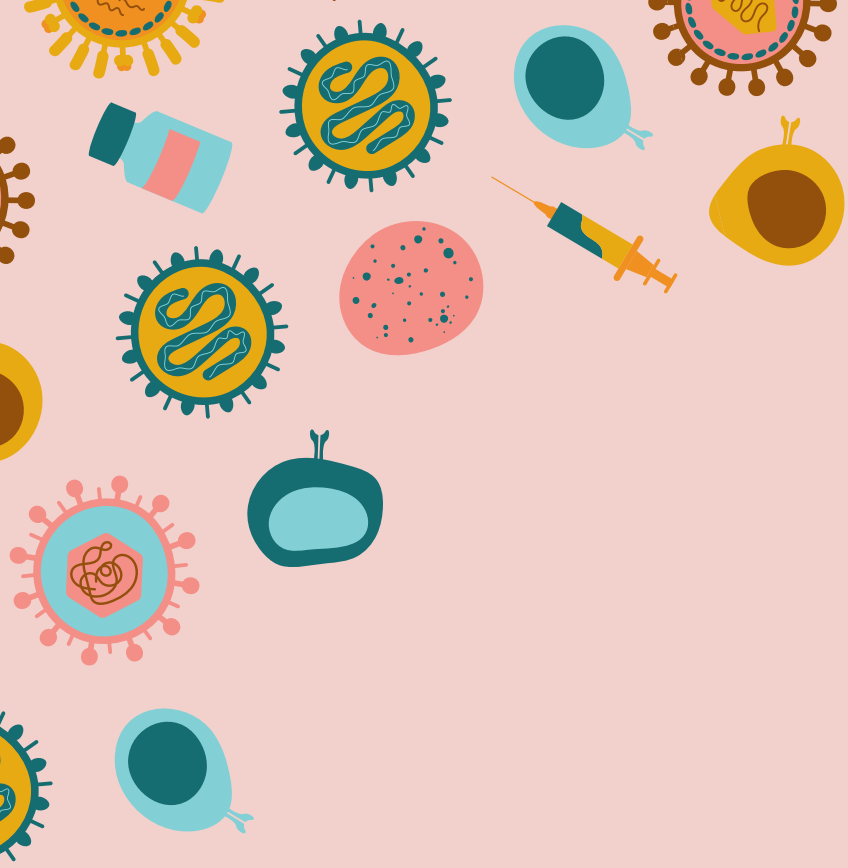
TABLE OF CONTENTS

INTRODUCTION



General introduction and scope of this thesis



POTENTIAL ROLE OF T CELLS IN VACCINATION AND INFECTION

Widespread immunization programs have dramatically reduced the impact of infectious diseases in our population. The introduction of childhood vaccination is without doubt one of the greatest successes in improving public health [16]. However, the fight against infectious diseases will remain an ongoing battle. Especially viruses will form a continuous threat and newly emerging and reemerging viruses stress the need for the development of new vaccines to protect the population. But also vaccine failure in risk groups, like older adults, is an increasing problem, due to the rapidly growing older population [17]. With age, functional impairment of the immune system occurs, reflected by weaker responses against new infections and vaccinations [18, 19]. Additionally, waning of vaccine-induced immunity is observed over time, also resulting in reduced protection against infectious diseases [20]. Especially respiratory infections, like influenza virus, respiratory syncytial virus and SARS-CoV-2 form an increasing challenge, as they lead to more severe disease in older adults, resulting in hospitalization or even death [21-24].

The ability of a vaccine to induce a protective immune response is usually measured in terms of induced antibody titers. One of the main mechanisms of antibodies is to neutralize invaders by binding to surface proteins of the virus. This way, they prevent the virus from infecting host cells. Despite the successes that have been achieved by inducing antibodies via vaccination, this strategy also has its limitations. Some viruses, like influenza virus, accumulate mutations in their surface proteins and can thereby escape from the antibody response. In addition, antibody titers typically decay over time, which may result in waning of the protective response [25]. T cells may fill the gaps when viruses are able to escape the antibody response [26]. T cells can potentially recognize a broader set of viral proteins, also the ones present on the inside of a virus particle. Internal proteins of the virus are more likely to be conserved between different virus strains, as their structure is more constrained and mutations in these proteins may have a direct impact on the viability of the virus [27]. In addition, T cells play an important role in the clearance of a virus by eliminating infected cells, and thereby lower the disease burden [28]. It has even been suggested that T cells may be a better correlate of protection against influenza virus in older adults than antibodies [26]. For both influenza virus and respiratory syncytial virus (RSV) infections it has already been shown that the presence of high numbers of virus-specific T cells is associated with less severe illness [29-32]. Induction of T cells by vaccination may

thus be a solid strategy to provide better protection in older adults. However, like the antibody response, also T cells are subject to impaired functioning with age, although it is not known to what extent and whether it has the same impact as seen for antibodies [33, 34]. However, as T cells recognize the more conserved parts of a virus, a possible strategy to protect older adults could be by stimulating the T cells at an earlier age, before immune aging sets in.

IMMUNE AGING OF T CELLS

One well-described hallmark of T-cell aging is the change in the composition of the T-cell pool. With age, the production of T cells by the thymus decreases due to thymic involution, a phenomenon that is thought to set in even before adulthood [35]. The resulting decrease in naive T-cell numbers and possibly diversity is thought to affect the response against newly encountered pathogens in older adults [36]. Other age-related observations include changes in the phenotype of the T cells, which can result in a decrease in their proliferative and activation potential [37]. These phenotypical changes are mostly observed in the memory T-cell pool, as T cells in older adults tend to be in a more differentiated state and express higher levels of senescence-associated markers. These age-related changes are all thought to contribute to the decreased protection of T cells against viruses [38, 39]. Insight in the gradual loss of naïve T cells and the maintenance of memory T cells during life may aid the development of T-cell based vaccines that provide long-term protection.

T CELLS IN ACTION

When a cell is infected with a virus, it presents virus-derived peptides (epitopes) on the outside of the cell via the major histocompatibility complex (MHC) molecules, also known as human leukocyte antigen (HLA) molecules [3]. T cells screen these molecules with their T-cell receptor (TCR) (Fig. 1). Different TCRs recognize different virus-derived peptides, making the immune response induced by T cells highly specific. Recognition means that a TCR binds the MHC-peptide complex with sufficient affinity, which can subsequently lead to activation of the T cell. Although the affinity of a specific TCR for the MHC-peptide complex is an important parameter for the strength of the T-cell response, secondary signals are needed for the T cell to become activated [40]. When T cells are activated, they will start to proliferate and differentiate into cells that can, among many other functions, kill infected cells. Especially $CD8^+$ T cells are well-known for their killing capabilities. Killing can occur via 1) secretion of cytotoxic granules, containing

perforin and granzymes, 2) secretion of cytokines such as IFNγ and TNF or 3) FAS/FASL interaction, leading to apoptosis of the target cell. By killing the infected cells, the intracellular pathogens get eliminated and further spreading of the virus is prevented.

The first time a virus is encountered, this process of activation and expansion takes 10-14 days. When the infection is 'cleared', T cells will differentiate into specialized memory T cells which will remain present in the body for years or even decades. During a second encounter with the same virus, these memory T cells will immediately react to prevent further spread of the virus and thereby provide protection against disease. With vaccination, an infection is mimicked in a safe and controlled manner by exposing a person to only a part or an attenuated version of a virus. This way, memory T cells are induced and are ready to respond once the real virus is encountered [41, 42].

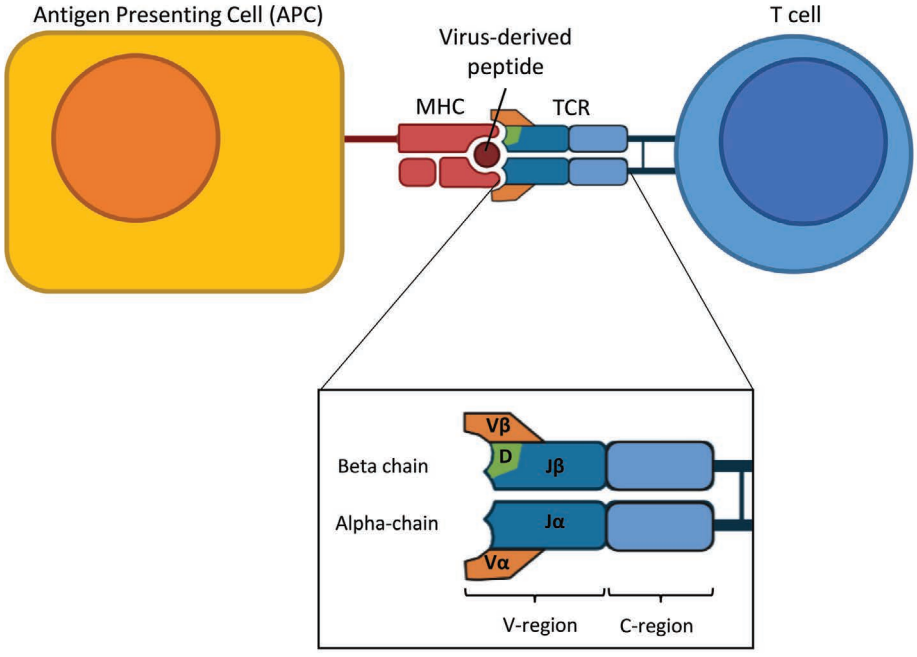


Figure 1: Schematic overview of TCR engagement with the peptide-MHC complex.
A functional T-cell receptor (TCR), represented on the surface of a T cell, recognizing a virus-derived peptide presented on an MHC molecule at the surface of an antigen presenting cell (APC). The TCR heterodimer consists of an α-chain and a β-chain. Both chains consist of a variable (V)-region and a constant (C)-region. The V-region of the TCR-α chain is comprised of a variable (V)-segment and a joining (J)-segment, depicted in orange and dark blue respectively. The V-region of the TCR-β chain includes an additional diversity (D)-segment, depicted in green.

IMPORTANCE OF TCR REPERTOIRE DIVERSITY

Because of the vast number of pathogens in the world, many different TCRs are needed, all with a different specificity, to ensure recognition of all possible foreign epitopes. At the same time, T-cell responses to self- epitopes need to be avoided in order to maintain "self-tolerance". Therefore, a highly diverse population of T cells is needed with highly specific TCRs in order to distinguish virus-derived epitopes from self-epitopes [43]. The sum of all these different T cells in an individual is called the TCR repertoire. It is generally assumed that a large TCR repertoire diversity is beneficial, because it increases the chance to recognize different virus-derived epitopes. In addition, in a more diverse T-cell repertoire, the chance that a TCR with a high affinity for an epitope is present is higher [44]. Indeed, it was shown that a more diverse T-cell repertoire was associated with better protection against disease severity of herpes simplex virus type 1 in mice [44, 45]. A broader T-cell repertoire could also lead to better protection against viruses that mutate and thereby evade the T-cell response, as it increases the chance that a T cell recognizing the mutated epitope (escape variant) is present [46-48]. This has been observed for the response against Epstein Barr virus (EBV) [49], hepatitis C [50, 51] and simian immunodeficiency virus (SIV) [52]. Thus, the diversity of the TCR repertoire is an important correlate of protection against virus infections [53].

HOW IS DIVERSITY OF THE TCR REPERTOIRE ESTABLISHED?

Our genome is not large enough to encode for all the different TCRs that are needed to protect us against all existing pathogens while ensuring self-tolerance. Instead, a highly sophisticated and stochastic process evolved to establish the necessary level of diversity of the TCR repertoire: the process of V(D)J recombination.

TCRs are heterodimers, that consist of an α- and a β-chain (Fig. 1) [56]. Both chains consist of 2 regions, the constant region, which is anchored to the cell surface, and the variable region, the part of the receptor that recognizes the peptide-MHC complex. As the name suggests, the variable region is the part where the uniqueness of the TCR is embedded. The variable region of the α chain consists of a variable-segment (V-segment, not to be confused with the variable region) and a joining-segment (J-segment, the part linked to the constant region) (Fig. 1) [57]. The β chain consists of an additional diversity-segment (D-segment, located in between the V and J segment), which gives the β chain an even higher level of diversity (Fig. 1) [58]. The junction between V and J (for α) or V, D and J (for β)

is also called the complementarity determining region 3 (CDR3) and is one of 3 hypervariable loops present in both chains. The CDR3 is the most diverse part of the TCR and plays an essential role in recognition of peptides. CDR1 and CDR2 are present in the variable-segment and interact predominantly with the MHC complex [57].

The process of V(D)J recombination occurs in the thymus. In brief, during this process different alleles of the different gene segments are combined (first level of diversity). The next step in generating a unique T-cell receptor is the addition and substitution of nucleotides in the CDR3 region [59]. Due to these nucleotide incorporations, even TCRs with the same V and J segments, can have very different CDR3 regions. Finally, pairing of an α and a β chain is needed to form a functional TCR; different combinations of α and β chains lead to the last level of diversity of the TCR. For more information and a schematic overview of this process, see Fig. 2 [44].

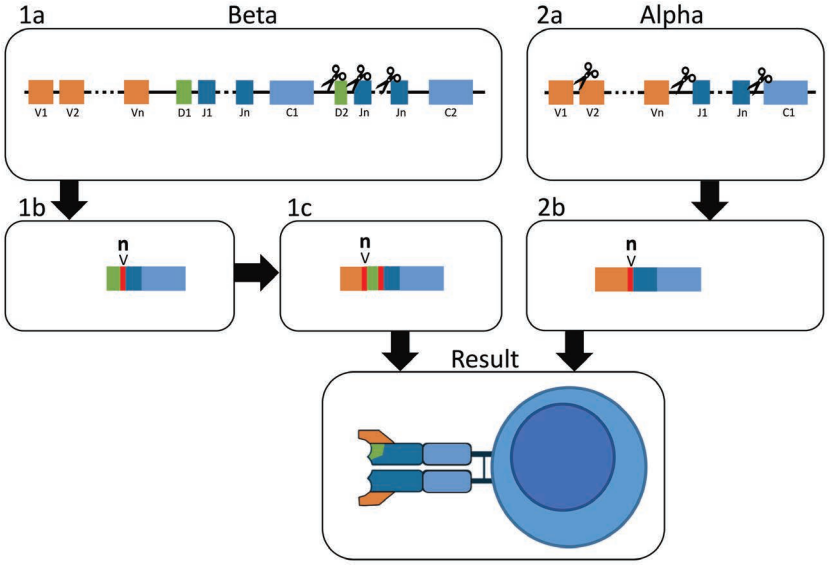


Figure 2: Simplified overview of V(D)J recombination of the α and β chains of the T-cell receptor.
The TCR-β gene locus showing the variable-segment (V, in orange), joining-segment (J, in dark blue), diversity-segment (D, in green) and the constant region (C, light blue) (1a). During somatic diversification, one of each segment is randomly selected. Recombination-activating genes (RAG, depicted as scissors) 1 and 2, cut the different gene segments at conserved and specific recombination signal sequences (RSS), after which recombination occurs. First, DJ recombination of the β-chain occurs (1b). Next, the V-gene segment is combined with the DJ segment (1c). Extra variability is introduced by terminal deoxynucleotidyl transferase (TdT) leading to incorporation of non-templated nucleotides (red)[54, 55]. Recombination of the TCR-α gene locus (2a+b) is analogous to the TCR-β chain, but without the D-segment. Finally, the α-chain and β-chain are combined, leading to a mature T-cell receptor.

After formation of their TCR, T cells are subject to a selection process before they are released from the thymus and go into the periphery [60]. Thymic selection is very important to make sure the T cells have the ability to recognize foreign epitopes and not self-peptides [61]. T cells undergo a two-step selection process to reach this goal. The first step, the positive selection step, ensures that the TCR can recognize and bind MHC-peptide complexes expressed by the host's cells. TCRs need to bind the MHC molecules with sufficient affinity in order to pass this positive selection step. The second step, also known as the negative selection step, ensures that TCRs are not strongly self-reactive. During this step, T cells that react too strongly to self-peptide-MHC complexes are eliminated [62]. Only 3-5% of the T cells that are generated in the thymus are eventually selected and released into the periphery, to scan for the potential recognition of pathogens [4]. Further shaping of the TCR repertoire occurs in the periphery and is discussed later on [63].

KEY ASPECTS OF THE T-CELL REPERTOIRE

1. Cross reactivity

In theory, the process of V(D)J recombination could create more than 1021 different TCRs [64]. Although the exact diversity of the TCR repertoire is unknown, it is estimated to be in the order of 106 [65], 107 [1], more than 108 [66], or even up to 1011 [67]. Nevertheless, the number of unique TCRs in an individual is relatively small compared to the number of different foreign epitopes that can be presented. To be able to recognize all possible epitopes, every T-cell clone should be able to recognize multiple epitopes, and therefore have some level of cross-reactivity [68].

Cross-reactivity is defined as the capacity of a TCR to recognize more than one peptide. It is even estimated that one T cell can recognize up to 105-106 peptides [68, 69]. There are different levels of cross-reactivity: a single TCR may be capable of recognizing structurally related peptides [70], but TCRs may also recognize peptides that show limited to no sequence homology [71, 72]. Cross-reactivity of TCRs is suggested to be explained by different mechanisms, including 1) high flexibility of the CDR3 loop, through which the TCR can adapt to a slightly different structure or different docking angle [73, 74] and 2) structural mimicry, in which the contact surfaces of the epitope the TCR recognizes are very similar [75]. Although T-cell cross-reactivity increases the chance that a T-cell response is made, and thus also provides flexibility in recognizing escape mutations [70, 76], this is a fine balance, as recognition of self-peptides should always be prevented [43].

2. Shared T-cell receptors

Despite the large potential diversity of the TCR repertoire, significant overlaps between TCR repertoires of different individuals are typically observed. These overlapping TCRs are often referred to as shared or public TCRs [2, 77-79]. Public responses have been observed against different viruses, including EBV [80, 81], CMV [82, 83], and HIV [84], and are associated with a relatively high affinity for the peptide-MHC complex [85, 86]. It has long been thought that public TCRs exist because of structural constraints, as the shape of the peptide-MHC complex enables effective binding of only a limited number of TCRs [87]. This cannot explain the presence of public TCRs in the naive T-cell repertoire, however [88].

A possible explanation for the presence of public TCRs in the naive T-cell pool is that there is a bias in the TCR recombination process. Firstly, convergent recombination may underlie the occurrence of shared TCRs in the naive T-cell pool: multiple independent recombination events can produce the same TCR nucleotide sequence, which may thereby become more abundant in the repertoire [89, 90]. Secondly, the recombination process may have evolved to produce TCRs that have a higher chance to be selected during thymic selection [91]. It was recently shown that the chance of a TCR to be shared between individuals is linked to the generation probability of the TCR sequence. Public TCRs may be more easily produced because they have undergone fewer nucleotide insertions during the V(D)J recombination process [92]. It is thought that a large number of public TCRs are created before birth, as these clones have fewer to no randomly inserted nucleotides. The expression of the enzyme responsible for these insertions starts after birth [93]. However, the exact purpose of these public TCR, or whether they even have a purpose, remains unknown.

TCR REPERTOIRE AS A POSSIBLE BIOMARKER FOR HEALTH AND DISEASE STATUS

Despite the presence of these overlaps between TCR repertoires of different individuals, TCR repertoires differ remarkably between individuals. This is in part due to the random process of TCR development as described earlier, as well as the genetic (MHC) background of individuals (see box 1: MHC background and TCR repertoire diversity for more information). Furthermore, the composition of an individual's memory TCR repertoire is thought to change over time. With every new infection or vaccination, T cells specific for the encountered antigens will expand and form new memory T cells [57]. Also time itself may influence the TCR repertoire, as with age, thymic involution leads to a decrease in the production of

new T cells. This is thought to result in "holes" in the T-cell repertoire. Thus, at older age, certain virus-derived epitopes may not be recognized because their specific T cells are no longer available in the T-cell repertoire [94]. As every individual's TCR signature is so unique and reflects the infections and vaccinations that have been encountered, it is tempting to think how the TCR repertoire could be used as a personal biomarker reflecting an individual's immune status [95-97].

Determining the TCR diversity or the presence or absence of certain TCRs may 1) help to predict the potential to respond to infections and/or vaccinations, 2) serve as an indicator of a person's infection history, and 3) be used to trace an immune response [98]. Ideally, by analyzing a person's TCR repertoire, individuals at risk for certain infectious diseases or vaccine failure may be identified [99]. Despite the large amount of information present in the TCR repertoire, current diagnostics and vaccine strategies do not yet take such information into account. This may in part be due to the very complexity related to analyzing and translating the information present in the TCR repertoire. Further research is needed to study how the TCR repertoire changes over time and what these changes may implicate.

Box 1: MHC background and TCR repertoire diversity.

MHC molecules play an important role in the induction of T-cell responses, as these molecules present virus-derived peptides on the cell surface of infected cells. There are two classes of MHC molecules: class I and class II. CD8+ T cells recognize peptides presented by MHC class I molecules, whereas CD4+ T cells recognize peptides presented by MHC class II molecules. For MHC class I, there are 3 different genes, called HLA-A, HLA-B and HLA-C. MHC class II molecules occur as MHC-DP, DQ and DR. For each MHC gene, a very large number of different alleles exist in the human population. The population diversity of MHC molecules is so large, that it is very rare to find two individuals with the exact same set of MHC molecules.

The population diversity of MHC molecules influences the composition of the TCR repertoire, as different MHC molecules present different peptides. Already during selection of T cells in the thymus, differences in MHC molecules will lead to different self-epitopes being presented and hence different T cells surviving positive and negative selection [3, 4]. During immune responses, different individuals (which will typically have different MHC molecules on the surface of their cells) will mount different T-cell responses against the same pathogen. Since MHC molecules are co-dominantly expressed, individuals with a heterozygous MHC background (carrying two different alleles of e.g. HLA-A) are thought to have a more diverse TCR repertoire, and a higher chance to respond to pathogens [6]. In line with this, MHC heterozygosity has been shown to lead to better resistance against infections in mice [8] and improved control of HIV-infection [10, 11].

ANALYZING THE ANTIGEN-SPECIFIC T-CELL REPERTOIRE

Unraveling the TCR repertoire in depth may be of importance for the development of new vaccine strategies that focus on the induction of T-cell responses. One way to get deeper insight in the TCR repertoire is to characterize different antigen-specific TCR-repertoires and their dynamics. So far, antigen-specific TCR repertoire analyses have provided information about clonal expansions, biased usage of certain V-segments, biases in CDR3 length, cross-reactivity and shared clonotypes for certain TCR specificities, e.g. in the CMV-specific repertoire. This information has made it possible to assign TCRs to their antigen specificity [96]. Despite these developments, only for a small number of TCRs, the antigenic specificity is known and there are still many unanswered questions about the dynamics of the antigen-specific TCR repertoire.

For example, it remains unknown how diverse the TCR repertoire needs to be for optimal protection against infections and whether insufficient repertoire diversity could be restored. While the effect of aging on the T-cell repertoire has mostly been described for the total memory T-cell pool, its effect on antigen-specific T-cell repertoires and the implications for vaccine strategies remain unknown. Studies investigating (long-term) maintenance of the responding TCR repertoire after infection or vaccination may provide valuable information on when to vaccinate. Furthermore, how the diversity of the antigen-specific TCR repertoire changes after repeated exposure to antigens may provide information on the effect of booster vaccinations. Likewise, it is important to understand the effects of heterologous infections on the composition and shaping of antigen-specific T-cell repertoires.

SCOPE OF THIS THESIS

In this thesis we study how the virus-specific T-cell repertoire changes over time and how this is influenced by aging, vaccination and infection history. For this, we focus on the antigen-specific $CD8^+$ T-cell repertoire.

For those who are interested, in **Chapter 2**, we briefly introduce the different techniques that are used to analyze the TCR repertoire. After that, the first part of this thesis focusses on how the antigen-specific TCR repertoire is maintained. In **Chapter 3** we give an overview of the current knowledge on changes in the antigen-specific T-cell repertoire with age. We also review what is known about the influence of infection history of both homologous and heterologous infections on the antigen-specific T-cell repertoire, and discuss why this information is important in the design of new vaccination strategies. We report that the antigen-specific T-cell repertoire is stable for at least a couple of years, while the influence of (new) antigenic stimulations on the antigen-specific T-cell repertoire remains inconclusive, underlining the need for further research into the effect of age and infection history on the antigen-specific TCR repertoire.

To further elucidate the effect of age and CMV-infection on the antigen-specific T-cell repertoire, we dive deeper into this matter in Chapters 4 and 5. In **Chapter 4**, we sequence the CMV-specific and EBV-specific T-cell repertoires in healthy adults of different ages and with different CMV serostatus to investigate how age and CMV-infection influence the EBV-specific T-cell repertoire. We show that with age, the diversity of the EBV-specific T-cell repertoire decreases, an effect that is even more pronounced in CMV^+ individuals. In **Chapter 5** we study the hypothesis that CMV-induced changes in the T-cell population of older adults may influence the response and repertoire composition of T cells against other viruses, by looking at influenza-specific T cells after influenza infection. In contrast to the commonly held view, we show that CMV does not influence the quality of the influenza-specific T-cell response.

For **Chapter 6**, we have the opportunity to longitudinally follow the mumps virus (MuV)-specific T-cell response against three different MuV-epitopes after mumps virus infection in previously vaccinated and unvaccinated individuals. Little is known about the characteristics of the MuV-specific T-cell response, while this response may be of importance in preventing the vaccine failure that has been observed in the last decades. We show that the antigen-specific T-cell repertoire

remains stable for up to 9 months after infection, despite large increases in MuV-specific T-cell frequencies.

For both Chapters 7 and 8 we switch to mouse models, to investigate how the TCR repertoire is shaped, as this cannot easily be tested in humans.
In **Chapter 7** we investigate whether time since previous influenza A virus (IAV) infection influences the T-cell response against a new heterosubtypic IAV infection at middle age. Our findings suggest that boosting of the pre-existing T-cell response at middle-age is not influenced by time since previous infection. However, boosting does result in a decrease in diversity of the antigen-specific T-cell repertoire.

In **Chapter 8** we study how amino acid alteration of virus-derived peptides leading to stronger binding to the MHC, influences the T-cell response against the natural peptides [100]. We evaluate how these peptide alterations shape the antigen-specific T-cell repertoire and whether such altered peptides could be used as a new vaccination strategy. Our data show that altered peptides might induce a lot of non-relevant T cells and clonal expansions, and may even lead to enhanced immunopathology.

Lastly, in **Chapter 9** we summarize the findings presented in this thesis and discuss our conclusions and how these may play a role in the design of new vaccine strategies.

Methodologies for TCR repertoire analysis

PAST AND PRESENT OF TCR REPERTOIRE ANALYSIS

There are several methods to perform TCR sequencing, all of which have their own advantages and disadvantages [101]. These methods keep evolving and especially the advances in high throughput sequencing (HTS) over the past decades have made it possible to perform deep analysis of the T-cell repertoire [102].

In the early days of TCR repertoire studies, monoclonal antibodies specific for the variable (V)-segment of the T-cell receptor were used for the characterization of the repertoire to determine the frequencies of different V-segments by flow cytometry [9, 103, 104]. Although this led to some early observations of repertoire skewing and diversity changes due to infection or aging, no information about the CDR3 region, the most variable part of the TCR [105], could be obtained. Later on, CDR3 spectratyping led to new insights in the diversity of the TCR repertoire [65], by providing information on the length of the CDR3 regions of different TCRs [106]. The first method using CDR3 sequencing was based on cloning and Sanger sequencing [103]. The use of this technique resulted in pioneering studies in which sharing of TCRs was observed between individuals [83, 107], and disease-specific T-cell expansions were identified [82, 85]. Due to the high labor intensity of the method, this resulted in a relatively low throughput of sequences, which made this method less suitable for obtaining a meaningful overview of TCR diversity in large unsorted T-cell populations [103, 108].

Nowadays, HTS has become the most common way to analyze the TCR repertoire [9, 109]. This technique has led to a major shift in the quantification of the diversity, dynamics and functioning of the TCR repertoire [110]. Sample preparation for TCR sequencing consists of several steps, and for every step in the process different methods can be used. During every single step, alterations and errors can be introduced in the data [111]. A comparison of 9 different methods to prepare T-cell samples for TCR sequencing, identified differences in the accuracy of the generated data, as well as mixed results on the reproducibility of the experiments [112]. As these different HTS-methods impose unique biases when capturing the TCR repertoire, it is important to first identify the scientific question or clinical problem, to then choose the best sample preparation procedure for the question at hand.

STARTING MATERIAL

In order to sequence the TCR repertoire, both genomic DNA (gDNA) and RNA can be used as starting material. Using gDNA may be favored as 1) it is more stable than RNA [9, 113], and 2) the resulting sequencing data correlate more directly with cell numbers as there is only one gDNA template per cell, while there can be multiple RNA copies per cell [114-116]. When using RNA as starting material, an extra step of cDNA synthesis (reverse transcription) is necessary, which could introduce technical errors in the data. Nevertheless, most TCR sequencing studies use RNA as the starting material. One advantage of the use of RNA is that only the final TCR product will be sequenced, whereas gDNA also consists of introns and sometimes silenced segments are included, which may lead to errors [9, 113]. Furthermore, RNA is present in several copy numbers in the cell, which enhances the chance of its detection, thereby making the method more sensitive than sequencing procedures based on gDNA [9]. An important drawback of this, is that RNA measurements are not directly related to cell numbers. Regardless of the starting material that is used, its quality is essential for the outcome of the experiment [117]. It has previously been shown that a too low RNA input can lead to disturbed insights into the TCR repertoire because of the difficulty to detect rare TCRs, creating a bias towards the more abundant TCRs [112].

MULTIPLEX VERSUS RACE AMPLIFICATION

Before the coding material can be sequenced, it needs to be amplified. The two currently used methods to do this are multiplex PCR and rapid amplification of cDNA ends (RACE).

To capture all different TCR transcripts in a sample, the multiplex PCR uses multiple different forward primers for all known V genes [9]. This is necessary because of the large sequence diversity in the V genes. The set of reverse primers is based on the starting material. A set of reverse primers for the different J genes is used when the starting material is gDNA, whereas the primers are specific for the C region when the starting material is RNA [1, 2, 118]. A disadvantage of the multiplex technique is that there is often an amplification bias towards specific V genes in a sample, based on stronger binding of certain primers [9, 114, 119].

The RACE technique is used to obtain the full-length cDNA from RNA via cDNA synthesis [105, 120]. The most used technology to do this is the SMART technique (switching mechanism at the 5'end of RNA template) [9]. The technique is based

on the enzyme reverse transcriptase, which adds non-templated cytosine (C) nucleotides to the 3'end of the cDNA (when it reaches the 5' of the mRNA template) (Fig. 1) [121]. A "template switch oligonucleotide" can bind to these C's, as it consists of complementary guanines (G's). The template switch oligonucleotide will then act as a template, so additional nucleotides can be added to the first cDNA strand by the reverse transcriptase: this is the template switch step [121]. This additional sequence is used as an annealing site for the primers of the subsequent PCR. An advantage of this technique is that only one primer pair is needed, as for the other primer one can choose a primer binding the poly A tail or a TCR-specific primer that binds the constant (non-variable) region. The advantage is that this does not lead to amplification bias of certain V-segments.

Nested PCR based on the 5'RACE and switch-oligo approach (only for RNA) makes use of the incorporation of an adaptor molecule at the 5' end of the cDNA during cDNA synthesis. The forward primer for a subsequent PCR is designed to bind to the 5' adaptor sequence, while the reverse primer is designed to bind to the C-region of the transcript. Hence, only one primer pair is required to cover the complete spectrum of possible V genes. Subsequent nested PCRs performed in the same fashion may increase outcome specificity. Finally, adaptor ligation is performed. The procedures shown in Fig. 1 are only an example of the different methods that are available.

CELL POPULATIONS VERSUS SINGLE CELL

When analyzing TCR repertoires in larger T-cell populations, bulk analyses are mostly used, meaning that all T cells in one sample are sequenced together. An advantage of bulk sequencing is that it provides a lot of information about the repertoire as a whole, and it is possible to analyze the diversity of the TCR repertoire in depth [9]. However, using bulk sequencing it is not possible to provide information about the pairing of the α and β chains of a TCR. Typically, the β chain is sequenced, as it contains more diversity than the α chain, due to the inclusion of the diversity (D)-gene segment [117].

Using single cell sequencing makes it possible to capture both the α and the β chain of each TCR, and thereby provides more precise insight in the diversity of the TCR repertoire [122, 123]. Single cell sequencing also solves many of the earlier described problems. For example, with single cell sequencing, there is no influence of different levels of RNA expression, and as multiple reads are generated per cell, the sequence data are more reliable [124].

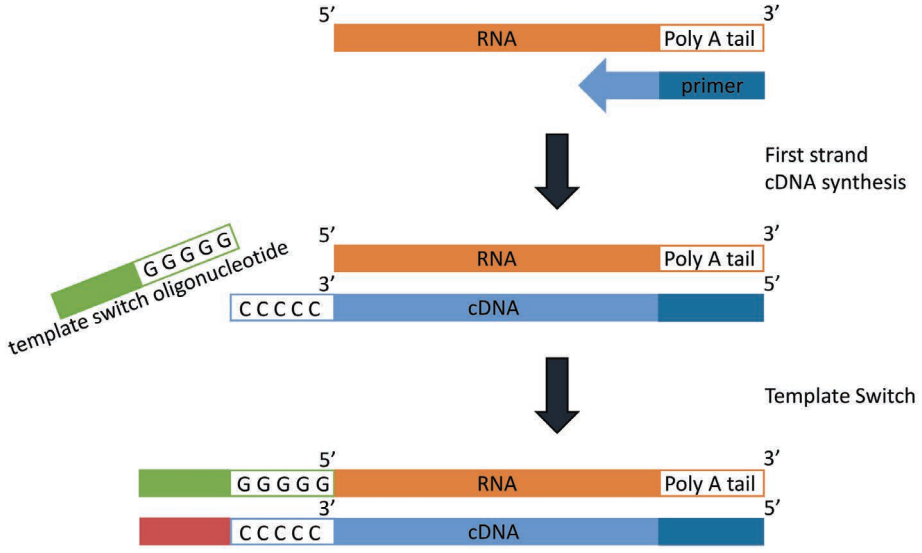


Figure 1: Simplified overview of 5'RACE cDNA synthesis.
This figure shows an overview of the 5'Race methodology used to convert mRNA (depicted in orange) into cDNA (depicted in light blue) using reverse transcriptase. Note that the precise protocol can vary. The first step is to produce a cDNA copy of the total, or a region of the, mRNA molecule. In this example a primer binding the poly A tail (depicted in dark blue, for transcription of the total mRNA) is used. The enzyme called reverse transcriptase will generate the cDNA from the end of the primer towards the 5' end of the mRNA template and will then add non-templated nucleotides (depicted as "C C C C C"). A template switch oligonucleotide (depicted in green) will then anneal to these non-templated nucleotides, making it possible to incorporate additional sequences into the first-strand cDNA. This additional sequence (depicted in red) can then be used as a primer annealing site for subsequent PCR rounds. At the end, the specific cDNA will be amplified by PCR, using primers that can anneal to the known sequences of the primer (dark blue) and the oligonucleotide (green).

Several techniques have been developed to isolate single cells, which are reviewed by De Simone et al. [125]. Nowadays, it is also possible to combine RNA-sequencing with TCR-sequencing, giving even more information about the phenotype and function of the T cells of interest [126, 127]. The largest disadvantage of single cell sequencing is its high cost, which when finances are limited may result in relatively low throughput of cells and hence possible biases in the data.

ERROR CORRECTION

One of the biggest challenges of analyzing the sequencing data is to identify whether sequence differences between T-cell clones are due to errors or based on real differences. Especially the recovery of rare TCR sequences from the sequences that originated from sequencing errors is difficult, as TCR sequences

may differ only based on a single nucleotide. During the last years, different software tools have been developed to handle sequencing errors and to recover these low frequency TCR sequences [110].

Recently, a strategy has been developed to correct some of the technical errors that can be introduced during the process of TCR sequencing. By implementing unique molecular identifiers (UMIs) during cDNA synthesis, it is possible to correct for amplification biases and sequencing errors [128]. This is especially important when performing bulk sequencing. UMIs are random sequences, which enable recovery of the original distribution of TCRs [129]. As they are implemented during cDNA synthesis, often in the template switch oligonucleotide, it is only possible to use them if the starting material is RNA.

Using UMIs makes it is possible to: 1) distinguish similar TCR sequences originating from different RNA molecules [129], as by counting the different UMIs instead of sequencing reads of the identical TCR sequences gives a more accurate quantification of the TCRs; and 2) correct PCR and sequencing errors, by looking at different sequences with the same UMI. When using UMIs, it is thus possible to make a distinction between technical errors and biological differences in the sequencing data and helps identify the rare T-cell clones [9]. However, note that when using UMIs there is still the problem of different amounts of mRNA copies per T cell, whereas also errors introduced during the cDNA synthesis cannot be resolved.

The use of UMIs is currently the golden standard in TCR repertoire analyses. Unfortunately, using UMIs comes at the cost of lower sensitivity as the insertion of UMIs increases the length of the template switch oligonucleotide, which results in a decreases in the efficacy of the cDNA synthesis [9, 112]. Therefore, when focusing on rare T-cell clones, non-UMI based methods may be more appropriate. Altogether, the most important thing is that there is uniformity between how samples are processed and analyzed when determining the TCR repertoire.

PART 1

MAINTENANCE OF THE T-CELL REPERTOIRE

How age and infection history shape the antigen-specific CD8⁺ T-cell repertoire: implications for vaccination strategies in older adults

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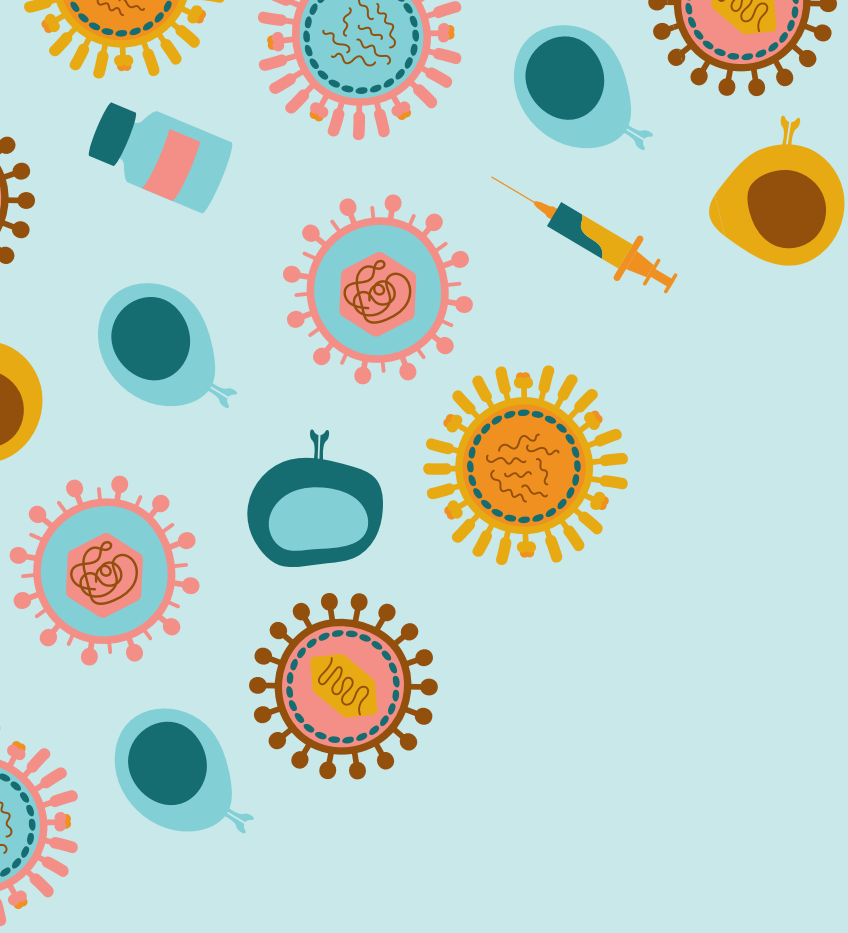


ABSTRACT

Older adults often show signs of impaired CD8⁺ T-cell immunity, reflected by weaker responses against new infections and vaccinations, and decreased protection against reinfection. This immune impairment is in part thought to be the consequence of a decrease in both T-cell numbers and repertoire diversity. If this is indeed the case, a strategy to prevent infectious diseases in older adults could be the induction of protective memory responses through vaccination at a younger age. However, this requires that the induced immune responses are maintained until old age. It is therefore important to obtain insights into the long-term maintenance of the antigen-specific T-cell repertoire. Here we review the literature on the maintenance of antigen-experienced CD8⁺ T-cell repertoires against acute and chronic infections. We describe the complex interactions that play a role in shaping the memory T-cell repertoire, and the effects of age, infection history and T-cell avidity. We discuss the implications of these findings for the development of new vaccination strategies to protect older adults.

INTRODUCTION

Cytotoxic CD8⁺ T cells are important in the clearance of virus-infected cells and are therefore of interest for the development of vaccination strategies against infectious diseases. An important correlate of protection against infectious diseases is the recruitment of T cells carrying high-affinity antigen-specific T-cell receptors (TCRs) [130]. The diversity of the recruited T-cell repertoire is also directly linked to disease outcome [52, 131], and narrow T-cell repertoires are associated with more frequent occurrence of viral escape [47]. Higher TCR diversity has been suggested to be important for a protective immune response, as it increases the chance of both high avidity clones as well as cross-reactive clones, able to recognize antigen variants, to be present in the responding repertoire [40, 53].

Diminished CD8⁺ T-cell responses to new infections and vaccination in older adults are thought to be due to a combination of decreased numbers and functional capacity, amongst which the priming capacity, of naïve T cells with age [36]. Another important role is assigned to a decline in the diversity of the T-cell repertoire with age [132]. Although estimating T-cell diversity is challenging for various reasons (Box 1), the diversity of the total CD8⁺ T-cell pool has indeed been shown to decrease with age in both longitudinal and cross-sectional studies [133-135]. As total T-cell diversity declines proportionally to naive T-cell numbers [133], the decrease in diversity of the total CD8⁺ T-cell pool is associated with decreased diversity in the naive CD8⁺ T-cell pool [66, 136] (Fig. 1). Indeed, it was shown that in aged mice the antigen-specific T-cell repertoire induced after antigen exposure was of lower diversity compared to that in young mice, which in turn was associated with lower numbers of naive precursor cells [137-141]. In humans, diminished responses and narrower antigen-experienced T-cell repertoires have also been observed at older age, for example in response to influenza A virus (IAV) infection [39, 142-144] or vaccination [19]. If decreased diversity of the naive T-cell pool indeed forms the bottleneck for the induction of protective memory T-cell responses in older adults, instead of vaccinating elderly people, it may be better to induce protective immune responses by vaccination earlier in adulthood. It would then be essential that such immune responses are maintained well into old age. Little is known about how long antigen-specific T cells are well maintained in the memory pool of older adults. Some studies have suggested that the diversity as well as the absolute number of cells in the CD8⁺ memory T-cell compartment remain relatively stable with age [35, 66]. This is in fact surprising for two reasons: 1) the memory pool is constantly fed with new T cells after every new antigen encounter, and 2) memory T cells that reencounter their antigen,

like chronic viruses, expand and thereby add cells to the memory T-cell pool. It has been suggested that with every T-cell entering the memory pool, antigen-experienced T-cells already present in the memory pool may be eliminated (Fig. 1), leading to memory attrition [145, 146]. Especially cytomegalovirus (CMV) is known to frequently reactivate and challenge the immune system, thereby maintaining high frequencies of antigen-specific T-cells [147–149]. The presence of these CMV-specific clonal expansions has been proposed to lead to changes in the antigen-specific T-cell repertoire against other viruses [150] and could therefore affect the maintenance of other antigen-specific T-cell clones in the memory pool.

This review provides an overview of studies addressing the maintenance of antigen-specific CD8⁺ T-cell repertoires in the memory pool. We address the influence of age on the dynamics of the antigen-experienced T-cell repertoire after infection. In addition, we cover how infection history and TCR affinity shape the antigen-specific T-cell repertoire. Finally, we discuss the implications of these findings for the development of vaccination strategies to protect older adults against infectious diseases.

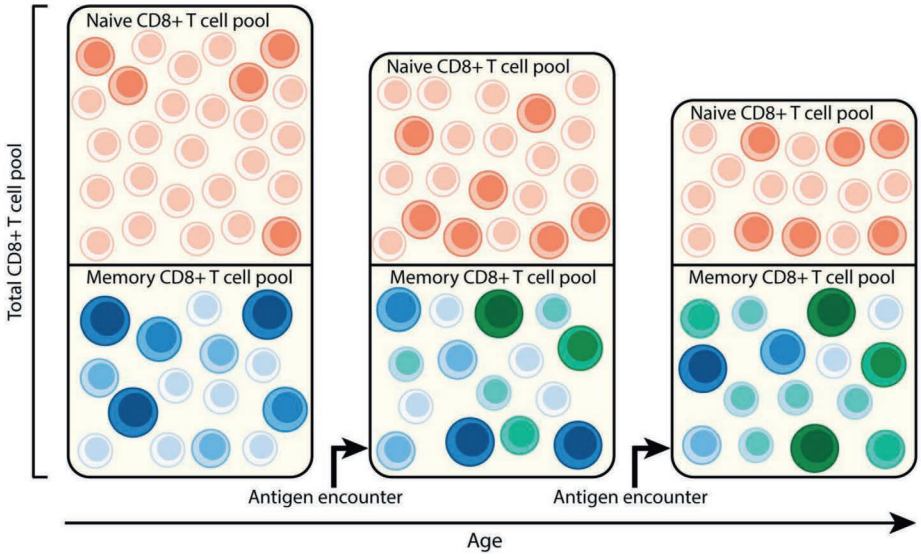


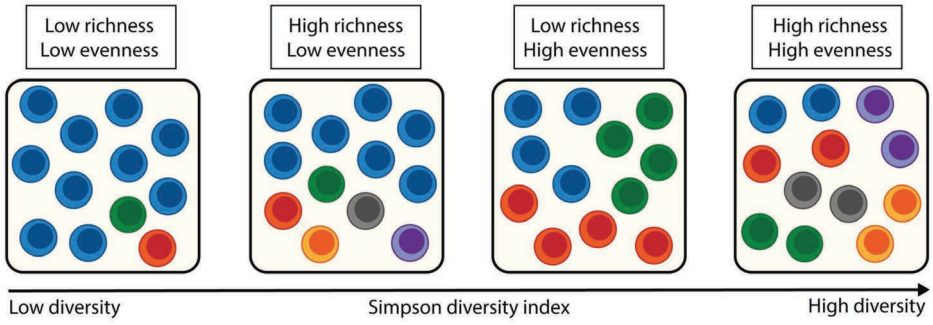
Figure 1: Influence of age on the human CD8⁺ T-cell repertoire.
Every circle represents a distinct T-cell clone, the larger the circle, the larger the clonal size. The diversity of the CD8⁺ T-cell repertoire decreases with age. This is mostly due to a decrease in the size of the naive T-cell pool (shown in red). As the size and diversity of the memory T-cell pool (shown in blue) remains relatively stable during adult life [66]), despite the fact that new memory T cells (shown in green) are continuously added to the memory T-cell pool, this may lead to displacement of early induced memory T-cell clones

Box 1: Challenges of studying T-cell receptor diversity

T-cell repertoire diversity is an important determinant of the efficacy of the $CD8^+$ T-cell response. However, determining the diversity of the T-cell repertoire is challenging at various levels, making the comparison between T-cell repertoire studies even more challenging. First, clinical samples used to determine T-cell repertoire diversity represent only a tiny fraction of the total T-cell pool present in the body. In theory, V(D)J recombination can result in more than 1021 unique T-cell receptors. However, the number of different clonotypes remains unknown, but is estimated to lie between 107 [1, 2] and 1010 [5]. A clinical sample contains only a few milliliters of blood, while blood only contains 2 percent of all the T-cells present in the body. This especially affects the identification of rare clonotypes [1].

Second, the differences in protocol used to estimate repertoire diversity can lead to discrepancies in conclusions. Especially when focusing on the antigen-specific T-cell repertoire, discrepancies are created by using different ways to detect enough antigen specific T-cells, as back in the days the T-cell population were often enriched through ex vivo stimulation. It was previously shown that repertoire analyses after ex vivo stimulation can lead to very different diversity measurements compared to direct ex vivo analyses [7]. Thanks to the use of tetramers and single cell analyses, it becomes easier to determine the direct ex vivo repertoire of small cell populations. Also, the use of different techniques can result in discrepancies in conclusions, due to the level of depth that is accomplished. In the past, TCR-Vβ antibodies were used to detect the presence of specific variable-gene subgroups with in the β chain of the T-cell receptor using flowcytometry. Nowadays, the usage of high throughput sequencing (HTS) is the norm, making it possible to analyze millions of different TCR sequences at once, mostly focusing on the β-chain of the TCR. HTS provides much greater and more accurate quantification of the repertoire compared to the older techniques. However, also for HTS there are several available options and differences are seen throughout the whole protocol, e.g. the starting material, PCR-technique and library preparation [9]. Furthermore, the data retrieved from HTS is very sensitive to PCR and sequencing errors. A step further in determining the diversity of the repertoire is the use of single cell analyses, which gives us more information on how the TCRα and TCRβ chain interact. Combining this with RNAseq analysis provides even more information, not only about the TCR usage, but also showing the functional state of T-cells bearing a specific receptor. However, only a limited number of cells can be analyzed, making the diversity estimations difficult.

Last, diversity is still a poorly defined concept. One way to look at the diversity of a population is by looking at its richness and evenness. Richness is the actual number of unique TCR sequences in a population, whereas evenness reflects the relative frequencies of the different TCR sequences observed. The first measure is particularly sensitive to the number of rare clonotypes, which are easily overlooked when using small samples. Conversely, evenness is strongly affected by clonal expansions (Box Figure). For a good estimation of the diversity, both components are needed and are therefore integrated in several diversity indices, like Simpson diversity index or Shannon diversity index. Both indices are often used in the field; however, both have their own shortcomings and do not always properly reflect the repertoire distribution. For example, Simpson diversity index puts more weight on dominant species. This means that clonal expansions influence the outcome of this index largely and that a decrease in diversity may be seen, due to clonal expansions, while the richness of the population did not change. Shannon diversity index on the other hand, puts more weight on richness of the sample. Note that a high richness does not always implies a high diversity, as combined to a low evenness will lead to a low diversity (See Box Figure). Therefore, caution is needed when using diversity measurements, as diversity may be too complex to be noted by only one number [12-15].



Box Figure: How richness and evenness make up the diversity.
This figure shows how richness and evenness affect the diversity index when estimating the diversity of the repertoire. The four boxes show an increasing diversity, with varying diversity scores, based on Simpson diversity index. Note that the diversity indices of population 2 and 3 are very close, when diversity would be calculated with Shannon diversity index their order would be reversed.

PART 1: CHANGES IN THE ANTIGEN-SPECIFIC T-CELL REPERTOIRE WITH AGE

To assess how the antigen-specific T-cell repertoire in the memory pool changes with age, studies that analyze single epitope-specific repertoires are needed. Most antigen-specific studies in humans have focused on $CD8^+$ T cells directed against epitopes of influenza virus and the chronic viruses CMV and Epstein-Barr virus (EBV). Antigen-specific T cells against these viruses are present in relatively high frequencies in the blood, making them easily detectable by tetramer staining or peptide stimulation. There is also quite a lot of literature on T-cell repertoire diversity in HIV-infection, however, as the interpretation of these data is complicated by the high mutation rate of the virus, heterogenicity in rates of disease progression and the use of antiviral therapy, we here focus on the antigen-specific response to the herpesviruses CMV and EBV and the memory phase of influenza virus-specific T cells during homeostasis.

1.1 The CMV-specific T-cell repertoire

CMV is a herpesvirus, known for its latent presence in the body. The virus is thought to frequently reactivate, leading to restimulation of the anti-CMV immune response. Hallmarks of CMV infection are the presence of clonal expansions and large numbers of highly differentiated cells in the $CD8^+$ T-cell memory pool [151]. These clonal expansions are often directed to the pp65 protein and can be focused on only one or a small number of epitopes, like the HLA-A2 restricted

NLVPMVATV (A2-NLV) epitope. The antigen-specific T-cell repertoire against the A2-NLV epitope has already been described more than two decades ago using TCR-Vβ antibodies, recognizing the different variable-segments of the β-chain of the T-cell receptor (**Box 1**). This revealed that the T-cell response against A2-NLV shows preferential usage of Vβ8 and Vβ17 [152, 153]. Skewed Vβ usage in the CMV-specific T-cell repertoire has also been observed in other studies [83, 154, 155] and is not restricted to the pp65 antigen [81, 82, 153, 156, 157]. Sequencing of cultured A2-NLV specific T-cell clones revealed the presence of the same clones at different time points over a time span of 18 months [153]. When these results were compared to a group of older donors [82], notable similarities were observed, including similar skewing towards Vβ8 segment usage and even the occurrence of the same clonotypes, also known as public clonotypes. It was suggested by both studies that CMV-specific T-cell clones can be maintained during life.

A later study suggested that the repertoire is less stable than originally thought. In fact, within the A2-NLV Vβ8$^+$ T-cell repertoire, the clonotype usage turned out to be much less stable than previously anticipated [107]. One T-cell clone was detected in 72% of the sequences in young donors (28-37 years), while its dominance decreased to below 40% in middle-aged and older adults. Interestingly, the TCR of another dominant clone that was only observed in older adults had a higher affinity for the A2-NLV epitope than the clone dominating in young donors [107]. This suggests that at older age, higher avidity T-cell clones become more dominant. Following the CMV-specific T-cell repertoire after primary infection showed that in the memory phase high avidity clones became dominant, which were subdominant early after primary infection [85]. Together this suggests that there is a shift in clonal dominance based on TCR affinity, even though the biased Vβ segment usage in the CMV-specific T-cell repertoire seems to be maintained over time.

Longitudinal studies have suggested that a large part of the A2-NLV-specific T-cell repertoire is conserved for at least 2 [158] to 4 years [159]. 60-100% of the clones found in middle-aged and older adults persisted over a period of 2 years [158]. A more in-depth analysis of the CMV-specific T-cell repertoire after 4 years, showed that a large proportion of the T-cell repertoire was stable over time, with persistence of the most dominant T-cell clones [159]. This stable repertoire has been suggested to be formed very early after primary infection, as the responding T-cell repertoires 6-16 weeks after infection were comparable to those in the same donors 3 to 5 years later [160]. Although these longitudinal studies have suggested that the antigen-specific T-cell repertoire is relatively stable over time,

the time frames of these studies are probably too short to show a shift in the T-cell repertoire as suggested by cross-sectional studies. In summary, the CMV-specific T-cell repertoire seems to consist of persistent T-cell clones, at least for relatively short periods of time, while with age, shifts in dominance may very well occur, probably selected based on their TCR affinity.

1.2 The EBV-specific T-cell repertoire

Although both CMV and EBV are chronic herpesviruses, known for their latent presence in the body, they have their own dynamics when it comes to infection and reactivation. EBV has a more restricted cell tropism than CMV [161, 162] and is thought to reactivate less often [163, 164]. Although EBV-specific T-cell frequencies are also relatively high, T-cell expansions specific for EBV are less pronounced than for CMV [165, 166]. Whether these differences also lead to different T-cell repertoire diversities for these two viruses remains debated. The T-cell repertoire against the frequently studied HLA-A2 restricted EBV epitope GLCTLVAML (A2-GLC) tends to be broader than the repertoire recognizing the A2-NLV epitope of CMV [159]. However, some similarities are observed between the repertoires, as both are oligoclonal, highly skewed and containing public T-cell clones (Price 2005). Furthermore, like the CMV-specific T-cell repertoire, the EBV (A2-GLC)-specific T-cell repertoire is characterized by dominant usage of particular Vβ segments, in this case Vβ20-1 and Vβ29-1 (previously known as Vβ2 and Vβ4) [81, 159, 167].

Several studies have suggested that the EBV-specific T-cell repertoire is very stable. For example, the A2-GLC-specific T-cell repertoire recovered in melanoma patients after transient lymphocyte depletion showed a similar bias in Vβ-segment usage as the A2-GLC-specific T-cell repertoire in healthy donors, including the same dominant T-cell clonotypes [167]. This suggests that once the EBV-specific T-cell repertoire is established, its clonal composition remains rather stable. In line with this, in a patient undergoing primary EBV infection after renal transplantation, the repertoire against two HLA-B35 restricted epitopes was highly stable from 18 weeks after infection up to 3 years later [160]. However, it was suggested that the clones that were detected during the chronic phase were not the most dominant clones during the peak of the primary infection. Sequencing A2-GLC-specific T-cell clones early after primary infection (7 till 15 days) and 2 years later actually showed, based on Vβ usage, very different clonotypes dominating the primary and the memory response [80]. These data thereby suggest that the T-cell response against EBV changes during the first phase of the infection, to become stable in the chronic phase up to at least 3 years after infection.

In contrast to the stable Vβ usage observed in longitudinal studies, a cross-sectional study reported differences in Vβ-segment usage between the EBV-specific T-cell repertoire of young and older adults. Although both age groups had a polyclonal and diverse TCR-Vβ repertoire specific for EBV, in the older adults the frequency of TCRs from the Vβ9 family was higher, while in the younger adults, T-cell clones from the Vβ13-1 family dominated [156]. In summary, the EBV-specific T-cell repertoire shows a relatively stable usage of Vβ segments after the peak of the primary response.

1.3 The influenza virus-specific T-cell repertoire

Although influenza A virus (IAV) infection is an acute infection, re-infection may occur several times during life [168, 169]. The effect of age on the IAV-specific T-cell repertoire has been studied extensively, as there is an age-associated increase of complications due to IAV infection [170]. Although IAV is known for the high mutation rate of its surface proteins, the internal proteins are relatively stable, leading to restimulation of IAV-specific $CD8^+$ T cells. The best studied IAV-specific epitope is the HLA-A2 restricted GILGFVFTL epitope (A2-GILG), present in the M1 protein that is highly conserved between different IAV strains [171].

The A2-GILG-specific T-cell repertoire shows a more skewed distribution based on Vβ-usage compared to the CMV A2-NLV-specific and EBV A2-GLC-specific T-cell repertoires. This may in part be explained by the reduced accessibility of the GILG epitope when presented by the HLA-A2 molecule [172]. Up to 90% of the A2-GILG-specific T cells express the Vβ19 segment and a conserved arginine-serine motif, also referred to as the "RS-motif", in the CDR3 region of the responding T cells is often observed [173-175]. Because such a large fraction of TCRs express the Vβ19 segment, several T-cell repertoire studies have focused on the dynamics of the $Vβ19^+$ T-cell clones within the A2-GILG-specific T-cell repertoire. One study reported a decrease in diversity when comparing the A2-GILG $Vβ19^+$ T-cell repertoires of middle-aged and older adults using different diversity measures [176]. A comparable study, on the other hand, did not find a significant loss of GILG-specific T-cell diversity with age. Although TCR richness was lower in older adults, the overall diversity of the repertoire was not. It was suggested that the overall diversity (which takes into account both richness and evenness) of the responding repertoire did not decrease with age, because loss in richness was counterbalanced by increased evenness of the repertoire [177] (see box 1).

A longitudinal study among 3 healthy volunteers over a time span of 7-10 years showed that many characteristics of the A2-GILG $Vβ19^+$ T-cell repertoire were

very stable over 7-10 years [178]: identical clonotypes were found, the repertoire always showed the same level of skewing and diversity indices of the A2-GILG specific T-cell repertoire did not change significantly. Nevertheless, some shifts within the Vβ19^{+} T-cell clones were observed, as there was a loss of clonotypes with the RS-motif with age [178].

Also for the TCRα chain, dominance of certain V-segments (i.e. Vα12 and Vα27, often expressed in combination with the Vβ19 segment) has been observed in the IAV -specific T-cell repertoire [176, 179]. The A2-GILG-specific T-cell repertoires of older adults showed more dominant usage of the Vα27 segment and less dominant usage of the Vα12 segment than those of middle-aged adults. However, within the Vα27^{+} flu-specific T-cell repertoire, TCR richness decreased with age. Thus, also the TCRα usage of the flu-specific T-cell repertoire changes considerably over time.

Remarkably, studies focusing on the complete A2-GILG-specific T-cell repertoire and those focusing on the Vβ19 repertoire have reached different conclusions. Studies focusing only on the Vβ19 expressing GILG-specific T-cell repertoire seem to miss a large part of the responding repertoire, especially in older individuals. Although almost all T-cell receptors against A2-GILG in young adults expressed the Vβ19 segment, in older adults this was only the case for 55% of the responding TCRs. A similar decrease was observed for the expression of the conserved "RS-motif" [144]. Direct ex vivo paired analyses of the TCRα and β chains of the A2-GILG specific T-cell repertoire showed that older adults tended to have a reduced TCR diversity within the Vβ19^{+} clonotypes, while their more private/non-Vβ19^{+} T-cell receptors consisted of a broader usage of Vβ and Vα segments compared to young adults [144, 179]. Despite these differences in TCR usage, the *in vitro* proliferative capacity of the A2-GILG specific T-cell repertoires of young and old adults were comparable after peptide stimulation [144]. Thus, Vβ19^{+} T-cell clones recognizing IAV become less dominant in older adults, leading to a more diverse usage of other Vβ segments within the flu-specific T-cell repertoire.

Because the A2-GILG-specific T-cell repertoire has been analyzed in so much detail, the data also allow to study changes of other TCR characteristics, such as CDR3 length and amino acid usage of the TCR chains. In general, a characteristic of the A2-GILG-specific T-cell repertoire is the 11 amino acid length of the CDR3 loops. Interestingly, it was shown that this characteristic becomes less prominent with age, giving rise to a broader range of longer CDR3 loops [144, 176, 179]. Furthermore, in older adults, the frequencies of glycines and alanines in the CDR3 region of the A2-GILG-specific T-cell repertoire appeared to be higher than in

middle-aged persons. The usage of more alanines and glycines and longer CDR3 loops is thought to lead to a more flexible CDR3 region resulting in a more cross-reactive T-cell receptor [180, 181]. This would suggest that the A2-GILG-specific T-cell repertoire would be more cross-reactive in older adults, which could lead to better recognition of escape variants of the epitope [70], although these are not expected to occur very often, considering the stable nature of the epitope A2-GILG epitope.

In conclusion, Vβ19 skewing of the A2-GILG-specific T-cell repertoire is mainly present in young adults and seems to be less prominent in older adults, leading to a more diverse flu-specific T-cell repertoire with age. However, especially in the case of IAV, it is unknown whether the loss of dominant T-cell clones is due to aging of the immune system, or whether restimulation due to reinfection plays an important role in the formation of the T-cell repertoire. It is therefore also important to obtain insights into the effect of infection history on the composition of the antigen-specific T-cell repertoire.

PART 2: EFFECTS OF INFECTION HISTORY ON THE ANTIGEN-SPECIFIC T-CELL REPERTOIRE

It is generally thought that homologous infection can lead to selective expansion of antigen-specific T cells. How the antigen-specific repertoire is shaped after repetitive stimulation is not completely understood, but the TCR- affinity for the peptide-MHC complex is thought to play an important role. Next to homologous infections, also heterologous infections may influence the antigen-specific T-cell repertoire. The effect of such heterologous infections on the antigen-specific T-cell repertoire likely depends on the degree of antigenic similarity between infections, although it has also been described for infections with very minimal (Oberle et al. 2016) or even no overlap [182, 183]. When investigating the maintenance of the antigen-specific T-cell repertoire with age, it is therefore important to understand how its diversity is influenced by both homologous and heterologous infections.

2.1 Homologous virus infections

Restimulation of the immune system by the same virus can occur both for acute and chronic infections, by re-infection or reactivation respectively. It is widely accepted that during primary infection, T-cell clones expressing high-affinity TCRs tend to be selected to respond. The effect of repetitive antigen stimulation on the T-cell repertoire is however still debated, and in fact two opposing effects have been proposed (Fig. 2):

1. A decrease in diversity and increased skewing of the antigen-specific T-cell repertoire, as mainly dominant, high avidity clones in the repertoire may expand upon antigen re-encounter [184, 185].
2. An increase in diversity of the antigen-specific T-cell repertoire, due to overstimulation and subsequent loss of the most dominant high avidity clones, leading to an increased representation of clones that used to be subdominant. [177, 186, 187].

The first effect, selection of high avidity clones upon antigen reencounter, has been observed in an acute *Listeria monocytogenes* infection model in mice, where a more focused antigen-specific T-cell population emerged after a second challenge [188]. The new antigen-specific T-cell pool appeared to have a higher avidity for *the L. monocytogenes*-specific peptide and a more intense tetramer-staining compared to the T-cell population after primary infection [184]. A highly focused T-cell repertoire consisting of high-avidity T-cell clones has also been observed for the CMV-specific T-cell repertoire in humans. Again, the highly focused A2-NLV-specific T-cell repertoire was due to the presence of dominant clones with a relatively high TCR-affinity for the peptide [83]. In line with this, another study found that the most differentiated and expanded T-cell clones specific for CMV and EBV, were the clones with the highest avidity [86].

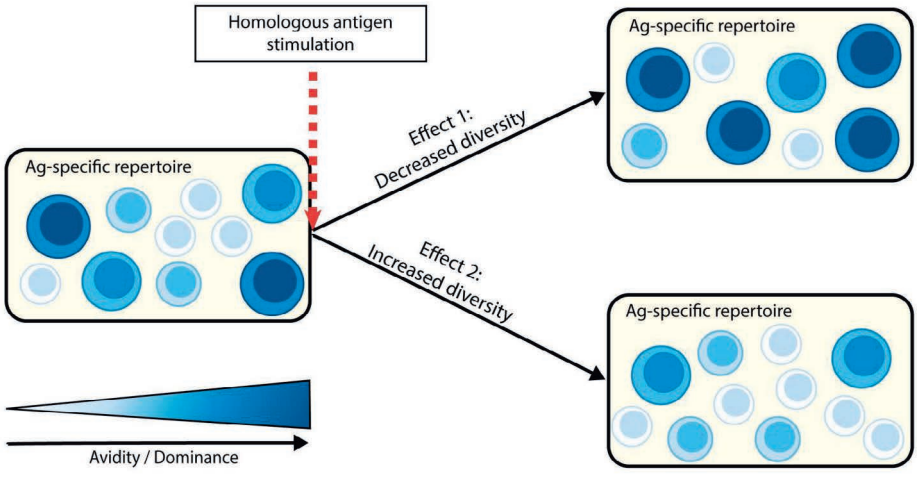


Figure 2: Possible effects of homologous antigen stimulation on the antigen-specific T-cell repertoire.
This figure shows two (quite opposing) effects that may impact the composition of the antigen-specific T-cell repertoire after homologous antigen stimulation. Size of circles indicates clonal size, darkness indicates avidity/dominance of the clones. Effect 1 refers to the selection of the most dominant, high avidity clones after stimulation, leading to a skewed antigen-specific T-cell repertoire, while effect 2 refers to the loss of the most dominant, high avidity clones, leading to a more diverse antigen-specific T-cell repertoire

In contrast, some other findings support the view that clonal selection of low-avidity clones through loss of high avidity clones (effect 2) occurs in both humans and mice [189] in the context of chronic CMV infection. In humans, a negative correlation was observed between the frequencies of TCRs recognizing the HLA-B8 QIKVRVDMV (B8-QIK) CMV-epitope and their affinity. In mice infected with an MCMV strain expressing the H2Kb-SIINFEKL epitope under an MCMV inflationary promotor (IE2), high-avidity T-cell clones had a more senescent phenotype. The authors proposed that selection of low-affinity TCRs is due to the chronic nature of the virus, which induces a senescent phenotype of the high avidity clones [189]. While they propose that in acute infections, clonal selection favors T-cell clones with high avidity. It remains unclear why even for a single pathogen such opposing results have been reported, and to what extent the use of different epitopes, and the frequency of viral reactivation could contribute to these differences.

The results of Schober *et al*. are in line with the basis of a modelling study, which proposed that repetitive stimulation due to virus reactivation could lead to loss of high avidity clones [186]. *Ex vivo* analyses showed that during primary EBV infection, high-affinity EBV-specific TCRs were selected, while 1 year later a decrease in affinity was observed. Clones that were less likely to expand during primary infection contributed more to the repertoire one year later [186]. To explain these observed effects, factors such as cellular senescence needed to be introduced in the model, suggesting that loss of previously dominant high avidity clones through senescence may play an important role in shaping the T-cell repertoire. A comparable result was described for the IAV Vβ19^{+} A2-GILG-specific T-cell repertoire, as *ex vivo* lower diversity was observed in older compared to middle aged individuals. Model analysis of these data showed that the parameters explaining the *ex vivo* results included a combination of senescence and selective expansion of clones [177], leading to a repertoire that progressively consists of clonotypes that used to be less dominant.

Analysis of the effects of viral reencounter in a more controlled way in mice has suggested that the antigen-specific T-cell repertoire remains relatively stable. A frequently used model in mice is repetitive infection with the (acute) lymphocyte choriomeningitis virus (LCMV) Armstrong strain. The T-cell repertoire against LCMV has a preferential usage of certain Vβ segments, including Vβ8.1 and Vβ8.2. The recall response consisted of large expansions of LCMV-specific T cells and a very similar skewing in Vβ usage was observed after primary and secondary infection. Based on these findings it was suggested that the LCMV-specific T-cell

repertoire remained relatively stable [190-192]. Epitope dominance seemed to play a considerable role in shaping the LCMV-specific response, as after secondary infection a profound shift in epitope immunodominance was observed [191]. This data suggests that in the case of LCMV infection after at least 1 recall challenge, no specific (high avidity) clone selection can yet be detected.

Also repetitive infection of mice with IAV generally shows that the levels of diversity of the responding T-cell repertoires after primary and secondary infection are comparable [193-195]. These studies included a more detailed analysis of the antigen-specific repertoire compared to studies on LCMV infection and therefore differences in clonal composition after primary and secondary IAV infection could be revealed. Some clones were found after both infections and others were only found after primary or secondary infection [193]. Transferring antigen-experienced influenza virus-specific (Db-NP366) Vβ13$^+$ T cells into mice, followed by IAV infection led to an increase of one particular clone with a relatively low start frequency. As a consequence, the frequency of one or two clonotypes that were dominant in the first mouse reduced [196]. This effect is somewhat comparable to what is observed for the A2-GILG specific repertoire in older adults, where a loss of clones consisting of the public "RS-motif" is observed within the dominantly used Vβ segment [176]. These data suggest that changes that tend to be described to aging in humans, may also be due to repetitive stimulation.

In conclusion, which affinity-based mechanism mostly occurs *in vivo* after homologous antigen stimulation remains unclear. The dominant mechanisms may be different per virus and epitope, as experimental evidence has been found for both effects even for the same virus. Apart from affinity based mechanisms, more recently it has been suggested that stochastic expansion of T cells may contribute to the maintenance of T-cell diversity in the memory pool. It was shown in mice that different MCMV-specific clonotypes underwent proliferation irrespective of their avidity, leading to a stable repertoire up to a year post infection. It was suggested that T-cell proliferation occurs in a stochastic manner, leading to the maintenance of both dominant and subdominant clonotypes, thereby contributing to a diverse antigen-specific repertoire [197].

2.2 Heterologous virus infections

The immune response induced by a previously encountered pathogen can even alter the immune response to new unrelated virus infections, a phenomenon also known as heterologous immunity [182]. In the light of heterologous immunity, infection with CMV has been of particular interest, because of the notoriously

large clonal expansions it induces. Percentages of CMV-specific T cells up to 40% of the total memory T-cell compartment have been reported [82, 198]. One of the first studies investigating the influence of CMV on the total $CD8^+$ T-cell repertoire, showed that the number of clonal expansions in CMV^+ individuals was more than 30% higher than in CMV^- donors [82]. Even though the specificity of these clonal expansions was not determined, the effect of CMV serostatus on the diversity of the memory T-cell pool was evident [82]. Later studies showed that the expanded clones in the memory T-cell pool of CMV^+ individuals were CMV-specific [95]. Therefore, it has been suggested that CMV infection may also influence T-cell repertoire diversity, especially within the memory T-cell population [150].

In the last decades, the theory that CMV-infection would have a negative influence on unrelated T cells, dominated the field. The large CMV-specific clonal expansions were thought to outcompete other T cells by competing for growth and survival factors [199-201]. This hypothesis was supported by the finding that a decline in T-cell repertoire richness with age is only observed in the memory T-cell population of CMV^+ individuals [66]. One study so far attempted to compare the diversity of the non-CMV-specific memory T-cell pools of 550 donors, either CMV^+ or CMV^-, by leaving out the most numerous 0.1% of the clones in the memory compartments from the analysis. These clones were removed as they were clearly larger in CMV^+ compared to CMV^- individuals, and for a few donors it was shown that these clones reacted to CMV-specific antigens. The diversity scores of the remaining "non-CMV-specific" memory T-cell pools of CMV^+ and CMV^- individuals turned out to be comparable [202]. This study suggests that the T-cell pool accommodates large CMV-specific T-cell clones by simply expanding, and that CMV-specific clonal expansions do not compromise the $CD8^+$ T-cell repertoire against other antigens.

To asses the impact of previous CMV infection on the diversity of the T-cell response to a new unrelated pathogen, the OVA-specific $V\beta12^+$ T-cell repertoire in response to Listeria infection expressing ovalbumin was compared between $MCMV^+$ and $MCMV^-$ mice. An unexpected positive effect was observed: the diversity of the OVA-specific T-cell repertoire in aged $MCMV^+$ mice turned out to be higher than in aged $MCMV^-$ mice. How this more diverse repertoire was established in the $MCMV^+$ mice remains unclear. Interestingly, sequencing of the naive T-cell repertoires of the mice showed comparable diversity, suggesting that similar numbers of precursor T cells were available [183]. The more diverse OVA-specific T-cell repertoire in $MCMV^+$ mice also led to a better IFNγ response to OVA peptide stimulation than in $MCMV^-$ mice. These findings are surprising

in light of earlier studies, which showed a significantly weaker response against heterologous infections, including LCMV and IAV, following MCMV-infection [139, 203, 204]. If anything, these data emphasize the complicated influence of CMV infection on the T-cell pool.

Heterologous immunity can also occur for virus infections that get cleared and nevertheless influence the antigen-specific T-cell repertoire to other pathogens. In LCMV infected mice, the LCMV-specific T-cell repertoire is typically strongly skewed towards expression of Vβ8.1, which is also observed after secondary LCMV-infection [191]. However, infection of mice with LCMV, followed by infection with pichinde virus (PV) and vaccinia virus (VV), and then followed by secondary LCMV infection, led to reduced frequencies of LCMV-specific T cells and to an altered usage of Vβ segments [182]. Effects of PV infection on LCMV infection have been observed more often, and it has been suggested that PV infection leads to a narrowed LCMV-specific repertoire (Selin 1994; Cornberg et al. 2010; Welsh et al. 2010). This heterologous immunity is probably due to the relatively high level of sequence similarity between the different virus-specific epitopes, causing some TCRs to recognize both epitopes, also known as cross-reactivity.

In humans, cross-reactivity has been observed for the EBV-specific A2-GLC and IAV-specific A2-GILG epitopes, epitopes which only have 3 amino acids in common, however most of the involved TCRs showed to have a low affinity for both peptides [71, 205, 206]. It has been suggested that cross-reactivity between antigens may narrow the responding T-cell repertoire, as the subset of T cells already present in the memory pool specific to one epitope can grow out in response to a new epitope, thereby limiting the recruitment of new naive T cells into the memory T-cell pool [181]. On the other hand, as has been proposed for homologous infections, one could argue that heterologous infections may lead to a decrease or an increase in the diversity of the antigen-specific repertoire. It was shown by computer simulation that the affinity of the TCR is probably an important factor determining whether cross-reactivity leads to broadening or narrowing of the antigen-specific T-cell repertoire. The more dissimilar the epitopes, as seen for A2-GLC and A2-GILG for example, the more likely broadening of the repertoire would occur, whereas if the epitopes are more similar, as seen for LCMV and PV, narrowing of the repertoire would be a more likely outcome [72].

In conclusion, for both homologous and heterologous infections an influence on the antigen-experienced T-cell repertoire can be expected. It remains unclear, however, whether a positive or negative effect will occur. Furthermore, whether the

effects observed in mice are comparable to those occurring in humans remains unknown. Differences can be due to the infection order. For example, mice are typically infected with MCMV long before they receive a heterologous infection, while humans gradually build up memory against other infections, making it much more challenging to study the effect of CMV in the human setting.

IMPLICATIONS FOR VACCINE STRATEGIES IN OLDER ADULTS

It is generally assumed that the diversity of the naive T-cell repertoire in elderly people is one of the limiting factors for the induction of T-cell responses to new antigens. Vaccinating at an earlier age during adulthood may lead to better responses at older age, but to protect the older adults it is essential that such T-cell clones are maintained over time. Therefore, in this review we investigated 1) the effect of age on the antigen-specific repertoire, 2) the impact of repetitive stimulation on the antigen-specific T-cell repertoire and 3) the influence of heterologous immunity, especially CMV-infection, on the T-cell repertoire against other antigens.

The antigen-specific repertoire dynamics in the memory pool described for CMV, EBV and IAV suggest that the repertoires against EBV and CMV are stable for at least a couple of years, while for IAV a decrease of the most dominant TCRs is observed with age. In addition to age, infection history seems to be a key player shaping the repertoire dynamics against these three viruses over time. Studying the maintenance of the T-cell repertoire against an infection we only encounter once could provide better insight into the longevity of the immune response. A frequently used model for an acute challenge in humans is yellow fever (YF) vaccination. This live-attenuated vaccination is thought to be one of the most effective vaccines currently available for the induction of T cells [207, 208], as even decades later YF-specific T cells can still be observed in the blood [209]. However, data on the dynamics of the T-cell repertoire against YF over a longer time period remain limited. In one donor, most of the clones identified after the booster vaccination were comparable to the clones observed after the primary infection 18 months earlier. In another donor who received the first vaccination 30 years earlier, it was shown that most YF-specific clonotypes were of low frequency or undetectable when sequencing the bulk memory T-cell pool. Shortly after booster vaccination, however, expansion of YF-specific T-cell clones was observed via sequence analysis, suggesting that YF-specific T cells remained present for 30 years [210].

Based on the available data, the effect of repetitive stimulation on the repertoire remains inconclusive, as both expansion and loss of dominant, high avidity clones has been observed. The effect of homologous restimulation is interesting in the light of booster vaccinations and vaccinations that are regularly given (e.g. seasonal flu vaccination), as these induce comparable repetitive T-cell antigen stimulation. This may be a vaccine strategy to overcome diminished responses at older age. However, it remains unknown when boosting results in the maintenance of a diverse repertoire or in the expansion of high affinity clones, and in fact it is not even known which of these outcomes would be favorable. Although the hypothesis of Schober *et al.* [189] that discrepancies between studies are due to the chronic or acute nature of the viruses is very attractive, such discrepancies are even observed between studies focusing on acute or chronic infections only.

Heterologous infections could also lead to a decrease or increase in the diversity of unrelated T-cell repertoires. Especially the effect of CMV-infection on the memory response to unrelated antigens should be studied in more detail in humans, as CMV occupies a large part of the memory T-cell pool. Given that CMV-specific T-cell responses are maintained at such high frequencies with age, CMV is regarded as a promising candidate to use as viral vaccine vector. The relative stability of the CMV-specific repertoire is an additional advantage for using CMV as a vector. Although the developments are still at an early stage, studies using CMV as viral vaccine vector against HIV, SIV and tuberculosis show broad and robust T-cell responses [211].

Other factors apart from aging and infection history may also play an important role in the dynamics and stability of antigen-specific T-cell repertoires, including the type of antigen, the processing and presentation of the antigen [212], the dose of the antigen [177] and the recruitment of T cells to the site of infection. The relative impact of these factors on the resulting T-cell repertoire still have to be determined. Nevertheless, alternative vaccination strategies linked to these factors have been proposed to improve the $CD8^+$ T cell-response in elderly, including the use of stronger adjuvants, higher antigen doses [25, 213, 214] or modified pMHC-interactions [100]. However, these strategies may in turn exert an effect on the responding T-cell repertoire. It was indeed shown in mice that the use of different adjuvants can alter the composition of the T-cell repertoire, due to changes in affinity thresholds for TCR selection [215].

Although previous studies provided insight in T-cell repertoire dynamics, several questions remain: How to link diversity measurements to T-cell efficacy? Why

do certain T-cell specificities survive while others disappear? And are certain TCRs needed to elicit a protective immune response? In line with these questions, there is a lot of interest for public T-cell clones and the preferred usage of certain Vβ segments, assuming that these provide an evolutionary selective advantage [57]. It has been proposed that certain Vβ segments provide a more favorable interaction between the TCR and the pMHC-complex [172, 216]. It remains unclear, however, to what extent the occurrence of public TCRs can be explained by their functional role in the recognition of certain epitopes, and to what extent they have a higher generation probability during VDJ recombination [88, 90, 92]. Finally, there is a need for long-term longitudinal studies, mostly to figure out basic questions like: How long is repertoire diversity to a new antigen maintained? How is it maintained after reexposure? And is it maintained in combination with other unrelated infections?

CONCLUSION

Knowledge on the dynamics of the antigen-specific T-cell repertoire could lead to important insights that can be used to optimize vaccine strategies to protect older adults. Although some challenging questions still need to be answered, important steps have been made in the past years. The first studies investigating how to identify an individual's infection history by dissecting the antigen-specific T-cell repertoire using antigen-specific TCR motifs are very promising [96, 97]. Such information could potentially lead to the design of a more personalized vaccination program, using alternative vaccination strategies including earlier vaccination to optimize T-cell responses at older age.

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Age and CMV-infection jointly affect the EBV-specific CD8⁺ T-cell repertoire

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ABSTRACT

CD8⁺ T cells play an important role in protection against viral infections. With age, changes in the T-cell pool occur, leading to diminished responses against both new and recurring infections in older adults. This is thought to be due to a decrease in both T-cell numbers and T-cell receptor (TCR) diversity. Latent infection with cytomegalovirus (CMV) is assumed to contribute to this age-associated decline of the immune system. The observation that the level of TCR diversity in the total memory T-cell pool stays relatively stable during aging is remarkable in light of the constant input of new antigen-specific memory T cells. What happens with the diversity of the individual antigen-specific T-cell repertoires in the memory pool remains largely unknown.

Here we studied the effect of aging on the phenotype and repertoire diversity of CMV-specific and Epstein-Barr (EBV)-specific CD8⁺ T cells, as well as the separate effects of aging and CMV-infection on the EBV-specific T-cell repertoire. Antigen-specific T cells against both persistent viruses showed an age-related increase in the expression of markers associated with a more differentiated phenotype, including KLRG-1, an increase in the fraction of terminally differentiated T cells, and a decrease in the diversity of the T-cell repertoire. Not only age, but also CMV infection was associated with a decreased diversity of the EBV-specific T-cell repertoire. This suggests that both CMV infection and age can impact the T-cell repertoire against other antigens.

INTRODUCTION

CD8⁺ T cells play an important role in the control and clearance of viral infections. One of the key components of a protective T-cell response is the recognition of viral epitopes via the T-cell receptor (TCR). T-cell receptors are formed via the random process of somatic V(D)J-recombination, leading to a large collection of TCRs with different specificities [59]. It is generally assumed that the diversity of the T-cell receptor repertoire is positively correlated with the level of protection against infectious diseases [53]. The diversity of the total CD8⁺ T-cell repertoire decreases with age [133-135], which is mainly caused by a decrease in naive T-cell numbers [133] as well as a decreased TCR diversity within the naive T-cell pool [66, 136]. Together with less efficient priming of T cells [36], this may explain why both CD8⁺ T-cell protection against viral infections and vaccine efficacy decrease with age [19, 39].

Although the level of TCR diversity within the *total* CD8⁺ T-cell memory pool seems to remain stable with age [66], the composition of the memory T-cell pool keeps changing at the antigen-specific level. Exposure to new antigens during life leads to recruitment of new T-cell specificities into the memory pool, and existing memory T-cell clones may expand or contract. The diversity of T cells that are already present in the memory T-cell pool may be affected by the arrival of new memory T-cell specificities, due to competition for T-cell growth and survival factors. The relative stability of the diversity of the *total* CD8⁺ T-cell memory pool therefore does not imply that *individual* antigen-specific T-cell repertoires are stably maintained with age. To study such changes in the memory T-cell pool, we investigated how the diversity of the antigen-specific TCR repertoires against cytomegalovirus (CMV) and Epstein Barr virus (EBV) changes with age.

Both CMV and EBV cannot be cleared from the body and repeatedly challenge the immune system, leading to high, and therefore easily detectable, frequencies of antigen-specific CD8⁺ T cells over all ages in the majority of individuals [165, 166]. Previous longitudinal studies focusing on the effect of aging on the antigen-specific repertoire have suggested that the T-cell repertoires against EBV and CMV remain relatively stable, at least during the first few years after primary infection, as the same T-cell clones were identified at different timepoints [80, 158-160]. Consistent with this, several cross-sectional studies into the CMV and EBV-specific T-cell repertoires reported similar Vβ-skewing in young and older adults, and even identical TCR sequences between individuals of different age groups [82, 107, 156]. Although these studies have led to the view that antigen-

specific T-cell repertoires are rather stable with age, it remains unknown, if the diversity of antigen-specific T-cell repertoires is maintained [217].

The cellular immune response against CMV is even more pronounced than the response against EBV, and can reach up to 40% of the $CD8^+$ T-cell pool in the blood [82, 198]. Furthermore, CMV-infection leads to changes in the $CD8^+$ T-cell pool similar to those observed with aging, including the presence of large fractions of terminally differentiated cells [218, 219] and a more skewed and less diverse TCR repertoire [82, 132]. It has been suggested that the large numbers of CMV-specific T cells can compete with non-CMV-specific T cells [199-201], leading to memory attrition [146]. Studies on the effect of CMV-infection on non-CMV-specific T cells are not unambiguous, however. Several studies have shown that mice infected with murine CMV (MCMV) have impaired responses to heterologous infections [139, 203, 204, 220]. In contrast, another study observed a positive effect of MCMV infection on the diversity of the T-cell repertoire specific for a heterologous infection [183]. Studies in humans showed similarly contradicting results: while one study showed that CMV^+ individuals had lower absolute numbers of EBV-specific T cells than CMV^- individuals [165], another study found that the diversity of the non-CMV-specific memory T-cell repertoire was comparable in CMV^- and CMV^+ individuals, thereby suggesting that the memory T-cell pool simply expands to accommodate the large frequencies of CMV-specific T cells [202]. Thus, the effect of CMV infection on non-CMV-specific T-cell responses and their repertoire diversity, and how this is linked to aging, remains poorly understood [217].

To gain insight into the maintenance of the repertoire of antigen-specific T cells, we studied the effect of aging on the phenotype and TCR repertoire composition of T cells specific for two immune-dominant CMV and EBV epitopes. In addition, we investigated how the EBV-specific T-cell repertoire is influenced by CMV-infection and how this is linked to aging. We observed that the richness of the CMV-specific and EBV-specific T-cell repertoire declines with age, independent of CMV serostatus. CMV infection led to a further decrease in diversity of the EBV-specific T-cell repertoire. This suggests that CMV infection and age both play an important role in the diversity of the antigen-specific T-cell repertoires.

METHODS & MATERIALS

Study design

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 a cohort of unvaccinated controls or pre-vaccination participants, were used from a study carried out in 2009-2011 (the Pandemic influenza vaccination trial, Netherlands Trial Register NL1952) [221]. The study was approved by the Central Committee on Research Involving Human Subjects of the Netherlands. Samples of older adults (n=57), ≥60 years of age, were control samples from a study carried out in 2014-2015 (Influenza-like-illness-3, NL4666) (Kaaijk et al 2020 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. See Sup. Fig. 1 for a flowchart of the selection criteria of the donors used for the analysis.

Cytomegalovirus (CMV)-specific and Epstein Barr (EBV)-specific antibodies

For healthy young adults, CMV-specific antibody levels were measured using a commercial ELISA kit (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^-. None of the participants included in this study scored between the 8 and 12 U/ml range.

For older healthy adults, CMV-specific antibody levels and EBV-specific antibody levels were simultaneously measured in serum by an in-house-developed multiplex immunoassay [222]. Individuals with a CMV-specific antibody level of ≤4 RU (relative units)/ml were considered to be CMV^- and individuals with an antibody level >7.5 RU/ml were considered CMV^+. None of the participants included in this study had a CMV-specific antibody level between 4 and 7.5 RU/ml. Individuals were considered EBV^- with an antibody level of ≤ 16 RU/ml, whereas those with an antibody level of >30 RU/ml were considered EBV^+. None of the older participants included in this study had an EBV-specific antibody level between 16 and 30 RU/ml. Note that the EBV-status of the younger individuals remained unknown, therefore only individuals with high $EBV^{A2\text{-}GLC}$-specific T-cell frequencies were used in our analysis.

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.

Antigen-specific T cells by flow cytometry

HLA-A2 positive healthy individuals were selected for subsequent EBV-specific and CMV-specific T cell analysis, by staining PBMCs for expression of HLA-A2 with the HLA-A2(BB7.2)-V450 antibody (BD Bioscience). From the HLA-A2 positive individuals, ±4 million PBMCs were stained using the HLA-class I dextramer containing the GLCTLVAML epitope of the BMLF1 protein of EBV (A*0201/GLCTLVAML-APC, Immudex) or the NLVPMVATV epitope of the pp65 protein of CMV (A*0201/NLVPMVATV-APC, Immudex), for 20 minutes at room temperature to assess their virus-specific T-cell frequencies.

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) [223] on 30 randomly selected dextramer-positive $CD8^+$ T cells per sample and labeled with epitope-specificity, age and CMV-serostatus. The tSNE clustering was performed on all these data combined (including both antigen-specificities). Perplexity of the clustering was set at 100. Cofactors for ArcSinH transformation were calculated using the flowVS package for (https://www.bioconductor.org/packages/release/bioc/html/flowVS.html). Both packages were slightly adapted to allow for FlowCytometric data analysis and integrated in an in-house developed pipeline.

Isolation of antigen-specific T cells for T-cell receptor analysis

$CD8^+$ T cells were isolated from PBMCs using a negative selection microbeads kit (Miltenyi Biotec) according to the manufacturer's protocol. Next, $CD8^+$ T cells were labelled at room temperature for 20 minutes with the A*0201/GLCTLVAML-APC dextramer and with the A*0201/NLVPMVATV-APC dextramer for CMV^+ individuals. Subsequently surface staining was performed using the following mAbs: CD3(UCHT1)-PerCP (Biolegend), CD4(OKT4)-BV510 (Biolegend) and CD8(RPA-

T8)-FITC (Biolegend). $CD3^+CD4^-CD8^+dextramer^+$ cells were then sorted by FACS Melody (BD) directly into RNAlater (Ambion Inc. Applied Biosystems) and stored at -80 °C for subsequent TCRβ clonotype analysis.

Preparing TCRβ cDNA libraries for sequencing

TCRβ analysis was performed as described previously [128], with minor modifications. Briefly, mRNA was isolated with the RNA microkit (Qiagen) according to the manufacturer's protocol. Isolated mRNA was used for cDNA synthesis with 5'RACE template switch technology to introduce a universal primer binding site, and unique molecular identifiers (UMIs) were added at the 5' end of the cDNA molecules using the SMARTScribe Reverse Transcriptase (TaKaRa). cDNA synthesis was followed by an AMPure XP bead-based clean-up (Beckman Coulter). Purified cDNA molecules were amplified in two subsequent PCR steps (25 cycles in PCR1 and 20 cycles in PCR2) using the Q5® High-Fidelity DNA Polymerase (New England BioLabs), with an AMPure XP bead-based clean-up in between. PCR products were size-selected on gel and purified using the Nucleospin PCR clean-up kit (Machery-Nagel). The PCR products were sequenced via Illumina MiSeq paired end 2x250 nucleotide (nt) sequencing.

TCRβ clonotype analysis

Raw sequencing data were processed using the 12nt UMIs to correct for amplification biases and error-correction of reads. RTCR [224] was used to identify both the UMI sequence and clonotype information from the reads. Because of the relatively small number of cells per sample, additional filtering steps were followed to minimize cross-sample contamination and biases introduced by errors in the UMI sequence. Sequences were only accepted if their UMI was observed in at least 40 sequencing reads. Sequences with identical UMIs in multiple samples were removed if they did not occur in at least 1000 sequencing reads or if their absolute frequency was lower than 10% of the maximum frequency in the other samples. UMIs were clustered within each sample if they were within a Hamming distance of 3. More detailed information about the processing and filtering of reads is provided in the Supplement.

Clonotypes were defined by their CDR3 amino acid sequence and V and J segment. Our sequencing reads do not always allow to distinguish between very similar V-segments, e.g. TRBV12-3 and TRBV12-4, which are annotated as V12-3/4 in the figures and supplementary tables.

For measuring diversity, the richness (defined as number of distinct clonotypes) of each sample was determined using normalized sample sizes (i.e., by iteratively sampling, without replacement, a given number of UMIs from the full set of UMIs that were identified in the sample). This approach accounts for the fact that the number of RNA molecules sampled may differ between cells. Diversity was calculated using the previously described Simpson's diversity index [225]. This index ranges between 0 and 1, with 0 representing minimal diversity and 1 representing maximal diversity. Sequence generation probabilities (CDR3$^+$ V and J segments) were calculated using the default recombination model of OLGA [226]. Known antigen specificity of sequences was assessed using the VDJdb (Shugay et al., 2017; retrieved on 29 October 2020). Sequences from CMV$^{A2\text{-}NLV}$- and EBV$^{A2\text{-}GLC}$-specific samples were counted as a match if their V gene + CDR3 amino acid sequence + J gene was listed as a human TCRβ sequence specific for the NLVPMVATV or GLCTLVAML epitope, respectively.

Statistical analysis of Flow cytometry data

Differences between the groups (for example CMV$^-$ compared to CMV$^+$) were assessed using Mann-Whitney U tests. 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 the study population

Healthy HLA-A2 positive individuals were on average 57.8±19.0 years old (n=91, range 21-82 years) and 57.1% of these individuals were CMV$^+$. Samples were obtained from two different cohorts, one containing young adults (21-52 years old) (n=34), and one containing older adults (≥60 years old) (n=57), of whom respectively 55.9% and 57.9% were CMV$^+$ (Table 1). No significant differences in age or sex were observed between CMV$^-$ and CMV$^+$ individuals. Sup. Fig. 1 gives a flowchart of the selection criteria of the donors used for the analysis.

Changes in the phenotype of antigen-specific CD8$^+$ T cells with age

To study the association between age and the antigen-specific CD8$^+$ T-cell frequencies against EBV and CMV, we performed a dextramer-staining with one immuno-dominant epitope of CMV (NLVPMVATV) derived from the pp65 protein and one immune-dominant epitope of EBV (GLCTLVAML) derived from the BMLF-1 protein, both presented on the HLA-A2 molecule.

Table 1

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=57)	**CMV$^-$ (n=24)**	**CMV$^+$ (n=33)**	**Statistics**
Age (mean ± SD)	71.2±6.4	70.8±6.6	71.5±6.4	ns
Sex (% women)	43.9%	37.5%	48.5%	ns
CMV-serostatus (CMV$^+$)	57.9%	.	.	.

Table 1: Characteristics of the study population. *Overview of the sex and CMV-serostatus distribution of the young adults (Rosendahl Huber et a. 2018) and older individuals (Kaaijk et al. submitted). Differences in percentage of CMV serostatus between groups was tested with Chi-squared test (p=ns for all age groups). Differences in age between groups was tested with unpaired T-test.*

We investigated the T-cell frequency against these two epitopes at different ages. The frequencies of CMV$^{A2\text{-}NLV}$-specific T cells tended to be slightly higher than the frequencies of EBV$^{A2\text{-}GLC}$-specific T cells (median of 0.220% versus median of 0.120%; P=0.0990 (ns)) (Fig. 1A). We observed less inter-individual heterogeneity in the CMV$^{A2\text{-}NLV}$-specific T-cell frequencies of younger compared to older adults; in the older adults, in whom these frequencies ranged from very low (from 0.01%) to very high (up to 17%) (Fig. 1B, left panel). EBV$^{A2\text{-}GLC}$-specific T cell frequencies were not significantly associated with the age of the individuals (Fig. 1B, right panel).

To assess the association between age and the phenotype of CMV$^{A2\text{-}NLV}$ and EBV$^{A2\text{-}GLC}$-specific T cells, we performed a cluster analysis (tSNE) based on the expression of the memory T-cell markers CD27, CCR7, CD95, CD45RO and CXCR3, on CD57 and KLRG-1, which are associated with a more differentiated phenotype and on the inhibitory receptor PD-1, and CD5, which plays a role in TCR signaling [227]. For the tSNE analysis the same amount of antigen-specific T cells (i.e. 30) per sample was used.

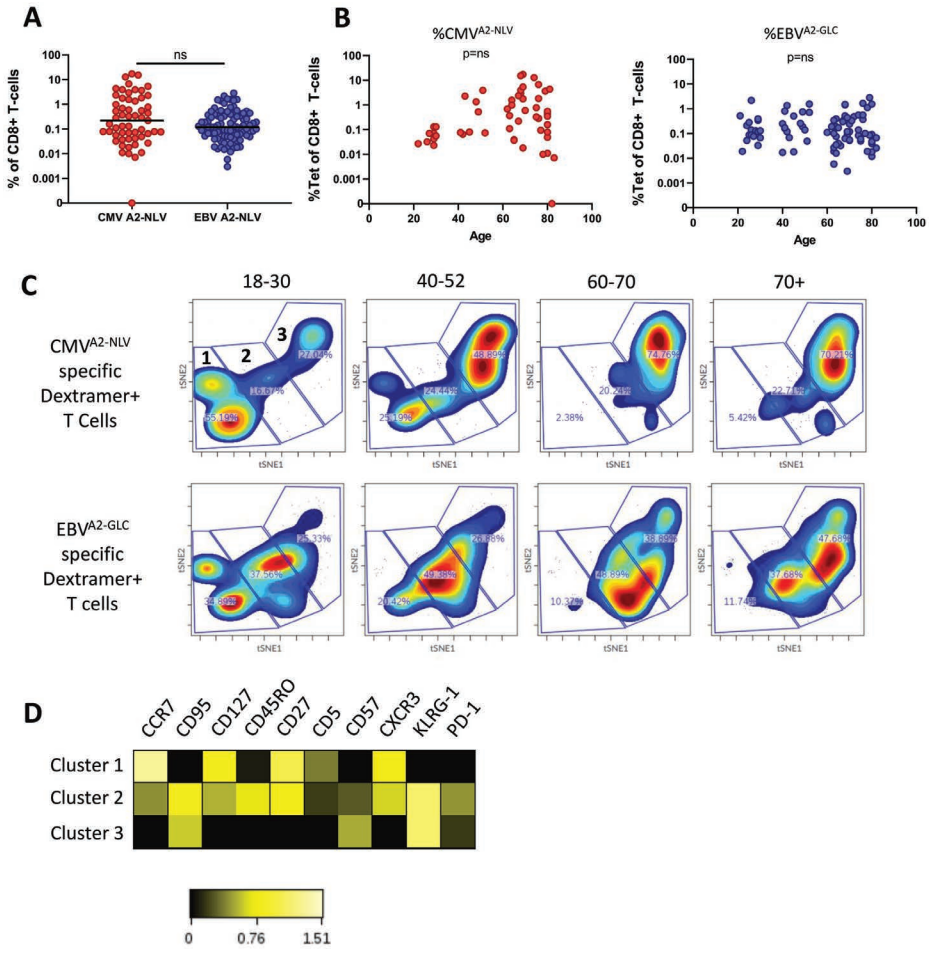

Figure 1: Changes in the phenotype of antigen-specific CD8⁺ T cells with age.
A) Percentage of CMV^{A2-NLV}-specific CD8⁺ T cells (red, n = 56) and EBV^{A2-GLC}-specific CD8⁺ T cells (blue, n = 99). Horizontal lines represent group median. B) Percentage of CMV^{A2-NLV} (left) and EBV^{A2-GLC}-specific (right) CD8⁺ T cells as a function of age. C) t-SNE analysis of CMV^{A2-NLV}-specificand EBV^{A2-GLC}-specific CD8⁺ T cells of donors (n = 47 and n = 77, respectively) categorized in four age groups. Clustering is based on MFI of CD5, PD-1, CD57, KLRG-1, CXCR3, CCR7, CD45RO, CD95, CD27, and CD127 of both epitopes. From each sample 30 cells were used. Three large clusters were manually identified. D) Heatmap of expression of markers of the three t-SNE clusters. Clustering of t-SNE based on both CMV^{A2-NLV} and EBV^{A2-GLC}-specific CD8⁺ T cells. Heatmap was based on the Log10 ratio of the median expression of the markers, normalized per marker to its column's minimum. Difference between epitopes was compared by Mann Whitney U-test. Correlations were tested with Spearman's rank correlation coefficient, ns stands for a non-significant p-value.

The very same clustering was applied on the samples in the four age groups and per epitope-specificity. We observed clear differences in the subset distribution between these groups for both CMV$^{A2\text{-}NLV}$ and EBV$^{A2\text{-}GLC}$-specific T cells (Fig. 1C). We identified 3 large clusters (1 to 3, Fig. 1C, upper left panel), in which cluster 1 contains Central memory type markers CCR7^{high}, CD27^{high}, KLRG-1^{low}, CD57^{low} cells, while cluster 3 contains the more differentiated cells, expressing CCR7^{low}, CD27^{low}, and KLRG-1^{high}. Cluster 2 forms an intermediate cluster based on the expression of these markers (Fig. 1D). Despite relatively large inter-individual variation (Sup. Fig. 2A), our data suggest a shift from cluster 1 to cluster 3 for both CMV$^{A2\text{-}NLV}$ and EBV$^{A2\text{-}GLC}$-specific T cells with age (Fig. 1C + Sup. Fig. 2A). This shift in clusters occurs earlier and becomes more pronounced with age for CMV$^{A2\text{-}NLV}$-specific T cells than for EBV$^{A2\text{-}GLC}$-specific T cells.

Phenotypic changes of CMV-specific and EBV-specific T cells are differently associated with age

We next explored how these phenotypic changes associated with age by quantifying the expression of various markers in the individual samples of CMV$^{A2\text{-}NLV}$-specific and EBV$^{A2\text{-}GLC}$-specific T cells. This allowed us to use the expression data of all the dextramer^{+} T cells. The composition of the memory population for the different ages based on conventional gating supported our observations of the cluster analysis. The memory subsets were defined based on the expression of CD27 and CD45RO in which the CD27^{-}CD45RO^{-} subset is referred to as Temra cells. We found a positive association between age and the percentage of Temra cells for both CMV$^{A2\text{-}NLV}$-specific T cells (p=0.0032, r=0.4167, slope of 0.75%/year (p=0.0006)) and, albeit to a lesser extent, EBV$^{A2\text{-}GLC}$-specific T cells (p=0.0010, r=0.0.3796, slope of 0.25%/year (p=0.1045)) (Fig. 2A). The fraction of Temra cells correlated positively with the frequency of CMV$^{A2\text{-}NLV}$-specific T cells, but not with the percentage of EBV$^{A2\text{-}GLC}$-specific T cells (Sup. Fig. 3A). For EBV$^{A2\text{-}GLC}$-specific T cells, the proportion of effector memory cells (Tem, CD27^{-}CD45RO^{+}) increased significantly with age (p<0.0001, r=0.5001) (Sup. Fig. 3B, right panel). The cluster analysis based on MFI from Fig. 1 also showed a gradual decrease in CCR7 expression with age, which was confirmed by plotting the geometric mean of the fluorescence intensity (gMFI) of CCR7 on a continuous scale for both CMV$^{A2\text{-}NLV}$-specific (p<0.0001, r=0.0.4411) and EBV$^{A2\text{-}GLC}$-specific T cells (p=0.0020, r=0.3604) (Fig. 2B).

Next, we analyzed the expression of markers associated with a more differentiated phenotype and exhaustion-associated markers on the antigen-specific T cells more closely. Both CMV$^{A2\text{-}NLV}$- and EBV$^{A2\text{-}GLC}$-specific T cells are associated with high expression of the inhibitory marker KLRG-1.

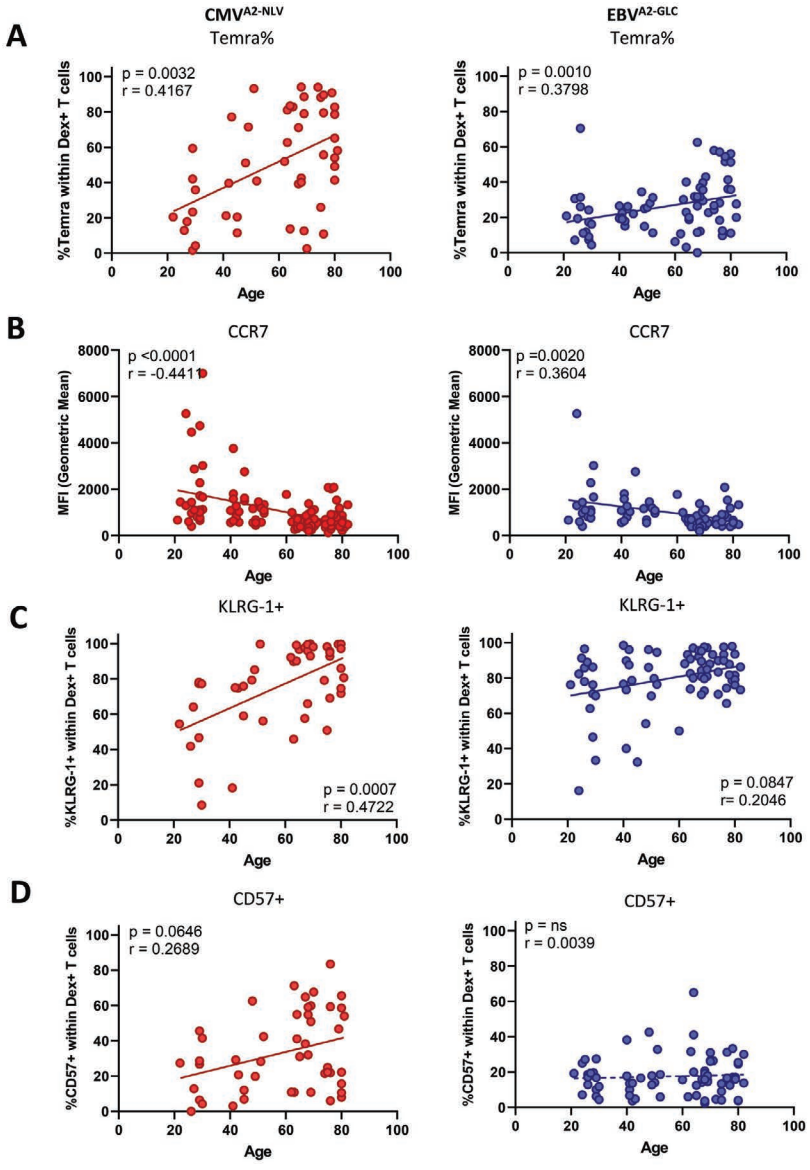

Figure 2: The phenotype of CMV-specific and EBV-specific T cells is affected differently by age.

A–D) Phenotype analysis of both CMV$^{A2\text{-}NLV}$*-specific (red) and* EBV$^{A2\text{-}GLC}$*-specific CD8$^+$ T cells (blue) plotted against age. A) Percentage of Temra cells (CD27–, CD45RO–). B) Geometric Mean of the fluorescent intensity of CCR7. Percentage of KLRG-1$^+$ (C) and CD57$^+$ (D) within Dextramer$^+$ CD8$^+$ T cells. Solid lines indicate a slope significantly ($p < 0.05$) different from a slope of 0, whereas a dotted line indicates no significant difference. Only donors with a sufficient T-cell response (at least 25 cells) were used for the phenotypical staining. Correlations were tested with Spearman's rank correlation coefficient.*

CD57 is a senescence marker known to be specifically highly expressed by CMV-specific T cells, compared to T cells against other chronic viruses [151, 228]. We indeed found high percentages of KLRG-1^{+} cells for both CMV$^{A2\text{-}NLV}$-specific (mean of 76.6%) and EBV$^{A2\text{-}GLC}$-specific T cells (mean of 79.8%), and the percentage of KLRG-1^{+} cells was positively associated with age, both CMV$^{A2\text{-}NLV}$-specific ($p=0.0007$, $r=0.4722$) and EBV$^{A2\text{-}GLC}$-specific T cells ($p=0.0847$, $r= 0.2046$) (Fig. 2C). The percentage of CD57^{+} T cells showed a positive trend with age for CMV$^{A2\text{-}NLV}$-specific T cells ($p=0.0645$, $r=0.2689$), while it was low across all ages for EBV$^{A2\text{-}GLC}$-specific T cells (Fig. 2D). We observed a significant positive association between the frequency of CMV$^{A2\text{-}NLV}$-specific T cells and the percentage of KLRG-1^{+}, CD57^{+} cells in the CMV$^{A2\text{-}NLV}$-specific T cell population (Sup. Fig. 3A, upper panels). The frequency of EBV$^{A2\text{-}GLC}$-specific T cells, in contrast, was only positively associated with the percentage of KLRG-1^{+} EBV$^{A2\text{-}GLC}$-specific T cells (Sup. Fig. 3A, lower panels), and not with the percentage of CD57^{+} EBV$^{A2\text{-}GLC}$-specific T cells.

We also investigated the percentage of CMV$^{A2\text{-}NLV}$-specific and EBV$^{A2\text{-}GLC}$-specific T cells expressing PD-1, which in the context of chronic (active) infection is often used as an exhaustion marker [229]. We found no significant correlation between the percentage of PD-1^{+} cells in the antigen-specific T-cell pool and age, both for EBV-specific and for CMV-specific T cells (Sup. Fig. 3C).

The CMV$^{A2\text{-}NLV}$-specific repertoire is less diverse than the EBV$^{A2\text{-}GLC}$-specific repertoire

We then investigated the TCR repertoire of CMV$^{A2\text{-}NLV}$-specific and EBV$^{A2\text{-}GLC}$-specific T cells in our samples by sequencing the TCRβ-chain. We used unique molecular identifiers (UMIs) to correct for sequencing errors and unequal PCR amplification, and performed an additional filtering procedure to exclude sequences that were likely due to contamination between samples or mutation in the UMI sequence (see Supplement for more details). We proceeded with the samples in which at least 10 UMI-TCR pairs remained after filtering. The distribution of the TCRβ sequences per individual are shown in Fig. 3 (see Sup. Fig. 4 for samples with less than 10 UMI-TCR pairs, all identified TCR sequences are provided in Sup. Table 1 and Sup. Table 2A+B), with colors indicating the TCRβ sequences that are shared between individuals.

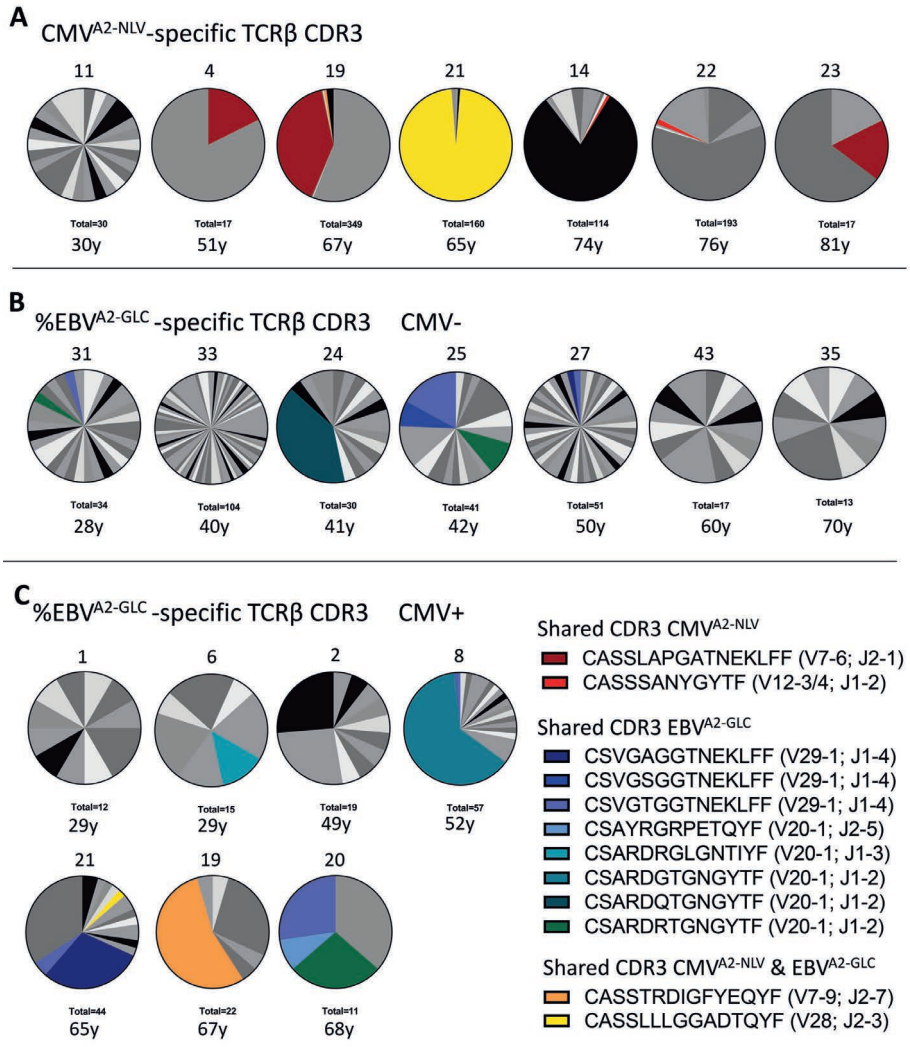

Figure 3: Characterization of the antigen-specific TCRβ repertoire.
(A) Distribution of TCRβ sequences in samples of CMV$^{A2\text{-}NLV}$-specific CD8$^+$ T cells (n = 7). (B,C) T-cell repertoire of EBV$^{A2\text{-}GLC}$-specific CD8$^+$ T cells of both CMV- (B) and CMV$^+$ individuals (C). Each pie depicts the repertoire of a different sample, with its Donor ID on top and the total number of UMI-TCR pairs identified at the bottom, as well as the individual's age. Colors represent shared TCRβ sequences between donors. Gray scales depict unique TCRβ sequences. Note two shared sequences between the CMV$^{A2\text{-}NLV}$ and EBV$^{A2\text{-}GLC}$ sample of two single individuals (yellow and orange). As this sharing was limited to these single donors and involved a very abundant TCRβ in either one of the samples, we expect that this overlap occurred during the sorting of the cells, probably due to unspecific binding of the dextramer.

We observed two different TCR sequences that were shared between individuals in the CMV$^{A2\text{-}NLV}$-specific repertoire samples, and eight in the EBV$^{A2\text{-}GLC}$-specific repertoire samples (Sup. Fig. 5A+B). A substantial fraction of the observed TCR sequences were also present in the VDJ database (VDJdb) of reported antigen-specific TCR sequences [230] (Sup. Fig. 5C). We found that the sequences that were shared between individuals within our study or between an individual of our study and the VDJdb had an over 16-fold higher generation probability than those that were not shared (Sup. Fig. 5D). This supports the idea that the likelihood of TCR generation plays an important role in the presence, abundance and sharing of antigen-specific TCR sequences [88, 90, 92].

To investigate the diversity of the antigen-specific T-cell repertoire, we used several measures of TCR diversity. The absolute richness, i.e. the total number of distinct clonotypes observed in a sample, was significantly higher for the EBV$^{A2\text{-}GLC}$-specific T cells than for the CMV$^{A2\text{-}NLV}$-specific T cells ($p=0.0289$) (Fig. 4A), even though the frequency of EBV$^{A2\text{-}GLC}$-specific T cells was lower than that of CMV$^{A2\text{-}NLV}$-specific T cells. However, the absolute richness is largely influenced by the total number of UMI-TCR pairs in a sample, i.e., the number of cDNA molecules that were sequenced. To overcome this potential bias, we therefore also used several alternative measures of TCR diversity. First, we calculated the unique clonotype ratio, by dividing the number of unique clonotypes by the total number of TCR sequences in each sample. Next, we calculated a normalized richness by counting the number of distinct TCR sequences in equally sized subsamples of the actual samples (by taking the mean richness of 10 randomly chosen subsamples). We also calculated the Simpson's diversity index as a sample size-independent measure of the TCR diversity in each sample [225]. While richness quantifies the variety of different TCRs, Simpson's diversity index quantifies the evenness of the frequency distribution across the TCRs. Even after excluding differential sample sizes as a confounding factor, we found a higher TCR diversity in the EBV$^{A2\text{-}GLC}$-specific compared to the CMV$^{A2\text{-}NLV}$-specific T-cell repertoire (Fig. 4B). We repeated these analyses on the subset of samples that contained at least 20 UMI-TCR pairs, which did not change the results qualitatively.

To understand what explains the higher diversity in some of the samples, we investigated the correlation between T-cell frequencies and the diversity of the repertoire for the CMV$^{A2\text{-}NLV}$-specific and EBV$^{A2\text{-}GLC}$-specific T-cell data combined. We found a negative correlation between all diversity measures and the frequency of antigen-specific T cells (Fig. 4C+D). This suggests that individuals with high

frequencies of antigen-specific T cells had large clonal expansions, leading to a decrease in TCR repertoire diversity.

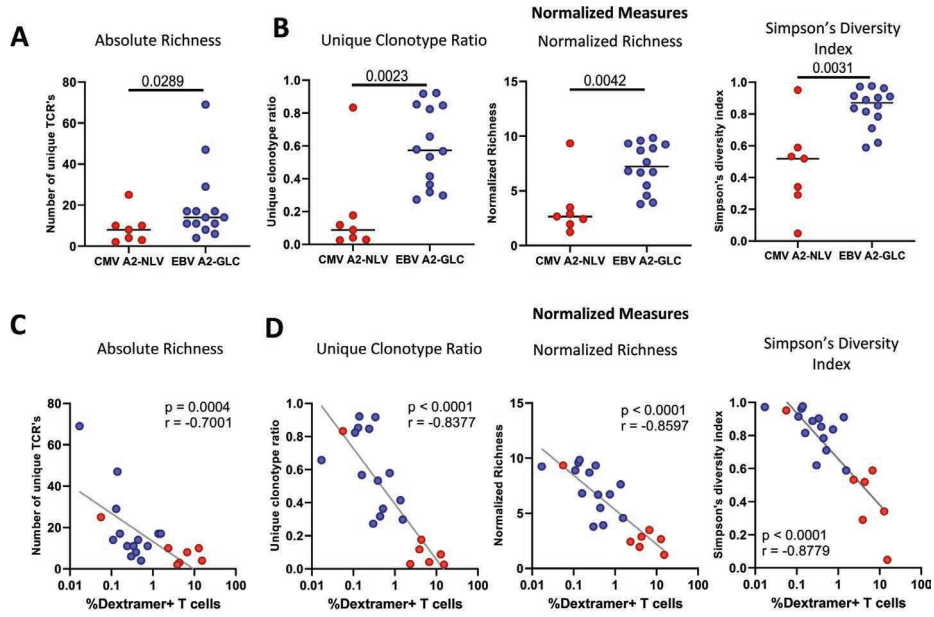

Figure 4: The CMV$^{A2\text{-}NLV}$-specific TCRβ repertoire is less diverse than the and EBV$^{A2\text{-}GLC}$-specific TCRβ repertoire.
Repertoire diversity calculations of the CMV$^{A2\text{-}NLV}$ and EBV$^{A2\text{-}GLC}$-specific CD8^{+} T-cell repertoire. A) Richness calculations based on the number of different TCRβ sequences. B) Measures normalized for differential sample sizes: Unique Clonotype Ratio (left), normalized richness (middle), and Simpson's Diversity Index (right panel). C,D) Correlation plot of the repertoire richness (C) and normalized diversity (D) and the frequency of the CMV$^{A2\text{-}NLV}$-specific (red) and EBV$^{A2\text{-}GLC}$-specific (blue) T cells. Horizontal lines in (A+B) show group medians. The unique clonotype ratio was calculated by dividing the number of unique clonotypes by the total number of TCR sequences. Normalized richness was calculated by counting the number of distinct TCR sequences in subsamples of 10 sequences. The mean of 1,000 subsampling iterations is shown. Differences between epitopes were compared by Mann Whitney. Correlations were tested with Spearman's rank correlation coefficient

Both age and CMV-infection are associated with a lower diversity of the EBV$^{A2\text{-}GLC}$-specific T-cell repertoire

To investigate whether age is associated with the diversity of the antigen-specific T-cell repertoire, we analyzed the normalized richness and the Simpson's diversity index of the repertoire of both CMV$^{A2\text{-}NLV}$-specific and EBV$^{A2\text{-}GLC}$-specific T cells in relation to the age of the individuals. The normalized richness of the antigen-specific T-cell repertoires against both CMV$^{A2\text{-}NLV}$ and EBV$^{A2\text{-}GLC}$ showed a negative trend with age (p=ns, r=0.1786 and p=0.0645, r=-0.5105 respectively), although

the decrease with age observed for the CMV$^{A2\text{-}NLV}$-specific samples was largely based on the datapoint of one young adult (Fig. 5A). The Simpson's diversity index showed the same negative trend, although this was also not significant (p=ns for CMV$^{A2\text{-}NLV}$ and p=0.0925 for EBV$^{A2\text{-}GLC}$ (Fig. 5A). This suggests that the diversity of the CMV$^{A2\text{-}NLV}$-specific and EBV$^{A2\text{-}GLC}$-specific T-cell repertoire decrease with age.

As there are indications in mice that CMV-infection can affect the T-cell response against heterologous virus infections [203, 204, 220], we then stratified the EBV$^{A2\text{-}GLC}$-specific T-cell data according to the individuals' CMV status. We found a higher diversity of the EBV$^{A2\text{-}GLC}$-specific T-cell repertoire in CMV- compared to CMV^{+} individuals based on both normalized richness (p=0.0111) and Simpson's diversity index (p=0.0070) (Fig. 5B). Linear regression analysis of these data suggests a lower diversity of the EBV$^{A2\text{-}GLC}$-specific T-cell repertoire in the presence of CMV over the entire observed age range (Fig. 5B). A significant decrease in diversity of the EBV$^{A2\text{-}GLC}$-specific T-cell repertoire with age was only observed in CMV^{+} individuals.

A likely explanation for the decreased diversity of the EBV$^{A2\text{-}GLC}$-specific T-cell repertoire in CMV^{+} individuals could be memory attrition, i.e. large frequencies of CMV$^{A2\text{-}NLV}$-specific T cells outcompeting non-CMV-specific T cells. If there is indeed a role for memory attrition, one would expect that 1) the percentage of EBV$^{A2\text{-}GLC}$-specific T cells is lower in CMV^{+} compared to CMV- individuals, and 2) that the percentage of EBV$^{A2\text{-}GLC}$-specific T cells correlates negatively with the percentage of CMV$^{A2\text{-}NLV}$-specific T cells. CMV^{+} individuals indeed had significantly lower percentages of EBV$^{A2\text{-}GLC}$-specific CD8^{+} T cells compared to CMV- individuals (p= 0.0300) (Sup. Fig. 6A).

The lower fraction of EBV$^{A2\text{-}GLC}$-specific T cells was seen at all ages (Fig. 5C). However, the percentage of EBV-specific T cells was not negatively associated with the percentage of CMV$^{A2\text{-}NLV}$-specific T cells. If anything, there was a trend towards a positive correlation between both antigen-specific T-cell frequencies (p=0.0598, r2=0.2654) (Fig.5D). Thus, the decreased frequency and diversity of EBV$^{A2\text{-}GLC}$-specific T cells in CMV^{+} individuals does not seem to be due to memory attrition.

Based on the same t-SNE cluster analysis as shown in Fig. 1B, we compared the EBV$^{A2\text{-}GLC}$-specific CD8^{+} T-cell population of CMV- (n= 32) and CMV^{+} (n=40) individuals (Sup. Fig. 6B). For this analysis, data from donors of all ages were pooled.

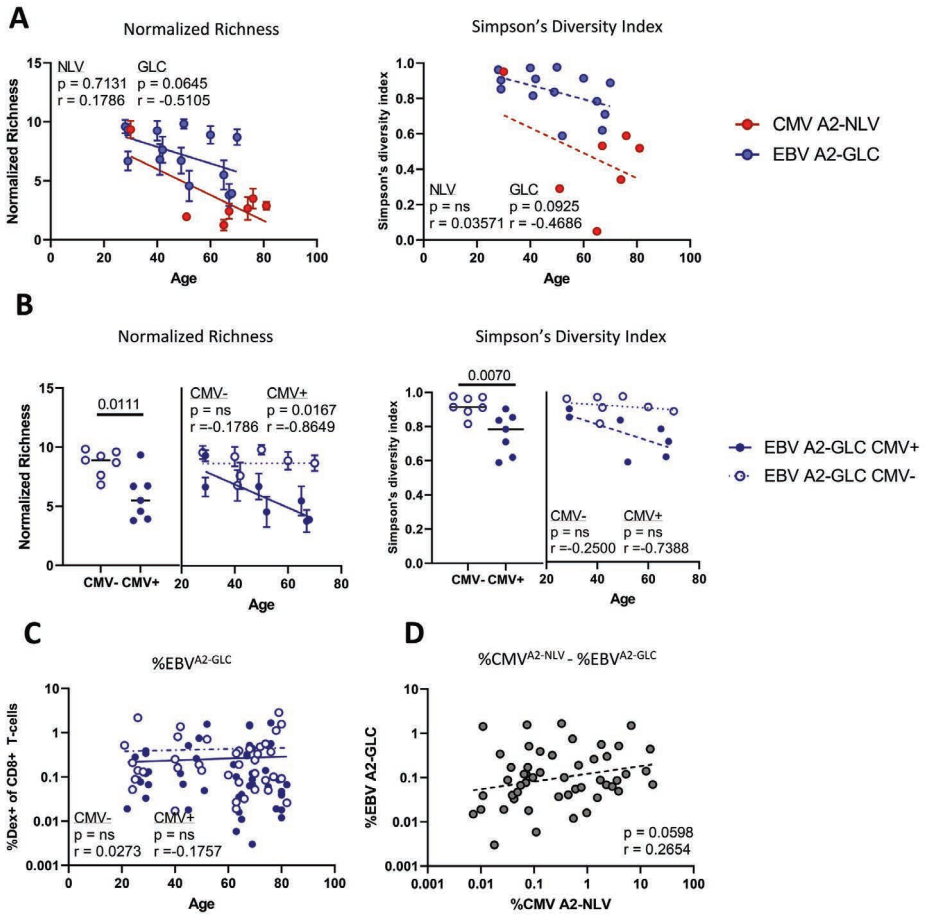

Figure 5: The CMV^{A2-NLV}-specific TCRβ repertoire and the EBV^{A2-GLC}-specific TCRβ repertoire decrease with age, whereas the diversity of the EBV^{A2-GLC}-specific.
TCRβ repertoire is lower in the CMV⁺ individuals than in the CMV– individuals. A) Correlation plots of normalized richness score (left) and Simpson's diversity index (right panels) of CMV^{A2-NLV}*-specific and* EBV^{A2-GLC}*-specific T-cell repertoires and age. Whiskers denote standard deviations over 1,000 subsampling iterations. B) Normalized richness score and Simpson's diversity index of the* EBV^{A2-GLC}*-specific T-cell repertoire divided in CMV– (blue, open circles) and CMV⁺ individuals (blue, filled circles). C) Percentage of* EBV^{A2-GLC}*-specific CD8⁺ T cells in all CMV– (blue open circles) and CMV⁺ individuals (blue filled circles). D) Correlation of* CMV^{A2-NLV} *and* EBV^{A2-GLC}*-specific CD8⁺ T-cell frequencies within donors. Frequencies are log transformed for statistical analysis. Solid lines indicate a slope significantly ($p < 0.05$) different from a slope of 0, whereas a dotted line indicates no significant difference. All correlations were tested by Spearman's Rank correlation coefficient.*

For none of the three earlier identified clusters did we find any significant differences between CMV⁺ and CMV- individuals (Sup. Fig. 6B). As the t-SNE gates were rather rough, we wondered whether there would be any differences

in the phenotype of EBV$^{A2\text{-}GLC}$-specific T cells when analyzing the data in more detail. Based on conventional gating, we observed no substantial CMV-related differences based on for example the percentage of CD57^{+} or KLRG-1^{+} cells. Only the percentage of PD-1^{+} expressing EBV$^{A2\text{-}GLC}$-specific T cells was significantly higher in CMV- as compared to CMV^{+} individuals (p=0.0255), while the EBV$^{A2\text{-}GLC}$-specific T-cell population in CMV^{+} individuals had a significantly higher percentage of effector memory T cells than in CMV- individuals (p=0.0106) (Sup. Fig. 6C). Thus, CMV-infection may induce subtle changes in non-CMV-specific T cell populations, like those specific for EBV.

DISCUSSION

In this study, we investigated the effect of age and CMV-infection on the phenotype and diversity of the antigen-specific T-cell repertoire. We focused on CMV$^{A2\text{-}NLV}$-specific and EBV$^{A2\text{-}GLC}$-specific T cells, as these antigen-specific T cells are readily detectable in the T-cell pool at all ages. The antigen-specific T cells against both persistent viruses showed an age-related increase in the expression of several markers associated with a more differentiated phenotype, including KLRG-1, an increase in the fraction of terminally differentiated T cells and a decrease in the diversity of the antigen-specific T-cell repertoire. CMV-infection has also been proposed to reduce the diversity of the total memory T-cell pool [82, 95]. However, the effect of CMV on the diversity of other antigen-specific T-cell repertoires remains poorly understood. Here we show that CMV infection is associated with a lower diversity of the EBV$^{A2\text{-}GLC}$-specific T-cell repertoire. Although the exact mechanism behind this association remains unknown, our data suggest that the decreased diversity of the EBV$^{A2\text{-}GLC}$-specific T-cell repertoire in CMV^{+} individuals is not due to memory attrition.

We found that antigen-specific T cells against CMV$^{A2\text{-}NLV}$ and EBV$^{A2\text{-}GLC}$ are different at the phenotypic level; CMV$^{A2\text{-}NLV}$-specific T cells have higher percentages of Temra cells, as defined by CD27- and CD45RO-, and higher expression of CD57 than EBV$^{A2\text{-}GLC}$-specific T cells. These findings are in line with other studies, showing that EBV$^{A2\text{-}GLC}$-specific T cells are predominantly CD45RO^{+} [231] and more often express CD27 [232]. The presence of terminally differentiated CMV$^{A2\text{-}NLV}$-specific T cells is probably explained by the presence of large clonal expansions, which are typical for CMV infection [151].

The relatively high expression of markers associated with a more differentiated phenotype and the relatively low TCR repertoire diversity of the CMV$^{A2\text{-}NLV}$-specific

T-cell population becomes even more pronounced in individuals at older age. Based on the high frequencies of CMV$^{A2\text{-}NLV}$-specific T cells in older adults, this age-effect is probably also linked to the presence of large clonal expansions consisting of terminally differentiated cells. It has been suggested that the increase in CMV-specific T-cell numbers with age is due to periodical infectious reactivation [233]. Although EBV$^{A2\text{-}GLC}$-specific T cells also show changes associated with age, these are less pronounced than for CMV$^{A2\text{-}NLV}$-specific T cells. This may be related to differences in cellular tropism between the two herpes viruses [161, 162], or to possible differences in viral reactivation frequencies [163, 164].

It is tempting to speculate that the features of the T-cell responses against CMV$^{A2\text{-}NLV}$ and EBV$^{A2\text{-}GLC}$ that we observed are characteristic for the immune response to these two viruses in general. Although these two epitopes tend to be immunodominant, and thereby represent a fair share of the T-cell response in many individuals, it was recently shown that different combinations of HLA-alleles can influence the immunodominance of an epitope [234]. This may also explain the large variation in antigen-specific T-cell frequencies that we observed in the four age groups. It remains to be investigated whether our results also apply to other epitopes for these two viruses, and to individuals in which these responses are less dominant.

Previous studies on the effect of aging on the antigen-specific T-cell repertoire have mostly focused on the maintenance of TCR sequences that are shared between individuals or that occur at different timepoints [80, 160] or on a biased usage of Vβ-segments [107]. As these studies observed dominant T-cell clones both in a longitudinal setting and in a cross-sectional setting across different ages, they led to the view that the antigen-specific T-cell repertoires against CMV and EBV are relatively stable over time. Even though the T-cell repertoire analyses were performed on a relatively small number of samples, our direct assessment of the TCR diversity of EBV$^{A2\text{-}GLC}$-specific T cells, and to a lesser extent of CMV$^{A2\text{-}NLV}$-specific T cells, showed a clear decrease in diversity with age, suggesting that the antigen-specific T-cell repertoires against these viruses are not as stable as previously thought. These seemingly contradicting conclusions may be due to the process of convergent contraction of the T cell repertoire, in which lower frequency clonotypes are lost over time, while only few T cells persist [197].

Observations of these persisting T-cell clones over time would suggest that the antigen-specific T-cell repertoire is relatively stable, even though the richness and diversity of the repertoire may decrease with age. To study whether convergent

contraction of the T-cell repertoire is indeed happening, a longitudinal study should be performed, focusing on the richness and diversity of the antigen-specific T-cell repertoire.

It remains unknown *why* the diversity of antigen-specific T-cell repertoires decreases with age and whether age is the real driver of the decrease in T-cell diversity or whether other factors play an important role. We cannot exclude the possibility that the older individuals of the study population had been infected at an older age, possibly leading to an antigen-specific T-cell repertoire of lower diversity because the diversity of the naive (precursor) pool is known to decrease with age [133, 136]. However, the recent finding that only a very small percentage of individuals seroconvert for CMV at later age [235], as well as the finding that more than 90% of the population is infected with EBV during adolescence [236, 237], makes this explanation unlikely. In our view, a more likely explanation for the reduced diversity in the antigen-specific T-cell repertoire of older individuals would be that older individuals have been infected for a longer time, and have lost more T-cell clones over time, for example due to exhaustion after restimulation [217].

Our results indicate that CMV-infection is associated with a lower diversity of the $EBV^{A2\text{-}GLC}$-specific T-cell repertoire. As it is generally thought that T-cell receptor diversity is positively correlated with the level of protection against infectious diseases, one would expect that this decreased diversity would lead to a decreased EBV-specific T-cell response in CMV^{+} individuals. If our findings also hold true for other antigens, this would suggest that CMV^{+} individuals are less protected against other infections.

This is in line with most mouse studies, which show a negative effect of CMV-infection on the T-cell efficacy against heterologous infections [139, 203, 204, 220]. *How* CMV-infection would lead to this lower diversity remains unknown. Although memory attrition has often been suggested to play a role, our data do not support this idea, as we observed a positive correlation between the frequencies of $EBV^{A2\text{-}GLC}$- and $CMV^{A2\text{-}NLV}$-specific T cells. The explanation for this positive correlation might be that some individuals are better T-cell responders than others. The observation that CMV-infection leads to lower frequencies of antigen-specific T cells is not surprising, as it is a relative measure, which is easily skewed by the high percentages of CMV-specific T cells. Likewise, in another study of our group it was shown that influenza-specific T-cell frequencies in older individuals were lower in CMV^{+} compared to CMV- individuals. Importantly, however, this did not result in lower influenza-specific IFNγ responses in CMV^{+} individuals (van den

Berg, submitted), suggesting that CMV did not negatively impact the influenza-specific T-cell response; although sample sizes were unfortunately too small to confirm this based on the diversity of the influenza-specific TCR repertoire. It remains puzzling why these different results are observed between studies, and it would be interesting to understand whether e.g. the acute or chronic nature of a pathogen plays a role.

T cells isolated from blood represent only a small fraction of the total T-cell pool in the body, as it has been estimated that blood contains only 2 percent of all the T cells in the body [238]. Although many T cells travel through different compartments in the body, it remains to be investigated whether antigen-specific T-cell characteristics, like their phenotype and repertoire diversity, that are observed in the blood can be extrapolated to T cells in other sites of the body. The presence of tissue-resident T cells, which hardly circulate through the blood, complicates this even further. In the case of CMV and EBV, however, the blood may in fact be the most informative site to follow the antigen-specific T-cell response. It has previously been suggested that most CMV-specific T cells are present in the blood [239]. In line with this, a recent human study showed that most terminally differentiated memory $CD8^+$ T cells, including CMV-specific T cells, are confined to the intravascular circulation and do not circulate through the thoracic duct lymph [240]. Also for EBV, a bloodborne virus infecting B cells, the blood may in fact be the most representative compartment to study the antigen-specific T-cell response. It remains to be investigated whether the changes we observed in the antigen-specific T-cell repertoire in the blood also apply to antigen-specific T cells in other sites of the body. So far, studies have shown both a minimal overlap in the naïve T-cell pool between spleen and lymph nodes[241], as well as high degrees of overlap when focusing on the memory T-cell pool between blood and the thoracic duct lymph [240], between peripheral blood and lymph nodes [198] and between spleen and lymph nodes [242].

In contrast to the commonly-held view that the antigen-specific T-cell repertoires against CMV and EBV are relatively stable with age, we here show that they both clearly decrease with age. Our data suggest that not only age but also CMV-infection is associated with the diversity of the $EBV^{A2\text{-}GLC}$-specific T-cell repertoire. Insights into how antigen-specific T-cell repertoires evolve with age and under the influence of other infections, like latent CMV, are important for the development of novel vaccination strategies to protect older adults against infectious diseases. One of the proposed strategies to prevent older adults is to induce protective immune responses through vaccination at a younger age. This would require

stability of the induced immune response in order to provide protection later in life. Our data suggest that the antigen-specific T-cell repertoire is not as stable as previously thought. Unfortunately, this implies that vaccination may also come too early. Ideally, one would like to vaccinate before the age-associated decline in naive T-cell repertoire diversity, but late enough to ensure that a substantial level of protection is maintained until later in life. Further research is needed to investigate why some cells are maintained while others are not, and to define the optimal moment of vaccination to protect the elderly.

ACKNOWLEDGEMENTS

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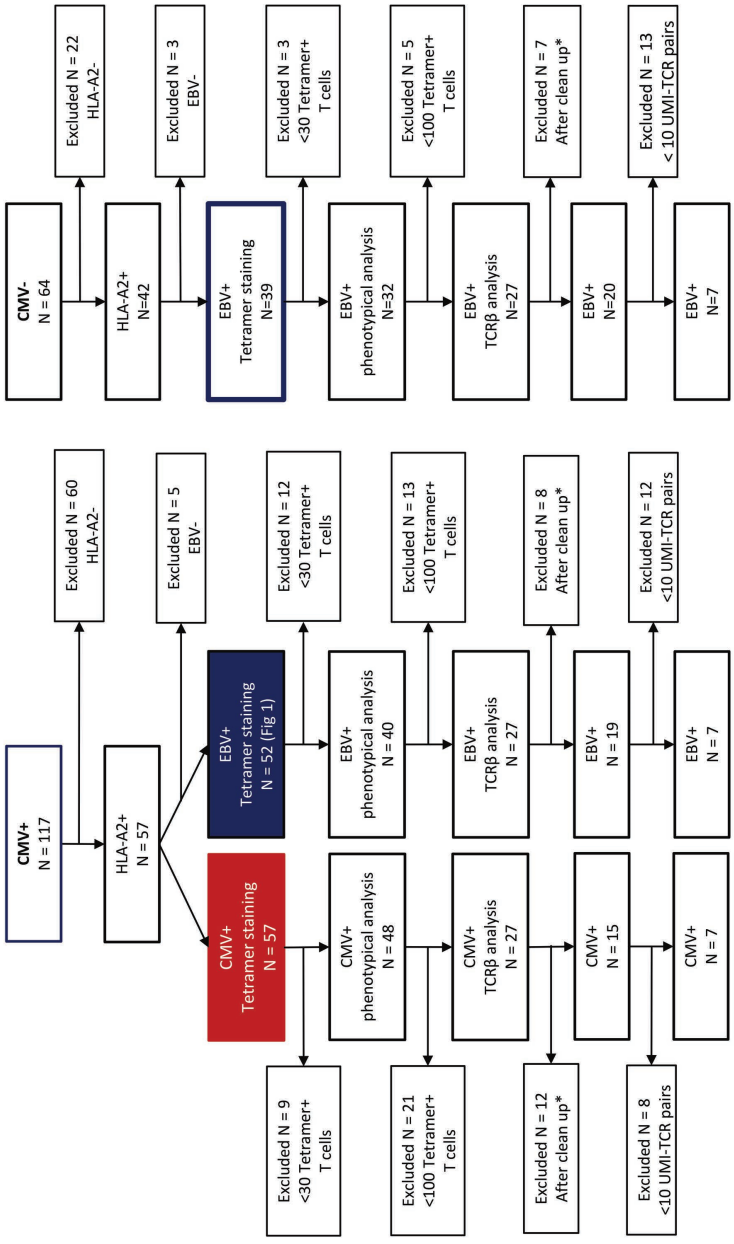
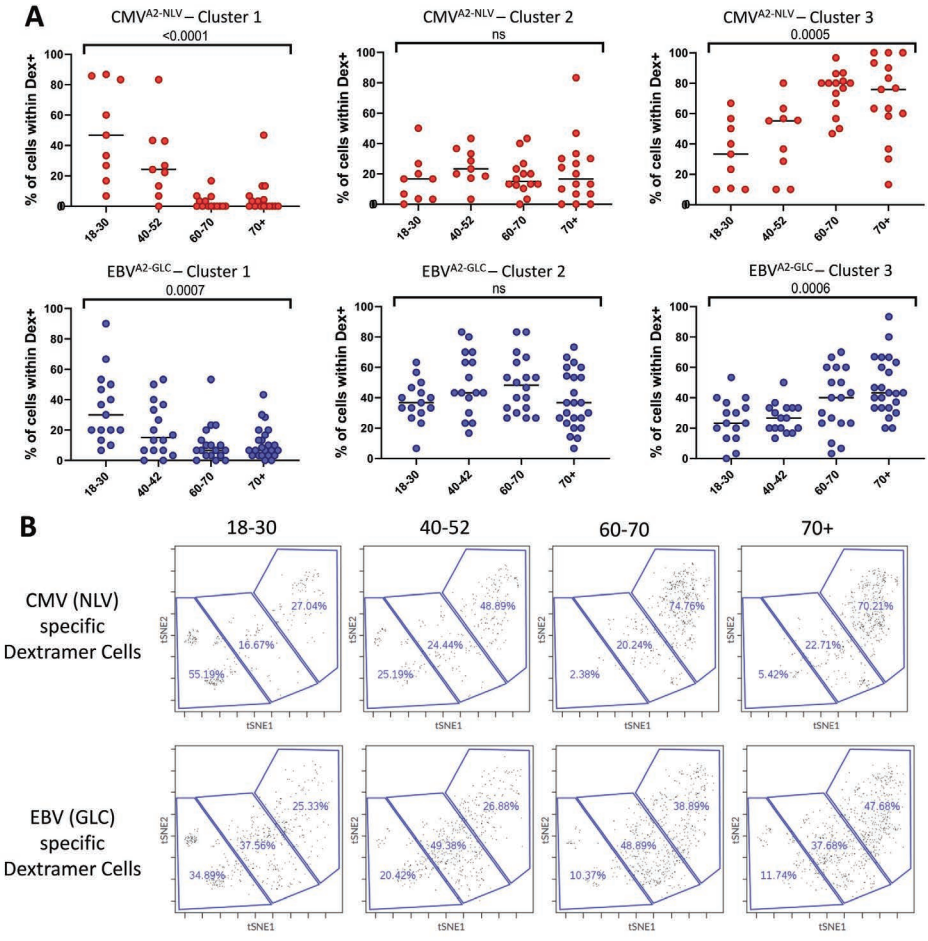
SUPPLEMENTALS

The Supplementary Tables with the TCR sequences are available online at: **https://www.frontiersin.org/articles/10.3389/fragi.2021.665637/full#supplementary-material**

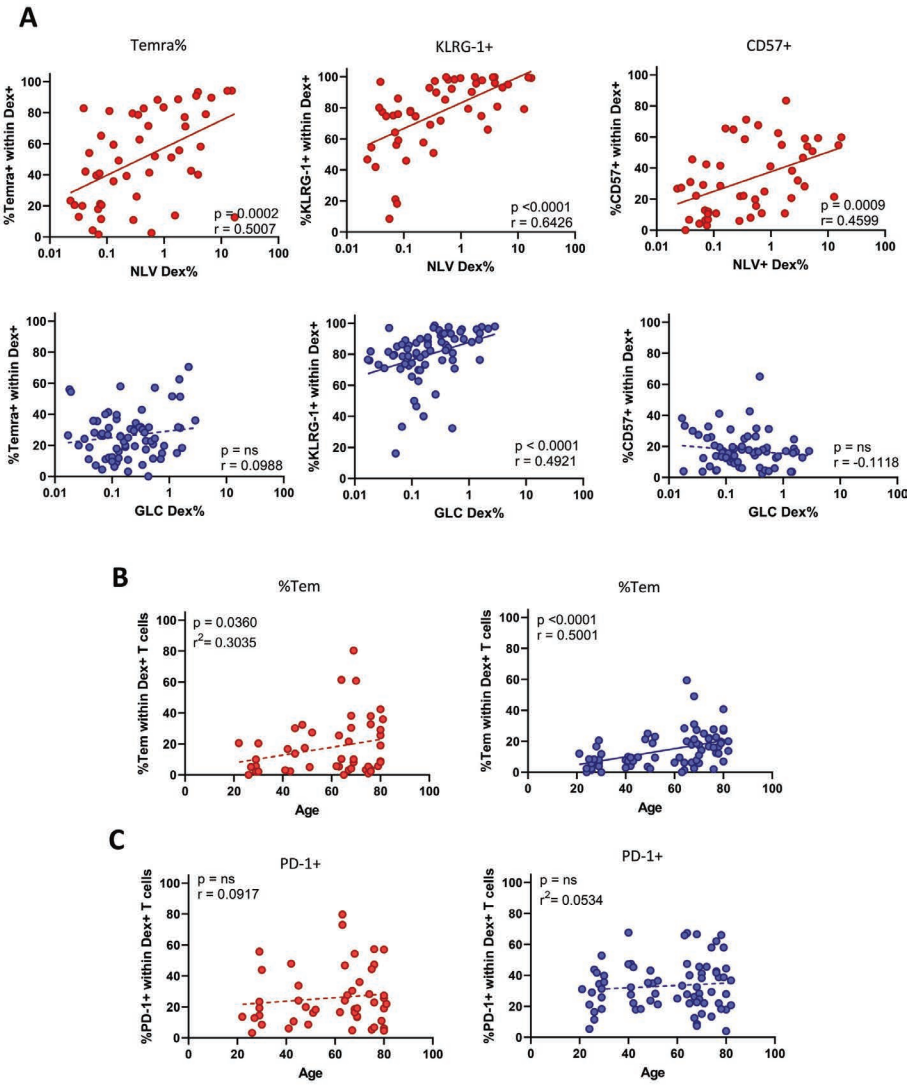


Supplementary Figure 1: Participants Flowchart.
*Overview of the participants and criteria used for the study. For the CMV+ individuals 54 participants (age 18-52) came from the study of Rosendahl Huber et al, whereas 63 participants (age 60 years and older) were derived from Kaaijk et al. For the CMV- individuals this was 28 and 36 respectively. *Data shown in Supplementary figure 2.*



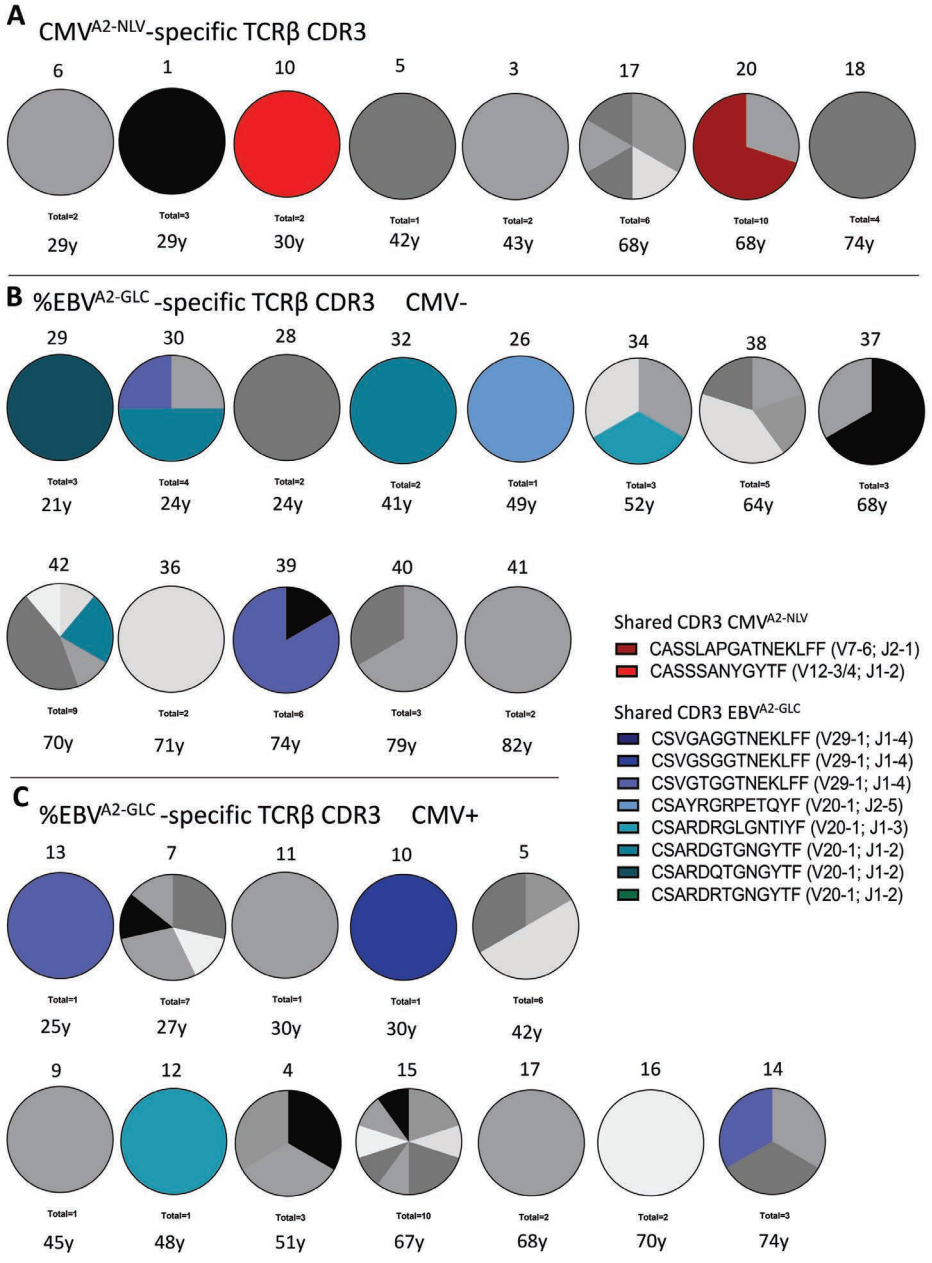
Supplementary Figure 2: Additional information associated with the t-SNE cluster analyses.

A) Percentages of CD8⁺ T cells per t-SNE cluster (1, 2 and 3) for the different age groups of both CMV^{A2-NLV}-specific (red, upper panels) and EBV^{A2-GLC}-specific (blue, lower panels) CD8⁺ T cells. Horizontal lines show group medians. Differences between groups was tested with the Kruskal-Wallis test. B)) t-SNE analysis of CMV^{A2-NLV}-specific (n=48) and EBV^{A2-GLC}-specific (n=72) CD8⁺ T cells of donors categorized in 4 age groups, shown as dots. Clustering is based on MFI of CD5, PD-1, CD57, KLRG-1, CXCR3, CCR7, CD45RO, CD95, CD27 and CD127. Equal numbers of cells were used per sample



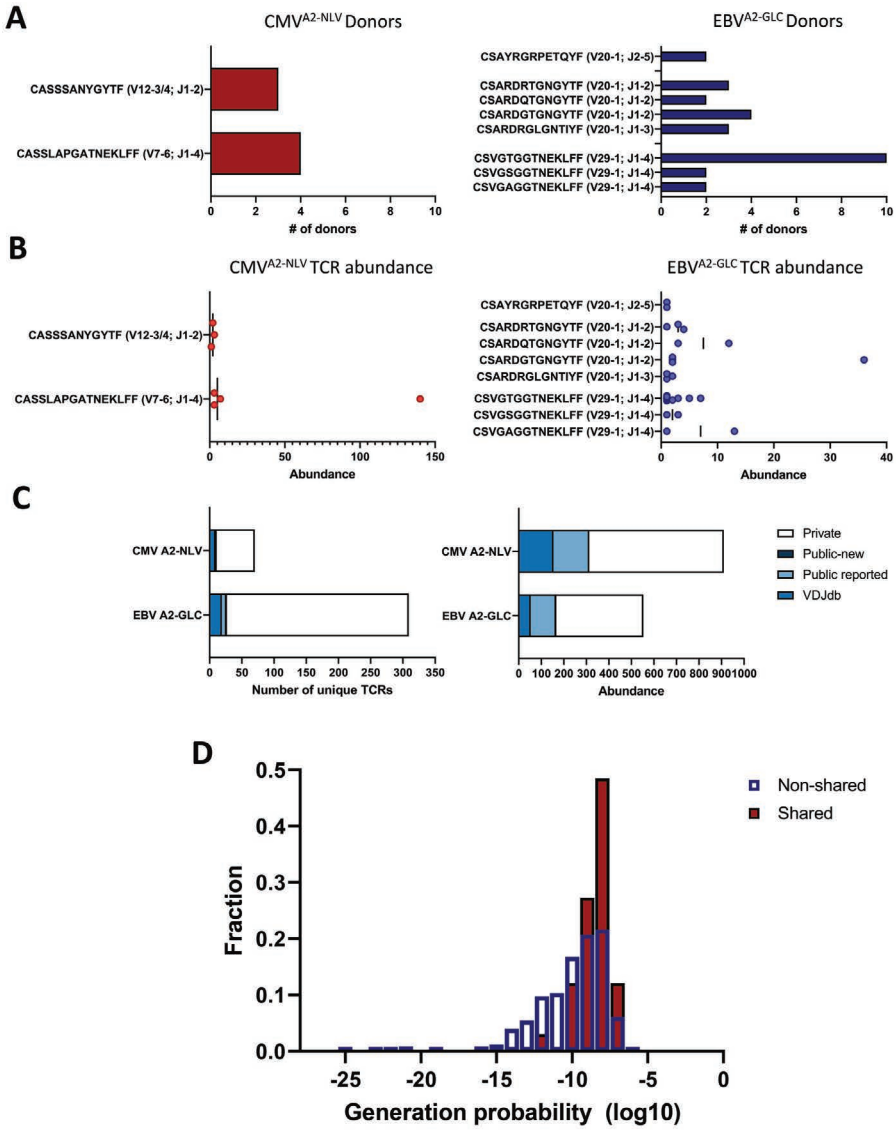
Supplementary Figure 3: Expression of the a more differentiated phenotype is associated with higher expression of the frequencies of CMV$^{A2\text{-}NLV}$-specific and EBV-$^{A2\text{-}GLC}$-specific T cells.

A) Correlation plots of percentage of Temra cells (left panel) CD57$^+$ (middle panel) and KLRG-1$^+$ (right panel) of CMV$^{A2\text{-}NLV}$-specific (red, upper panels) and EBV$^{A2\text{-}GLC}$-specific (blue, lower panels) CD8$^+$ T cells and the frequencies of Dextramer$^+$ T cells. Frequencies were log transformed. Correlations were tested with Spearman's rank correlation. Only donors with a sufficient T-cell response (at least 25 cells) were used for the phenotypical staining. B+C) Percentage of Effector memory T cells (Tem, CD27-CD45RO$^+$) (A) and PD-1$^+$ T cells (B) of CMV$^{A2\text{-}NLV}$-specific (red, left panel) and EBV$^{A2\text{-}GLC}$-specific (blue, right panel) CD8$^+$ T cells plotted with age. Solid lines indicate a slope significantly ($p<0.05$) different from a slope of 0, whereas a dotted line indicates no significant difference.

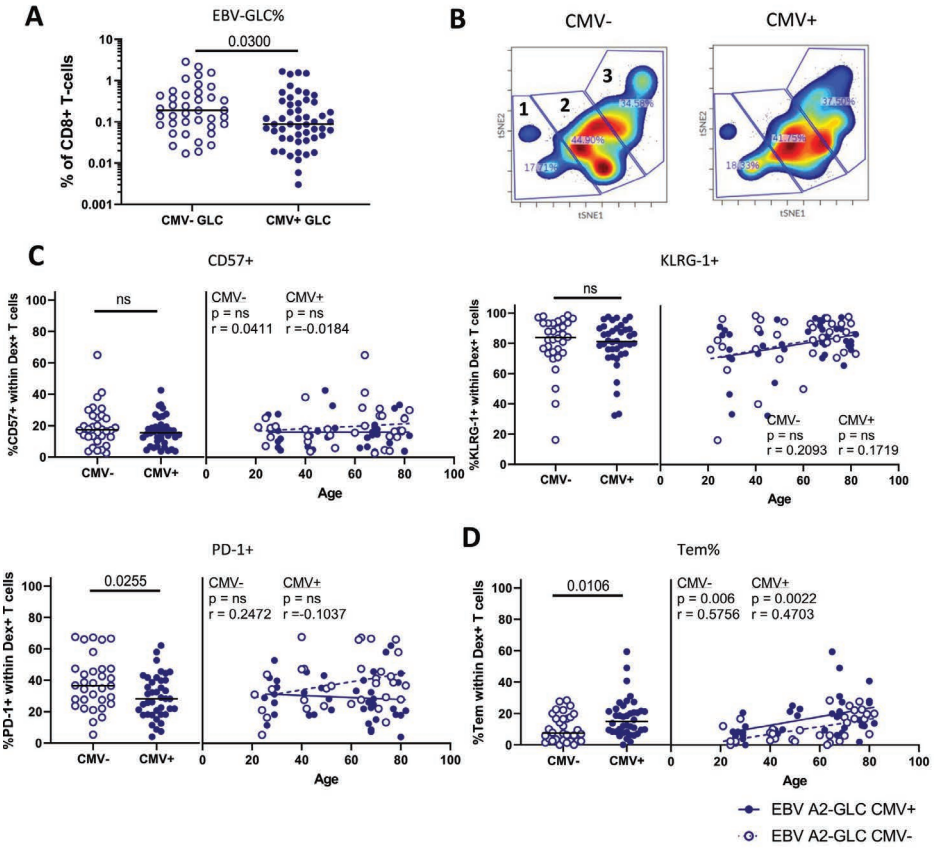


Supplementary Figure 4: Characterization of the antigen-specific TCRβ repertoire consisting of less than 10 UMI-TCR pairs.

A) T-cell repertoire of CMV$^{A2\text{-}NLV}$-specific CD8^{+} T cells (n=8). B,C) T-cell repertoire of EBV$^{A2\text{-}GLC}$-specific CD8^{+} T cells of both CMV- (B) and CMV^{+} individuals (C). Each pie depicts the repertoire of a different donor. Colors represent shared CDR3 sequences between donors. Grey scales depict unique CDR3 sequences.



Supplementary Figure 5: Shared sequences consist of a higher generation probability. *A) Number of donors sharing CDR3 sequences in their CMV$^{A2\text{-}NLV}$-specific (red, upper panel) or EBV$^{A2\text{-}GLC}$-specific (blue, lower panel) T-cell repertoire. B) Total number of different UMIs coupled to the shared CDR3 sequences found in the different donors of in their CMV$^{A2\text{-}NLV}$-specific (red, upper panel) or EBV$^{A2\text{-}GLC}$-specific (blue, lower panel) T-cell repertoire. C) Number of total CDR3 sequences that are private (white) or shared within our data base (dark blue), and also reported in the VDJdb (light blue) or sequences not shared in our database but reported in the VDJdb (blue). D) Generation probability distributions of sequences that are shared between donors within our data, or with the VDJdb (shared) and the other sequences (non-shared). Only sequences matching with the CMV$^{A2\text{-}NLV}$ and EBV$^{A2\text{-}GLC}$ epitopes were labeled as shared.*



Supplementary Figure 6: Phenotypical differences between the EBV$^{A2\text{-}GLC}$-specific T cells in CMV- and CMV^{+} individuals.

A) Percentage of EBV$^{A2\text{-}GLC}$-specific CD8^{+} T cells In CMV- (blue, open circles) and CMV^{+} (blue, filled circles) individuals. Median is presented in the figures. Difference was compared by Mann Whitney U test. B) t-SNE analysis of EBV$^{A2\text{-}GLC}$-specific CD8^{+} T cells based on MFI of CD5, PD-1, CD57, KLRG-1, CXCR3, CCR7, CD45RO, CD95, CD27 and CD127 in CMV^{-} (n=32) and CMV^{+} individuals (n = 40). 30 cells were used from each sample. 3 large clusters were identified. C) Percentage of CD57^{+} (upper left panel) KLRG-1^{+} (upper right panel) and PD-1^{+} (lower left panel) and fraction of Effector Memory T cells (D) of EBV$^{A2\text{-}GLC}$-specific CD8^{+} T cells shown for CMV^{-} (blue, open circles) and CMV^{+} (blue, filled circles) individuals (left panels) and correlated against the age of the donors (right panels). Median is presented in the figures. Differences were compared by Mann Whitney U test.

CLEANING SEQUENCE DATA

Introduction

Since we analyzed antigen-specific subsets of the T-cell repertoire, the samples consisted of relatively small numbers of T cells. This may cause problems when applying standard methods for sequencing and analyzing T-cell repertoire diversity. To overcome these issues, we applied various filtering steps to minimize the potential impact of unavoidable experimental errors on the results presented in the main text.

An important step in many TCR-analysis protocols is the use of unique molecular identifiers (UMIs). These are introduced before extensive multiplication by PCR and hence allow to map PCR-products to their ancestral cDNA-molecule. This way, one can correct for biases by uneven PCR amplification and other errors that are likely to arise in some of the sequences. While removing biases, the results may still be affected by two other factors. The first is that if a sample contains a very abundant UMI-TCR pair (which originated from just one cDNA molecule), even the smallest contamination between samples may cause this UMI-TCR pair to also occur in another sample. This shared TCR sequence would then be interpreted as a clonotype present in multiple individuals. The other problem is that during PCR and sequencing of the product, errors may be introduced in the UMI sequence. This will lead to an inflated number of distinct UMI sequences that are observed with a given TCR, and hence to an overestimation of the abundance of individual TCR sequences in a sample.

When analyzing samples containing millions of unsorted T cells one may accept these factors to play a role, as their relative impact is expected to be small. However, since the number of sequences in our samples is limited, small biases could have a major impact on the results. Therefore, we performed additional filtering steps to make sure that our results are not affected by these confounding factors. We did this in a step-wise approach by using the abundance, sharing and nucleotide sequence of the UMIs. Note that this cleaning method does not correct for possible contamination in the sort process of the cells, which may occur for example via unspecific binding of T cells to the dextramers. This is probably the explanation for the shared sequences in the $CMV^{A2\text{-}NLV}$-specific and $EBV^{A2\text{-}GLC}$-specific samples as observed for donors 19 and 21 (see the legend of Fig. 3).

A) Pairing, UMI-identification and V/J-alignment

Demultiplexed samples were first merged using tool Paired-End reAd mergeR (PEAR, Zhang Bioinformatics 2014). Since assembly efficiency was variable between different samples, we decided to also include the non-assembled reverse read in the analysis. The 12nt UMI sequences in the reads were identified using the 'Checkout' algorithm in Recover T Cell Receptor (RTCR, Gerritsen Bioinformatics 2016). For all samples together, 1.06 million UMI sequences could be found in a total of 34.3 million reads. Within each sample, the reads were then collapsed by their UMI into consensus sequences using the 'umi_group_ec' method of RTCR. We then used the 'run' function of RTCR to align the sequences to the reference TRBV and TRBJ genes. Only the alignment information was used, ignoring the further clustering steps that RTCR performs by default. We proceeded with the ~ 34% UMI-based consensus sequences in which the V as well as the J gene could be identified.

B) Within-sample cleaning

Since we separately took the merged and non-assembled reverse reads into account, individual UMIs could generate two consensus sequences. We confirmed that in the vast majority of such cases, where both V and J genes could be identified, these were identical. We selected the consensus sequence with highest support, i.e., based on the highest number of reads in such cases. Next, we removed all UMIs that were observed in just a single read, because they are most likely erroneous, leaving $6.6*10^4$ consensus sequences for all samples together. We then analyzed Hamming Distances between UMI sequences observed within and between samples. This showed that there were about 50 times more similar (measured as Hamming Distance ≤ 3) UMI pairs within than between samples. We noticed that consensus sequences of similar UMIs within a sample had identical V and/or J genes in most cases. This indicates mutation of UMI sequences, which would lead to an inflated abundance estimation of the corresponding TCR sequences. We corrected for this by clustering pairs of UMIs within a Hamming Distance of 3, and subsequently removing the UMIs with the lowest read counts. This approach excluded many likely UMI-mutants that were supported by a much lower number of reads (mean 19) than the remaining sequences ($2.0*10^3$). RTCR was run individually on each of the remaining $7.1*10^3$ UMI-based consensus sequences to identify their CDR3 sequence while also retaining the UMI sequence.

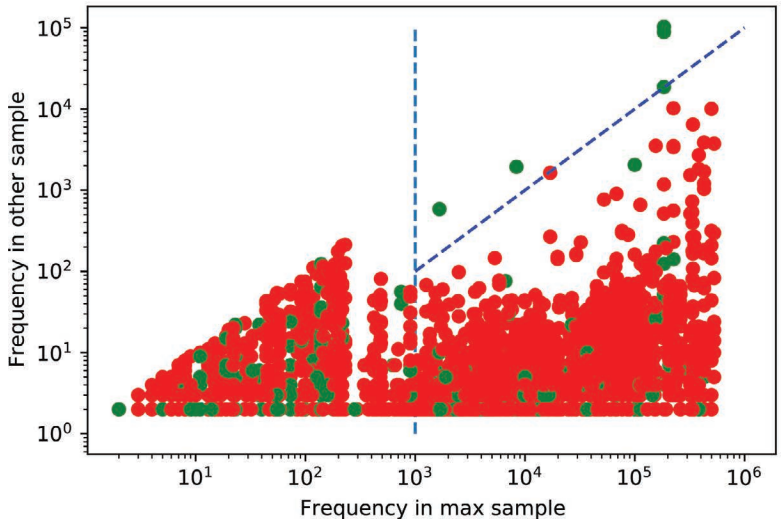


Figure: Read counts of UMI sequences that are shared between samples. Red dots indicate pairs with identical UMI and CDR3 sequence in two samples (indicating contamination), green dots represent pairs with identical UMI but different CDR3 sequence. The vertical black dashed line represents the read count threshold of 1000, below which all sequences with the corresponding UMI are deleted. The diagonal blue line indicates the 10% of maximum frequency threshold. UMI duplicates above this threshold are both kept (upper right quadrant), below the threshold only the UMI with maximum number of reads is accepted (lower right quadrant).

C) Cross-sample cleaning

A substantial fraction of the UMIs (21%) was observed in more than one sample. Although overlap of UMIs between samples is theoretically possible, this was observed much more often than expected by chance, suggesting cross-sample contamination. Indeed, 93% of the UMIs that were shared between at least two samples showed identical CDR3 amino acid sequences in the corresponding TCRB sequences. We reasoned that contamination by abundant UMI-TCR pairs is expected to be represented by the same UMI-TCR pair at a much lower frequency in other samples. For less abundant UMI-TCR pairs, the frequency differences between samples are smaller, making it often impossible to tell which sample contained the genuine UMI-TCR pair and which the contamination. Hence, we only accepted overlapping UMIs between samples if 1) the frequency of the UMI in one of the samples was more than 1000 reads and 2) the frequency of the UMI in the other sample was more than 10% of the maximum frequency. The few overlapping UMIs that hence remained, all had different CDR3 sequences in both samples, suggesting that they were not due to contamination. We proceeded with these remaining overlapping UMI-TCR pairs and all others that were not shared between samples.

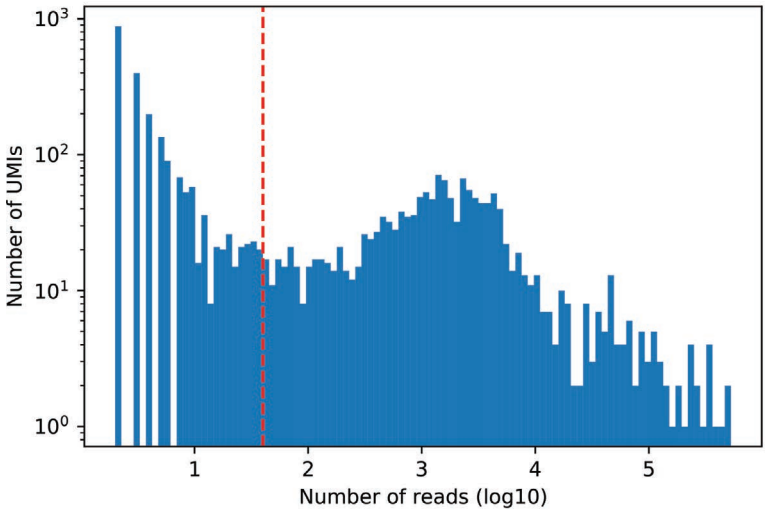


Figure: Histogram of all UMI read counts in each sample. The minimum read count threshold (40) is indicated with the horizontal red line.

D) Read count threshold

Although we applied within-sample UMI-clustering and between-sample cleaning of UMI-overlap, still erroneous sequences could have remained, for example by mutation of UMI sequences that arose by contamination. Indeed, there were multiple samples containing abundant UMI-CDR3 pairs and also many identical CDR3 sequences with different UMIs. Most of these other UMIs were supported by only few reads. By inspecting individual samples as well as all samples together, we often observed a bimodal distribution of read counts. Assuming that the UMIs supported by fewer reads are enriched for erroneous sequences, we only used UMI-CDR3 pairs above a read count threshold. We decided to only keep UMI-CDR3 pairs supported by at least 40 reads to further exclude confounding effects in our data. Note that we also tested the effect of an even stricter threshold of 600 reads, but this did not change our results qualitatively. The distributions of UMI distances within and between samples indicates that our cleaning procedure was successful.

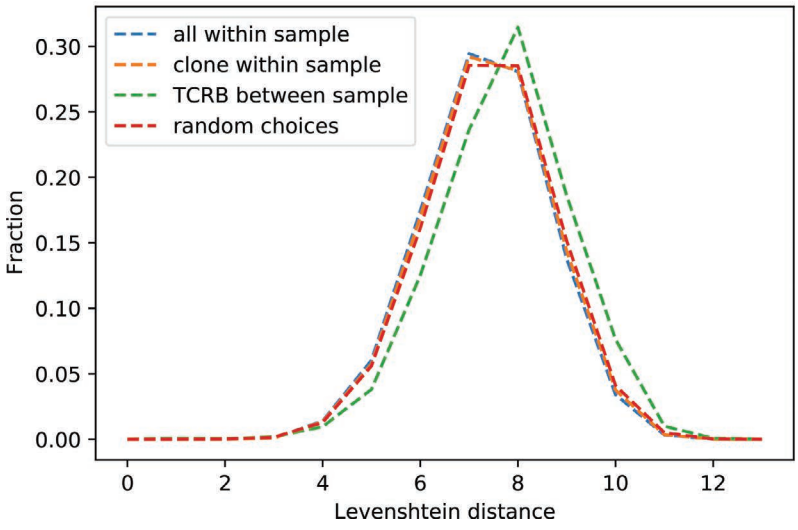


Figure: Distributions of UMI distances after data cleaning. Pairwise comparison between sets of UMI sequences using Levenshtein Distance. Blue: all UMIs are compared to all other UMIs in the same sample. Orange: UMIs are compared to all within-sample UMIs corresponding to identical CDR3 amino acid sequence. Green: similar, but comparing to UMIs in another sample with identical CDR3. Red: pairwise comparison of randomly chosen UMI sequences from all samples. The similarity of all distributions indicates that the cleaning method removed erroneous sequences from our data. Note that there were only a limited number of CDR3s shared between samples, which may explain the visible difference to the other distributions.

Latent CMV-infection is associated with lower influenza virus-specific memory T-cell frequencies, but not with an impaired T-cell response to acute influenza virus infection

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ABSTRACT

Latent infection with cytomegalovirus (CMV) is assumed 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. CMV is expected to impact especially older adults, who are already at higher risk of severe disease and hospitalization upon infections such as influenza virus (IAV) infection. Here, we investigated the impact of CMV-infection on IAV-specific T-cell frequencies in healthy individuals (n=96) and the response to IAV infection in older adults (n=72).

IAV-specific memory T-cell frequencies were lower in healthy CMV^{+} older individuals compared to healthy CMV^{-} older individuals. Upon acute IAV infection, CMV-serostatus or CMV-specific antibody levels were not negatively associated with IAV-specific T-cell frequencies, function, phenotype or T-cell receptor repertoire. This suggests that specific T-cell responses upon acute IAV infection are not negatively affected by CMV. In addition, we found no association between CMV-infection and inflammatory cytokine levels in serum during acute IAV infection nor between cytokine levels and the height of the IAV-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 IAV-specific T-cell responses early upon acute IAV infection.

In conclusion, although associated with lower frequencies of memory IAV-specific T cells in healthy individuals, CMV-infection does not seem to form a threat for mounting a proper T-cell response during acute IAV infection in older adults.

INTRODUCTION

The worldwide population is ageing rapidly. With age, deleterious changes in the immune system arise, referred to as 'immunosenescence', which impair responses against infectious diseases and vaccinations [19, 243]. Age-related changes of the immune system mainly occur in the T cell pool, including an increase in the number of $CD45RA^+$ memory T cells [218, 219] and decreased diversity of the T-cell receptor repertoire [158, 244]. About 2 decades ago, latent infection with cytomegalovirus (CMV) was implicated as a possible driving force of these age-related changes [245-247]. CMV-seropositivity was identified as part of the so-called 'immune risk profile', predictive of early mortality in older adults [199, 245]. Moreover, CMV-seropositivity is the largest non-heritable factor influencing differences among humans in the immune profile [248]. CMV is generally thought to establish this large effect by its frequent attempts to reactivate during life-long carriage, thereby gradually affecting te immune system [249]. Since the 2000s, it is often hypothesized that CMV might impair the human immune response to a heterologous challenge [201, 244, 249], as was shown in mice [203, 204, 220].

With age, the risk for serious complications and hospitalization after influenza virus (IAV) infection increases [170]. Vaccination is an important tool to prevent infection, however also the efficacy of influenza vaccination decreases with age [19, 250]. The role of CMV infection on influenza vaccination efficacy has been well studied. These studies have yielded conflicting results as both a negative effect of CMV [251-253], a positive effect [243] as well as no effect of CMV on the vaccine response have been reported [254, 255]. A systematic review by our group including a meta-analysis showed no clear evidence for a negative effect of CMV infection on the antibody response to influenza vaccination [256].

Both CMV and ageing primarily affect the T-cell compartment [35, 149, 218, 219, 257]. During IAV infection, an effect of CMV would therefore mainly be expected on the T-cell response. T cells play an important role in clearance of the IAV [32]. These T-cell responses are predominantly specific for the internal viral proteins, such as matrix protein-1 [258], which are conserved among influenza strains that have undergone antigenic drift. Indeed, pre-existing and early IAV-specific T-cell responses are associated with lower disease severity of influenza [29, 30], while delayed T-cell responses to IAV are thought to induce prolonged inflammation and delayed viral clearance and recovery [30, 259]. Upon activation, IAV-specific $CD8^+$ T cells show increased expression of activation markers [260, 261], produce

proinflammatory cytokines and kill virus-infected cells by releasing perforin and granzyme B [258, 262].

Whether CMV infection attenuates the immune response to IAV infection in older adults remains unclear. 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 which can take up to 30%-90% of the $CD8^+$ T-cell memory pool [82, 165, 263], may fill the 'immunological space' [199-201]. Thereby CMV infection may hamper the induction of other immune responses [199-201]. IAV-specific T cells may thus be outcompeted by CMV-specific T cells in their competition for proliferation and survival factors [201]. Secondly, it has been suggested that CMV is linked to 'inflammaging', the lingering low-graded level of inflammation occurring with ageing [264]. The production of pro-inflammatory mediators has been shown to enable CMV reactivation [265, 266]. Upregulation of TNF-α, IL-6 and CRP have been observed in CMV-infected individuals, as well as increased production of IL-10 [248, 267, 268]. 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 IAV, which also fits with the observed lower levels of granzyme B [26, 269, 270]. Importantly, even though CMV has been suggested to diminish the T-cell response to IAV, there is no clinical evidence of a direct link between CMV infection and the T-cell response against IAV infection in humans.

We had the unique opportunity to study the effect of CMV infection on IAV in humans in the in a relatively large cohort of natural influenza infected older adults. We first investigated the effect of CMV infection on the presence of IAV-specific memory T cells in healthy young and healthy older individuals. Next we assessed the effect of latent CMV infection on the IAV-specific T-cell response in older adults undergoing an acute IAV infection. Our data show that CMV-infection is associated with reduced frequencies of IAV-specific T cells in healthy older adults, whereas in healthy younger adults no association with CMV-infection is observed. Nevertheless, CMV infection does not hamper the T-cell response to acute IAV 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 selected based on age and sex from a study carried out in 2009-2011 (the Pandemic influenza vaccination trial, Netherlands Trial Register NL1952) [221]. The study was approved by the Central Committee on Research Involving Human Subjects of the Netherlands. Samples of older adults (N=65), ≥60 years of age, were control samples from a study carried out in 2014-2015 (Influenza-like-illness-3, Netherlands Trial Register NL4666) (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.

IAV A infected older adults during IAV 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 influenza season of 2014-2015 (NL4666, Kaaijk et al., submitted). Study design of the Influenza-like-illness-3 study was comparable to previous studies as described in Van Beek et al., 2017 [271]. In short, 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 [272] 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 [271]. IAV infection was laboratory confirmed, and subtyped by PCR and sequencing in n=72 individuals by methods described previously [271]. The 72 IAV 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 not determined), 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 (IBL international GMBH) according to manufacturer's instructions or by an in-house-developed multiplex immunoassay, [222] depending on the cohort. For healthy young adults, CMV-specific antibody levels were measured using a commercial ELISA. Recommended cutoffs of the commercial ELISA kit were followed. 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 IAV-infected individuals, CMV-specific antibody levels were measured in serum by our in-house-developed multiplex immunoassay. Cutoff were based on previous calculations: 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 [273]. 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 IAV-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 dextramer for epitope GILG of the M1 protein of IAV (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) [223] with for every donor 10.000 $CD8^+$ T cells. Donors with less than 10.000 $CD8^+$ T cells were excluded from t-SNE analysis (n=3).

IAV-infected individuals

Of all HLA-A2 positive individuals undergoing IAV infection (n=36), both IAV-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 dextramer for the GILG epitope of the M1 protein of IAV (A*0201/GILGFVFTL-APC, Immudex) (±8 mln PBMCs) (detected in n=17) and the HLA-A2 dextramer 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), 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.

IAV-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 18 hours at 37 °C on 96-well membrane-bottomed plates (PVDF plate MSIPS4510, Millipore) coated with anti-IFNγ mAbs (Mabtech). 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 IAV-infected individuals within 72 hours, and 2 and 8 weeks after fever onset. Levels were measured by bead-based multiplex LEGENDplexTM (BioLegend) according to the manufacturer's instructions. The pro-inflammatory cytokines IL-6, IFNγ, IL-10 and CRP were analyzed for this study. Stimulations were performed in duplicate. Analysis was performed on a Canto II flowcytometer. 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 intra-assay averages to correct for batch effects.

Isolation of IAV-specific T cells for T-cell receptor analysis

$CD8^+$ T cells were isolated from PBMCs using a negative selection microbeads kit (Miltenyi Biotec). Next, $CD8^+$ T cells were labelled at room temperature for 20 minutes with the A*0201/GILGFVFTL-APC dextramer (Immudex) (GILG). Subsequently surface staining was added with the following mAbs: CD3(UCHT1)-PerCP (Biolegend), CD4(OKT4)-BV510 (Biolegend) and CD8(RPA-T8)-FITC (Biolegend) and $CD3^+CD4^-CD8^+GILG^+$ cells were sorted by FACS Melody (BD) directly into RNAlater (Ambion Inc. Applied Biosystems) and stored at -80 °C for subsequent TCRβ clonotype analysis.

Preparing TCRβ cDNA libraries for high throughput sequencing

T-cell receptor analysis was performed as described previously [119], with minor modifications. Briefly, mRNA was isolated with the RNA microkit (Qiagen) according to manufacturer's protocol. Isolated mRNA was used for cDNA synthesis with 5'RACE template switch technology to introduce a universal primer binding site, and unique molecular identifiers (UMI's) were added at the 5' end of the cDNA molecules using the SMARTScribe Reverse Transcriptase (TaKaRa). cDNA synthesis was followed by an AMPure XP bead-based clean-up (Beckman Coulter). Purified cDNA molecules were amplified in two subsequent PCR steps using the Q5® High-Fidelity DNA Polymerase (New England BioLabs), with an AMPure XP bead-based clean-up in between. PCR products were size selected on gel and purified using the Nucleospin PCR clean-up kit (Machery-Nagel). The PCR products were sequenced via Illumina MiSeq paired end using 2x250 bp sequencing.

Analysis of TCRβ clonotype analysis

Raw sequencing data was processed using the 12nt UMIs to correct for amplification biases and error-correction of reads. RTCR [224] was used to identify both the UMI sequence and clonotype information from the reads. Because of the

relatively small number of cells per sample, additional filtering steps were followed to minimize cross-sample contamination and biases introduced by errors in the UMI sequence. Sequences were only accepted if their UMI was observed in at least 40 sequencing reads. Sequences with identical UMIs in multiple samples were removed if they did not occur in at least 1000 sequencing reads and also if their absolute frequency was lower than 10% of the maximum frequency in the other sample. UMIs were clustered within each sample within a Hamming Distance of 3.

Severity of symptoms assessment

Symptom assessment was performed by self-reporting by the participants. As soon as a fever occurred, participants reported the already experienced symptoms up until then and subsequently reported the last day of each symptom until full recovery up until a maximum of timepoint three (8 weeks after start of fever). This way, the 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 of an IAV infected individual up until ten days before onset of fever were considered to be IAV infection related and included in the analysis in this paper. The duration of each symptom in days was normalized by transformation of these values to 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. Finally, to assess the overall severity of symptoms during IAV infection, the average of the Z-values of the seven symptoms was calculated.

Statistical analysis

Differences between groups (for example CMV^- compared to CMV^+) were assessed using Mann-Whitney *U* test, and comparisons within the same individuals (for example to compare time points in response upon influenza virus infection) with the Wilcoxon singed-rank test. Differences between groups in categorical variables were tested by chi-square test and corrected for multiple testing if applicable and indicated in figure legends. 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).

Table 1: Characteristics of the study population

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)	71.7±6.6	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

Differences were tested with Mann-Whitney *U* test for continues variables and chi-square test for categorical variables.

RESULTS

Characteristics of study population

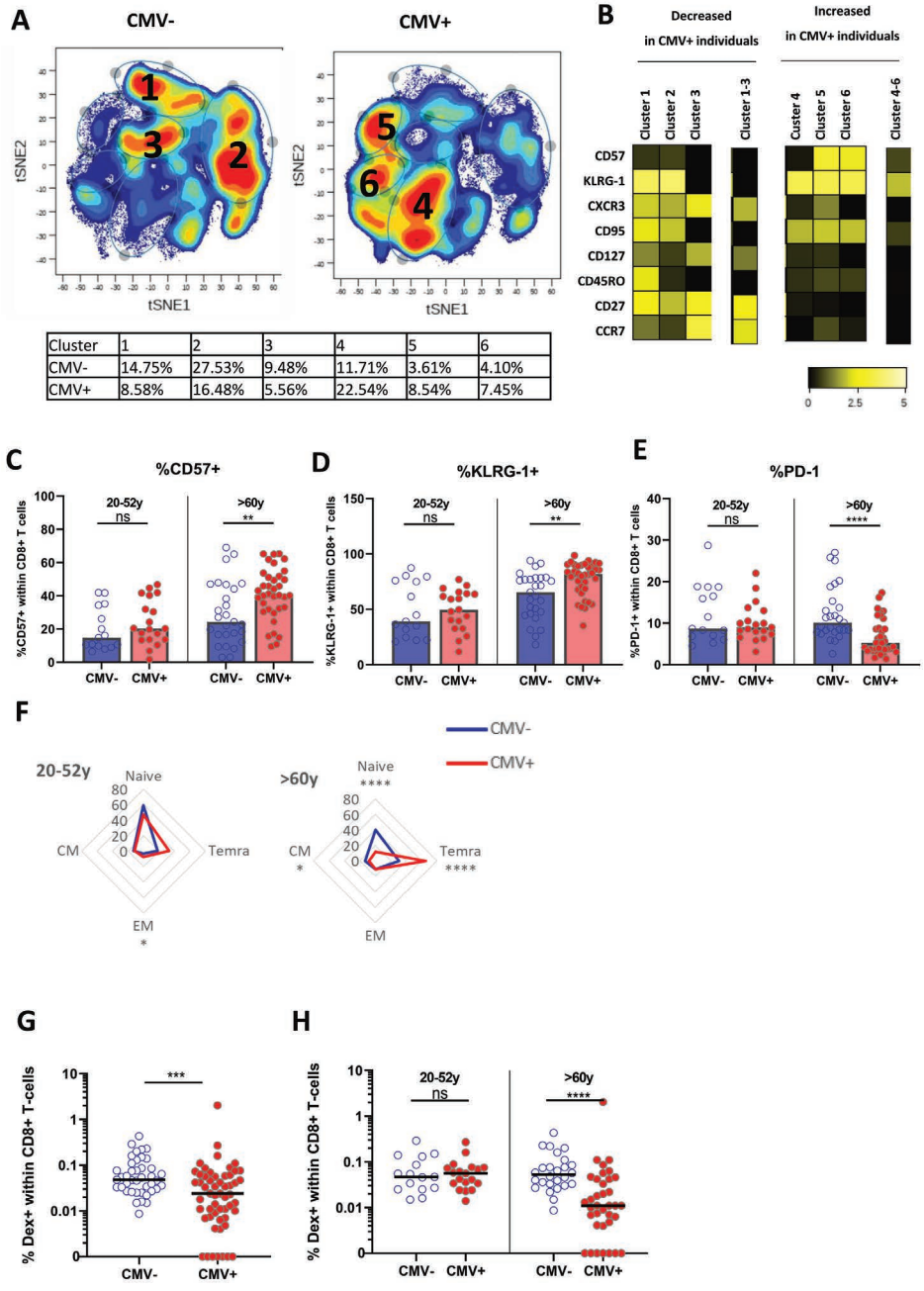
Healthy individuals were on average 59.2 years old (range 21-82 year) (n=99). They were categorized into young (21-52 years old) (n=34) and old (>60 years old) (n=65) individuals, of whom respectively 55.9% and 58.5% were CMV-infected (**Table 1**). No significant differences in age or sex were observed between CMV$^-$ and CMV$^+$ individuals (**Table 1**). In addition to healthy individuals, older adults (average 69.9 years, range 60-88 years) with confirmed IAV infection were included in this study (n=72). Also for the IAV-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=64/72), while some were infected with the H1N1 strain of influenza (n=8/72). All individuals in the H1N1 infected group turned out to be CMV$^-$.

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 [274]. Cluster analysis conformed the large differences between the CMV^- (n=40) and CMV^+ (n=56) group (Fig. 1A), and six different clusters were identified. Clusters 1-3 containing non-senescent CD27highCCR7high CD57low cells were predominantly present in CMV^- individuals (Fig. 1B, Sup. Fig. 1A). In contrast, clusters 4-6 containing the more differentiated cells expressing KLRG-$1^{high}CD57^{medium}$ were more pronounced in CMV^+ individuals (Fig. 1B, Sup. Fig. 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, but not in young, adults (Fig. 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 (Fig. 1E). In line with this, CMV infection was associated with significantly increased frequencies of TEMRA cells specifically in older individuals (Fig. 1F). Together, this indicates that CMV infection establishes large changes in the $CD8^+$ T-cell pool by inducing terminally differentiated and senescent T cells in older adults.

Frequency of IAV-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 IAV-specific T cells in healthy individuals was determined using HLA-A2 dextramers containing the matrix protein-1 GILG-epitope. The frequency of IAV-specific T cells was significantly lower in CMV^+ compared to CMV^- individuals (P=0.0005) (Fig. 1G). Importantly, this lower percentage of IAV-specific T cells was solely explained by a lower frequency in the older group, where some donors even had no detectable HLA-A2 GILG-specific T cells. Among the young adults no differences in the frequencies of IAV-specific T cells between CMV^- and CMV^+ individuals were observed (Fig. 1H). Thus, CMV infection results in lower frequencies of IAV-specific memory T cells, but only in older adults.



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: The effect of CMV on memory CD8⁺ T cells and IAV-specific CD8⁺ T cells in older adults.

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 = 96). B) Heatmap of expression of markers of t-SNE clusters. Differences between CMV⁻ and CMV⁺ individuals is tested in Supplementary Figure 1A. 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 naive and memory subpopulation in CMV⁻ and CMV⁺ individuals categorized in young (left panel) and old (right panel) adults. G) Percentage of IAV-specific CD8⁺ T cells in all CMV⁻ and CMV⁺ individuals. H) Percentage of IAV-specific CD8⁺ T cells in CMV⁻ and CMV⁺ individuals categorized in young and old individuals. Median is presented in each figure. Differences between groups were compared by Mann Whitney U test. **P < 0.01, ***P < 0.001, ****P < 0.0001. ns, non-significant.*

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

To investigate the effect of CMV infection on the T-cell response during IAV infection, we characterized the T-cell response to IAV infection during infection in older adults (N=72), i.e. within 72 hours after start of fever, and 2 and 8 weeks later. The frequency of IAV-specific CD8⁺ T cells was determined by dextramer staining for the HLA-A2 GILG-epitope. IAV-specific CD8⁺ T-cell frequencies were increased upon IAV 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 (8 weeks post infection, median of 0.08/total CD8⁺ T-cells) (Fig. 2A). The increase in IAV-specific CD8⁺ T-cell frequencies was mainly explained by the expansion of IAV-specific effector memory T cells (TEM) (Fig. 2B), while the frequencies in the other subsets (i.e. naïve (TN), central memory (TCM) and TEMRA) 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 (Fig. 2C). This was true for both H3N2 influenza-infected and H1N1 influenza-infected individuals, and no difference in increase in the IFNγ response between the two strains was observed (data not shown). When the IAV specific IFNγ response was calculated as percentage of CD3⁺ T cells instead of PBMCs, the IAV-specific IFNγ response also tended to be higher in response to IAV infection (2 weeks) compared to steady state (8 weeks), albeit not significant (p=0.059) (Sup. Fig. 2A left panel)

T-cell response to IAV infection in older adults is not impaired by CMV-infection

To investigate whether the T-cell response during IAV infection (N=72) is influenced by CMV infection, we analyzed CMV⁺ and CMV⁻ individuals separately. No significant association of CMV-seropositivity on IAV-specific CD8⁺ T-cell percentages or on the percentage of TEM cells among the IAV-specific T cells was observed (Fig. 2D,E). Likewise, IAV-specific IFNγ responses were not negatively associated with CMV-seropositivity (Fig. 2F) or with the level of CMV-specific antibodies in CMV⁺ individuals at any of the time points (Sup. Fig. 2B). Surprisingly, in the acute phase (<72 hours after fever onset), CMV⁺ individuals even showed a significantly higher IAV-specific IFNγ T-cell response than CMV⁻ individuals (P=0.013) (Fig. 2F).

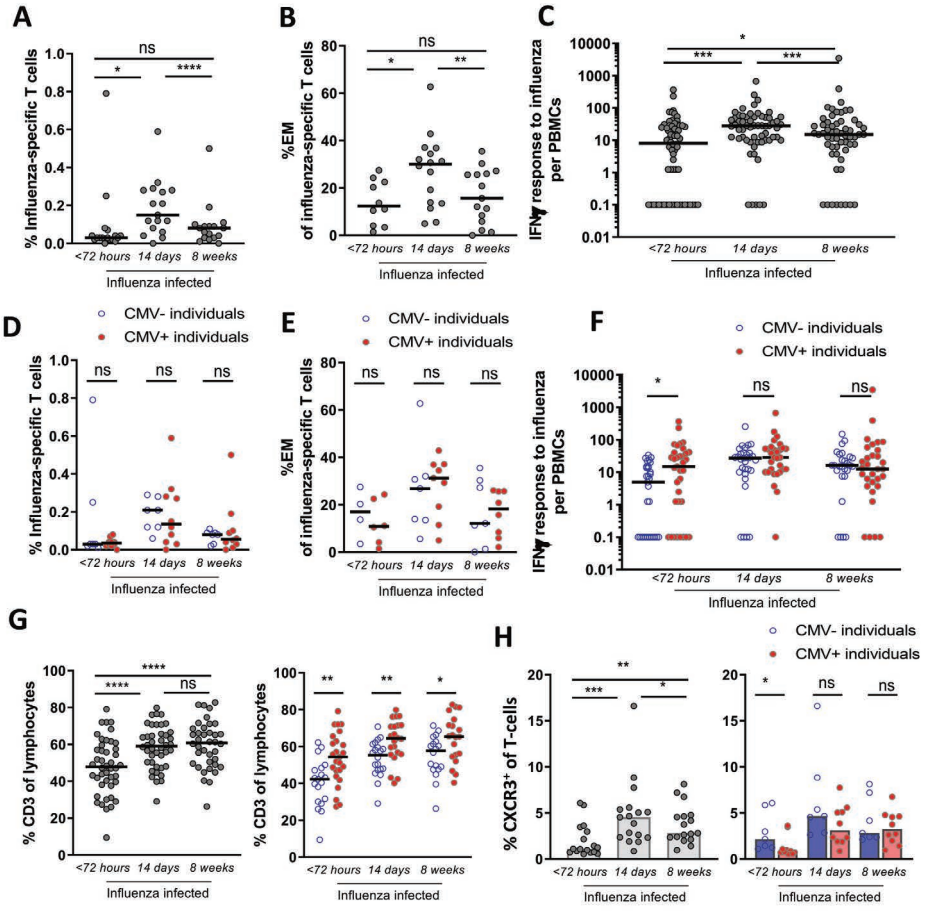


Figure 2: IAV-specific CD8⁺ T-cell response after infection shows no impairment by CMV seropositivity, but enhancement of the early IAV-specific CD8⁺ T-cell response.
*A) Percentage of IAV-specific CD8⁺ T cells upon influenza infection. Dextramer for matrix protein-1 GILG-epitope was used (B) The percentage effector memory (EM) cells of IAV-specific CD8⁺ T cells upon influenza infection. All HLA-A2-positive influenza-infected individuals (n =36) were stained for dextramers; IAV-specific T cells were detected at least at one time point in 17 individuals. C) IAV-specific IFNγ T-cell response upon influenza infection. D) Percentage of IAV-specific T cells upon influenza infection for CMV- and CMV⁺ individuals. E) The percentage effector memory (EM) cells of IAV-specific T cells upon influenza virus infection for CMV⁺ and CMV- individuals. F) IAV-specific IFNγ T-cell response upon influenza infection for CMV⁻ and CMV⁺ individuals. G) Frequencies of CD3⁺ T cells in the blood of IAV-infected individuals at < 72 hours, 2 weeks and 8 weeks after infection (left panel) and in CMV⁻ and CMV⁺ individuals (right panel). H) Percentage of CXCR3⁺ within total CD8⁺ T cells upon infection (left panel) and in CMV⁻ and CMV⁺ individuals (right panel). 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 1 x 106 PBMCs. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. ns, non-significant.*

Also when corrected for the percentage of T cells among all lymphocytes, the same trend was observed (Sup. Fig. 2A, right panel). This higher IAV-specific IFNγ T-cell response in CMV^+ individuals could not be explained by the moment of sampling within the 72 hours after fever onset (data not shown).

A possible explanation for the higher IAV-specific IFNγ T-cell response in CMV^+ individuals might be related to differences in T-cell migration. We therefore also investigated the total frequency of T cells in the blood and their expression of activation and migration markers upon IAV infection in CMV^- and CMV^+ individuals. The frequency of total $CD3^+$ T cells in the blood of influenza-infected individuals was significantly lower early after infection compared to 2 or 8 weeks later (Fig. 2G left panel). Comparing CMV^+ individuals with CMV^- individuals, the $CD3^+$ T-cell frequency was also consistently lower in CMV^- individuals (Fig. 2G right panel). Next, the migration of $CD3^+$ T cells outside the blood, early after influenza infection, was further assessed by comparing the relative decrease within CMV^- and CMV^+ individuals. The drop in $CD3^+$ T-cell frequency early after IAV infection was significantly larger in CMV^- than in CMV^+ individuals (Sup. Fig. 3A). Thus, the higher IFNγ IAV-specific responses in blood in CMV^+ individuals early after influenza virus infection (Fig. 2F) may at least partly be explained by higher frequencies of total T cells in CMV^+ as compared to CMV^- individuals. The higher frequencies of total T cells may be a result of less migration of T cells. Investigating migratory capacity of T cells, we indeed found that early after IAV infection CXCR3 expression by $CD8^+$ T cells, which is associated with migratory capacity of these cells to the lungs, was lower in CMV^+ individuals compared to CMV^- individuals (Fig. 2H). These data suggest that T cells in CMV^+ individuals may be less prone to be recruited to the lungs early after IAV infection and thereby accumulate in the blood leading to higher frequencies. However, on IAV-specific $CD8^+$ T cells, only a small trend of increased CXCR3 expression on ($p=0.11$) was observed in CMV^- compared to CMV^+ individuals (Sup. Fig. 3B) early after IAV infection. In addition, the activation status of IAV-specific T cells by the frequencies of $HLA\text{-}DR^+CD38^+$ or $CD127^-KLRG\text{-}1^+$ revealed no difference between CMV^- and CMV^+ individuals. IAV-specific T cells from CMV^- and CMV^+ individuals both showed increased expression of these activation markers early after IAV infection (<72 hours after start fever) as compared to 2 and 8 weeks later, irrespective of CMV serostatus. This pattern was not seen for CMV-specific T cells, which served as a control. This suggests that activation and rapid expansion of IAV-specific memory T cells early after infection occurs irrespective of CMV-serostatus (Sup. Fig. 3C). Overall, these data suggest that migration of T cells to the lungs might play a role in the enhanced

IAV-specific IFNγ response observed in CMV⁺ individuals early after IAV infection (Fig. 2F) although evidence remains circumstantial.

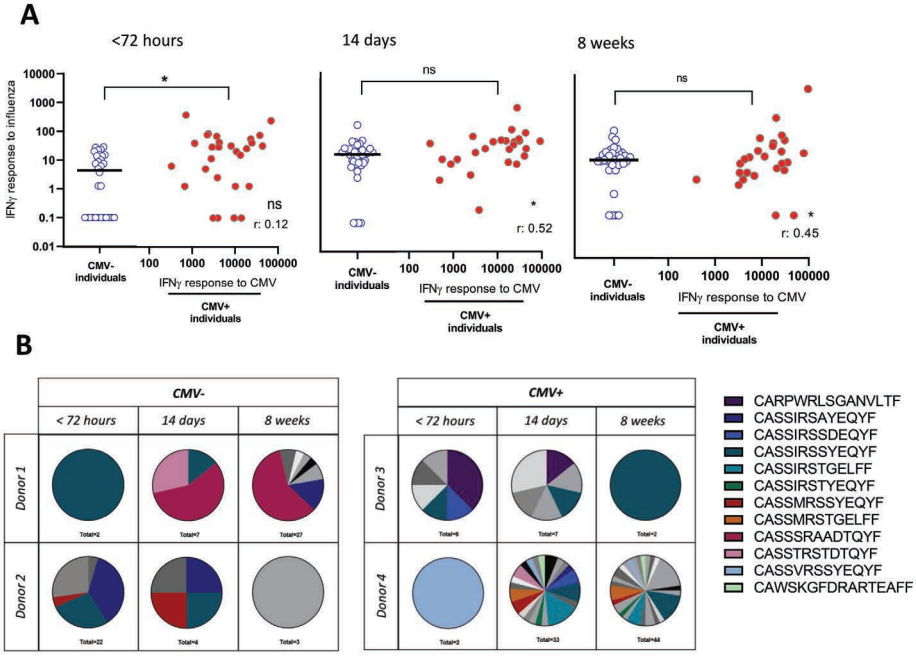


Figure 3: No reduced IAV-specific T-cell response in number of T cells or clonal diversity due to large expansions of CMV-specific T cells.
*A) IAV-specific IFNγ Tcell response in CMV⁻ and CMV⁺ individuals differentiated on the CMV-specific T-cell response. IAV-specific IFNγ T-cell responses are depicted 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. 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 1 x 106 PBMCs. B) T-cell repertoire of sorted HLA-A2-GILG–specific CD8⁺ T cells, detected by PCR of two CMV⁻ and two CMV⁺ individuals. Each pie-chart depicts the repertoire of a donor at a certain timepoint (< 72hours, 14 days or 8 weeks after infection). Colors represent shared CDR3 sequences between timepoints and donors. Grey scales depict unique CDR3 sequences. *P < 0.05. ns, non-significant.*

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

As CMV might only affect the immune system through competition for "limited immunological space" in individuals with large CMV-specific T-cell responses, we next investigated the association between CMV infection and the T-cell response to IAV by taking into account the magnitude of the IFNγ CMV-specific T-cell responses within CMV⁺ individuals. First, we assessed the IFNγ T-cell response to IAV infection.

No negative correlation between expanded CMV-specific T-cell responses and T-cell response to IAV infection was observed at any of the three time points (Fig. 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 IAV-specific IFNγ T-cell response at 2 and 8 weeks after fever onset (Fig. 3A) (R: 0.52, p=0.016 and R:0.45, p=0.014 respectively. In addition, we further assessed the quality of the IAV-specific T-cell response by investigating TCR diversity of the IAV-specific T-cell response, by sorting the HLA-A2-GILG-specific T cells and sequencing the TCRβ chain. The clonotype distribution was analyzed over time after IAV infection in two CMV^- and two CMV^+ individuals. In general it was observed that 89% of the different TCR sequences expressed the Vβ-19 segment, of which 74% consisted of the highly conserved -RS-motif (See Sup. Table 1A,B for the V and J segments). Interestingly, all donors showed a shift in dominance after IAV infection, however large heterogeneity was observed between the donors with some repertoires becoming more diverse and other less diverse over time. No indication for CMV-related differences were present, as no differences between the CMV^- and CMV^+ individuals were observed. Based on these data no differences were observed in the TCR diversity of the IAV-specific T-cell repertoire between CMV^- and CMV^+ individuals (Fig. 3B). These lines of evidence do not support the competition between CMV-specific memory T cells and a proper IAV-specific T cells during IAV infection.

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

As it is suggested that CMV is linked to "inflammaging", we questioned whether pro-inflammatory mediators may be enhanced in CMV^+ individuals during IAV-infection. We investigated the levels of pro-inflammatory cytokines in serum of CMV^+ and CMV^- individuals, and their potential association with the IAV-specific T-cell response. At the early phase of IAV infection, the inflammation-associated factors IL-6 and CRP were elevated in serum compared to 2 and 8 weeks later (Fig. 4A).

No significant differences in IL-6 and CRP levels were observed between CMV^+ and CMV^- individuals, also at the peak of the T-cell response (2 weeks later) and at steady state (week 8 after fever onset) (Fig. 4A). We also measured the IFNγ:IL-10 ratio, as it was suggested that a shift in this ratio leads to a decline in IAV-specific T-cell responses with age and is associated with decreased protection against IAV [26, 275]. No difference was observed (Fig. 4B) in the IFNγ:IL-10 ratio between CMV^+ and CMV^- individuals at any of the time points, even though both IFNγ and IL-10 levels were elevated at the earliest timepoint (Sup. Fig. 4).

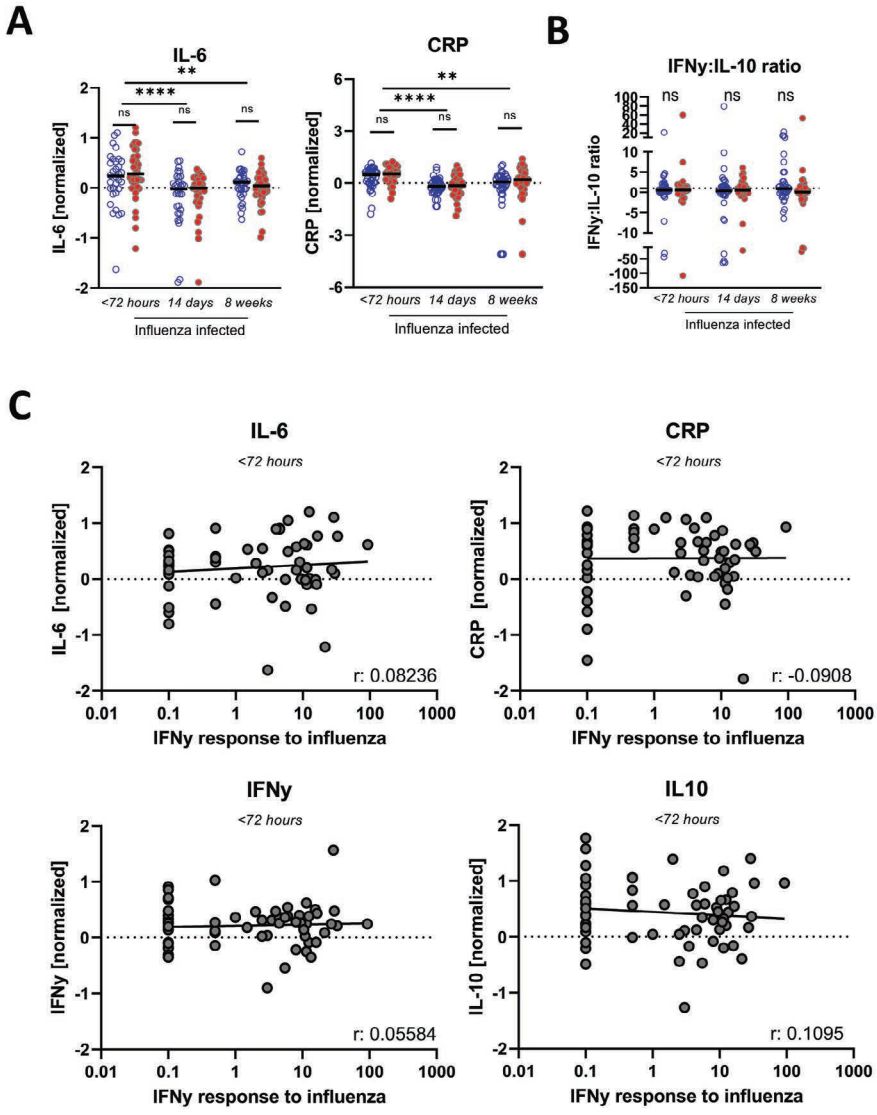


Figure 4: No sign of increased inflammation in CMV⁺ individuals based on cytokines levels in serum nor in relation to the IAV-specific T-cell response.
*A) Serum levels of "inflammaging markers" CRP and IL-6 upon influenza infection for CMV⁺ and CMV⁻ individuals at < 72 hours after fever onset, and 2 and 8 weeks later. B) The IFNγ:IL-10 ratio for CMV⁻ and CMV⁺ individuals upon influenza infection. C) Magnitude of the IAV-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. Correlations between IAV-specific IFNγ response and the different cytokines levels in serum were assessed by Spearman correlation. T-cell IFNγ responses are presented per 1 x 106 PBMCs. **P < 0.01, ****P < 0.0001. ns, non-significant.*

Although increased levels of the cytokines IL-6, CRP, IFNγ and IL-10 were observed in the acute phase of IAV infection, no significant association between these cytokine levels and the height of the IAV-specific T-cell response was observed <72 hours after start of fever (Fig. 4C) or 2 and 8 weeks later (data not shown). Together, this suggests that CMV infection in older adults does not affect cytokine levels in serum upon IAV infection.

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

Since early T-cell responses during IAV infection play an important role in limiting disease severity, we also investigated whether the height of the IAV-specific T-cell response was associated with severity of symptoms of IAV infection. In IAV-infected older adults, the number and duration of the following symptoms was assessed: fever (≥37.8 °C), cough, sore throat, runny nose, headache, pain while breathing and muscle pain. The severity of symptoms of IAV infection was positively associated with the height of the IAV-specific T-cell response at timepoint 3 (8 weeks after start of fever) (Fig. 5A), but not within 72 hours or 2 weeks after start of fever. This association was mainly based on the number of symptoms, and not on their duration (Sup. Fig. 5). Thus, the IAV-specific T-cell response 8 weeks after IAV infection, and not the early T-cell response, was linked to the severity of symptoms of the IAV infection in this cohort.

We next investigated whether CMV-infection was associated with more severe symptoms during IAV infection. No significant differences between CMV^- and CMV^+ individuals were observed in the number of symptoms during IAV infection (Fig. 5B), total duration of symptoms (Fig. 5C), or severity of symptoms as assessed by a combination of duration and number of symptoms (Fig. 5D). When investigating the IAV infection symptoms individually, CMV^+ individuals tend to suffer less from muscle pain (Fig. 5E) and although frequency of coughing was not different between CMV^+ and CMV^-, CMV^+ individuals tended to suffer less long from coughing as a symptom compared to CMV^- individuals (data not shown). When the frequency of individuals coughing was plotted over time, we indeed found an indication for a faster decline in the frequency of coughing individuals amongst CMV^+ individuals compared to CMV^- individuals (Fig. 5F). Of note, both the increased frequency of muscle pain and the longer persistence of coughing among CMV^- individuals could not be ascribed to a difference in IAV strain infection between CMV^- and CMV^+ individuals (data not shown). The severity of symptoms was also not associated with CMV-specific antibody levels (data not shown). Together, this suggests that CMV infection at least does not worsen the number and duration of IAV infection symptoms.

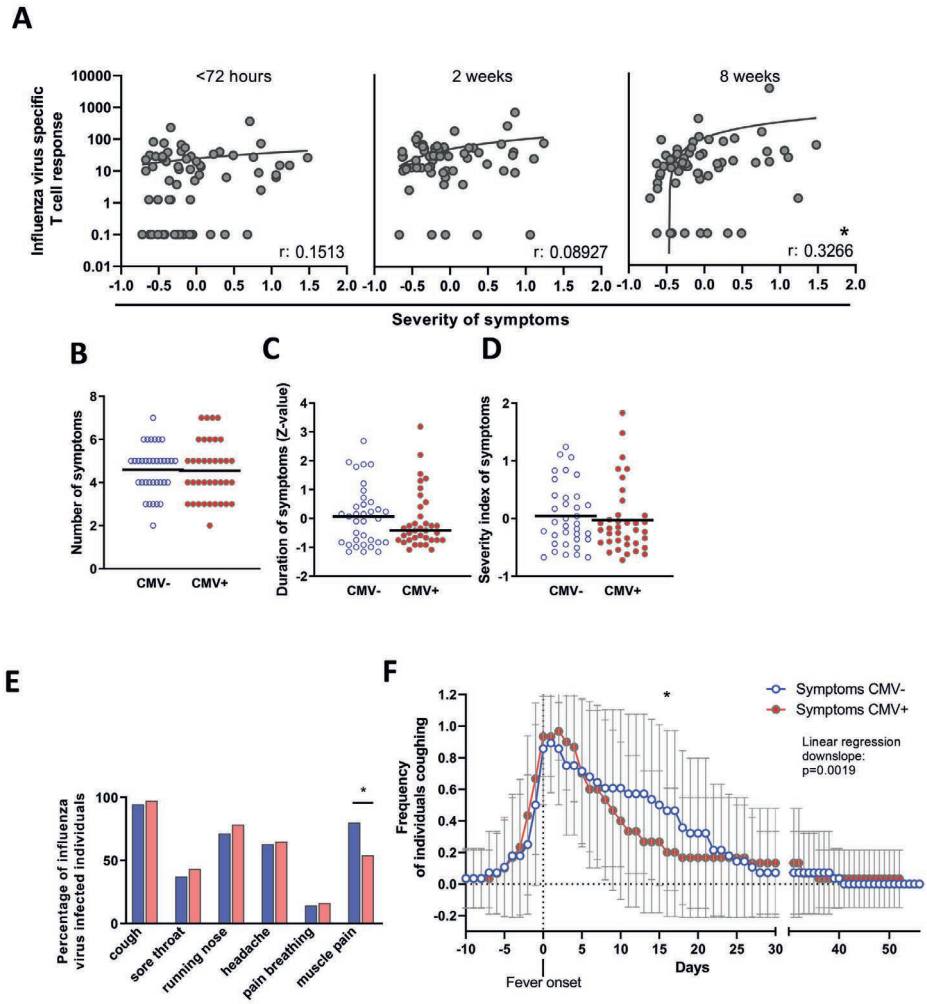


Figure 5: No negative effect of CMV-infection on number and duration of symptoms of influenza virus infection in older adults.

*A) IAV-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. Statistical differences between CMV- and CMV+ were tested by csquare 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 one of the six symptoms. Statistical differences between CMV- and CMV+ were tested by csquare test and corrected for multiple testing by Bonferroni correction. 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. *P < 0.05.*

DISCUSSION

In this study we studied the impact of CMV infection on the immune response to IAV. We found that CMV infection is associated with a more differentiated and senescent phenotype of $CD8^+$ T cells. In healthy younger individuals, no difference in IAV-specific T-cell frequencies were observed, but, CMV^+ older individuals had lower frequencies of IAV-specific memory $CD8^+$ T cells compared to CMV^- older individuals. Nevertheless, the induction of an IAV-specific T-cell response during active IAV infection in older adults was not impaired. Also, severity of IAV-associated symptoms was not negatively affected by CMV infection. We did not find any evidence for a negative effect of CMV infection on the IAV immune response by 'limited immunological space' or by increased levels of pro-inflammatory mediators. In contrast, there seemed to be a positive association between CMV infection and the IAV-specific T-cell response early after IAV infection (<72 hours after fever onset).

To the best of our knowledge, we are the first to investigate the potential effect of CMV infection on the T-cell response to a heterologous infection in humans. 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 in healthy individuals, by increasing the number of highly differentiated memory cells, especially in older adults [35]. We show that CMV infection leads to a decrease in the frequency of memory IAV-specific T cells in healthy older adults. Relatively low frequencies of IAV-specific T cells in older adults compared to younger adults have been reported before [39, 144] and are considered to be a key determinant of a diminished T cell response in IAV infection [143]. Note, that the observed frequencies and characteristics of the IAV-specific response are based on one HLA-A2 epitope (GILGFVFTL). Although this epitope is immunodominant and conserved between most influenza strains, it remains to be determined whether it reflects the total IAV-specific T-cell response.

Unfortunately, absolute T-cell numbers could not be investigated in this study, leaving the possibility that the decreased frequency of IAV-specific T cells merely reflected a relative increase in CMV-specific T cells in older adults. A decline in the frequency -and not in the number- of IAV-specific T cells may explain why decreased IAV-specific T-cell frequencies in healthy older adults do not seem to result in reduced T-cell responses upon acute influenza virus infection. This was also observed in the MCMV mouse model, were reduced frequencies were

observed, but the absolute counts of $CD8^+$ T cell against an heterologous virus infection were maintained in MCMV infected mice [276]. Alternatively, the number of IAV-specific T cells may have been lower in CMV^+ older adults. A similar effect has previously been reported for EBV-specific T cells [165]. During IAV infection, we observed that IAV-specific T cells in CMV^+ and CMV^- individuals responded equally well and no substantial changes in IAV-specific T-cell repertoire were observed in CMV^- and CMV^+ individuals. Previously, to influenza vaccination, other studies reported impaired IAV-specific T-cell responses by CMV infection [252, 277, 278]. In contrast, other groups showed a positive effect of CMV on the vaccine-induced IAV-specific T-cell response in humans [279]. In addition, in mice, MCMV infection was shown to enhance the diversity of the T-cell repertoire against Listeria monocytogenes expressing OVA [183], whereas we found no differences in the repertoire diversity between CMV^- and CMV^+ individuals. This could probably be explained by the small sample size of our repertoire analyses, or by the difference in epitope, as the HLA-A2-GILG specific T-cell repertoire is suggested to be more restricted compared to other antigen-specific repertoires [172]. We also found that the magnitude of the CMV-specific T-cell response was positively associated with the magnitude of the IAV-specific T-cell response 2 weeks and 8 weeks after fever onset of IAV infection. Together, this suggests that CMV^+ individuals have a sufficient amount of IAV-specific T cells able to respond to IAV infection.

Pre-existing T cells and timing of the IAV-specific $CD8^+$ T-cell response are thought to play an important role in the reduction of severity of IAV-related symptoms [29, 30]. An early T-cell response has been proposed to accelerate viral clearance, whereas a delayed and prolonged T-cell response may lead to high and prolonged levels of inflammation and increased severity of disease [30, 259]. 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 IAV-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 of symptoms, we found a significant positive correlation between the magnitude of the IAV-specific T-cell response and severity of symptoms 8 weeks after fever onset, and not early after fever onset or 2 weeks later. This suggests that a prolonged T-cell response, still present 8 weeks after fever onset, is increasing symptom severity of IAV infection.

As CMV infection was suggested to induce a more inflammatory environment [280], we also studied the potential association between CMV and inflammatory

markers and how this might be related to IAV-specific T-cell responses. Although the levels of pro-inflammatory cytokines and chemokines measured here were significantly increased in the acute phase of IAV infection (<72 hours after fever onset), they were similar between CMV^+ and CMV^- individuals. Since pro-inflammatory responses were similar between CMV^+ and CMV^- individuals, it may (partly) explain the lack of difference in the severity of clinical symptoms between these groups. Furthermore, these levels did not seem to be associated with the IAV-specific T-cell response at any time point, although, we cannot rule out the possibility that other immune modulatory factors may play a role.

Most studies claiming a negative effect of CMV on an immune response to a heterologous challenge have been performed in mice. Several mouse studies have shown that only lifelong infection with MCMV leads to decreased immunity against heterologous infections [203, 204, 220]. 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. 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 IAV-specific T-cell response. One of the reasons for the observed differences between mice and men, could be the order of infections by CMV and IAV. 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 IAV infection before they were infected with CMV, which may lead to the presence of IAV-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 IAV. 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 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.

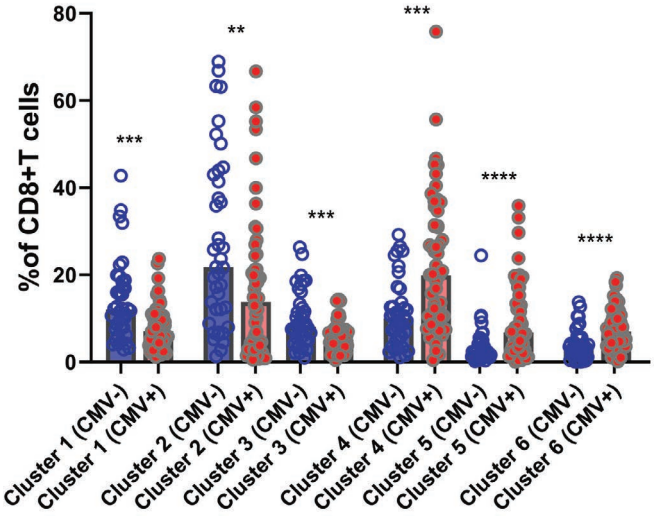
The increased IFNγ T-cell response to IAV infection that we observed in CMV^+ older individuals remains partially unexplained. Previously, enhanced influenza vaccine responses in humans and mice were explained by an increase in IFNγ in serum [243]. However, we did not observe a difference in IFNγ levels in serum between CMV^+ and CMV^- individuals in our study. However, an important difference between our study and the study by Furman *et al.* is the age of the individuals,

as the latter observed this increase in IFNγ levels mainly in younger adults. As the positive effect of CMV was observed in IAV IFNγ ELISpot assays, we cannot exclude the possibility that CMV infection may only affect $CD4^+$ T cells, which may respond in the assay as well. Another explanation for the difference in the early IAV-specific T-cell response between CMV^- and CMV^+ individuals could be related to the migratory capacity of the responding T cells, as we observed a trend towards enhanced CXCR3 expression of IAV-specific $CD8^+$ T cells in CMV- individuals. This may lead to early migration towards tissues such as the lungs. The effect of CMV on IAV-specific T-cell responses at the site of infection instead of the blood would be of great interest, and requires further research. Also, other factors like apoptosis or proliferation of the IAV-specific T cells may play a role in the difference in IAV-specific T-cell response between CMV- and CMV^+ individuals.

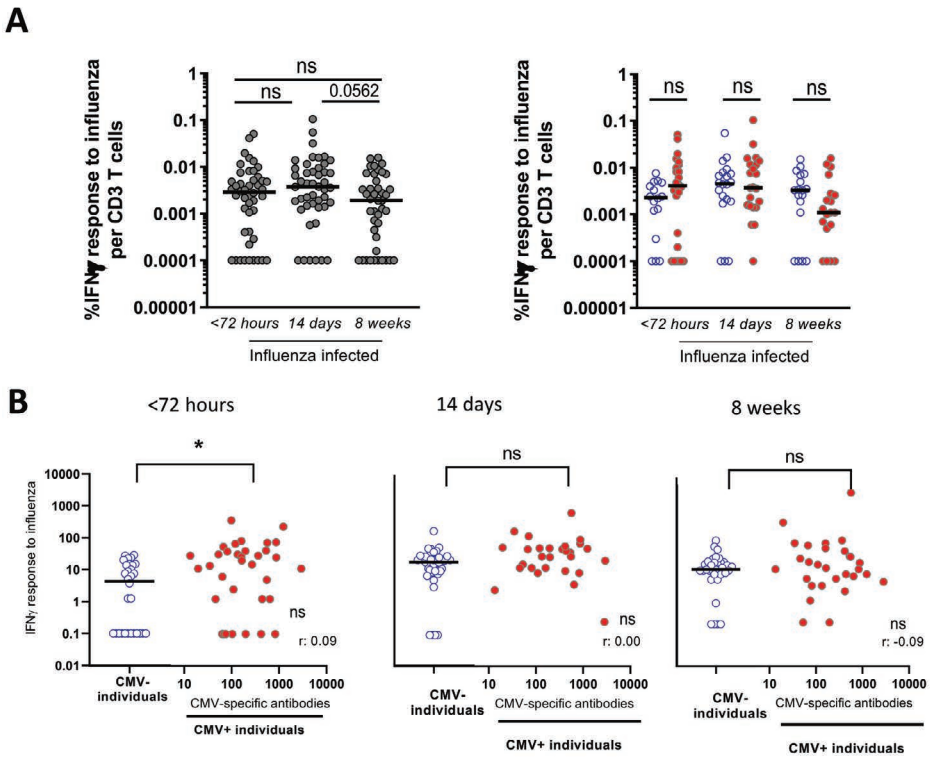
In conclusion, identification of the driving forces that induce age-related changes in the immune system is important to protect the growing population of older adults against infectious diseases. Especially IAV leads to more increased disease burden in older adults, e.g. severe symptoms and higher risk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

SUPPLEMENTALS

The Supplementary Tables with the TCR sequences are available online at: **https://www.frontiersin.org/articles/10.3389/fimmu.2021.663664/full#supplementary-material**

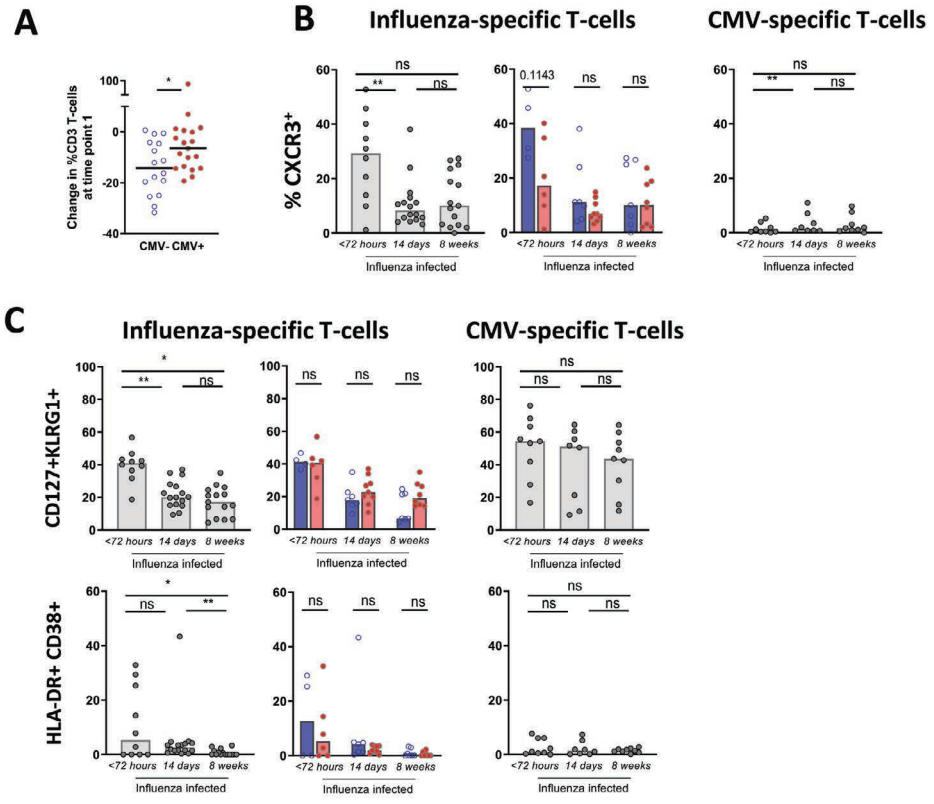


Supplementary Figure 1
*Percentage of CD8⁺ T cells per t-SNE cluster in CMV⁻ and CMV⁺ individuals. Percentage of CD8⁺ T cells per t-SNE cluster in CMV- (blue bars; N=40) and CMV⁺ (red bars; N=57) individuals based on MFI of CD57, KLRG-1, CXCR3, CD95, CD127, CD45RO, CD27 and CCR7. Data shown as median. Differences between groups were compared by Mann Whitney. Stars indicate statistical differences, e.g. * P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.*



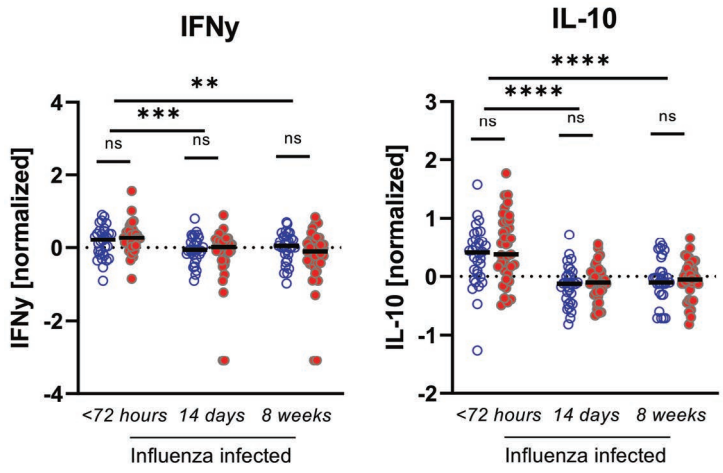
Supplementary Figure 2

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.



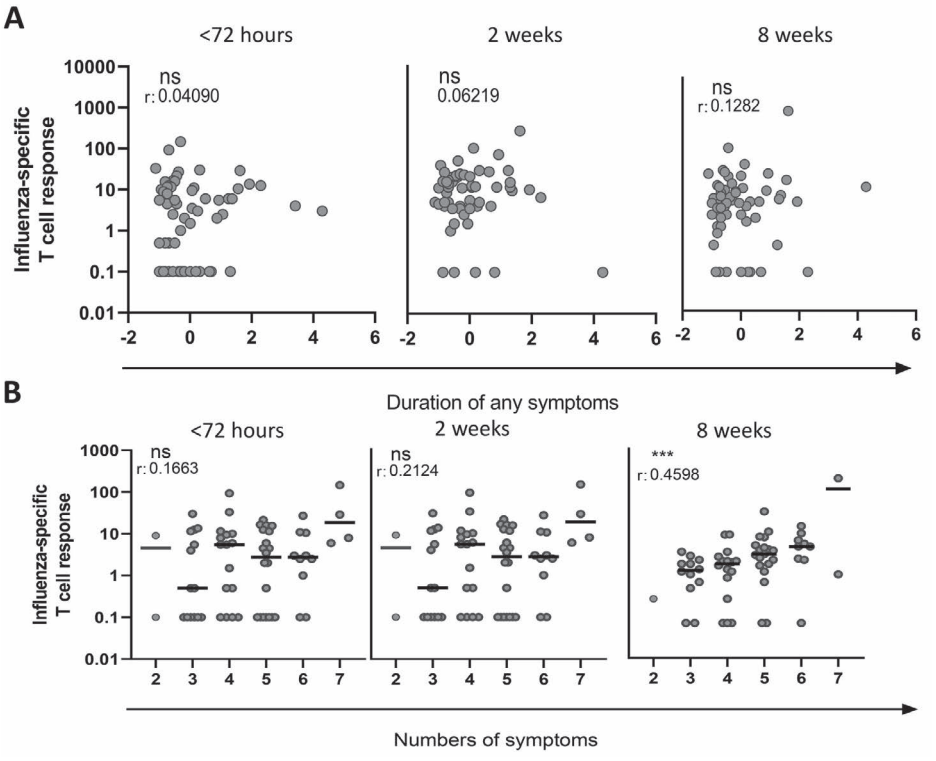
Supplementary Figure 3

Expression of activation and migration markers in T cells during influenza virus infection. A) The change in percentage of CD3⁺ T cells at time point 1 compared to time point 2 in CMV- and CMV⁺ individuals. B) 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). C) 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. 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.

Longitudinal characterization of the mumps-specific HLA-A2 restricted T-cell response after mumps virus infection

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ABSTRACT

Waning of the mumps virus (MuV)-specific humoral response after vaccination has been suggested as a cause for recent mumps outbreaks in vaccinated young adults, although it cannot explain all cases. Also $CD8^+$ T cells may play an important role in the response against MuV, however little is known about the characteristics and dynamics of the MuV-specific $CD8^+$ T-cell response after MuV-infection. Here we had the opportunity to follow the $CD8^+$ T-cell response to 3 recently identified HLA-A2*02:01-restricted MuV-specific epitopes from 1.5 till 36 months post MuV-infection in five previously vaccinated and three unvaccinated individuals. The infection-induced $CD8^+$ T-cell response was dominated by T cells specific for the ALDQTDIRV and LLDSSTTRV epitopes, while the response to the GLMEGQIVSV epitope was subdominant. MuV-specific $CD8^+$ T-cell frequencies in the blood declined between 1.5 and 9 months after infection. This decline was not explained by changes in the expression of inhibitory receptors or homing markers. Despite the ongoing changes in the frequencies and phenotype of MuV-specific $CD8^+$ T cells, TCRβ analyses revealed a stable MuV-specific T-cell repertoire over time. These insights in the maintenance of the cellular response against mumps may provide hallmarks for optimizing vaccination strategies towards long-term cellular memory response.

INTRODUCTION

Mumps is a viral infectious disease typically characterized by bilateral or unilateral swelling of the parotid glands. In some individuals, more severe complications such as orchitis, deafness, meningitis and encephalitis occur [281]. Therefore, many countries vaccinate their population against mumps, usually as a combination vaccine together with measles and rubella vaccine components (MMR-vaccine) [282, 283]. This has led to a dramatic decrease in the incidence of mumps virus (MuV) infection [282]. However, in the last decades, mumps outbreaks have been reported in various countries, despite high vaccination coverage [284, 285]. In the Netherlands, several mumps outbreaks occurred between 2009 and 2013, mainly among young adults, most of which did receive their two childhood MMR-vaccinations [286].

Just as in natural infection, both humoral and cellular responses are induced after MuV-vaccination [287, 288]. Humoral responses have been investigated extensively, and one of the causes of vaccine failure in young adults is thought to be waning of antibody levels, although it may not explain all cases [283]. Another explanation for re-emergence of mumps could be the antigenic mismatch between the vaccine and outbreak strains [289, 290]. The current Jeryl Lynn vaccine carries two viral isolates (JL-2 and JL-5) of the genotype A, whereas genotype G is the most recently circulating infectious mumps strain in the Netherlands [291, 292]. These genotypes have a relatively large phylogenetic distance with known antigenic differences [293, 294]. $CD8^+$ T cells are known to play an important role in the clearance of viruses and in disease outcome, and generally recognize the more con-served parts of a virus. Despite some mismatch epitopes between the vaccine and circulating MuV strains [295], many $CD8^+$ epitopes are conserved, and may therefore play an important role in the prevention of vaccine failure, despite antigen mismatch. However, current insights into the MuV-specific cellular response are limited.

Studies focusing on the T-cell response against mumps have mostly investigated the response after MuV-vaccination, however it is generally observed that vaccination-acquired immunity is not as efficient as the long term response induced after a natural infection [16, 34, 296]. This is also the case for the T-cell responses to MuV-vaccination and MuV-infection. Although MuV-specific T-cell proliferation and IFNγ production were detectable up to 21 years after vaccination, these responses were less pronounced than in naturally-infected individuals [287, 297, 298]. It was also reported that MuV-specific $CD8^+$ T cells after vaccination are less polyfunctional compared to those in naturally-infected individuals

[299]. Together these studies suggest that current MuV-vaccination evokes a less optimal cellular immune response compared to natural MuV-infection. One way to pre-vent MuV-vaccine failure, could be to induce a sustainable T-cell response more comparable to the response against natural infection. To this end, more knowledge is needed about the clonal dynamics and characteristics of the MuV-specific T cells in time after natural MuV-infection.

In this study we had the unique opportunity to investigate in great detail the MuV-specific $CD8^+$ T-cell response after natural MuV-infection in 5 previously vaccinated and 3 unvaccinated individuals. Our group recently identified HLA-A*02:01-restricted MuV-specific T-cell epitopes from the recent outbreak strain (genotype G) [300]. Here, we ex vivo analyzed MuV-specific $CD8^+$ T cells in mumps cases after infection, using recently developed dextramers with which high frequencies of MuV-specific $CD8^+$ T cells could be detected. We show that relatively large MuV-specific T-cell frequencies in the blood contracted significantly between 1.5 and 9 months after infection, to gradually further decline to low frequencies up to 36 months. Although we observed changes in both PD-1 and CXCR4 ex-pression between 1.5 and 9 months post infection, the expression of these T-cell inhibitory and homing markers 1.5 months post-infection did not explain the decline in MuV-specific T-cell frequencies in the convalescence phase. The largest fraction of cells had a central memory phenotype ($CD27^+$, $CD45RO^+$) that remained stable over time, whereas a shift was found to memory precursor cells based on CD127 and KLRG-1 over time after infection. Despite the observed changes, a relatively stable T-cell receptor repertoire in both vaccinated and unvaccinated individuals was found, indicating a sustainable T-cell response has been maintained at the long run.

MATERIALS AND METHODS

Study design

Samples were collected from mumps cases at 1.5 months, 9 months, 18 months and 36 months after infection, in two Dutch observational longitudinal studies, VAC263 [288] and IMMfact [301]. Written informed consent was obtained from all participants. All trial-related activities were conducted according to Good Clinical Practice, which includes the provisions of the Declaration of Helsinki. The studies were approved by the ethical committee METC Noord Holland and Review Board METC UMC Utrecht, respectively (clinical study numbers NL37852.094.11 and NL4679.094.13). Eight HLA-A2 positive mumps cases (21-53 years of age) were selected based on serotyping. Of these 8 subjects, 3 subjects were unvaccinated,

while the other 5 had been vaccinated with two doses of the MMR vaccine during childhood. IgG titers were adopted from the study of Kaaijk et al. [288].

PBMC isolation

Peripheral blood mononuclear cells (PBMCs) 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.

Analysis of MuV-specific T cells by flowcytometry

In the 8 HLA-A2 positive mumps cases, MuV-epitope-specific T-cell responses were analyzed by staining 4 million PBMC's in FACS buffer (2mM EDTA, 0.5% BSA in PBS) using HLA-class I dextramers for the epitopes ALDQTDIRV (ALD) of the M-protein (A*02:01/ ALD-FITC), GLMEGQIVSV (GLM) of the F-protein (A*02:01/ GLM-APC) and LLDSSTTRV (LLD) of the HN-protein (A*02:01/ LLD-PE), for 20 minutes at room temperature. Next, surface staining was performed in FACS buffer for 30 minutes at 4°C, using the following monoclonal antibody (mAb) mix: CD27(M-T271)-PerCP-Cy5.5, CD3(SK7)-APC-R700, CCR7(150503)-BrilliantViolet711, CD45RO(UCHL1)-BrilliantUV395, CD4(SK3)-BrilliantUV737, CD183/CXCR3(1C6)-BrilliantViolet421 (all BD), CD8a(RPA-T8)-BV510 (BioLegend), CD127(A019D5)-BrilliantViolet650 (BioLegend), CD184/CXCR4(12G5)-BrilliantViolet786 (BD), Fixable Viability staining-780 and KLRG1(13F12F2)-PE-Cyanine7 (eBioscience). Ac-quisition was performed on a LSRFortessaX20 and data analysis was performed using FlowJo (Treestar). Populations of antigen-specific cells that were smaller than 30 events were excluded from further analysis. Gating strategy is shown in Sup. Fig. 4.

Isolation of MuV-specific T cells for TCRβ analysis

For cell sorting, cells were stained using dextramers and the following mAb mix: CD3(SK7)-APC-R700, CD4(SK3)-BriliantViolet711 (Both BD) and CD8a(RPA-T8)-BrilliantViolet510 (Biolegend). $CD3^+CD4^-CD8^+dextramer^+$ cells were sorted on a FACSAria III, directly into fetal calf serum (FCS) pre-coated tubes containing RNAlater (Ambion Inc. Applied Biosystems) and stored at -80 degrees for subsequent TCRβ clonotype analysis. In addition, we stained with the following mAbs for further phenotypical analysis of the $dextramer^+$ T cells: CD152/CTLA4(BNI3)-BrilliantViolet786, Fixable Viability staining-780 (both BD), PD1(EH12.2H7)-PerCP Cy5.5, Tim3(F38-2E2)-BrilantViolet421, (both BioLegend), and TIGIT(MBSA43)-PE Cyanine7 (eBioscience). Due to limited amounts of PBMCs, the phenotypic analyses of the MuV-specific T cells was combined with cell sorting for 3 individuals (i.e. subject 12 of the vaccinated individuals and subjects 02 and

10 of the unvaccinated individuals). This led to a less extensive phenotyping, including CD27(M-T271)-PerCP Cy5.5, CD3(SK7)-APC-R700, CCR7(150503)-BrilliantViolet711, CD45RO(UCHL1)-BrilliantViolet421, CD4(SK3)-BrilliantViolet786 (SK3), and Fixable Viability staining-780 (all BD), CD8a(RPA-T8)-BrilliantViolet510, CD127(A019D5)-BrilliantViolet650 (both BioLegend), and KLRG1(13F12F2)-PE-Cyanine7 (eBioscience). Again, populations of antigen-specific cells that were smaller than 30 events were excluded from further analysis.

Preparing TCRβ cDNA libraries for sequencing

T-cell receptor analysis was performed as described previously[23], with minor modifications. Briefly, mRNA was isolated with the RNA microkit (Qiagen) according to the manufacturer's protocol. Isolated mRNA was used for cDNA synthesis with 5'RACE template switch technology to introduce universal primer binding sites, and unique molecular identifiers (UMI's) were added at the 5' end of the cDNA molecules using the SMARTScribe reverse transcriptase (TaKaRa). cDNA synthesis was followed by an AM-Pure XP bead-based clean-up (Beckman Coulter). Purified cDNA molecules were amplified in two subsequent PCR steps using the Q5® High-Fidelity DNA Polymerase (New England BioLabs), with an AMPure XP bead-based clean-up step in between. PCR products were size-selected on gel and purified using the Nucleospin PCR clean-up kit (Machery-Nagel). The PCR products were sequenced via Illumina MiSeq paired-end 2x250 nt sequencing.

TCRβ clonotype analysis

The raw sequence data was processed using the 12nt UMIs to correct for sequencing errors and unequal PCR amplification. RTCR [24] was used to identify both the UMI sequence and clonotype information from the reads. An additional filtering step was performed to exclude TCR sequences that were likely due to contamination in the sequencing protocol, and to minimize biases introduced by errors in the UMI sequence. In short, sequences were only accepted if their UMI was observed in at least 40 sequencing reads. Sequences with identical UMIs in multiple samples were removed if they did not occur in at least 1000 sequencing reads and also if their absolute frequency was lower than 10% of the maximum frequency in the other sample. Within each sample, UMIs within a Hamming Distance of 3 were clustered. More detailed information about the processing and filtering of the sequence reads is explained in Lanfermeijer et al. [302].

Alignment of MuV-peptides

Sequences for the alignment of the epitopes were obtained via uniprot.org. The corresponding Genbank accession numbers are: AF345290 for Jeryl Lynn 2,

AF338106.1 for Jeryl Lynn 5, JX287390 for Genotype G5, JX287385.1 for Genotype G06, KY969483.1 for Genotype H and MH892406 for Rubulavirus 2.

Statistical analysis of Flow cytometry data
Differences between the groups (for example vaccinated versus unvaccinated) were assessed using Mann-Whitney U tests. Paired data (differences between timepoints or differences between epitopes) were compared using the Wilcoxon Rank test (non-parametric).

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
From a cohort of MuV-infected individuals, a total of eight HLA-A2 positive individuals were selected for this study. Five out of these eight cases received two doses of the MMR vaccine during childhood (at 14 months and 9 years of age), while the other three subjects were unvaccinated. In sera of all subjects MuV-specific IgG antibodies were found (Table 1). The unvaccinated individuals had significantly lower IgG antibody concentrations 1.5 months after infection than the vaccinated individuals (p=0.0357) (Sup. Fig. 1), but this difference was neither present at 9 months post-infection, nor in the larger cohort [8]. There was no significant difference in severity of symptoms related to MuV-infection between vaccinated and unvaccinated individuals (Table 1).

Decrease in MuV-specific $CD8^+$ T-cell frequencies over time after infection
Previously, our laboratory identified several MuV-specific HLA-A*02:01-restricted epitopes, including the matrix protein-derived peptide ALDQTDIRV (ALD, residues 10-116), the fusion protein-derived peptide GLMEGQIVSV (GLM, residues 253-262), and the hemagglutinin-derived peptide LLDSSTTRV (LLD, residues 505-513) (Sup. Table 1). These three MuV-specific epitopes are immunogenic, as illustrated by relatively high frequencies of specific $CD8^+$ T cells in MuV-infected individuals [300] and are conserved between several mumps strains, including the vaccine strains and the circulating (genotype G) outbreak strain (Sup. Fig. 2). Dextramers loaded with these MuV-peptides were used to detect MuV-specific $CD8^+$ T cells after infection in previously vaccinated and unvaccinated mumps cases (Fig. 1A).

Table 1. Study population.

Donor	Sex	Age (yrs)	Time points	Vaccination status	IgG concentration, 1.5 months after infection (RU/ml)	IgG concentration, 7-10 months after infection (RU/ml)	Clinical Symptoms
263-**05**	M	21	1.5m, 9m & 36m	Vaccinated	4436	3010	Parotitis, swollen neck glands, fever, cold, cough
263-**12**	M	25	1.5m & 9m	Vaccinated	7265	5928	Parotitis, swollen neck glands, fever
263-**22**	M	26	1.5ms & 9m	Vaccinated	21683	7108	Orchitis
274-**66**	F	30	1.5m, 9m, 18m & 36m	Vaccinated	34843	10579	Parotitis, swollen neck glands
274-**83**	F	20	1.5m, 9m & 18m	Vaccinated	12785	23669	Parotitis, fever, permanent unilateral deafness
263-**02**	F	26	1.5m & 9m	Unvaccinated	358	1856	Parotitis, swollen neck glands, abdominal pain, cold, otitis
263-**10**	M	40	1.5m & 9m	Unvaccinated	2396	663	Orchitis, parotitis, swollen neck glands, fever, sore throat
263-**19**	F	53	1.5m & 9m	Unvaccinated	449	3704	Swollen neck glands, fever, cough, vertigo, temporary deafness

The dextramers allowed us to analyze the MuV-specific T-cell response at the epitope-specific level in depth at several timepoints after infection, starting at 1.5 months after MuV-infection. Percentages of MuV-specific T cells in the blood decreased significantly over time after infection. Between timepoints 1.5 and 9 months after infection, the largest decrease in MuV-specific T-cell frequencies against all three epitopes was observed (p=0.0078) (Fig. 1B). MuV-specific T-cell frequencies remained low from 9 months on, but were still detectable up to 36 months after infection. No link between the decline of the MuV-specific T-cell response and waning of the anti-body titers could be observed, whereas also age did not influence the frequencies of dextramer$^+$ T cells (data not shown). No major differences in the magnitude or contraction of the MuV-specific T-cell frequencies were observed between previously vaccinated and unvaccinated individuals.

From peptide elution experiments with HLA-A*02:01 positive antigen-presenting cells, it is known that the abundance at which these peptides are presented by the HLA-A*02:01 molecule differs greatly between these three epitopes [26]. While the ALD-epitope was found to be presented at the highest abundance, the GLM-epitope was predicted to have the strongest binding to the HLA-A*02:01 molecule (Sup. Table 1). We wondered whether this would influence the immunodominance of the CD8$^+$ T-cell response induced by these three peptides. At 1.5 months after infection, T-cell frequencies against the LLD-epitope (HN-protein) and the ALD-epitope (M-protein) were significantly higher than those against the GLM-epitope (F-protein) (Sup. Fig. 3). By 9 months post infection, these differences between T-cell frequencies against the different epitopes had disappeared. We also plotted the relative contribution of T-cell frequencies against the ALD, GLM and LLD epitopes to the "total" MuV-specific T-cell response (sum of the T-cell frequencies induced by the three epitopes) (Fig. 1C). In 5 out of 8 mumps cases, the T-cell response against the ALD epitope was the most dominant response, while in the other 3 cases the response against LLD was dominant. The GLM peptide induced a subdominant response in all MuV-infected cases. We observed no significant difference in immunodominance between vaccinated and unvaccinated individuals and no obvious shifts in dominance over time after infection (Fig. 1C). Thus, although the MuV-specific T-cell frequencies clearly contracted between 1.5 and 9 months after MuV-infection, this did not impact the relative dominance of the specific T-cell responses within individuals.

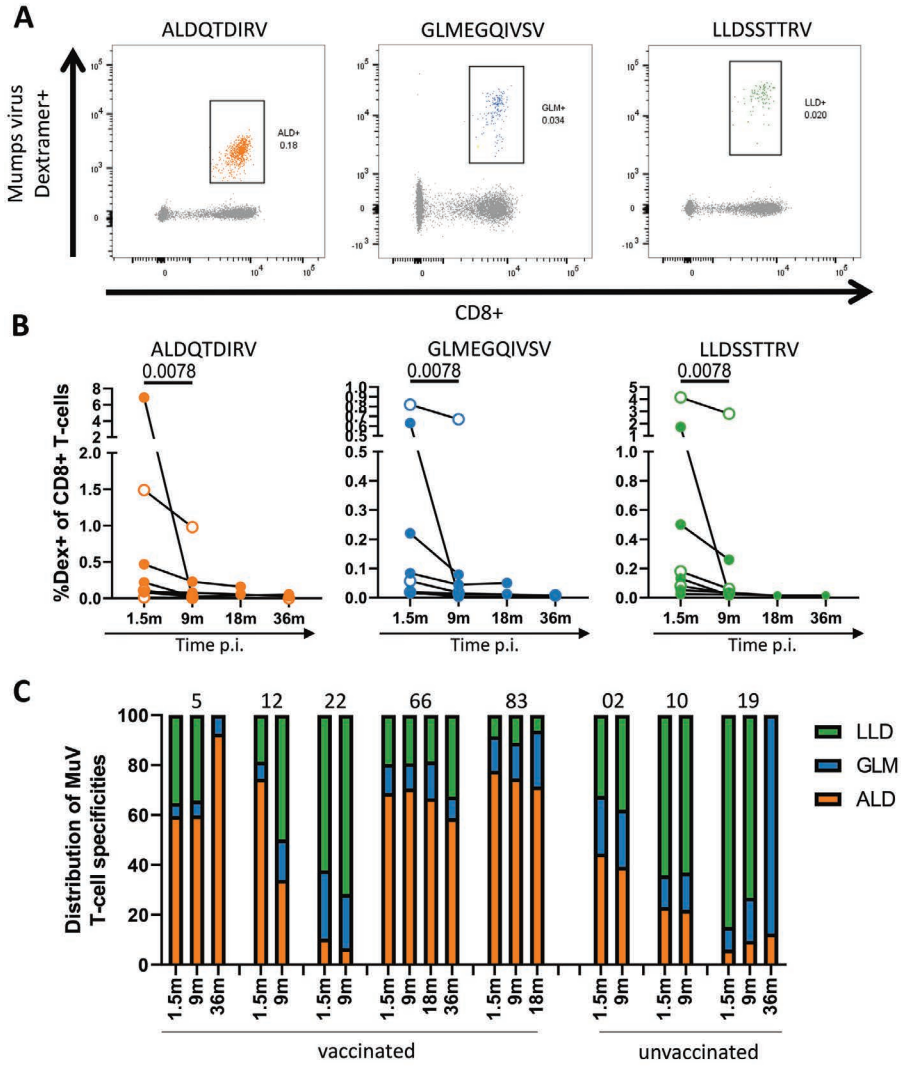

Figure 1: Frequencies of the MuV-specific CD8⁺ T cells wane after infection.
A) Representative dextramer staining and quantification of the MuV-specific CD8⁺ T cells against ALD (left), GLM (middle), and LLD (right). B) Percentages of the MuV-specific CD8⁺ T cells against ALD (left), GLM (middle), and LLD (right) in HLA-A2-positive individuals upon MuV infection. Solid circles indicate vaccinated individuals, whereas open circles indicate unvaccinated individuals. p.i., post-infection. The percentage of dextramer⁺ CD8⁺ T cells of donor 5 have been published before in the study of de Wit et al. 2020 [300]. C) The relative contribution of the three MuV epitopes to the "total" (= sum of the frequencies of the three) MuV-specific CD8⁺ T-cell response over time. Donor numbers are depicted above the graphs. Wilcoxon rank test was used to compare T-cell responses of individuals over time.

Phenotype of MuV-specific CD8$^+$ T cells shifts from short lived effector to memory cells

We next investigated the maintenance of the phenotype of MuV-specific memory CD8$^+$ T cells over time. To this end, we gated the memory T-cell subsets based on the expression of CD27 and CD45RO (Gating strategy is shown in Sup. Fig. 4). Every individual showed a consistent pattern in the distribution of the subsets for the 3 epitopes, however, the patterns differed between individuals (Sup. Fig. 5). In general, the largest fraction of cells had a central memory phenotype (CD27$^+$, CD45RO$^+$), while for the other memory subsets, large variations were observed between donors.

Focusing on the memory T-cell subsets based on the expression of CD127 (IL-7Ra) and killer cell lectin-like receptor G1 (KLRG-1), showed a different pattern (Fig. 2A + Sup. Fig. 6 AB). The markers CD127 and KLRG-1 distinguish between early effector cells (EEC; CD127$^-$, KLRG-1$^-$), short-lived effector cells (SLEC; CD127$^-$, KLRG-1$^+$), double positive effector cells (DPEC; CD127$^+$, KLRG-1$^+$) and memory precursor effector cells (MPEC; CD127$^+$, KLRG-1$^-$). Overall, between 1.5 and 9 months after infection, the fraction of EEC and SLEC within the MuV-specific T cells decreased ($p=0.0267$ for EEC and $p=0.079$ for SLEC), while the fraction of DPEC and MPEC increased ($p=0.0038$ for DPEC and $p=0.0017$ for MPEC) (Fig. 2B). The fraction of MPEC remained rather low for all three peptides at all timepoints. For both gating strategies, we found no significant differences in the composition of the MuV-specific T-cell pool between vaccinated and unvaccinated, as well as no effect of age.

The decline of MuV-specific CD8$^+$ T-cell frequencies is not explained by the expression of inhibitory markers

In chronic infections, the expression of inhibitory receptors on the cell surface of virus-specific CD8$^+$ T cells is mostly associated with functional exhaustion, due to continuous antigenic stimulation [303, 304]. The exact role of inhibitory receptors in acute infections remains unclear, but it has previously been suggested that these receptors regulate the primary response [229, 305, 306] and may limit immunopathology. Especially upregulation of PD-1 has been observed in response to activation of virus-specific T cells [307], but insight in the expression at later timepoints after infection are missing. Here, we measured the fraction of MuV-specific T cells expressing the inhibitory receptors PD-1, TIM3 or TIGIT.

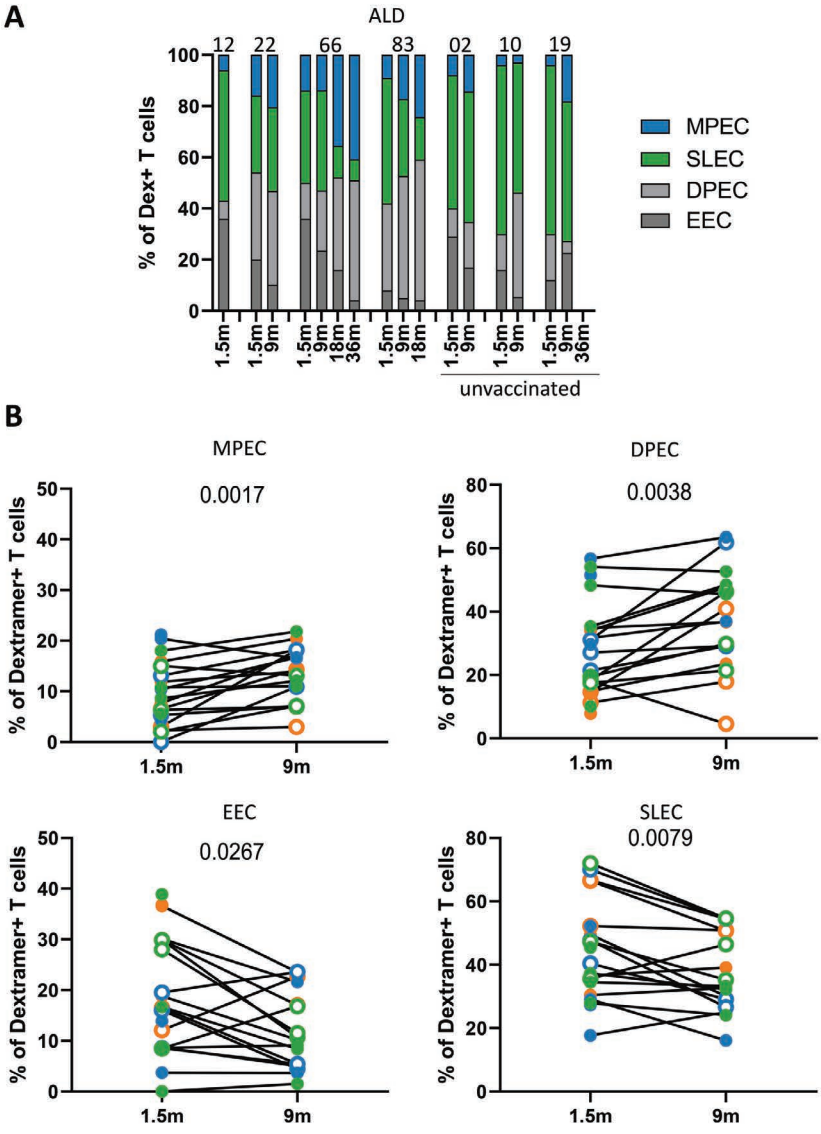

Figure 2: MuV-specific CD8⁺ T cells differentiate from effector cells towards memory cells over time after infection.

A) Bar graph showing the subset distribution based on CD127 and KLRG-1 expression of the MuV-specific CD8⁺ T cells against the ALD epitope. Donor numbers are depicted above the graphs. B) Fraction of the memory subsets based on CD127 and KLRG-1 expression of the MuV-specific CD8⁺ T cells at 1.5 months and 9 months after MuV-infection. The memory precursors cells(MPEC; CD127⁺, KLRG-1⁻), short lived effector cells (SLEC; CD127⁻, KLRG-1⁺), double positive cells (DPEC; CD127⁺, KLRG-1⁺) and the early effector cells (EEC; CD127⁻, KLRG-1⁻). CD8⁺ T cells specific for the ALD epitope are depicted in orange, for the GLM epitope in blue and for the LLD epitope in green. Solid circles indicate vaccinated individuals, whereas open circles indicated unvaccinated individuals. Differences between timepoints were tested by Wilcoxon Rank test.

Despite the large variation in the percentage of PD-1 expressing MuV-specific $CD8^+$ T cells between donors and between epitope-specificities, the percentage of PD-1-expressing MuV-specific T cells was relatively high at 1.5 months after MuV-infection (average of 50.78% ± 24.84%) (Sup. Fig. 7A, gating of a representative donor is shown in Sup. Fig. 7B) and significantly lower at 9 months post-infection (average of 43.10% ± 15.76). The percentage of both $TIGIT^+$ and $TIM3^+$ MuV-specific $CD8^+$ T cells did not change significantly between these two time points (Sup. Fig. 7A). We observed no significant differences in the expression of these inhibitory receptors between vaccinated and unvaccinated individuals. In line with the study of Ahn et al. [308] the percentage of $PD\text{-}1^+$ MuV-specific T cells was associated with the differentiation status of the MuV-specific T cells, as we found a significant negative correlation with the percentage of both CM ($CD27^+$, $CD45RO^+$) and MPEC ($KLRG\text{-}1^-$, $CD127^+$) at 1.5 and 9 months post MuV-infection (Sup. Fig. 8), Furthermore, the percentage of $PD\text{-}1^+$ MuV-specific T cells showed a negative correlation with thee TEMRA ($CD27^-$, $CD45RO^-$) phenotype, whereas no correlation with the EM ($CD27^-$, $CD45RO^+$) phenotype was observed.

To investigate whether there could be a role of these inhibitory receptors in restraining the response, we studied whether the decline in MuV-specific T cells between 1.5 and 9 months post-infection, indicated by the fold-change in MuV-specific T-cell frequencies, correlated with the expression of inhibitory receptors. We hypothesized that if inhibitory receptors lead to stronger contraction of the immune response, the higher expression levels of inhibitory receptors at 1.5 months post-infection should associate with a stronger decline in T-cell frequencies. In contrast, we observed a significant positive association between the expression of PD-1 at 1.5 months post infection and the fold-change in the height of the T-cell response ($r=0.7273$, $p=0.0096$), meaning that the MuV-specific T-cell responses with the lowest level of PD-1 expression 1.5 months after infection contracted the most (Sup. Fig. 7C). For TIGIT and TIM-3 no correlation between the expression of the receptors at 1.5 months post infection and the decrease in MuV-specific frequencies was observed (data not shown). Although the expression of PD-1 decreased over time post MuV-infection, the role of the expression of inhibitory markers after the acute phase of the immune response remains to be elucidated.

The decline of MuV-specific $CD8^+$ T-cell frequencies is not explained by increased expression of bone marrow homing markers

It has been suggested that virus-specific memory T cells, including those against MuV, are resting in the bone marrow for prolonged periods of time, even when their frequencies in the blood have already declined [309]. To study the migration

of the MuV-specific T cells out of the blood, towards other tissues, we measured the expression (based on mean fluorescent intensity) of the T-cell migration-associated markers CCR7, CXCR3 and CXCR4 on MuV-specific CD8⁺ T cells. CCR7 drives homing of T cells towards secondary lymphoid organs (SLO) [310], CXCR3 to the site of inflammation [311], and CXCR4 facilitates homing to the bone marrow [312].

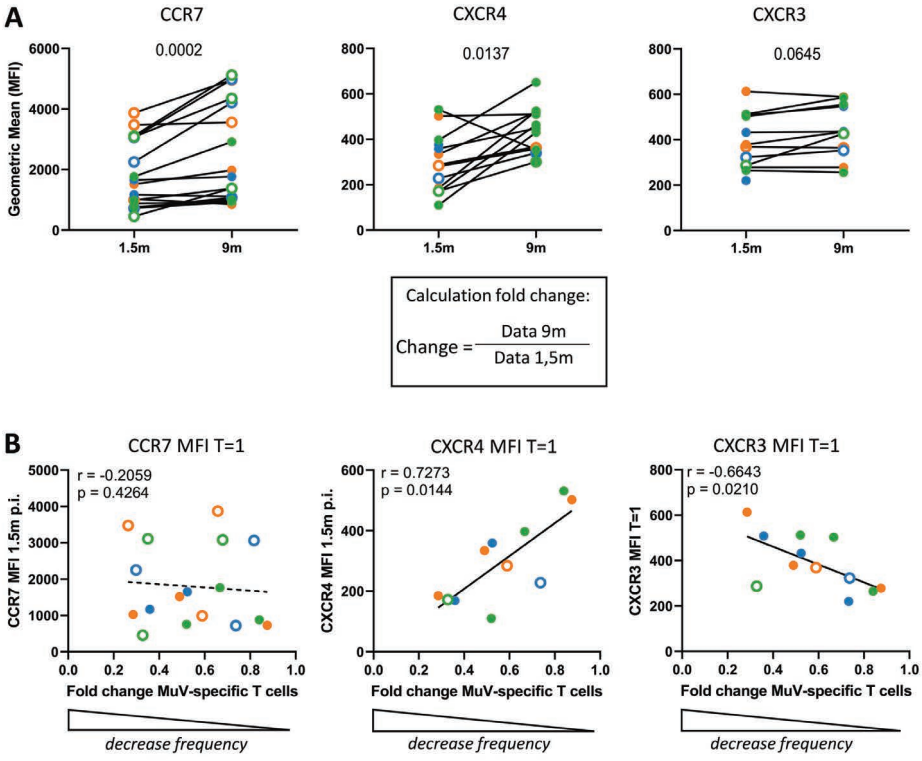

Figure 3: MuV-specific T cells tend to home to the bone marrow.
A) The expression of chemokine receptors CCR7 (left), CXCR4 (middle) and CXCR3 (right) based on their MFI value (geometric mean fluorescence intensity) of the MuV-specific T cells at 1.5 months and 9 months after MuV-infection. B) Association between the expression of CCR7 (left) or CXCR4 (right) (geometric mean of MFI) at timepoint 1.5 months post infection and fold change of dextramer frequencies at timepoints 1.5 months and 9 months after infection. Fold changes were calculated by dividing the expression or frequencies found at 9 months after MuV infection by the expression or frequencies 1.5 months after MuV infection, the calculated fold changes were all below 1, indicating a decrease. Expression of chemokine receptors on CD8⁺ T cells specific for the ALD epitope are depicted in orange, for the GLM epitope in blue and for the LLD epitope in green. Solid circles indicate vaccinated individuals, whereas open circles indicate unvaccinated individuals. Differences between timepoints were tested by Wilcoxon Rank test. Associations were tested by Spearman's correlation.

The expression of both CCR7 and CXCR4 increased significantly between 1.5 and 9 months post-infection for T cells specific for all three epitopes (p=0.0002 and p=0.0137, respectively) (Fig. 3A, left and middle panel), while the expression of CXCR3 remained stable between these two timepoints (Fig. 3A, right panel). Next, we investigated whether there was an association between the expression of tissue homing markers and the decline in T-cell frequencies in the blood between 1.5 and 9 months post-infection. Our hypothesis was that if homing to the tissues would play a role in the decline in T-cell frequencies in the blood, high expression of the homing markers at 1.5 months post-infection would be associated with a stronger decline in MuV-specific T-cell frequencies for that person at 9 months post-infection. Indeed, CXCR3 expression at 1.5 months post infection did show the higher the expression of the homing marker, the stronger the decline in T-cell frequencies. In contrast, the lower the expression of CXCR4 at 1.5 months post infection, the stronger the decline in MuV-specific T-cell frequencies observed (r=0.7273, p=0.0144) (Fig. 3B). No association between CCR7 expression at 1.5 months post infection and the decrease in MuV-specific $CD8^+$ T-cells frequencies was observed. Thus, the high expression of CXCR3 and low levels of CXCR4 at 1.5 months post infection associate with the decrease in T-cell frequencies, which implies migration to the inflammation site and bone marrow at 1.5 month. At 9 months, only CXCR4 expression is enhanced, which suggests migration to the bone marrow.

MuV-specific TCRβ repertoire is maintained in the memory phase

To investigate how the MuV-specific T-cell repertoire evolves over time after MuV-infection, we were able to analyze the T-cell receptor (TCR) sequences of MuV-specific T cells in the blood samples over time. By sequencing the TCRβ chain of ALD-specific, GLM-specific and LLD-specific T cells, we identified the Variable (Vβ) and Joining (Jβ) segment and the CDR3 region of the MuV-specific T-cell receptors (all identified TCR sequences are provided in Sup. Table 2A-C). At 1.5 months post-MuV infection, we observed a dominant usage of the Vβ-segment 15 (TRBV15) for the LLD epitope (87.7%, combined data of 6 donors) (4A, left panel). This large abundance is not only due to clonal expansions, as also a relatively high percentage (38,5%) of the different TCR sequences specific for LLD contained this segment (Fig. 4A right panel). The data on the V-segment usage of T cells recognizing the ALD and GLM epitopes were less conclusive, as these were based on fewer TCR sequences.

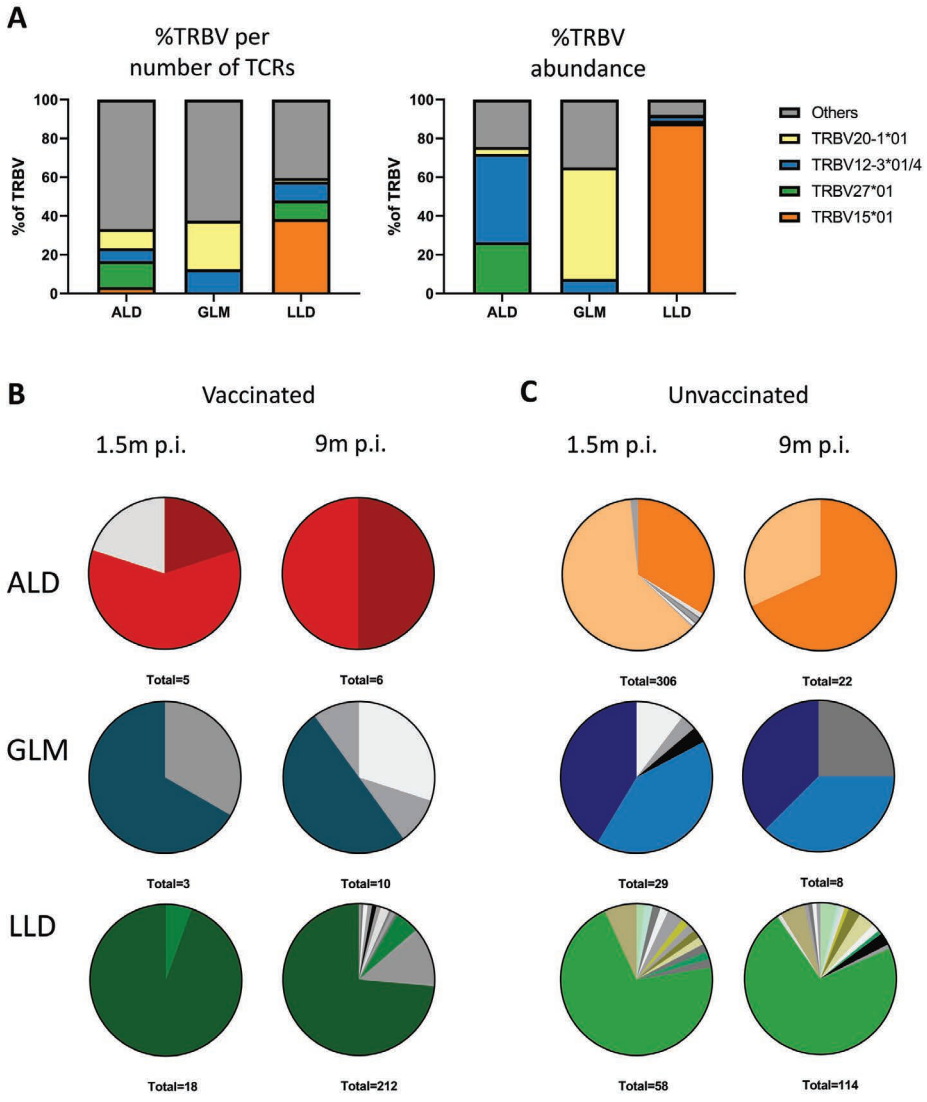

Figure 4: MuV-specific TCRβ repertoire is maintained in the memory phase.
A) Contribution of several Vβ families in the MuV-specific CD8⁺ T-cell repertoire based on number of sequences (left panel) and based on abundance (right) at 1.5 months of all donors (Table S2A–C). B,C) Characterization of the T-cell repertoire of MuV-specific CD8⁺ T cells against ALD, GLM, and LLD, detected by PCR of vaccinated (B) and unvaccinated (C) individuals. Each pie chart depicts the repertoire of a representative donor at a certain timepoint (1.5 months and 9 months after infection). Colors represent shared CDR3 sequences between timepoints and donors. Grey scales depict unique CDR3 sequences. Total number below a pie indicates the number of clones detected. p.i., post-infection.

The T-cell repertoires against the three MuV-specific epitopes that we analyzed were relatively stable between 1.5 and 9 months after MuV-infection, in that the same TCRβ sequences were found at both timepoints (Fig. 4B+C, Sup. Table 2A-C). Vaccination status did not influence this, as this was observed in both vaccinated (Fig. 4B) and unvaccinated individuals (Fig. 4C). In summary, although overall MuV-specific T-cell frequencies in the blood clearly decreased between 1.5 and 9 months post-MuV-infection, we found no evidence that this resulted in loss of specific clones between these two timepoints.

DISCUSSION

In this study, we investigated the frequency, phenotype and TCR repertoire of the MuV-specific $CD8^+$ T-cell response in the memory phase from 1.5 up till 36 months after onset of natural MuV-infection in previously vaccinated and unvaccinated adults. We focused on the T-cell response against three recently identified HLA-A2-restricted epitopes of mumps virus [300]. We found a significant decline in MuV-specific T-cell frequencies against all three peptides in the blood between 1.5 and 9 months after infection, whereas the frequencies remained stable up to 36 months. Phenotypically, changes in PD-1 expression and in expression of memory and homing markers were observed, but none of these changes could be linked to the decrease in T-cell frequencies, except for CXCR3.

The CXCR3 expression was found to be associated with fold change of MuV-specific T-cell frequencies over time after infection. Despite the observed changes in T-cell frequencies and phenotype, the characteristics of the MuV-specific $CD8^+$ T-cell response remained relatively stable at the TCRβ repertoire level.

We observed no obvious differences between childhood vaccinated and unvaccinated individuals in terms of T-cell frequencies, phenotype and stability at the clonal level after MuV-infection. Although T-cell responses have been observed up to 21 years after vaccination [297], the frequency and polyfunctionality of the $CD8^+$ T-cell response against mumps has previously been described to be suboptimal after vaccination, as compared to the response after MuV-infection [301]. Since we could not determine MuV-specific T-cell frequencies and characteristics prior to infection, we can only speculate about the presence of vaccine-induced memory T-cell immunity before infection in the vaccinated cases. Although the number of mumps cases analyzed in this study is small, the data suggest that the course of the $CD8^+$ T-cell response after MuV-infection is

similar in unvaccinated and childhood vaccinated adults. In addition, also no age-related correlation was found in the data.

The three MuV-specific epitopes used in this study are derived from different MuV-proteins. Nevertheless, the dynamics and characteristics of the T-cell responses against these three epitopes were comparable. There was only a difference in immunodominance, as we consistently found higher LLD-specific compared to GLM-specific $CD8^+$ T-cell frequencies both 1.5 months and 9 months post-MuV-infection. T-cell dominance did not seem to be influenced by the binding strength of the peptides to the HLA-A2:01 molecule (which was highest for the GLM peptide) or the abundance of the peptide (which was highest for the ALD peptide), as the response against the LLD-epitope was the most dominant response (Sup. Table 1).

The decrease in MuV-specific T-cell frequencies that we observed between 1.5 and 9 months after MuV-infection was similar for vaccinated and unvaccinated, and occurred at a relatively later stage of the contraction phase. The peak of expansion and subsequent start of the contraction most likely took place at an earlier timepoint after infection [313, 314]. It is generally assumed that T-cell frequencies after acute infection stabilize within 30 days post-infection [315]. However, the later phase of the T-cell response after acute infection has not frequently been explored and the exact length of the contraction phase remains to be elucidated. Therefore, it remains unknown whether the observed contraction is part of the end of the classical contraction phase, or whether the decline after 1.5 months post infection is specific for MuV-infection and delayed compared to the contraction of the T-cell response after other acute infections. Studies comparing the expansion and contraction phase of primary and secondary responses against acute infections actually showed that the secondary T-cell response consisted of a prolonged contraction phase compared to the primary response [315, 316]. A prolonged contraction phase may thus even result from involvement of the memory response. Based on the timepoints in this study this could unfortunately not be investigated. We also found no link between the contraction of the $CD8^+$ T-cell response and waning of the antibodies after infection.

Although we did observe a decrease in the expression of the inhibitory marker PD-1 and an increase in the expression of the homing markers CXCR4 and CCR7 on MuV-specific $CD8^+$ T cells over time, it is difficult to interpret the exact role of these markers in the memory phase after an acute infection. For PD-1, the observed decrease in expression could be a late effect of its early upregulation which is often observed upon activation of T cells at the start of the response

[307]. In case of the homing markers, we were surprised to find an upregulation of CCR7 between 1.5 and 9 months post-infection. We expected this marker to play a more dominant role during the acute phase of infection, as CCR7 is related to homing to secondary lymphoid organs, which most likely plays a role during T-cell priming [317, 318].

We were interested in the expression of CXCR4, as previous studies have suggested that the bone marrow contains large fractions of memory T cells, including MuV-specific T cells [309, 319-321]. These antigen-specific memory T cells in the bone marrow were shown to be resting and to be maintained for long periods of time, probably for decades, even when they were no longer detectable in the blood [309]. Surprisingly, we found that higher expression of CXCR4 at 1.5 months post-infection was associated with a lower decrease in MuV-specific T-cell frequencies in the blood. We cannot exclude the possibility that in individuals with MuV-specific T cells with a relatively high level of CXCR4 expression, MuV-specific T cells were already migrating to the bone marrow before our first timepoint. Alternatively, homing of MuV-specific T cells to the bone marrow may have occurred between the two time points at 1.5 and 9 months post-infection, which would also explain the increased levels of CXCR4 expression at 9 months post infection. As the time interval between the two time points is relatively large, more samples between 1.5 and 9 months would be needed to follow the dynamics of the expression of CXCR4 in relation to the decrease in MuV-specific frequencies.

Although no significant change in CXCR3 expression was observed between 1.5 and 9 months post-infection, we did find an association between the level of CXCR3 expression at 1.5 months post-infection and the fold decline of MuV-specific T-cell frequencies. CXCR3 expression is known to be associated with migration to the site of inflammation [322]. We may speculate that the T cells at 1.5 months still need to be present at the site of inflammation, whereas homing to the bone marrow probably takes place at a later timepoint.

The overall decrease in MuV-specific T-cell frequencies between 1.5 and 9 months fits well with the observed decrease in the EEC and SLEC fraction of the MuV-specific T cells over time, as these two populations play a role early after infection and subsequently contract [323]. However, it can be noted that the population of MuV-specific EECs and SLECs remains rather high at 9 months post MuV-infection. The relatively high frequency of short-lived and early-effector cells seems a bit counterintuitive 9 months post-infection. The presence of high percentages KLRG-1^{+}/CD127^{-} (SLEC) in the memory phase has been described in chronic infection,

but not for acute infections [323]. In mice, Renkema et al showed that the KLRG-1^{+}/CD127^{-} population did not contract after infection, and are actually Long lived effector cells (LLECs). This population was further characterized by lack of expression of CD62L, CD27 and CCR7 [324]. Analysis of KLRG-1^{+} MuV-specific T cells at 9 months indeed showed that part of the response lack CD27 or CCR7 (data not shown). MPECs, on the other hand, have an increased propensity to persist in the memory phase. Indeed, MuV-specific T cells showed an increase in the fraction of MPECs over time, suggesting that these cells contribute to the MuV-specific long-term memory response. Therefore, the MuV-specific T cells at 9 month post-infection may be considered to have a more prominent memory and long-lived effector phenotype, rather than short-lived effector cells.

Despite the strong decrease in MuV-specific T-cell frequencies in the memory phase of the response to MuV-infection, MuV-specific TCR repertoire remained relatively stable between 1.5 and 9 months post MuV-infection. Longitudinal data on the diversity and stability of TCR repertoire usage of virus-specific T cells after acute infection is limited. Vaccination with live-attenuated yellow fever virus (YFV) is often used as a model for acute infection, as it induces a strong T-cell response which can be detected in the blood even decades after infection [207, 208]. In a study focusing on the antigen-specific T-cell repertoire after YFV-vaccination, it was shown in one donor that the yellow-fever virus specific T-cell receptor sequences observed 45 days after primary vaccination were comparable to those observed after secondary vaccination, 18 months later, suggesting that practically all clones were maintained in the memory pool and responded during secondary vaccination [210]. Similar results have been observed after chronic infection with Epstein Barr virus (EBV). It was shown that a stable EBV-specific TCR repertoire was established after infection, as reflected by similar Vβ segment usage and recurrence of the same dominant T-cell clonotypes at different timepoints after the acute phase of the infection [80, 159, 160]. As EBV-specific T-cell frequencies remain relatively high during life, for EBV it could be shown that the responding TCR repertoire remained stable for at least 3 years after infection [207, 208, 210]. Our data is in line with these studies and suggests that also after acute infection the MuV-specific TCR repertoire is stable for at least a few months after infection and during contraction.

As we observed a stable T-cell response against MuV, which was still detectable up till 36 months after infection, the question remains to what extent the induced MuV-specific CD8^{+} T cells after infection will contribute to protection against MuV-infection in the long-term. Due to the low number of MuV-specific T cells we were

unable to measure functional responses, which will be of great value for further studies. Another question that remains unanswered based on this data is, why the vaccinated individuals in this cohort were infected and developed mumps disease. One possibility would be that the T-cell response induced by the MuV-vaccine is not as stable as the response induced by MuV-infection. Our earlier findings suggest that the relatively reduced magnitude and polyfunctionality of the MuV-vaccine induced $CD8^+$ T-cell response could play a role [301]. Insight in the pre-existing MuV-specific T cell response in vaccinated individuals would have been very informative. Unfortunately, in this study we cannot assess the pre-existence of childhood MuV-vaccination induced memory T-cells, as pre-infection samples were not available.

Taken together, our data on the characterization of the T-cell responses of MuV-infected individuals suggest the development of a sustainable T-cell memory population. However, most of our adult mumps cases were childhood vaccinated. This raises the question whether the vaccine-induced $CD8^+$ T-cell response was still present in these individuals, as we found no clear differences in the response between vaccinated and unvaccinated individuals after MuV-infection. Future studies should point out how we could enhance the effectivity and longevity of the $CD8^+$ T-cell response after MuV-vaccination. The results of this study add to the body of knowledge on effectivity and longevity of $CD8^+$ T-cell responses induced by natural infection, and may be helpful to optimize vaccination strategies aimed at obtaining long-term cellular memory.

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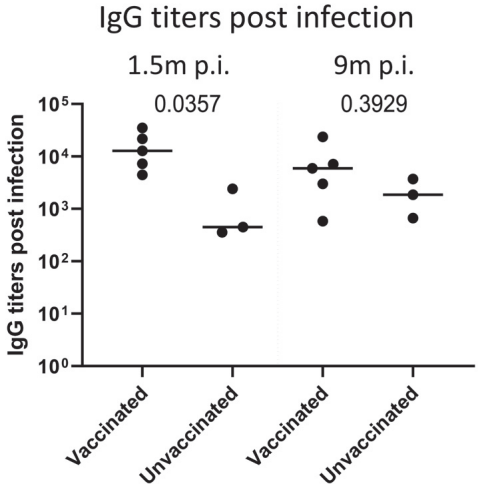
SUPPLEMENTALS

The Supplementary Tables with the TCR sequences are available online at:
https://www.mdpi.com/article/10.3390/vaccines9121431/s1

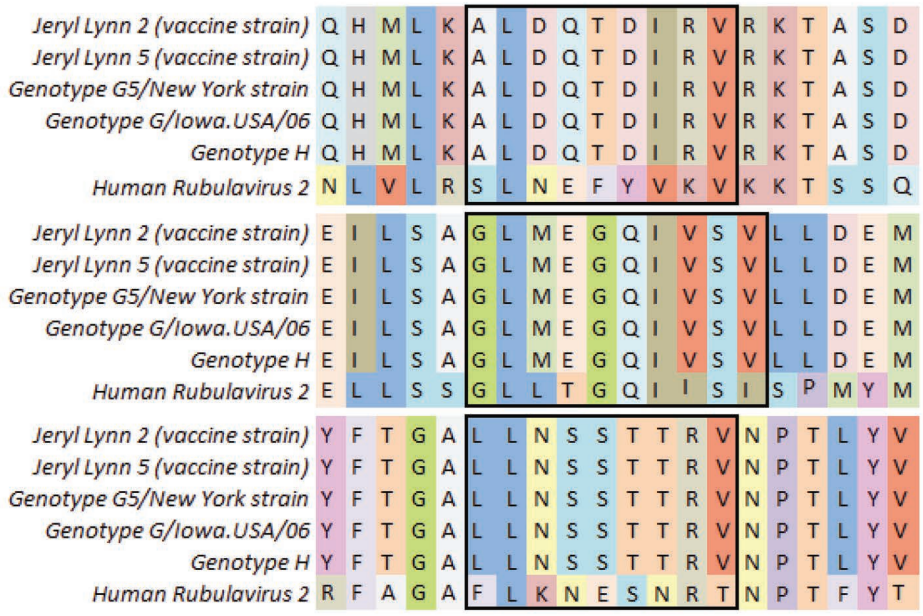
Supplementary table 1: Peptide information.

Epitope	MuV protein	Location	Average Tet% 1.5m	Average Tet% 9m	HLA-A2:01 Binding (%Rank)	Abundance (copy number/cell)
ALDQTDIRV	M	108-116	1.17 ± 2.36	0.17 ± 0.33	1.2992	781
GLMEGQIVSV	F	253-262	0.23 ± 0.32	0.11 ± 0.23	0.0234	3
LLDSSTTRV	HN	505-513	0.85 ± 1.44	0.41 ± 0.97	0.4569	14

Data about HLA-A2:01 binding was adopted from de Wit et al. 2020.

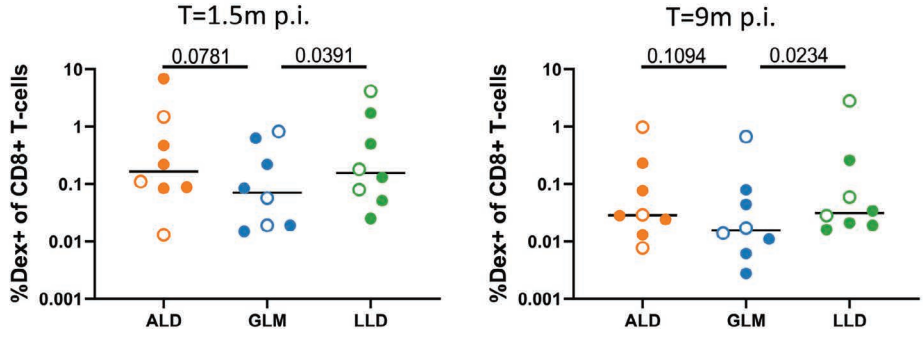


Supplementary Figure 1: Unvaccinated MuV-infected individuals have lower IgG levels 1.5 months after infection compared to vaccinated individuals.
IgG concentration (RU/ml) comparison between childhood-vaccinated (solid circles) and unvaccinated(open circles) mumps cases at 1.5 months (left) and 9 months (right) after infection. The IgG concentrations have been published before in the study of de Wit et al. 2020 [300]. Differences were calculated using the Mann-Whitney test.



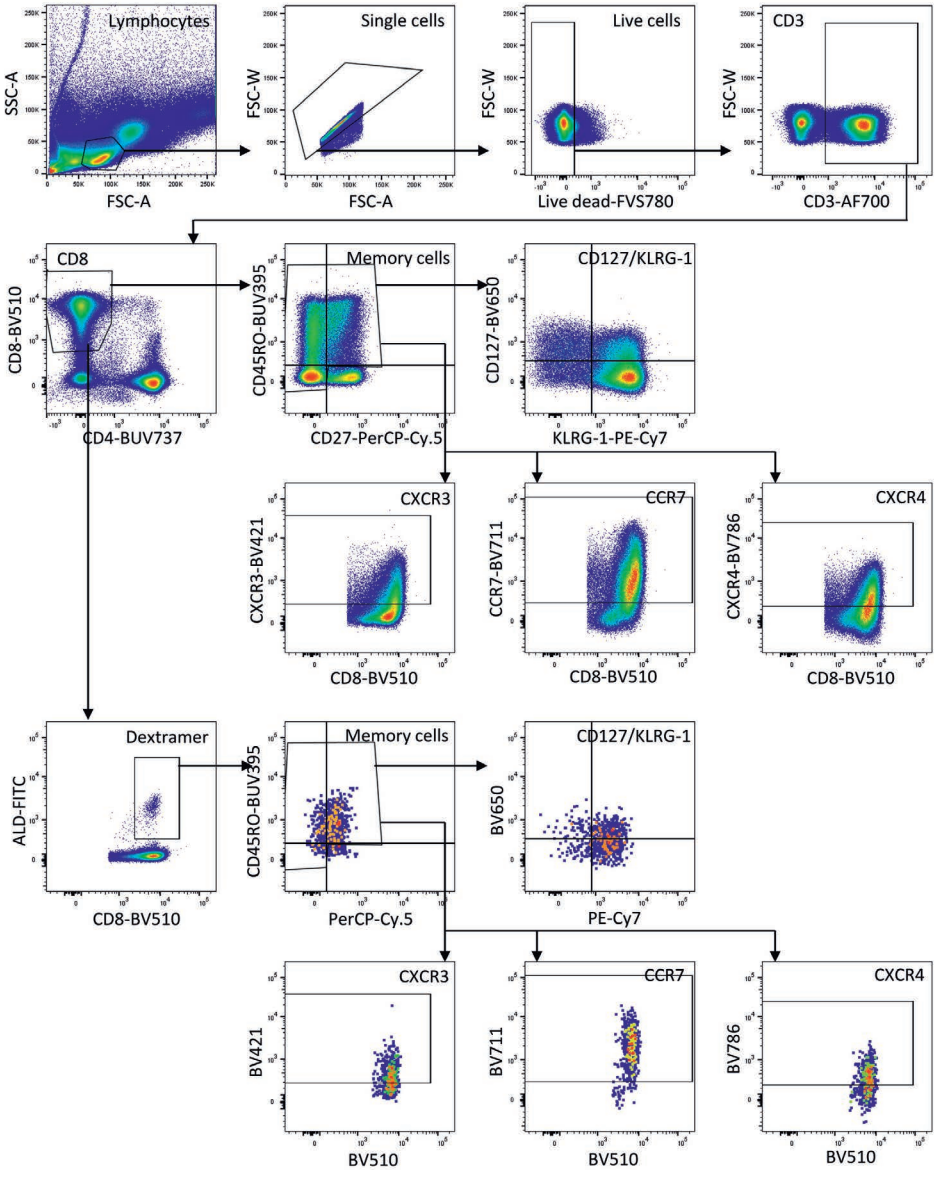
Supplementary Figure 2: The MuV-specific CD8⁺ epitopes are conserved within various mumps strains.

Shown are the alignments of amino acid sequences of the three MuV-specific peptides (indicated by the black boxes) and their surrounding amino acids for vaccine strains Jeryl-Lynn and outbreak strains Genotype G. Human Rubulavirus 2 is used as a comparison as it belongs to the same genus as mumps virus. Positions of the epitopes: ALDQTDIRV (M-protein, residues 108-116), GLMEGQIVSV (F-protein residues 253-262), and LLDSSTTRV (HN-protein, residues 505-513).



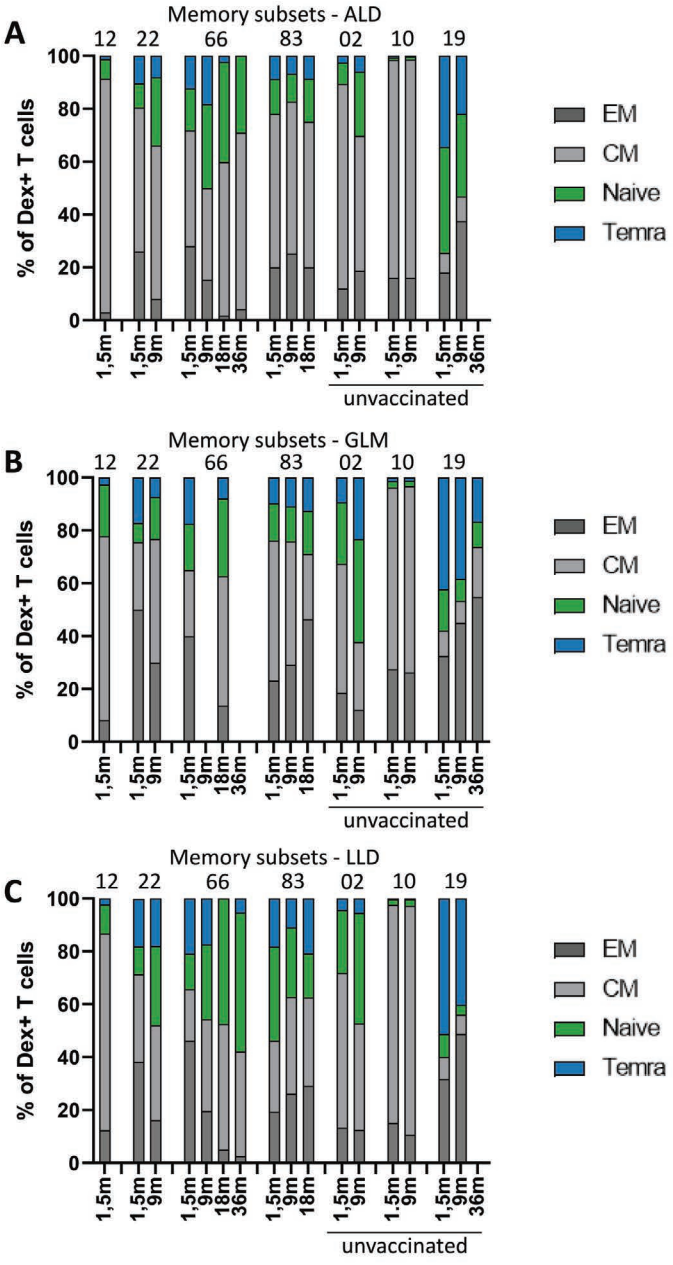
Supplementary Figure 3: Gating strategy of the phenotypical analyses of the MuV-specific response.

Frequencies of the MuV-specific response against the three epitopes at the timepoints 1.5 months post MuV-infection (left panel) and at 9 months post MuV-infection (right panel). CD8⁺ T cells specific for the ALD epitope are depicted in orange, for the GLM epitope in blue and for the LLD epitope in green. Solid circles indicate vaccinated individuals, whereas open circles indicated unvaccinated individuals. Wilcoxon Rank test was used to compare T-cell responses of individuals.

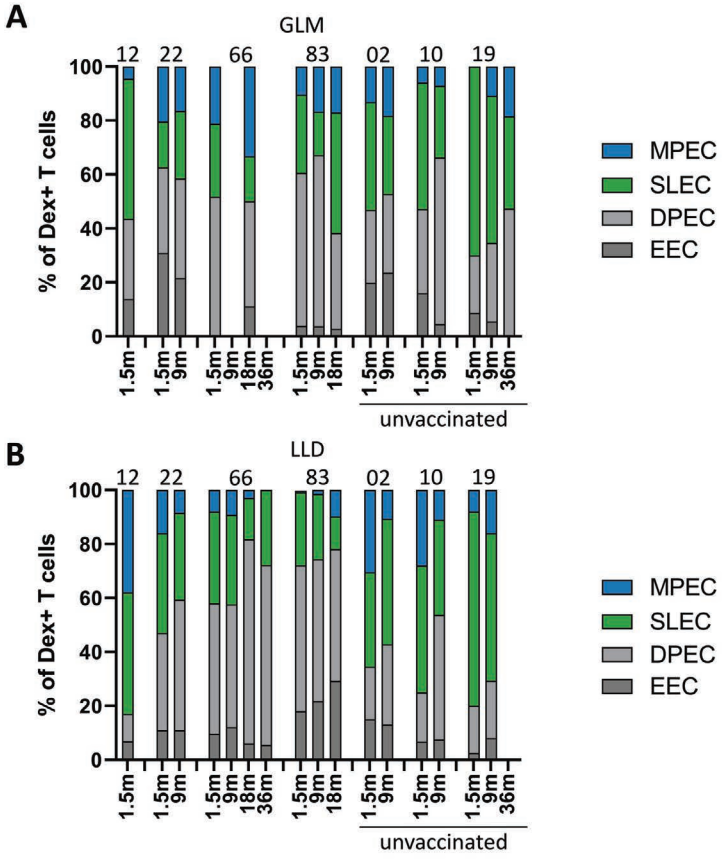


Supplementary Figure 4: Gating strategy of the phenotypical analyses of the MuV-specific response.

Flowcytometry gating as used for the different cell populations in the Dextramer⁺ population. Gating was based on the CD8⁺ T cell population and next applied to the Dextramer⁺ populations.

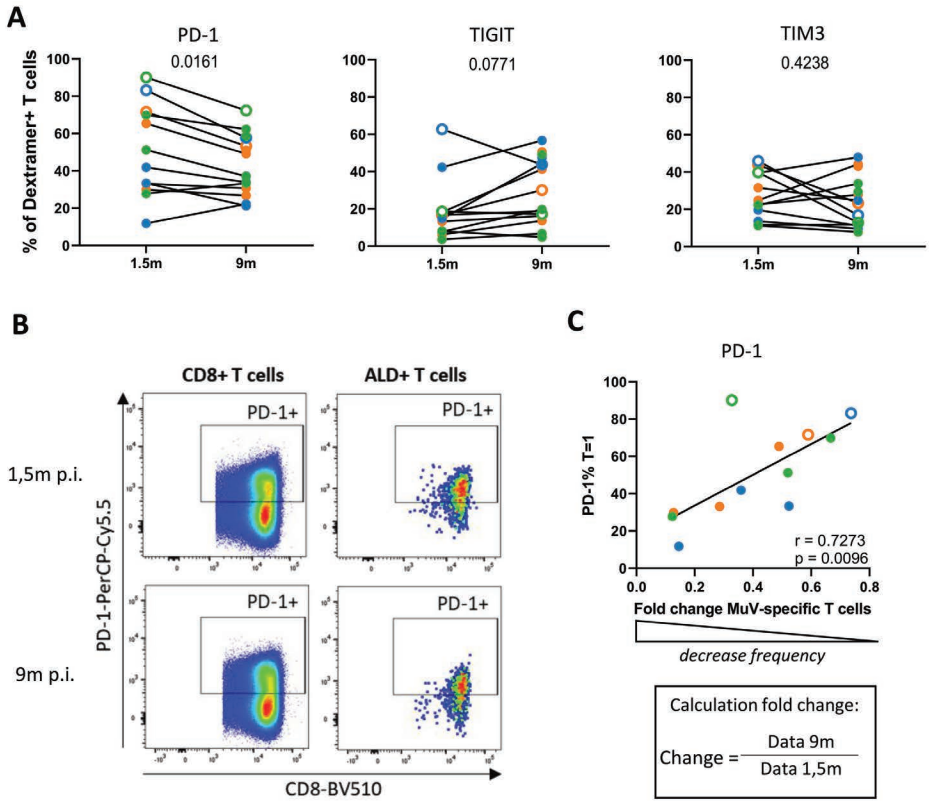


Supplementary Figure 5: Memory subsets of MuV-specific CD8⁺ T cells show mostly patient-specific patterns instead of longitudinal dynamics.
Fraction of the memory subsets based on CD27 and CD45RO expression of the MuV-specific CD8⁺ T cells against ALD (A), GLM (B) and LLD (C) over time. The naïve T cells (CD27⁺, CD45RO⁻) are predicted in dark gray, Central memory (CM, CD27⁺ CD45RO⁺) in light gray, Effector memory (EM, CD27⁻, CD45RO⁺) are shown in green and the Temra cells (CD27⁻, CD45RO⁻) are shown in blue.



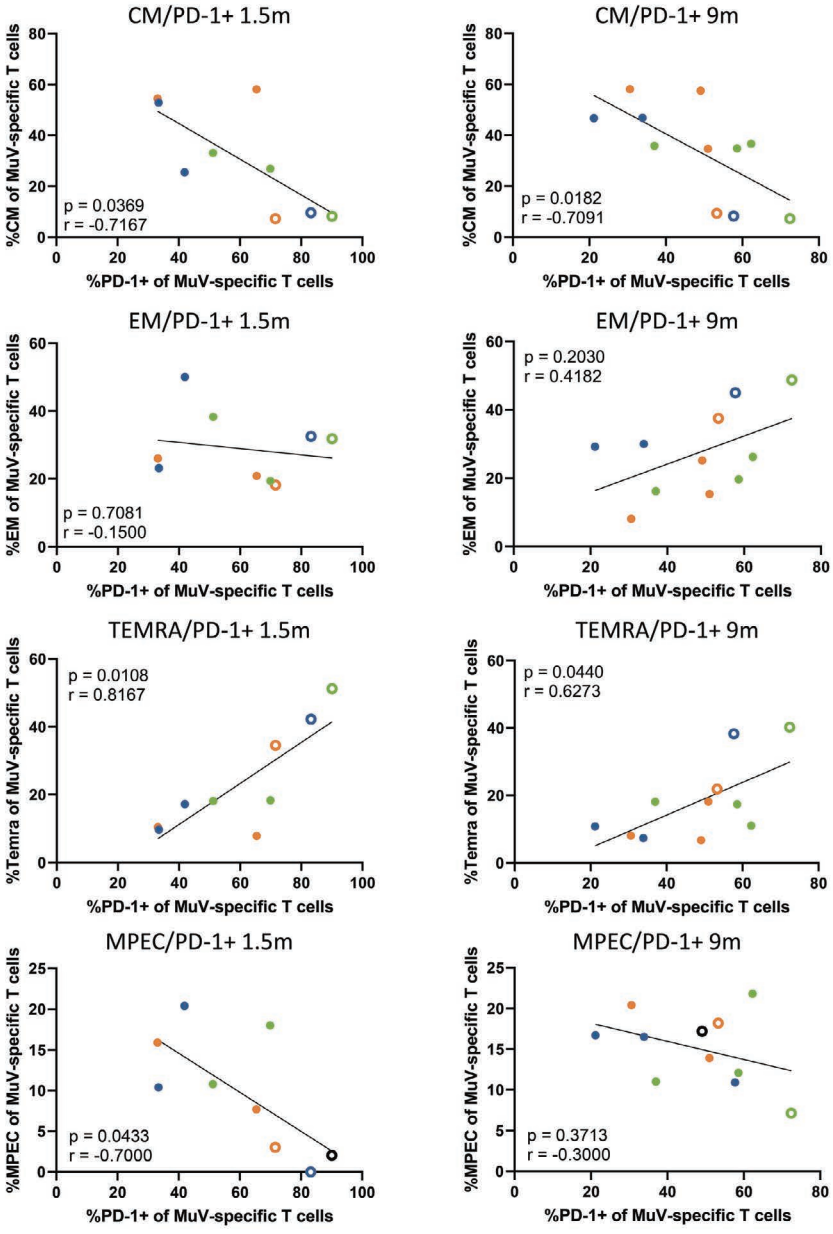
Supplementary Figure 6: MuV-specific CD8⁺ T cells differentiate from effector cells towards memory cells over time after infection.

MuV-specific CD8⁺ T cells differentiate from effector cells towards memory cells over time after infection. Fraction of the memory subset based on CD127 Table 127. and KLRG-1 expression of the MuV-specific CD8⁺ T cells against GLM (A) and LLD (B) over time. The memory precursors (MPEC; CD127⁺, KLRG-1⁻) are depicted in blue, Short lived effector cells (SLEC; CD127⁻, KLRG-1⁺) in green, double positive cells (DPEC; CD127⁺, KLRG-1⁺) are shown in light grey and the early effector cells (EEC; CD127⁻, KLRG-1⁻) are shown in dark grey. Donor numbers are depicted above the graphs.



Supplementary Figure 7: PD-1⁺ expression of the MuV-specific CD8⁺ T cells decreases over time after infection.

A) Fraction of de PD1⁺, TIGIT⁺ and TIM3⁺ MuV-specific T cells at 1.5 months and 9 months after MuV-infection. B) Gating of PD-1⁺ T cells on both CD8⁺ and ALD⁺ T cells of a representative donor at both timepoint 1.5 months and 9 months after MuV-infection. C) Association between the expression of PD-1⁺ MuV-specific T cells at 1.5 months after infection and the fold change in MuV-specific frequencies between 1.5 and 9 months after MuV infection. CD8⁺ T cells specific for the ALD epitope are depicted in orange, for the GLM epitope in blue and for the LLD epitope in green. Solid circles indicate vaccinated individuals, whereas open circles indicated unvaccinated individuals. Differences between timepoints were tested by Wilcoxon Rank test.Fold changes were calculated by dividing the expression or frequencies found at 9 months after MuV infection by the expression or frequencies 1.5 months after MuV infection, the calculated fold changes were all below 1, indicating a decrease.

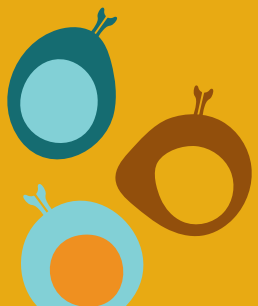


Supplementary Figure 8: PD-1⁺ expression is associated with the memory phenotype of the MuV-specific CD8⁺ T cells.

Association between the % of PD-1⁺ MuV-specific CD8⁺ T cells with the following subsets: CM (CD27⁺, CD45RO⁺), EM (CD27⁻, CD45RO⁺), TEMRA (CD27⁻, CD45RO⁻) and MPEC (KLRG-1⁻, CD127⁺). CD8⁺ T cells specific for the ALD epitope are depicted in orange, for the GLM epitope in blue and for the LLD epitope in green. Solid circles indicate vaccinated individuals, whereas open circles indicated unvaccinated individuals. Correlations were tested with Spearman's rank correlation coefficient.

PART 2

SHAPING THE T-CELL REPERTOIRE



The memory CD8⁺ T-cell response in mice is not influenced by time since previous infection

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Submitted

ABSTRACT

To protect older adults against influenza A virus (IAV) infection, novel approaches should be sought to overcome the decrease in protective immune responses with age. One suggested strategy is to boost T cells that recognize conserved epitopes by vaccinating at middle age, when the immune system is still fit. However, at the time of vaccination some individuals may have been more recently exposed to IAV than others and the time interval between previous infection and vaccination could affect the T-cell response. Here, we studied in a mouse model whether priming with IAV at different moments during life affects the T-cell response after a heterosubtypic IAV booster infection at older age. Before the booster infection (at 12 months of age), 6 months-primed mice displayed lower IAV-specific $CD8^+$ T-cell responses in the spleen and lung than 9 months-primed mice. Nevertheless, we observed comparable responses after the booster infection, based on IFNγ-responses, and IAV-specific T-cell frequencies and repertoire diversity. Lung-derived T cells of 6 and 9 months-primed mice expressed similar levels of tissue-resident memory T-cell markers 30 days post-booster infection. These data suggest that the IAV-specific T-cell response after boosting is not influenced by the time post priming.

INTRODUCTION

Influenza A virus (IAV) infection leads to higher morbidity and mortality with increasing age [170]. Therefore, yearly vaccination is used as a strategy to protect the older population against IAV infection. This has reduced the severity of disease and lowered the hospitalization rate amongst older adults [170, 325]. However, the efficacy with which immune responses are induced by IAV-vaccination also decreases with age, and vaccine failure occurs in a significant part of the older population [19]. Novel strategies are needed to improve protection of the older population. One strategy would be to vaccinate earlier in life, at middle age, when individuals are still able to mount a strong immune response to vaccination. Unfortunately, for the current IAV vaccines – which primarily induce humoral responses against the globular domain of the surface proteins hemagglutinin (HA) and neuraminidase (NA) – this strategy is not suitable. Due to mutations in these domains over time (antigenic drift), the virus tends to escape from the vaccine-induced antibody response [326, 327]. The antibody response is thus unlikely to still mediate sufficient protection against circulating IAV strains several years after vaccination.

In contrast to most HA- and NA-specific antibodies, T cells can recognize relatively conserved epitopes of IAV. Pre-existing IAV-specific T-cell responses are associated with decreased severity of disease [29, 30, 32] and it has been suggested that in older adults, the T-cell response against IAV is a better correlate of protection than the antibody response [26]. While T-cells are thus interesting vaccine-targets, the T-cell compartment unfortunately undergoes significant changes with aging, leading to decreased responses. In older adults, memory T cells tend to have impaired proliferation and lytic capacities [328], IAV-specific T-cell numbers are lower, and dominant public T-cell clones are observed less frequently [144, 179]. Primary IAV-infection in old mice has been shown to lead to lower IAV-specific T-cell responses and a less diverse antigen-specific T-cell receptor (TCR) repertoire compared to IAV infection in young mice [137]. It may therefore be beneficial to boost the T-cell response at middle age to enhance protection against IAV at older age. In line with this, Valkenburg et al. showed that the height of the IAV-specific T-cell response and its TCR repertoire diversity in old mice (22 months of age) could be retained at the same level as that in young mice if mice were primed with IAV at a very early age (6 weeks old) [329]. Boosting of the T-cell response might also benefit the maintenance of tissue-resident memory T (TRM) cells in the lung [330, 331]. Lung-TRM cells play a critical role in the protection against heterologous influenza infections and loss

of this population leads to reduced protection against heterosubtypic influenza infection in mice [331, 332]. Additionally, increasing age and repeated exposure to influenza can impact the formation and survival of TRM cells in the lung [330, 333]. Boosting the influenza T-cell response could potentially increase protection against IAV infection, but there is a high level of heterogeneity in infection history in the human population. This raises the question if an influenza vaccine-induced T-cell response is negatively affected when the time between vaccination and the previous infection is increased.

We set out to investigate how the time between antigen exposures influences the IAV-specific T-cell response at middle age by infecting middle-aged mice with a mouse-adapted IAV. Mice were primed with IAV at either 6 or 9 months of age and received a heterosubtypic IAV booster infection at middle-age, i.e. at 12 months of age. We examined the T-cell response in the spleen, lung and bone marrow, as well as the antigen-specific TCR repertoire after IAV-infection. Just before the booster, mice that were primed at a younger age (6m-primed mice) showed lower IAV-specific T-cell responses in spleen and lung than mice primed at a later age (9m-primed mice). Only in the BM the IAV-specific T-cell response was comparable between early and late primed mice. The differences in the T-cell response in spleen and lung before the booster did not result in an altered memory T-cell response after the second infection, in terms of T-cell frequencies, repertoire diversity and expression of TRM-markers. These findings suggest that boosting of a pre-existing T-cell response in middle-aged mice is not affected by the time since last infection.

MATERIALS & METHODS

Animals

Mice were inspected daily and were provided food and water ad libitum. Mice would have been euthanized prior to scheduled termination if they reached the humane endpoints, which were defined as >20% weight loss, pumping breath, inactive, feeling cold, bulging and ruffled coat. If mice would show >20% weight loss but none of the other humane endpoints, mice would not be sacrificed. None of the mice reached a humane endpoint during the study. However, one mouse was found dead the day after infection, and one during the study (unrelated to infection). For (scheduled) termination, mice were anesthetized with a isoflurane/oxygen-mixture and bled by orbital puncture. Influenza infections were likewise performed under anesthesia with isoflurane/oxygen-mixture to minimize suffering.

Study Design

5.5 month old female C57BL/6J (black/6) mice (Janvier labs) arrived at the Animal Research Centre (Bilthoven, The Netherlands) 2 weeks before start of the study for acclimatization. Based on data gathered from a pilot experiment, a power analysis suggested a minimal group size of 8 mice to find a 1% difference in tetramer-specific $CD8^+$ T-cell frequencies with a power of 0.8 between groups. Due to the relatively long duration of the study and the risk of age-related deaths, we increased the group size to 10 mice. The placebo group consisted of 6 mice. For practical reasons, the experiment was divided in two parts, each with 5 (experimental groups) or 3 (placebo group) mice per subgroup. Mice were randomly distributed over the groups and housed per subgroup in filtertop Macrolon III cages and accommodated with cage enrichment (igloo's and nestlets).

Mice were infected with H7N9 influenza A reassortant virus based on the backbone of the H1N1 influenza A/PR/8/34 virus (NIBRG-375, code: 17/136, NIBSC, London) intra-nasally (50 µl) at 6 months of age (6m-primed) or at 9 months of age (9m-primed) while the rest of the mice received PBS. At 12 months of age, all mice, except for the placebo group, were infected with H1N1 influenza A/PR/8/34 virus (PR8). Groups that did not receive a virus infection at 6, 9 or 12 months of age instead received PBS intra-nasally at these timepoints. The response against PR8 was measured before the booster (day 0), 10 days post booster (10 dpb) and 30 days post booster (30 dpb).

Viruses

A/PR/8/34 (PR8; NIBSC code 16/108) and H7N9/PR8 reassortant (NIBRG-268, NIBSC code 13/250) virus were obtained from the National Institute for Biological Standards and Control (NIBSC, Hertfortshire, UK). Influenza viruses were grown on MDCK cells in MEM medium (Gibco; Thermo Fisher Scientific) supplemented with 40µg/ml gentamicin, 0.01M Tricine and 2µg/ml TPCK treated trypsin (all from Sigma-Aldrich). At >90% cytopathic effect (CPE), the suspension was collected and spun down (4000x g for 10 minutes) to remove cell debris. Supernatant was collected, aliquoted and frozen at -80°C.

Sample collection

During dissection, heparin blood, spleen, lungs and bone marrow (BM) were collected and used the same day for measurements. Blood: Erythrocytes were lysed using ACK-buffer (NH4Cl 0.15M, KHCO3 0.01M, Na2EDTA 0.1 mM). Spleen: Spleens were homogenized, passed through 70 um filters (BD biosciences) and washed with RPMI 1640 containing 10% FCS, 100 U/ml penicillin, streptomycin, and

glutamate. Erythrocytes were lysed using ACK-buffer (NH4Cl 0.15M, KHCO3 0.01M, Na2EDTA 0.1 mM). Lung: After collection, lungs were minced into 1mm sized chunks using scissors and incubated in 3ml 2.4mg/ml collagenase A (Roche) and 1mg/ml DNAse (Roche) in RPMI1640 suspension for 30 minutes at 37 °C. Subsequently, the tissue suspension was diluted with 7ml washing medium (RPMI1640 + 1%FCS + 2mM EDTA + 1x P/S/G) and mashed over a 70um cell strainer using a plunger. The resulting suspension was centrifuged for 5' at 500x g to remove the collagenase after which erythrocytes were shocked using ACK-buffer. The cells were then washed, transferred over a 70um cell strainer and resuspended in 1ml of stimulation medium (RPMI1640 + 10% FCS + 1x P/S/G) for ELISpot, cytokine-FACS and dextramer staining. BM: Before cutting the femurs at both end, muscles and residue tissues surrounding the femur were removed. A 25-gauge needle and 10 cc syringe with ice-cold RPMI (10 ml) were used to flush the bone marrow out of the femur, into a 70 µm nylon cell strainer placed in a 50 ml Falcon conical tube. The tissue was smashed through the cell strainer and washed with RPMI (RPMI1640 + 10%FCS + 2mM EDTA + 1x P/S/G).

ELISpot

Pre-coated mouse IFNγ-ELISpot (ALP) plates (Mabtech) were used according to the manufacture's protocol. Cells of the spleen, lung and BM were stimulated with 0.1 nmol/well peptide ASNENMETM (ASN, NP366-374 H-2 Db), SSLENFRAYV (SSL, PA224-233 H-2 Db), SSYRRPVGI (SSY, PB1703-711 H-2 Kb) or MGLIYNRM (MGL, M1128-135 H-2 Kb) in ELISpot plates for 20 hours at 37 degrees. Controls consisted of medium, SINNFEKL (Ovalbumin 257-264 H-2 Kb) and pma/iono stimulation. All peptides were synthesized at DGpeptides (China), with a purity of >99%. Per condition, 400.000 cells (spleen, BM), 100.000 cells (spleen, lung) or 50.000 cells (lung) were stimulated with the relevant stimuli in 125µl stimulation medium. After 20 hours, plates were developed according to the manufacturer's protocol. Plates were dried for 1 night, after which they were analyzed using the ImmunoSpot® S6 CORE (CTL, Cleveland, OH). Maximum count was set at 500 spots per well and responses were corrected for background responses in medium-stimulated wells.

Cytokine responses by flow cytometry

In total, ±2 million lymphocytes were stimulated with ASN, SSL or SIIN peptide for 6 h. For the last 5 hours of stimulation, monensin (Biolegend) was added to the cells, followed by storage o/n at 4 °C. The next day, cells were washed twice with FACS buffer (2 mM EDTA, 0.5% BSA in PBS) and extracellular staining was performed in 100 µl FACS buffer with the following antibody mix: CD44(IM7)-BrilliantBlue515, CD103(2E7)-BrilliantBlue700, CD4(RM4-5)-AF700,

Fixable Viability Stain 780, CD8a(53-6.7)-BrilliantViolet510, CD62L(MEL-14)-BrilliantViolet650, CD49a(Ha31/8)-BrilliantViolet786, CD69(H1.2F3)-PE/Cy7, KLRG-1(2F1)-PE-CF594, CD3e(145-2C11)-Brilliant UV395 (all BD Biosciences) and CD127-(A7R34)-BV711 (Biolegend) for 30 mins at 4 °C. After washing, cells were fixated and permeabilized with BD Cytofix/Cytopmerm kit (BD Biosciences) according to the manufacturer's protocol. Cells were then stained intracellularly with IFNγ(XMG1.2)-APC, IL-2(RMP1-30)-BrilliantViolet421 and TNF(MP6-XT22)-PE (All BD) for 20 mins at 4 °C. After washing twice, the pellet was resuspended in FACS buffer and measured on an LSR Fortessa X-20 (BD). Data were analyzed using FlowJoTM Software v10.6.2 (BD).

Antigen-specific CD8⁺ T cells by flow cytometry

Approximately 2 million splenocytes, 2 million lung-derived lymphocytes, 4 million BM cells and lysed whole blood was used for dextramer staining. Cells were stained using the commercial H-2 Db ASNENMETM-APC and H-2 Db SSLENFRAYV-PE (Immudex) for 20 min at RT in the dark. Surface staining was added containing the following antibodies: CD44(IM7)-BrilliantBlue515, CD103(2E7)-BrilliantBlue700, CD4(RM4-5)-AF700, Fixable Viability Stain 780, CD8a(53-6.7)-BrilliantViolet510, CD62L(MEL-14)-BrilliantViolet650, CD49a(Ha31/8)-BrilliantViolet786, CD69(H1.2F3)-PE/Cy7, KLRG-1(2F1)-PE-CF594, CD3e(145-2C11)-Brilliant UV395 (all BD Biosciences) and CD127-A7R34-BV711 (Biolegend) and incubated for 30 min at 4 degrees. After washing twice, cells were resuspended in FACS buffer. Acquisition was performed on an LSR Fortessa X-20 (BD) and data analyses were performed using FlowJo v10.6.2 (BD).

UMAP

FlowSOM and UMAP analyses were performed in R [334] , using the CATALYST package [335]. Prior to clustering, the data was pre-processed in FlowJoTM Software v10.6.2 (BD) by first gating and then exporting the CD8⁺dextramer⁺ T-cell populations. The data was then imported into R where it was transformed using logicle transformation. Next, FlowSOM analysis was performed using 10x10 SOMCodes, merged into 12 metaclusters. FCS files containing less than 50 cells were not included in the analysis. Finally, UMAP analysis was performed. UMAP plots were colored using FlowSOM metacluster ID's.

Isolation of dextramer-specific T cells for T-cell receptor analyses

CD8⁺ T cells were isolated from PBMCs using a negative selection microbeads kit (Miltenyi Biotec) according to the manufacturer's protocol. Next, CD8⁺ T cells were labeled at room temperature for 20 min with H-2 Db ASNENMETM-APC and H-2 Db

SSLENFRAYV-PE (Immudex). Subsequently, surface staining was performed using the following mAbs: CD3(17A2)FITC, CD4(GK1.5)- BrilliantViolet510, CD8(53.6.7)-BrilliantViolet786 (All BD). $CD3^+CD4^-CD8^+dextramer^+$ cells were then sorted using a FACS Melody (BD) directly into RNAlater (Ambion Inc. Applied Biosystems) and stored at −80°C for subsequent TCRβ clonotype analysis.

Preparing TCRβ cDNA libraries for sequencing

mRNA was isolated with the RNA microkit (Qiagen) according to the manufacturer's protocol. Isolated mRNA was used in the 5' RACE-based SMARTer Mouse TCR α/β profiling kit (Takara Bio USA, Inc.) to perform sequencing of TCRs, following the manufacturer's protocol using only the TCRβ-specific primers. Cleanup was performed with AMPURE XP clean-up beads (BD). PCR products were sequenced via Illumina MiSeq paired end 2x300 nucleotide sequencing.

TCRβ clonotype analysis

Demultiplexed samples were first merged using tool Paired-End read merger [336]. Clonotype information was identified from the raw sequence data using RTCR [224] by aligning the sequences to reference TRBV and TRBJ genes. Clonotypes were defined by their CDR3 amino acid sequence.

TCR sequences were only accepted when they consisted of at least 100 sequencing reads, to clean the data from possible errors and contamination. This threshold was determined based on different cut offs, to make sure it would not lead to a qualitative bias in our results. Diversity was calculated using the previously described Simpson's diversity index (Venturi et al., 2007). This index ranges between 0 and 1, with 0 representing minimal diversity and 1 representing maximal diversity. Richness was calculated as the number of distinct TCR sequences in equally sized subsamples, by iteratively sampling (100.000 times), without replacement of 100.000 reads.

Statistics

Groups were compared using a 1-way or 2-way ANOVA, after which results were corrected for multiple testing using the two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli with a false discovery rate of 10% [337]. Multiple testing correction was performed per assay, tissue, day (e.g. IFNγ-ELISpot, lung, day 0). In cases where a 1-way or 2-way ANOVA could not be performed, groups were compared using the Mann-Whitney U test. For all analyses, p-values < 0.05 (after correction for multiple testing) were considered statistically significant. Data were analyzed using GraphPad Prism 9.1.0.

RESULTS

Study design

To study the effect of time since last infection on boosting the pre-existing IAV-specific CD8⁺ T-cell response in middle-aged mice, we primed mice with an H7N9 influenza A reassortant virus based on the backbone of the H1N1 influenza A/PR/8/34 virus (H7N9/PR8) either at 6 months (6m-primed mice) or at 9 months (9m-primed mice) of age. At this age, mice are mature and have a fully developed immune system [338]. To assure a homologous boost of the T-cell response to the internal proteins and no interference of previously induced neutralizing antibodies, we subsequently boosted these mice with H1N1 influenza A/PR/8/34 virus (PR8) at 12 months of age (0 days post booster (dpb), Fig. 1A). The age of 12 months was chosen to resemble middle-aged adults [338, 339], the target group of early influenza vaccination. We additionally included a group of naive mice that only received the PR8 infection at 12 months of age (previously uninfected mice). Immune responses were investigated prior to the booster (day 0) and 10 and 30 days dpb with PR8.

Lower baseline T-cell response in 6m-primed mice compared to 9m-primed mice

We compared responses in the spleens and lungs of 6m-primed and 9m-primed mice at day 0 to investigate whether time since last infection influenced the T-cell response before booster infection. Lung-derived lymphocytes and splenocytes were stimulated in an IFNγ-ELISpot assay with the influenza-specific CD8⁺ T-cell epitopes ASNENMETM (ASN, NP366-374 H-2 Db), SSLENFRAYV (SSL, PA244-253 H-2 Db), SSYRRPVGI (SSY, PB1703-711 H-2 Kb) and MGLIYNRM (MGL, M1128-135 H-2 Kb), ranked from highest to lowest level of immunodominance. Lung T-cell responses (measured by IFNγ-ELISpot) against SSL ($p=0.0159$) and SSY ($p=0.0025$), but not to ASN and MGL, were significantly higher in the 9m-primed mice compared to the 6m-primed mice (Fig. 1B). In the spleen, we detected significantly higher responses in 9m-primed mice against ASN ($p=0.0437$) and SSL ($p=0.0437$), with a similar trend towards higher responses in 9m-primed mice for SSY ($p=0.0614$) (Fig. 1C). Correspondingly, the sum of the individual peptide-specific T-cell responses was lower in the 6m-primed than in the 9m-primed mice in both lung ($p=0.0227$) and spleen ($p=0.0753$; Fig. 1D+E).

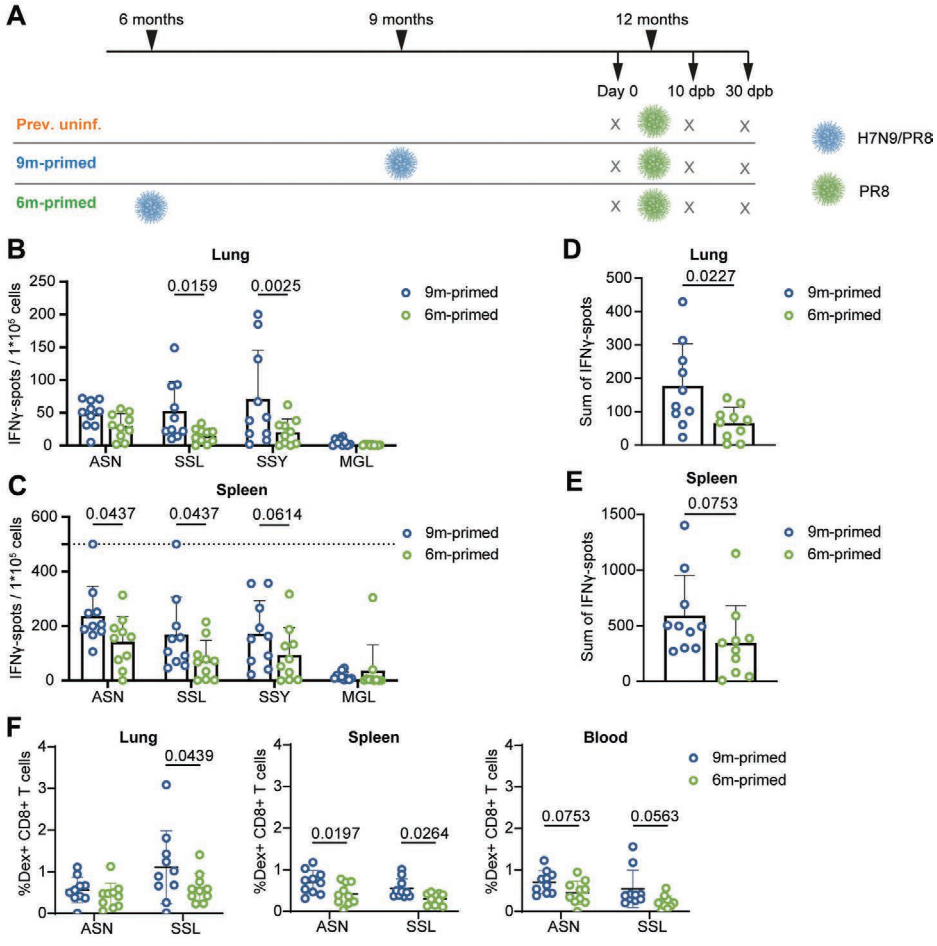


Figure 1: Lower baseline T-cell response in 6m-primed mice compared to 9m-primed mice.

A) Study layout depicting the prime-boost strategy. Mice were infected with H7N9/PR8 at 9 (9m-primed) or 6 (6m-primed) months of age. Mice not infected with H7N9/PR8 received a mock-infection with PBS instead (prev. uninf.). All groups received a booster infection with PR8 at 12 months of age. Mice were sacrificed at day 0 (before booster), and 10 and 30 days post booster (dpb). B-E) Cellular responses in IFNγ-ELISpot of lung-derived lymphocytes (B, D) and splenocytes (C, E) after restimulation with the IAV-specific epitopes ASN, SSL, SSY and MGL at day 0. The IFNγ-response is shown per peptide stimulation (B, C) or as a sum of the total response (D, E). ELISpot-responses are corrected for background (minus medium stimulation). No responses against a negative control OVA-peptide were detected. The horizontal dotted line depicts the upper limit of detection of the assay. ***F****) ASN-specific and SSL-specific T-cell frequencies measured in lung, spleen and blood at day 0 with use of dextramers and depicted as frequency of total CD8⁺ T cells.* ***B-F****) Results are depicted as individual mice (open circles) with mean and standard deviation. For statistical testing, only 6m-primed and 9m-primed groups were compared.*

We also measured frequencies of CD8$^+$ T cells recognizing the two most immunodominant epitopes, ASN and SSL, by dextramer staining in lung, spleen and blood. In line with the IFNγ-ELISpot results, SSL-specific CD8$^+$ T-cell frequencies were lower in the lungs of 6m-primed mice compared to the 9m-primed mice ($p=0.0439$), while in the spleen both ASN-specific ($p=0.0197$) and SSL-specific ($p=0.0264$) T-cell frequencies were lower in 6m-primed mice (Fig. 1F). The blood showed the same trend as observed in the spleen, although the differences were not significant (ASN $p=0.0753$, SSL $p=0.0563$). Together, these data suggest that the IAV-specific T-cell response at 12 months of age is lower in 6m-primed compared to 9m-primed mice, although the significance of the observed differences seems dependent on the epitope and compartment investigated.

Next we investigated the phenotype of ASN-specific and SSL-specific T cells in blood, spleen and lung at baseline before the second infection (i.e. day 0). We found that the fraction of central memory (CM) T cells (CD62L$^+$, CD44$^+$) within the ASN-specific and SSL-specific CD8$^+$ T cell populations was significantly higher in 6m-primed compared to 9m-primed mice (Sup. Fig. 1A+B). Correspondingly, the fraction of cells in the effector memory (EM) population (CD62L-, CD44$^+$) was lower in 6m-primed compared to 9m-primed mice. We also measured the expression of PD-1, as this inhibitory marker has been suggested to play a role in memory formation and to restrain early expansion of virus-specific CD8$^+$ T cells during acute respiratory infections [306]. The percentage of PD-1$^+$ T cells within the ASN-specific T cell population of 9m-primed mice was significantly higher than in 6m-primed mice (Sup. Fig. 1C). No such significant difference was observed for the SSL-specific T-cell population. In conclusion, not only the magnitude, but also the phenotype of the antigen-specific memory T-cell population changes over time, with a shift towards a central memory phenotype longer after priming.

Similar differences in T-cell responses to booster infection in 6m- and 9m-primed mice

Next we investigated whether the differences that we observed between the 6m-primed and 9m-primed mice would affect the recall response against PR8 infection at 12 months of age. In addition, we compared this to the response to a *de novo* response in 12-month old mice that had not been infected before (previously uninfected). We sacrificed half of the mice at 10 days post booster (dpb) to investigate the effector phase of the T-cell response, and the other half at 30 dpb to investigate the early memory response [313]. In most cases, the cellular immune response against the four peptides (ASN, SSL, SSY and MGL) at 10 and 30 dpb in both lung and spleen did not differ between 6m-primed and 9m-primed

mice, as determined by IFNγ-ELISpot (Fig. 2A+B). There were a few exceptions: The responses against SSY at 30 dpb in the lung (p=0.0048) and spleen (p=0.0100) were significantly lower in 6m-primed mice compared to 9m-primed mice. In the spleen, the MGL-responses at 10 (p=0.0493) and 30 dpb (p=0.0133) were also lower in 6m-primed mice compared to 9m-primed mice. Strikingly, while T-cell responses against ASN, SSL and SSY clearly increased after booster-infection in the lung and spleen of 6m-primed and 9m-primed mice (Sup. Fig. 2A-C), the responses against SSL, SSY and MGL in these mice did not exceed the response in previously uninfected mice at 10 dpb (Fig. 2A+B).

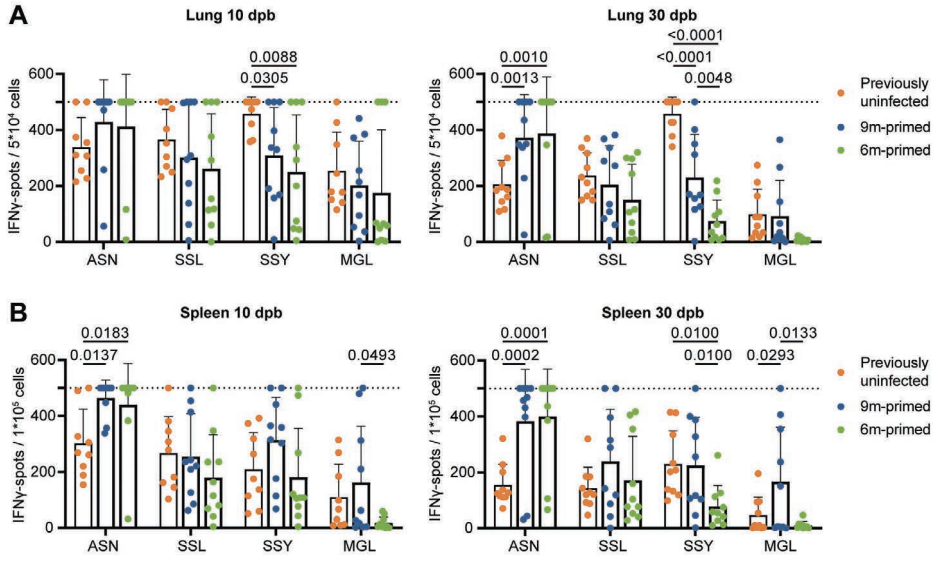


Figure 2: Differences before booster do not lead to differences in IFNγ-response against a new IAV infection.

A-B) Cellular responses in IFNγ-ELISpot of lung-derived lymphocytes (A) and splenocytes (B) after restimulation with IAV specific epitopes at 10 and 30 days post booster (dpd). ELISpot-responses are corrected for background (minus medium stimulation). No responses against a negative control OVA-peptide were detected. Horizontal dotted lines depict the upper limit of detection of the assay. Results are depicted as individual mice (filled circles) with mean and standard deviation.

After booster infection, the IFNγ-ELISpot assays often reached the upper limit of detection, due to which we might not have been able to detect potential differences between 6m-primed and 9m-primed mice. We therefore also measured IFNγ-responses by flow cytometry. IFNγ-responses after *ex vivo* stimulation of lung-derived lymphocytes and splenocytes with the ASN and SSL peptides showed similar responses between 6m-primed and 9m-primed mice at both 10 and 30 dpb (Fig. 3A+B). When comparing previously uninfected with primed mice, results were

in line with the IFNγ-ELISpot results. ASN-specific responses in lung and spleen were clearly higher in 6m-primed and 9m-primed mice compared to previously uninfected mice, while SSL-responses did not significantly differ between primed and previously uninfected mice (Fig. 3A+B).

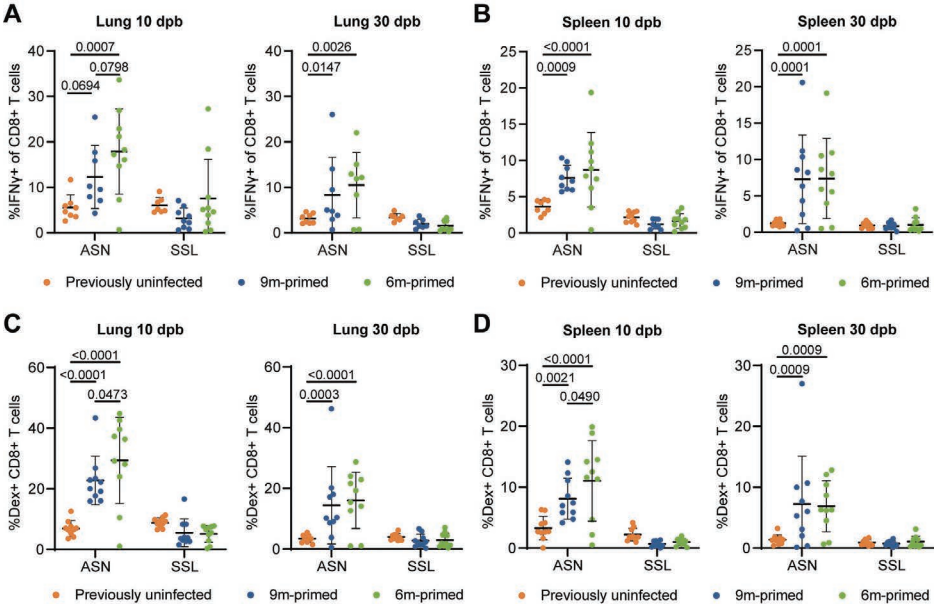


Figure 3: Differences before booster do not lead to differences in IFNγ-expressing and Ag-specific T-cell frequencies against a new IAV infection.
A, B) IFNγ-positive CD8⁺ T cells after stimulation with the ASN or SSL peptide in lung (C) and spleen (D) at 10 and 30 dpb. C- D) ASN- and SSL-specific T-cell frequencies were measured in lung (A) and spleen (B) with dextramers and depicted as frequencies of total CD8⁺ T cells at 10 and 30 days post booster (dpb). A-D) Results are depicted as individual mice (filled circles) with mean and standard deviation

We also analyzed the antigen-specific CD8⁺ T-cell frequencies by flow cytometry using dextramer staining. In agreement with the IFNγ-ELISpot results, SSL-specific T-cell frequencies in the lungs and spleen at 10 and 30 dpb did not differ significantly between 6m-primed and 9m-primed mice (Fig. 3C+D). In contrast, ASN-specific T-cell frequencies were higher in the lungs (p=0.0473) and spleen (p=0.0490) of 6m-primed mice at 10 dpb. The variation within groups was large however, with percentages ranging from 0-45% in the lung at 10 dpb. ASN-specific T-cell frequencies were clearly boosted in 6m-primed and 9m-primed mice compared to previously uninfected mice at 10 dpb. SSL-specific T-cell frequencies in contrast did not exceed the response in previously uninfected mice at 10 and 30 dpb. Despite our finding that the phenotype of IAV-specific T cells before booster

infection differed significantly between 9m-primed and 6m-primed mice, we did not find any significant differences in the percentages of EM and CM cells between the two groups during the recall response (Sup. Fig. 2D).

In conclusion, the higher T-cell response we observed in 9m-primed mice compared to 6m-primed mice before booster (day 0) only marginally affected the T cell-response 10 and 30 days after the booster infection. The differences between 6m-primed and 9m-primed mice that we detected by IFNγ-ELISpot were observed for the sub-dominant SSY and MGL peptides. Responses against ASN were clearly boosted in 6m-primed and 9m-primed mice, as they exceeded responses of previously uninfected mice in both spleen and lung. In contrast, we did not find significant differences for SSL-specific responses between the three treatment groups.

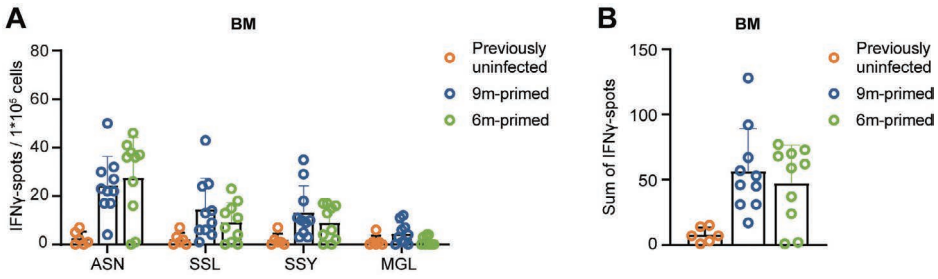


Figure 4: IAV-specific T-cell responses are similarly maintained in the bone marrow of 6m-primed and 9m-primed mice before booster.
A, B) IFNγ-responses against four IAV-specific epitopes in bone marrow (BM) of 9m-primed and 6m-primed mice before booster, as measured by IFNγ-ELISpot. The IFNγ-response is shown per single peptide stimulation (A) or as a sum of the total response (B). ELISpot-responses are corrected for background (minus medium stimulation). No responses against a negative control OVA-peptide were detected. Results are depicted as individual mice (open circles) with mean and standard deviation. For statistical testing, only 6m-primed and 9m-primed groups were compared.

IAV-specific T-cell responses are similarly maintained in the bone marrow of 6m-primed and 9m-primed mice

The bone marrow (BM) is generally regarded as a reservoir for long-term immunological memory and it has been shown that antigen-specific T-cell populations stay relatively constant in BM while they decrease in the spleen [321]. We wondered to what extent the IAV-specific T-cell response in the BM of 6m-primed and 9m-primed mice had been maintained before the recall response against PR8. Despite the observed differences in the baseline T-cell response against the four influenza epitopes in the spleen and lung (Fig. 1B-F), the baseline

response in the BM was comparable between 6m-primed and 9m-primed mice (Fig. 4AB). This suggests that the IAV-specific T-cell response in the BM is more stable over time than the responses in spleen and lung, supporting the hypothesis that the BM is a reservoir for long-term T-cell memory. After the booster infection, T-cell responses in the BM against ASN, SSL and SSY increased similarly in 6m-primed and 9m-primed mice and stayed stable until 30 dpb, while MGL-responses did not significantly increase after booster (Sup. Fig. 3).

Lung-derived T cells of 6m-primed and 9m-primed mice express similar levels of TRM markers after booster

Tissue resident memory T (TRM) cells in the lung have been shown to be important in protection against heterosubtypic influenza infections [331, 332]. TRM cells are usually classified based on expression of CD69, CD103 and/or CD49a. To verify that IAV infection induced TRM cells in the lung, we analyzed blood and lung lymphocytes of 6m-primed and 9m-primed mice for expression of TRM-markers by flow cytometry prior to the booster (day 0). Using the flow cytometry data of SSL-specific and ASN-specific T cells, we identified and visualized twelve clusters with different expression patterns by uniform manifold approximation and projection (UMAP) analysis (Fig. 5A). Of special interest are clusters 1, 2, 3 and 5 (Sup. Fig. 4A). Clusters 1 and 5, which are marked by high CD103 expression, were more abundant in the SSL-specific population of the lung compared to the blood, although this difference was not significant for cluster 5 ($p=0.8796$). Similarly, clusters 2 and 3, which are marked by high CD69 expression, were more abundant in the lung than in the blood (cluster 3 only for the SSL-specific T-cell population). Despite previous reports that CD49a-expression is associated with TRM cell retention in the lung [340, 341], we did not find clusters specifically representing CD49a-positive T cells in the lung. The more abundant expression of various TRM-markers in the lung was confirmed by direct measurement of the expression levels of CD49a, CD69 and CD103 by flow cytometry (Fig. 5B). While the number of T cells expressing CD49a did not differ between lung and blood, a significantly higher proportion of ASN-specific and SSL-specific T cells expressed CD69 or CD103 in the lung compared to the blood. Importantly, while $CD49a^+$ and $CD69^+$ T-cell frequencies did not differ significantly between lung-derived T cells from 6m-primed and 9m-primed mice, CD103 expression-levels were significantly reduced in 6m-primed mice ($p=0.0044$ for ASN, $p=0.0009$ for SSL). The proportion of antigen-specific T cells that expressed both CD69 and CD103 – a combination that is typically used to identify TRM cells – was likewise higher in lungs of 9m-primed mice. In addition, a higher proportion of blood and

lung SSL-specific and ASN-specific T cells tended to express CD49a compared to the CD8⁺ T-cell population as a whole (Sup. Fig. 4C).

Next, we assessed how expression of CD49a, CD69 and CD103 changed after a second influenza infection. We performed another UMAP analysis in which we included lung-derived ASN-specific and SSL-specific T cells from previously uninfected, and 6m-primed and 9m-primed mice 30 days after PR8 infection (Fig. 5C). The size of most clusters did not differ between 6m-primed and 9m-primed mice; only cluster 1 was slightly less pronounced in 6m-primed mice. Correspondingly, we did not find significant differences in the proportion of T cells expressing CD49a, CD69, CD103 or both CD69 and CD103, between these groups (Fig. 5CD and Sup. Fig. 4DE). Compared to 6m-primed and 9m-primed mice, previously uninfected mice possessed a higher percentage of lung-derived SSL-specific T cells at 30 dpb (Fig. 3B), of which a larger proportion was expressing CD49a, CD69, CD103 and a combination of CD69 and CD103 (Fig. 5D). Lung-derived ASN-specific T cells from previously uninfected mice also expressed more CD69, but were otherwise similar to ASN-specific T cells from primed-mice. In conclusion, at 30 dpb, expression of TRM-markers on lung-derived T cells in previously uninfected mice was at least as high as, or even higher than, in 6m-primed or 9m-primed mice. Additionally, while CD103 expression was initially lower on lung-derived T cells of 6m-primed compared to 9m-primed mice, this difference disappeared after a second influenza infection.

As TRM cells are potent IFNγ-producers [342-344], we wondered if expression of TRM-markers was related to cytokine expression. To study this, we reanalyzed the flow-cytometry data from Fig. 3A, which depicts IFNγ responses within CD8⁺ T cells at 10 and 30 dpb. We determined the percentage of cells expressing IFNγ within populations that were either negative or positive for CD49a, CD69 or CD103 and calculated the ratio of IFNγ-expressing cells in the subset that was positive versus the subset that was negative for a certain TRM-marker (e.g. ratio = %IFNγ⁺ within CD49a⁺ / %IFNγ⁺ within CD49a-). Cells that expressed CD49a and CD69 were more likely to also express IFNγ on both day 0 and 30 dpb (Fig. 5E). This was especially true for CD69: cells were 10-times more likely to produce IFNγ if they expressed CD69. CD103-expression was associated with higher IFNγ responses in the ASN-specific population on day 0, but not for SSL-specific CD8⁺ T cells or at 30 dpb. These findings indicate that expression of TRM-markers – at least to some extent – correlates with increased IFNγ expression. We did not observe any differences in frequency of IFNγ-expressing TRM cells between 6m-primed and 9m-primed mice 30 dpb.

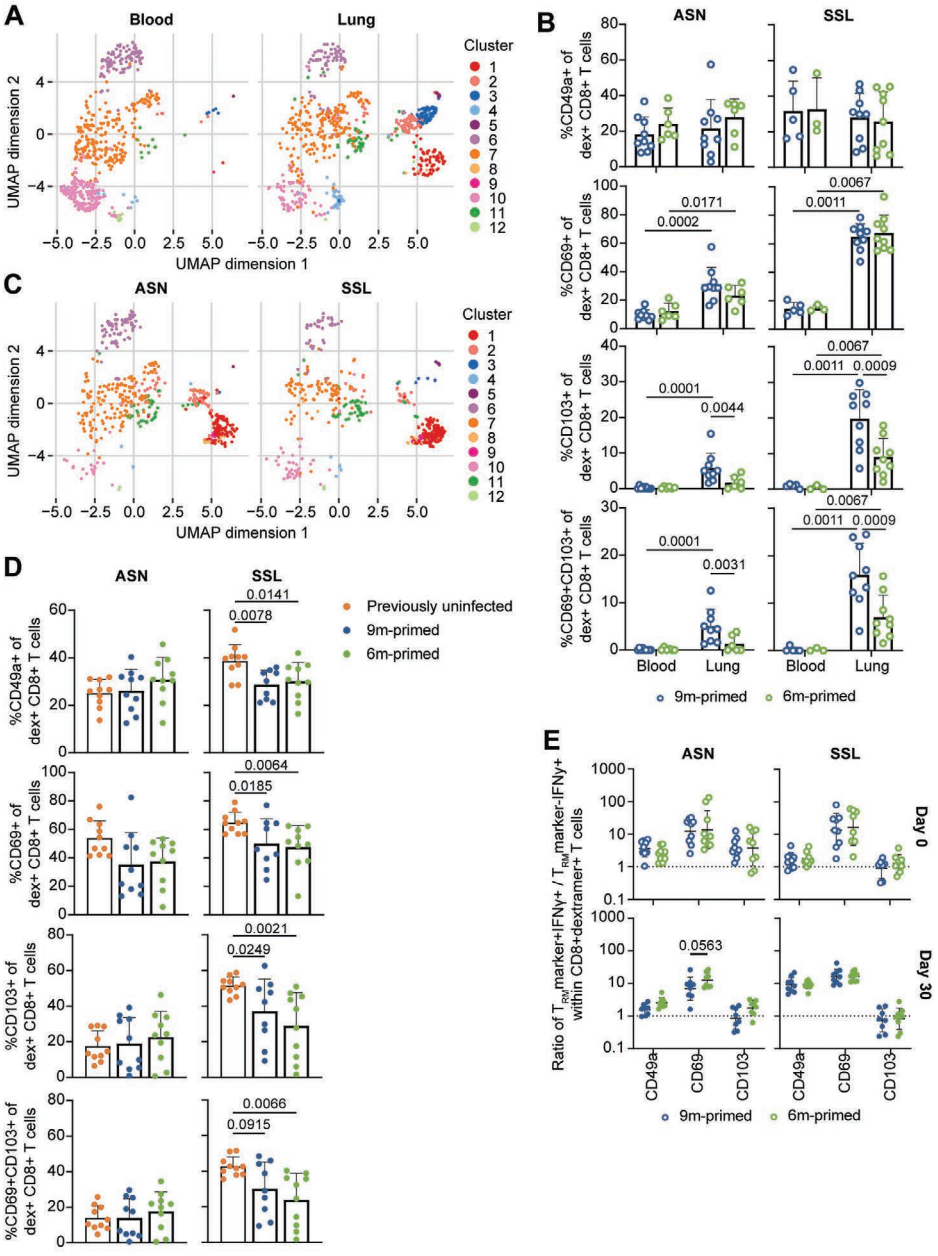


Figure 5: Expression of TRM-markers initially differs between 6m-primed and 9m-primed groups, but is similar after booster.

A) Uniform manifold approximation and projection (UMAP) analysis to compare blood- and lung-derived ASN-specific and SSL-specific CD8⁺ T cells of 6m-primed and 9m-primed mice before booster (day 0). Groups and antigen-specific CD8⁺ T cells are combined and analyzed separately for blood and lung-derived CD8⁺ T cells. B) Frequency of ASN-specific and SSL-specific CD8⁺ T cells at day 0 expressing the TRM-markers CD49a, CD69, CD103

or both CD69 and CD103. ***C)*** *UMAP analysis to compare lung-derived ASN-specific and SSL-specific CD8⁺ T cells of 6m-primed, 9m-primed and previously uninfected mice 30 dpb. Groups are combined and analyzed separately for ASN-specific and SSL-specific CD8⁺ T cells. D) Frequency of ASN-specific and SSL-specific CD8⁺ T cells of within lung lymphocytes 30 dpb expressing the TRM-markers CD49a, CD69, CD103 or both CD69 and CD103.* ***E)*** *IFNγ-production within subsets of ASN-specific and SSL-specific CD8⁺ T cells that are negative or positive for the TRM-markers CD49a, CD69 or CD103 at 0 and 30 dpb. Results are depicted as a ratio (TRM-marker⁺ IFNγ⁺ / TRM-marker- IFNγ⁺), in which a ratio > 1 means that cells positive for a certain TRM-marker are more likely to express IFNγ than cells not expressing that TRM-marker. For A-E, samples containing less than 50 ASN-specific or SSL-specific cells were excluded from analysis. Results are depicted as individual mice (open circles = before booster, closed circles = after booster) with (B, D) mean and standard deviation or (E) geometric mean and geometric standard deviation.*

IAV-specific TCR repertoire diversity is maintained between 3 and 6 months after infection

In older adults, a loss of dominant public clones against IAV has previously been observed, which might explain a decreased T-cell response to infection or vaccination at older age [144, 179]. To test whether a loss of IAV-specific clonotypes also occurs after boosting of the antigen-specific T-cell repertoire, we sorted ASN-specific T cells from splenocytes at baseline and 10 dpb and analyzed their TCRβ sequences. TCR diversity was compared by calculating the Simpson's diversity index [225], where an index of 1 represents maximal diversity and an index of 0 minimal diversity. The value of Simpson's diversity index reflects both the richness and the evenness of the TCR repertoire. Evenness is a measure of the distribution of clones in the TCR repertoire, where low evenness is indicative of skewing in the TCR repertoire due to the large expansion of selective clones. As a measure for evenness, we assessed the contribution of the most dominant clones by calculating the sum of the percentage of the top 5 largest T-cell clones to the total repertoire per sample. Repertoire richness was calculated as the number of distinct TCR sequences in equally sized subsamples, to compensate for the fact that the number of reads differs between samples.

First we compared the diversity of the ASN-specific T-cell repertoire between 6m-primed and 9m-primed mice before booster (day 0). No significant difference in TCR diversity was observed between the two primed groups (Fig. 6A, open circles). The similarity of the TCR repertoire between 6m-primed and 9m-primed mice at day 0 was reflected in similar levels of repertoire skewing (Fig. 6B) and richness (Fig. 5A) of the ASN-specific T-cell repertoire. Also a skewing towards Vβ13-1 was observed, which was comparable for the 6m-primed and 9m-primed mice (Sup. Fig. 5A).

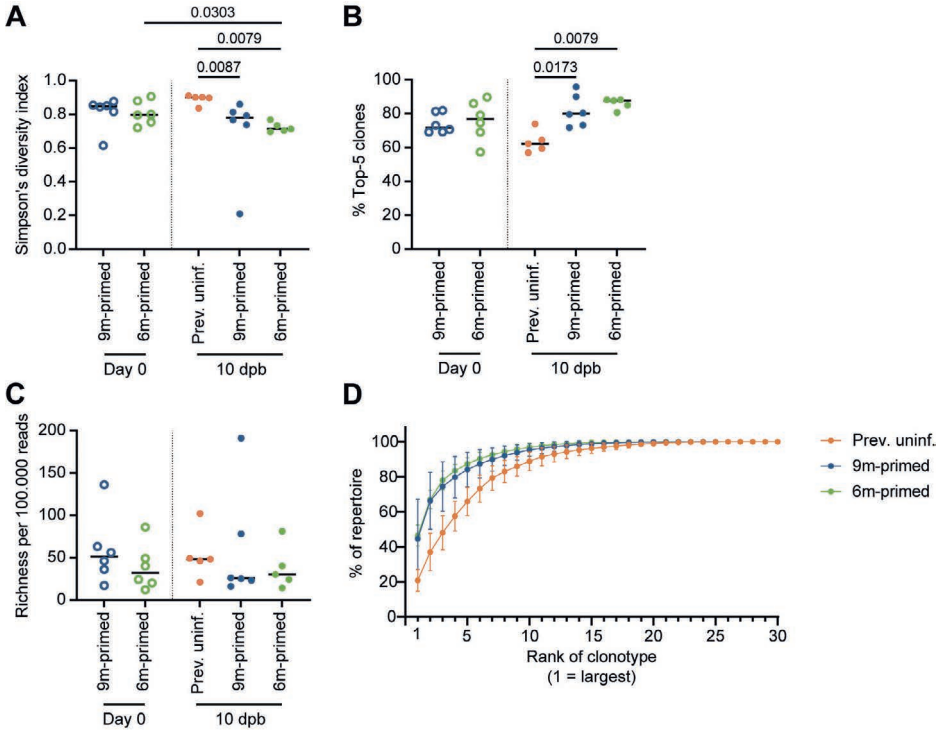


Figure 6: ASN-specific TCR repertoire diversity after boosting is not influenced by time between antigen exposure.

A) Repertoire diversity of the ASN-specific T-cell repertoire calculated with the Simpson's diversity index before booster (day 0, open circles) and 10 days post booster (dpb) (filled circles). B) Evenness of the ASN-specific T-cell repertoire calculated as the contribution of the top 5 largest clones per sample in percentages. C) Richness of the ASN-specific T-cell repertoire calculated by iteratively sampling (100.000 times), normalized to 100.000 reads per sample. D) Clonal distribution within the TCR repertoire, ranking the top 30 most prevalent clones starting from the largest clone, plotted as the cumulative frequency at 10 dpb. In A-C, results are depicted as individual mice (open circles = before booster, closed circles = after booster) with mean. In D, results are depicted as average per group (closed circles) with standard deviation

We also calculated the clonal distribution of the TCR repertoire per group, per timepoint. This was done by ranking the top 30 clones from large to small and calculating their contribution (in frequencies) to the repertoire (Sup. Fig. 5B). These data suggest that despite the lower number of antigen-specific T cells at day 0 of 6m-primed compared to 9m-primed mice, TCR repertoire diversity is maintained from 3 till 6 months after IAV-infection.

At 10 dpb, the diversity of the ASN-specific TCR repertoire in the previously uninfected group was significantly larger compared to that in the previously primed groups (p=0.0067 (9m-primed), p=0.0079 (6m-primed) (Fig. 6A, closed circles). This was related to significantly less skewing of the T-cell repertoire in the previously uninfected group compared to the two primed groups (p=0.0173 (9m-primed) and p=0.0079 (6m-primed)) (Fig. 6B). There was no significant difference in Vβ13-1 segment usage between the uninfected and primed groups (Sup. Fig. 5A). In previously uninfected mice, the top 5 clones constituted ±60% of the total repertoire, while the contribution of the top 5 clones was as high as ±75% and ±80% in 9m-primed and 6m-primed mice, respectively (Fig. 6B). This suggests that the decreased diversity observed in previously primed mice was due to an expansion of the most dominant clones. This was confirmed by the clonal distribution of the TCR repertoire we estimated based on the contribution of the top 30 largest clones in frequency, ranked from largest to smallest (Fig. 6D).

No significant differences in the ASN-specific TCR repertoire were observed between the 6m-primed and 9m-primed mice at 10 dpb based on repertoire diversity (Fig. 6A), evenness (Fig. 6B) or richness (Fig. 6C). In the 6m-primed group, TCR diversity decreased significantly between baseline and 10 dpb (p=0.0303), which was not observed in 9m-primed mice (Fig. 6A). Together with the observation that the TCR diversity at 10 dpb was higher in the previously uninfected group than in the primed groups, this suggests that boosting of the antigen-specific repertoire decreases the diversity of the responding T-cell repertoire, but that time between antigen exposure has no significant impact on the responding T-cell repertoire.

DISCUSSION

We examined how the IAV-specific T-cell response is influenced by boosting in middle-aged mice and specifically addressed the influence of time since previous infection. Our data show that despite the lower baseline T-cell response in 6m-primed compared to 9m-primed mice, the subsequent $CD8^+$ T-cell response and repertoire diversity upon reinfection did not significantly differ between these groups at 10 and 30 days after booster infection. Similarly, while baseline $CD103^+$ TRM cell numbers in the lung were lower in 6m-primed mice compared to 9m-primed mice, their numbers were boosted to similar levels after reinfection. Although boosting of the response to a previous influenza infection resulted in skewing of the TCR repertoire, we observed no influence of timing between the prime and boost on TCR repertoire diversity. This suggests that the IAV-specific T-cell repertoire is maintained over time.

To study the broad IAV-specific T-cell response, we selected four different IAV-specific epitopes that are known to induce T-cell responses in mice [345]. These epitopes are presented on either H2-Db (ASN & SSL) or H2-Kb (SSY & MGL) and differ in the level of immunodominance of the T-cell response. The response against these epitopes has already been studied extensively and is known to have different dynamics after heterologous infection [346]. Our data are in line with earlier studies in which ASN was found to induce the most immunodominant response after primary infection, followed by SSL and SSY [347], whereas MGL induces a subdominant response [348]. It has also been described that a shift in immunodominance occurs after a secondary infection, mostly because of a diminished SSL response [349], which we also observed. The dynamics of the recall responses against the IAV epitopes were independent of the time since primary infection. Only SSY-responses were significantly higher in the lung and spleen of 9m-primed mice at 30 dpb, as 6m-primed mice did not show an increase in the SSY response at all after the booster infection.

It was already known that the ASN-specific T-cell repertoire tends to be highly skewed towards Vβ8.3 (IMGT gene name: Vβ13-1) usage [195, 350]. We observed a similar bias for Vβ13-1 across all infection groups and time points. We found that the TCR repertoire diversity during the response against the secondary infection was not influenced by the time since last infection. The TCR repertoire diversity of the IAV-specific T-cell response decreased after boosting due to expansion of the most dominant clones. This phenomenon has also been observed for other pathogens, like listeria monocytogenes and CMV [83, 86, 188]. Adoptive transfer models have shown a slight narrowing of the repertoire in the recall response, although this was restored during the (second) contraction phase [351]. Nevertheless, our findings seem to be in contrast with previous studies on repeated IAV-infection, which found no significant differences between the T-cell repertoire after primary and secondary infection in terms of Vβ8.3 usage and showed no evidence for the emergence of new TCRβ sequences [194, 350]. The same has been found for the SSL-specific T-cell response, for which the breadth of the TCR repertoire did not change upon secondary infection [193]. Also the study of Valkenburg et al, in which mice were primed at 6 weeks of age, showed that boosting at a later age did not result in changes in the ASN-specific and SSL-specific repertoire diversity [329]. This seeming discrepancy with our study may be due to differences in the methods used to analyze the TCR repertoire, as 1) the high throughput sequencing that we used leads to higher sequencing depth and influences the estimated richness of the repertoire as more clones are detected, and 2) the previously described studies investigated the diversity within the Vβ13-1 segment, while we examined

the whole ASN-specific T-cell repertoire. Interestingly, we also did not observe a difference in the Vβ13-1 usage after boosting.

As the lung is the site of infection, one could question if the TCR repertoire is comparable between the spleen and lung. Due to a limited amount of T cells in the lung, we unfortunately could not investigate the TCR repertoire of ASN-specific lung T cells. Interestingly, a previous study showed more than 70% overlap between the SSL-specific T-cell repertoires after primary IAV-challenge in the lung and spleen (Turner SJ., 2003). After secondary challenge this overlap was even as high as 80%. This suggests that clonal frequencies measured in the spleen may be a good predictive read-out of the antigen-specific TCR repertoire in the lung. We generally observed similar T-cell response kinetics – in terms of IFNγ-responses over time – in the lung, spleen and blood after the booster infection. The only compartment that showed different T-cell response kinetics was the BM, which fits with previous studies showing that the number of antigen-specific T cells in the BM remains constant up to 1 year post infection [321]. Our data also suggest that antigen-specific T cells are maintained in the BM for at least 3 months, as at day 0 we found comparable responses between 6m-primed and 9m-primed mice. The maintenance of the IAV-specific response in the BM could be part of the explanation why the systemic IAV-specific response was comparable between 6m-primed and 9m-primed mice after the recall infection, despite lower baseline IAV-specific T-cell levels in 6m-primed mice in other compartments.

Lung TRM cells contribute to protection against heterosubtypic influenza infections, and boosting of IAV-specific lung TRM cells should thus increase the level of protection [330-333, 352]. Before booster infection, the expression of the TRM-markers CD49a and CD69 was similar between 6m-primed and 9m-primed mice, but CD103 expression was reduced in 6m-primed mice. This suggests that the $CD103^+$ phenotype of ASN-specific and SSL-specific $CD8^+$ T cells declines over time and that boosting of the T-cell response might be beneficial. Previous studies have demonstrated that CD49a is involved in retention of TRM cells in the lung tissue [340, 341]. Interestingly, we found that CD49a expression was not elevated in the antigenic-specific T-cell population in the lung tissue compared to blood, even at 6 months post infection. Our finding does not necessarily contradict previous findings, as CD49a might not directly mediate migration into the lungs, but primarily allows TRM cells to be retained after migration into the lung. It is also noteworthy that CD69 and CD103 were expressed by a larger fraction of SSL-specific compared to ASN-specific T cells. This is consistent with a previous study, reporting fewer ASN-specific T cells with a TRM phenotype compared to SSL-

specific T cells [353]. The authors suggested that this might be due to differences in the antigen availability as i) the presence of antigen contributes to TRM-cell formation [353] and ii) influenza antigens are known to be differentially expressed over time [349].

Several studies have shown that TRM cells are potent cytokine producers [342-344]. Expression of CD49a has been linked to enhanced cytokine production of TRM cells in the skin [354] and to a polyfunctional phenotype of lung TRM cells [355]. Our findings that antigen-specific T cells expressing CD49a, CD69 or CD103 in the lung are associated with IFNγ-production supports these previous reports. Lung TRM cells cannot be solely identified by expression of (a combination of) CD49a, CD69 and CD103, as some TRM cells have been described to be negative for these markers [355, 356]. To distinguish between lung TRM cells and circulating T cells, an intravenous (i.v.) staining method could be used [357]. We did not adopt this strategy because it was incompatible with the IFNγ-stimulation for flow cytometry and i.v. staining can give the false impression that T cells that are temporarily in the lung are tissue-resident.

Our data show that the IAV-specific $CD8^+$ T-cell response can be successfully boosted in middle-aged mice, independent of time since previous infection. This suggests that the timing between prime and boost does not play a major role when introducing vaccination at middle-age in humans, although it remains challenging to translate the 3-month difference between early (6 month) and late (9 month) priming to the human situation. The turnover rate of T cells in mice is much faster compared to that in humans [358] and the process of immune ageing in mature mice (>6 months) is approximately 25-times faster than in humans [338]. A period of 3 months in mice would thus translate to >6 years in humans, which is not disproportionate considering that adults are estimated to be infected with influenza every 5-10 years [359, 360].

If one would indeed want to protect the elderly by boosting the IAV-specific T-cell response at middle age, it is important to know how long the IAV-specific T cells can be maintained and provide sufficient protection against disease. In mice, it has been suggested that IAV-specific T cells can be maintained for a life time [361], which is approximately 2 years. The life-time of the IAV-specific T cell response in humans remains hard to quantify. The T-cell response against the HLA-A2 restricted epitope GILGFVFTL can be detected in the majority of $HLA\text{-}A2^+$ individuals, at respectable frequencies and has been suggested to be maintained for at least 13 years [362]. However, human adults are thought to be

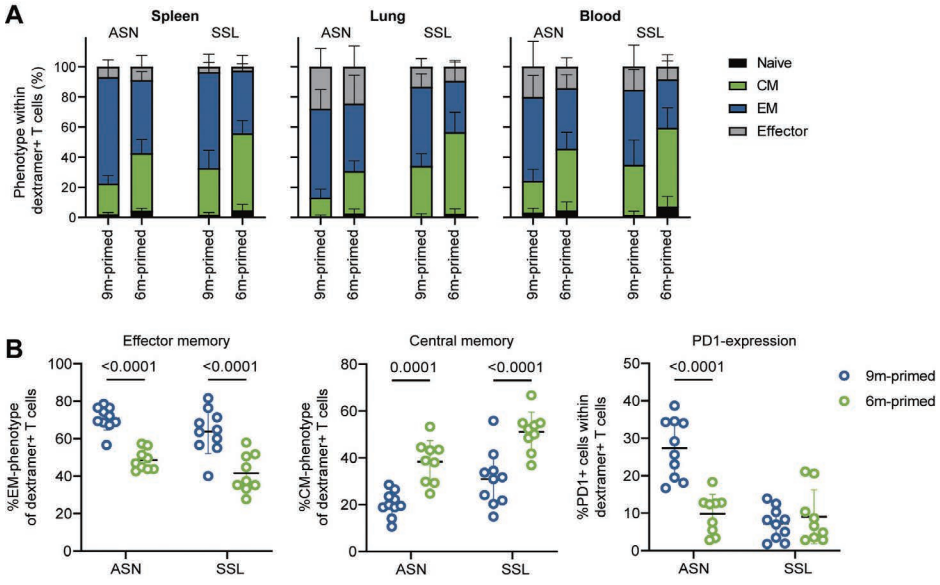
infected with IAV every 5-10 years, due to seasonal circulation of the virus [359, 363], meaning that their IAV-specific T-cell response would be boosted multiple times during life. As we found that boosting of pre-existing IAV-specific T cells leads, amongst other things, to a decrease in TCR diversity of the responding T cells, it would be interesting to investigate the effect of more than two repeated infections. Our data indicate that due to boosting, there is an expansion of the most dominant clones. It could be that more than two infections would lead to exhaustion of the most dominant clones, and would thereby result in the loss of dominant clonotypes as observed in humans, or even in more skewing as has been described for CMV-specific $CD8^+$ T cells [83, 144, 179]. Additionally, several reports indicate that excessive boosting of the T-cell response might have adverse effects. Studies using other pathogens or vaccinations have shown that boosting $CD8^+$ T cells repeatedly can result in a decrease in the cytolytic potential, cytokine production and proliferative capacity [364-366] and repertoire diversity of the responding T cells [83, 86, 188]. Further research is needed to unravel the exact effect of multiple boosts of the IAV-specific T-cell response.

Taken together, we found that the recall T-cell response to IAV did not significantly differ between early- and late-primed middle-aged mice. This suggests that the T-cell response after vaccination with a T-cell inducing influenza vaccine in humans may not be affected by the time since previous IAV infection. Although our study only covers one aspect of the heterogeneity between humans – the timing between two infections/vaccinations – this is promising for the development and testing of new IAV vaccines that focus on inducing cellular responses in the middle-aged population to provide protection at older age.

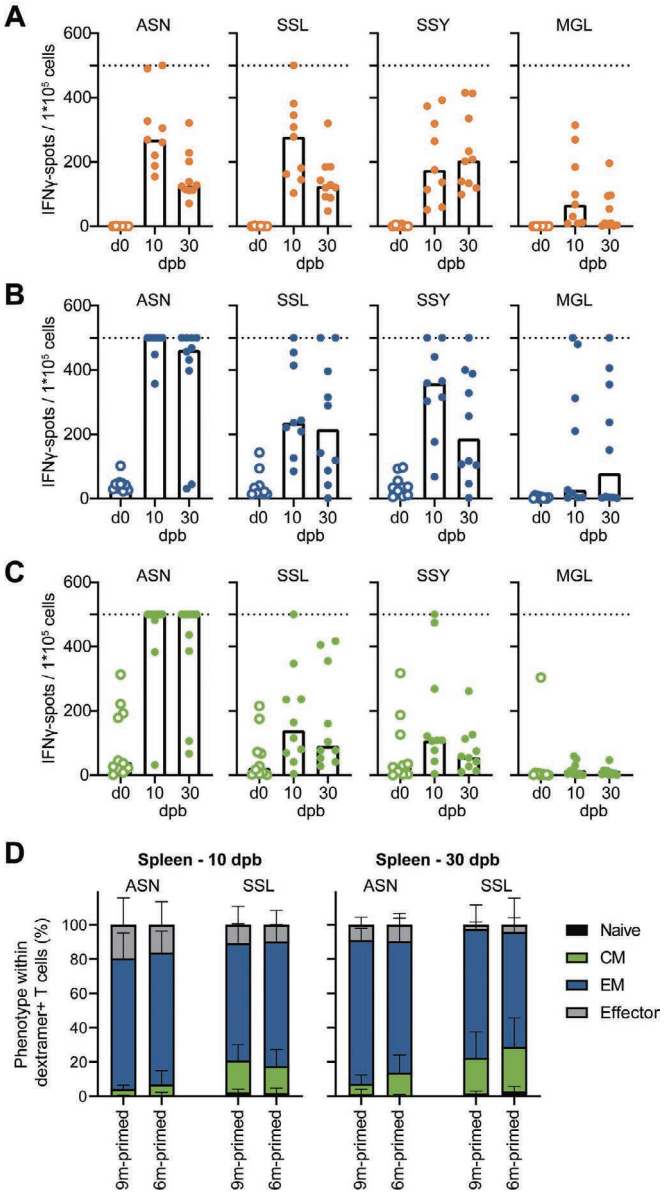
ACKNOWLEDGEMENTS

The authors are grateful to the employees of the Animal Research Centre for animal handling. We thank Noortje Smits, Ronald Jacobi and Jolanda Kool for their assistance in the lab and Teun Guichelaar for critically reading the manuscript.

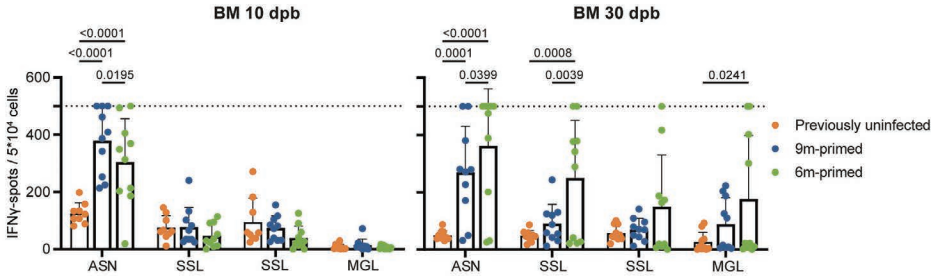
SUPPLEMENTALS



Supplementary Figure 1: Phenotype of the ASN and SSL-specific T cells before booster
A) Fraction of memory subsets based on CD62L and CD44 expression of the ASN-specific and SSL-specific CD8⁺ T cells in spleen, lung and blood. Bars depict mean naïve (CD62L⁺, CD44⁻), central memory (CM; CD62L⁺, CD44⁺), effector memory (EM; CD62L⁻, CD44⁺) and effector T cells (CD62L⁻, CD44⁻) with standard deviation. B) Percentage of EM, CM and ***C)*** *PD1⁺ within ASN-specific and SSL-specific CD8⁺ T cells depicted as individual mice with mean and standard deviation.*

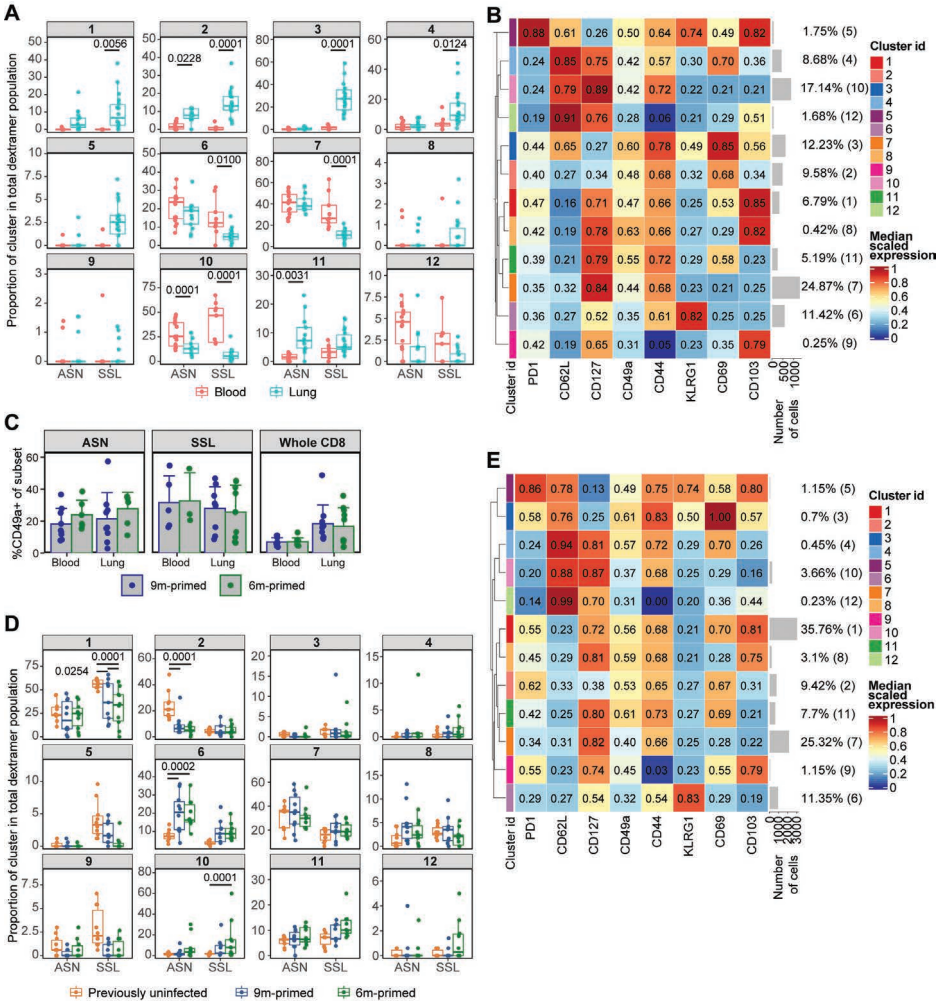


Supplementary Figure 2: Kinetics of T cell response after booster infection.
A-C) Cellular responses in IFNγ-ELISpot assays of splenocytes from A) previously uninfected mice, B) 9m-primed mice and C) 6m-primed mice at day 0 (d0) or 10 and 30 days post booster (dpb) after restimulation with IAV specific epitopes. Results are depicted as individual mice (open circles = before booster, closed circles = after booster) with mean (bar). Horizontal dotted lines depict the upper limit of detection of the assay. D) Relative size of T-cell subsets based on CD62L and CD44 expression within the ASN-specific and SSL-specific CD8⁺ T cells in spleen at 10 and 30 dpb. Bars depict mean naïve (CD62L⁺, CD44⁻), central memory (CM; CD62L⁺, CD44⁺), effector memory (EM; CD62L⁻, CD44⁺) and effector T cells (CD62L⁻, CD44⁻) with standard deviation.



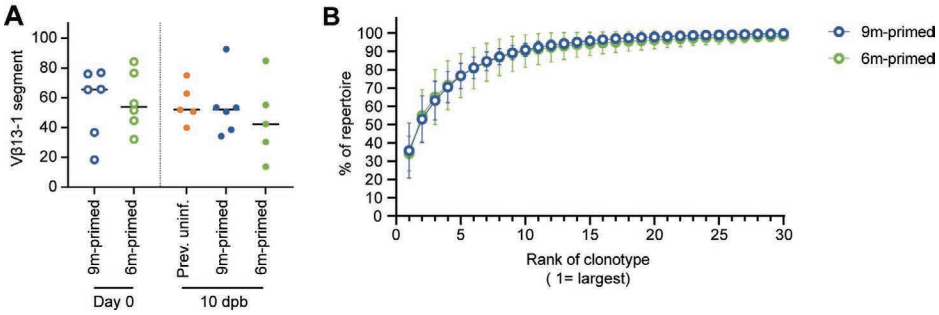
Supplementary Figure 3: IAV-specific T-cell responses at 10 and 30 days post booster (dpb) in the bone marrow.

IFNγ-responses measured by IFNγ-ELISpot in bone marrow against the IAV-specific epitopes of previously uninfected mice, and of 9m-primed and 6m-primed mice at 10 and 30 dpb. Results are depicted as individual mice (open circles = before booster, closed circles = after booster) with mean (bar) and standard deviation.



Supplementary Figure 4: uniform manifold approximation and projection (UMAP) analysis of ASN-specific and SSL-specific T cells from blood and lung.

A, B) UMAP of ASN-specific and SSL-specific CD8⁺ T cells of blood and lungs from 6m-primed and 9m-primed mice before booster infection (day 0). Data of 6m-primed and 9m-primed mice are combined for this analysis. A) Comparison of clusters between blood and lung populations. B) Heatmap of 12 clusters generated by UMAP. C) Expression of CD49a on ASN-specific, SSL-specific and whole CD8⁺ T cells from blood and lungs of 6m-primed and 9m-primed mice before booster infection (day 0). D, E) UMAP of ASN-specific and SSL-specific CD8⁺ T cells from lungs of previously uninfected, 6m-primed and 9m-primed mice after booster infection (day 30). D) Comparison of clusters between previously uninfected, 6m-primed and 9m-primed. E) Heatmap of 12 clusters generated by UMAP. A, C, D) Results are depicted as individual mice (circles) with (A, D) boxplots depicting the median with the first and third quartiles or (C) bars depicting the mean and standard deviation.



Supplementary Figure 5: Clonal distribution of ASN-specific repertoire before booster. *A) Usage of Vβ13-segment within ASN-specific CD8⁺ T cells isolated from spleen of previously uninfected, 6m-primed and 9m-primed mice at day 0 and 10 days post booster (dpb). Horizontal line depicts the median per group. B) Clonal distribution within the TCR repertoire, ranking the top 30 most prevalent clones starting from the largest clone, plotted as the cumulative frequency at day 0. In A, results are depicted as individual mice (open circles = before booster, closed circles = after booster) with mean. In B, results are depicted as average per group (closed circles) with standard deviation.*

Modified influenza CD8⁺ peptide vaccination induces non-relevant T cells and may enhance pathology after challenge

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Submitted

ABSTRACT

CD8⁺ T cells are promising targets for vaccination against influenza A virus (IAV) infection. Their induction via peptide vaccination is not trivial, because peptides tend to be weakly immunogenic. One strategy to overcome this is by vaccination with chemically enhanced altered peptide ligands (CPL), which have improved MHC-binding and immunogenicity. It remains unknown to what extent CPL-vaccination affects the resulting immune response. We studied the effect of CPLs derived from the influenza GILGFVFTL (GILG) epitope on the T-cell response. CPL-vaccination led to higher T-cell frequencies, but only a small percentage of the induced T cells recognized the GILG-wildtype (WT) peptide. CPL-vaccination resulted in a lower richness of the GILG WT-specific T-cell repertoire and no improved protection against IAV-infection compared to GILG-WT peptide-vaccination. One CPL even appeared to enhance pathology after IAV-challenge. CPL-vaccination thus induces T cells not targeting the original peptide, which may lead to potential unwanted side effects.

INTRODUCTION

Despite the availability of vaccines, influenza A virus (IAV) infection is still a worldwide health threat. Traditional influenza vaccines induce humoral immune responses against the highly variable viral surface proteins haemagglutinin (HA) and neuraminidase (NA) [367], which are relatively narrow and therefore mainly strain-specific. Via mutations in these surface proteins (antigenic drift), IAV can escape previously induced immunity. In contrast to humoral immune responses, cellular immune responses are often directed against more conserved parts of the virus. Cellular immunity may therefore provide cross-protection against seasonal drifting strains and against newly emerging influenza viruses with pandemic potential. This is corroborated by the observation that individuals with pre-existing IAV-specific T-cell immunity have an immunological advantage resulting in (partial) protection upon encounter with a new IAV infection [29, 30, 32]. Therefore, induction of cellular responses against the more conserved parts of the virus is of great interest for the design of new, broadly-reactive vaccines against IAV.

One way to induce cellular responses is via peptide vaccination. Peptides alone, however, tend to be weak immunogens. To overcome this, different approaches to induce robust T-cell responses by peptide-based vaccination have been tested, including i) vaccination with long synthetic peptides to simultaneously activate $CD8^+$ and $CD4^+$ T cells [368], ii) the use of strong adjuvants or immune stimulants, as seen in peptide-conjugate vaccines, and iii) optimization of the drug delivery system [369]. Previously, we conducted a study with chemically enhanced altered peptide ligands (CPLs) to induce IAV-specific T-cell responses [100]. By changing the residues of a peptide at or near the anchor into non-proteogenic amino acids, the binding affinity of the peptide to the MHC molecule can be increased, thereby prolonging the presentation of vaccine peptides on the cell surface. A prerequisite for the success of this approach is that most T cells induced by CPL-vaccination should be able to recognize the wildtype (WT) viral peptide. We hypothesized that prolonged presentation would lead to enhanced T-cell immunogenicity and a broader T-cell response, both at the clonal level (i.e. more T-cell receptor (TCR) diversity) and in terms of cross-reactivity against naturally occurring viral mutants, since T cells would have more time to recognize the peptide, allowing more clones to react. Furthermore, the incorporation of non-proteogenic amino acids may make CPLs resistant to proteolytic degradation. We indeed observed that several CPLs had enhanced MHC-binding affinity compared to natural IAV-peptides,

and that vaccination of mice with CPLs induced a higher IFNγ-response than vaccination with the WT peptide [100].

In the current study, we investigated additional features of the T-cell response after vaccination with CPLs, including the frequencies of peptide-specific $CD8^+$ T cells, and their IFNγ-expression, cross-recognition and TCR repertoire diversity. We focused on the response against the IAV-specific Matrix protein 1 GILGFVFTL (GILG) epitope, which is highly immuno-dominant in humans with HLA-A2. The GILG epitope is highly conserved between different IAV strains, emphasizing the advantage of inducing a robust response against this epitope [370]. We selected four different CPLs of the GILG epitope, all with enhanced binding affinity to the HLA-A2 molecule, but resulting in different T-cell responses after vaccination compared to vaccination with the WT peptide [100]. We evaluated the characteristics of the different responses that were induced *in vivo,* in a transgenic mouse model expressing a hybrid class I MHC gene, containing the α-1 and α-2 domain of the human HLA-A2.1 gene. We found that CPL-vaccination induced higher T-cell frequencies than vaccination with the WT peptide. The majority of the induced T cells, however, did not recognize the WT peptide, and are therefore considered non-relevant. Furthermore, vaccination with CPLs led to a more skewed T-cell repertoire against the WT peptide. Most importantly, we found that CPL-vaccination did not provide better protection against IAV-challenge than vaccination with the WT peptide. Vaccination with one of the CPLs even tended to cause enhanced pathology after IAV-challenge. These findings show that modified-peptide vaccine strategies may induce a substantial amount of non-relevant, and possibly even detrimental, T cells. Such strategies should thus be monitored carefully when used to protect against infectious diseases.

MATERIALS & METHODS

Ethical Statement

The study was approved by the Animal Welfare Body of Poonawalla Science Park, Animal Research Center (Bilthoven, The Netherlands) under permit number AVD3260020173890 of the Dutch Central Committee for Animal experiments. All procedures were carried out in accordance with EU legislation. Mice were inspected daily and were provided food and water ad libitum. Mice were housed by subgroup in filtertop Macrolon III cages and accommodated with cage enrichment (Igloo's and nestlets). If mice would have reached any of the humane endpoints prior to scheduled termination they would have been euthanized. Endpoints were defined as: > 20% weight loss, pumping breath, inactive, feeling

cold, bulging. None of the mice reached a humane end point during the study, however two animals did not wake up after they were anesthetized during virus infection and one animal was found dead in the cage, likely due to narcosis. When the experimental end point was reached, mice were anesthetized (isoflurane/oxygen) and bled by orbital puncture. Mice were anesthetized during influenza challenges by isoflurane in O2 to minimize suffering.

Study design

HLA-A2 transgenic female mice, B6.Cg-Immp2l-Tg (HLA-A/H2-D)2Enge/J (Jackson Laboratory, USA) arrived at the Animal Research Centre (Bilthoven, the Netherlands) at least 2 weeks before commencement of the study for acclimatization. Mice were aged between 5 and 10 weeks at day of arrival. The animals were semi-randomly distributed; older mice were used first and younger mice were used in the later experiments.

Dose finding experiment

For this experiment mice were vaccinated with different doses (10 nm – 30 nm – 90 nm – 270 nm) of the WT or MOD1 peptide (Table 1). Each treatment group consisted of four animals, while both the negative (mock vaccination) and the positive (virus challenge) control group consisted of 8 animals. For practical reasons the experiment was divided into four sub experiments, in which 1 mouse from the peptide vaccination group was vaccinated or sacrificed and 2 mice from the negative and positive control group. Mice were housed together based on sub experiment and based on the peptide with which they were vaccinated (WT or MOD1) with 1 mock-vaccinated mouse in every cage to reduce cage effects. The mice infected with IAV were housed separately from the vaccinated mice. On day 0, groups were vaccinated subcutaneously (s.c.) in the left groin fold. On day 21, mice received the booster vaccination s.c. in the right groin fold. Ten days after the booster vaccination, mice were sacrificed.

Mod1 – Mod4 (6 till 11 weeks of age)

Each treatment group consisted of 8 animals, which were either vaccinated with a peptide or infected with influenza virus (PR8 strain, 50 µl i.n.). The control group consisted of 4 animals and received a mock-vaccination consisting of DMSO. Peptide vaccinations consisted of the WT peptide or one of the 4 CPLs (MOD1, MOD2 MOD3 or MOD4, Table 1). Mice were vaccinated s.c. on day 0, in the left groin fold. At day 21, the mice received their booster vaccination, s.c. in the right groin fold, while the positive control group received a virus infection i.n. with 50 µl PR8 virus (1x103 TCID50). For the first part of the experiment, 8 mice per peptide

vaccination were euthanized 10 days after the booster vaccination (day 31), as well as 8 mice after the IAV infection, and 4 mice of the mock group. For practical reasons, the experiment was divided into two parallel groups (A and B) with a time difference of 1 week, with 4 mice (treatment group and virus infection) or 2 mice (mock vaccination) per subgroup.

We continued the experiment with MOD1 and MOD2 of the CPLs, to investigate early memory formation and protection against challenge. For this part, extra subgroups of mice were made in the groups receiving vaccination with the WT, MOD1 or MOD2 peptide (total of 16 mice per vaccination type). These mice received their vaccination or challenge at the same time as the mice described above, however, per vaccination, 8 of the 16 mice were sacrificed at day 51 (30 days after the booster vaccination); while the other 8 mice received a virus challenge i.n. with 50 ul PR8 ($1x10^5$ TCID50) at day 54 and were sacrificed at day 58 (5 days after the virus challenge). Both section moments were controlled by a mock vaccination (2 mice at day 51 and 8 mice after the virus challenge), and mice immunized by infection with IAV (8 mice at both day 51 and day 58).

Sample Collection

Of every mouse, spleen, heparin blood and serum were collected. Measurements on spleen and blood were performed at the day of withdrawal, serum was stored at -20 °C. Spleens were homogenized and passed through 70 um filters (BD biosciences), washed with RPMI 1640 containing 10% FCS and 100 U/ml penicillin, streptomycin, and glutamate. Erythrocytes were lysed using ACK-buffer (NH4Cl 0.15M, KHCO3 0.01M, Na2EDTA 0.1 mM). At day 59, besides the collection of the spleen, heparin blood and serum, also the lungs were collected. The right part of the lung was collected in formalin and used for histopathology, the left part of the lung was collected in Lysing Matrix A tubes and stored at -80 °C for virus titer analyses.

Peptide vaccination

Peptides were adjuvanted with Incomplete Freund's Adjuvant (IFA) (1/1 (V/V)) and CpG (50 µg/mouse) and supplemented with PBS. The mixture was vortexed for 1 hour. Mice were vaccinated with the indicated peptides at their respective doses in a volume of 100 µl. Before use, the freeze-dried peptides were dissolved in DMSO, aliquoted and stored at -20 °C. Mock vaccination consisted of DMSO and adjuvants.

CPLs were designed as described before and synthesized at the Department of Cell and Chemical Biology, Leiden University Medical Centre, by standard solid-phase peptide synthesis using Syro I and Syro II synthesizers [371]. The non-proteogenic peptides used in this study were selected based on their MHC-binding affinity and induced T-cell response as described in Rosendahl Huber et al. 2016 [100]. WT and natural variant peptides were synthesized at DGpeptides (China), with a purity of >99%.

Virus

Influenza A/PR/8/34 (PR8; NIBSC code 16/108) virus was obtained from the National Institute for Biological Standards and Control (NIBSC, Hertfortshire, UK). Influenza viruses were grown on MDCK cells in MEM medium (Gibco; Thermo Fisher Scientific) supplemented with 40µg/ml gentamicin, 0.01M Tricine and 2µg/ml TPCK treated trypsin (all from Sigma-Aldrich). At >90% cytopathic effect (CPE), the suspension was collected and spun down (4000x g for 10 minutes) to remove cell debris. Supernatant was collected, aliquoted and frozen at -80°C.

ELISpot Assay

Pre-coated mouse IFNγ-ELISpot (ALP) plates (Mabtech) were used according to the manufacturer's protocol. Splenocytes were stimulated with 0.1 nmol/well WT or modified peptide in ELISpot plates at 37 °C. Controls consisted of medium and PMA/ionomycin stimulation. Per well, 400.000 cells were added. After 24h, the plates were developed according to the manufacturer's protocol. Plates were dried for 1 night, after which they were analysed using the ImmunoSpot® S6 CORE (CTL, Cleveland, OH). Maximum count was set at 500 spots per well.

Flowcytometry

Approximately 2 million splenocytes and lysed whole blood cells were stained using the commercial A*0201/ GILG dextramer PE. In the same reaction, samples were also stained with an APC-labeled dextramer, loaded with a modified peptide, corresponding to the CPL vaccination. Surface staining was performed for 30 min at 4 °C with the following antibodies: CD3(17A2)-FITC, CD8a-PerCP/Cy5.5 (53-6.7), Fixable Viability Stain 780, CD127(SB/199)-BV421, CD62L(MEL-14)-BV786, CD44(IM7)-PE/Cy7, KLRG1(2F1)-PE/CF594, CD4(GK1.5)-BUV395 (All BD Biosciences). Data acquisition was performed on an LSRFortessa X-20 and data analysis was performed using FlowJo v10.6.2 (BD) software.

Isolation of WT-specific T cells for TCR repertoire analysis

CD8⁺ T cells were isolated from PBMCs using a negative selection microbeads kit (Miltenyi Biotec) according to the manufacturer's protocol. CD8⁺ T cells were subsequently labeled at room temperature for 20 min with the A*0201/GILG dextramer (PE, Immudex) and corresponding dextramers manufactured with the modified peptides (APC, Immudex). Subsequently, surface staining was performed using the following mAbs: CD3(17A2)-FITC, CD4(GK1.5)-BV510, CD8(53-6.7)-BV786 (All BD Biosciences). CD3⁺CD4−CD8⁺dextramer⁺ cells were then sorted using a FACS Melody (BD) directly into RNAlater (Ambion Inc. Applied Biosystems) and stored at −80°C for subsequent TCRβ clonotype analysis.

Preparing TCRβ cDNA libraries for sequencing

mRNA was isolated with the RNA microkit (Qiagen) according to the manufacturer's protocol. Isolated mRNA was used in the 5' RACE-based SMARTer Mouse TCR α/β profiling kit (Takara Bio USA, Inc.) to perform sequencing of TCRs, following the manufacturer's protocol using only the TCRβ-specific primers. Cleanup was performed with AMPURE XP clean-up beads (BD). PCR products were sequenced via Illumina MiSeq paired-end 2x300 nucleotide (nt) sequencing.

TCRβ clonotype analysis

Demultiplexed samples were first merged using tool Paired-End reAd mergeR (PEAR) [336]. Clonotype information was identified from the raw sequence data using the pipeline RTCR [224] by aligning the sequences to reference TRBV and TRBJ genes. Clonotypes were defined by their CDR3 amino acid sequence.

TCR sequences were only accepted when they consisted of at least 100 sequencing reads, to clean the data from possible errors and contamination. Different cut-offs were tested to make sure the choice of the exact cut-off did not influence our qualitative results. TCR diversity was calculated using the previously described Simpson's diversity index (Venturi et al., 2007). This index ranges between 0 and 1, with 0 representing minimal diversity and 1 representing maximal diversity.

Pathology

Pathology scoring of the lung was performed as previously described [372]. In brief, after fixation the right half of the lung was embedded in paraffin and sliced into 5µm thick sections. Haematoxylin and eosin-stained slides were examined microscopically at 5x, 10x and 20x magnification. Pathological scoring distinguished between 'damage', 'peri-inflammation' and 'intra-lymphocyte filtrate'. Damage related parameters included hypertrophy, hyperplasia, flattened

or pseudo squamous epithelia, necrosis and denudation of bronchi(oli) epithelium, hyperemia of septa and alveolar emphysema and haemorrhages. Inflammation related parameters included (peri)bronchi(oli)tis, interstitial infiltrate, alveolitis and (peri)vasculitis characterized by polymorphonuclear (PMN) cells and macrophages. Intra-lymphocytic infiltration-related parameters included lymphocytes, lymphoblasts, and plasma cells. Pathological findings were scored on a scale of 0 (no aberrations) to 5 (severe damage) of which the median was taken as the 'end score' for the damage, peri-inflammation or intra-lymphocyte filtrate for different components (e.g. bronchi, bronchiole, blood vessels, interstitium, alveoli) per mouse. Per mouse, at least 8 microscopic fields were scored. An end score was used to summarize the total pathology of the lung. The percentage affected lung tissue was estimated at 20x magnification. Microscopic slides were randomized and scored blindly.

Virus titer analysis

Virus titer analysis was performed on tissue of the left half of the lung. For analyses, tissue stored in Lysing Matrix A tubes was thawed and 500µl of Minimal Essential Media (MEM, Gibco) supplemented with 250 ug/ml gentamicin and TPCK-trypsin was added to each tube. The samples were then dissociated using FastPrep (MP Biomedicals). Samples were spun down for 10 minutes at 4000x g and 250µl of the supernatant was serially diluted and tested in 6-plo on MDCK cells. Cytopathic effect (CPE) was cored after 5 days of culturing and TCID50 values were calculated using the Reed & Muench method [373].

RESULTS

Chemically altered peptides

In an earlier study, IAV-specific peptides were chemically altered near or at the MHC-anchor residues using non-proteogenic amino acids [100]. This way the binding to HLA-A2 was enhanced, which in some cases resulted in a higher IFNγ-response upon CPL compared to WT-peptide vaccination in mice. In the current study, we selected four modifications (MOD1, MOD2, MOD3 and MOD4, see Table 1) of the GILGFVFTL peptide to acquire a deeper understanding of the effect of these modifications on the T-cell response after vaccination. These CPLs were selected based on their enhanced MHC-binding scores compared to the WT peptide (Table 1) and subsequent different outcomes in their enhancement of the IFNγ-response [100].

Table 1: Overview of peptide modifications and corresponding binding affinity to MHC.

	Amino Acid sequence + modification	Original name [100]	Binding (% of inhibition) after 4h	Binding (% of inhibition) after 24h
WT	GILGFVFTL		85±0	84±2
MOD1	[am-phg]ILGFVFTL	G1	97±4	98±4
MOD2	[3-pyra]ILGFVFTL	G8	94±3	93±1
MOD3	G[NLE]LGFVFTL	G16	91±3	90±5
MOD4	[Some]ILGFVFTL	G25	89±12	89±13

HLA-A*0201 binding affinity was determined by a competition assay as described in the study of Rosendahl Huber [100]. Binding was scored as percentage inhibition of tracer peptide binding after 4h and 24h.

Dose-response experiment MOD1

First we performed an extended dose-finding study with a broader range of peptide concentrations and more animals, to select a dose for further immunological analysis of the T-cell responses induced by the CPLs. To this end, we selected the WT peptide and one CPL (MOD1) that previously showed an improved response [100]. We prime-boosted HLA-A2 transgenic mice with either 10, 30, 90 or 270 nmol of the WT or MOD1 peptide, with an interval of 21 days between prime and boost (Fig. 1A).

Ten days post booster vaccination, we observed that the IFNγ-response of splenocytes induced by MOD1-vaccination already reached its maximum at 30 nmol in an ELISpot assay after *ex vivo* restimulation with WT peptide (Fig. 1B). At higher doses, the response started to plateau. There was no significant difference in the response induced by WT-vaccination or MOD1-vaccination for any of the doses. *Ex vivo* stimulations with MOD1 peptide induced a very high response in MOD1-vaccinated mice and a lower response in mice vaccinated with WT peptide (Fig. 1C).

When we analyzed the peptide-specific T-cell frequencies by staining with WT-loaded HLA-A2 dextramers, we observed a clear subset of WT-specific T cells in both WT and MOD1-vaccinated mice, in both spleen and blood (Fig. 1D+E, Sup. Fig. 1A). Splenocytes isolated from mock-vaccinated mice and IAV-infected mice served as staining controls (Sup. Fig. 1A+B). Comparable to what we observed in the IFNγ-ELISpot assay, WT-specific T-cell frequencies also approached their maximum at a vaccination dose of 30 nmol. Based on these results, we selected 30 nmol as the dose for an in-depth mechanistic analysis of the four selected CPLs.

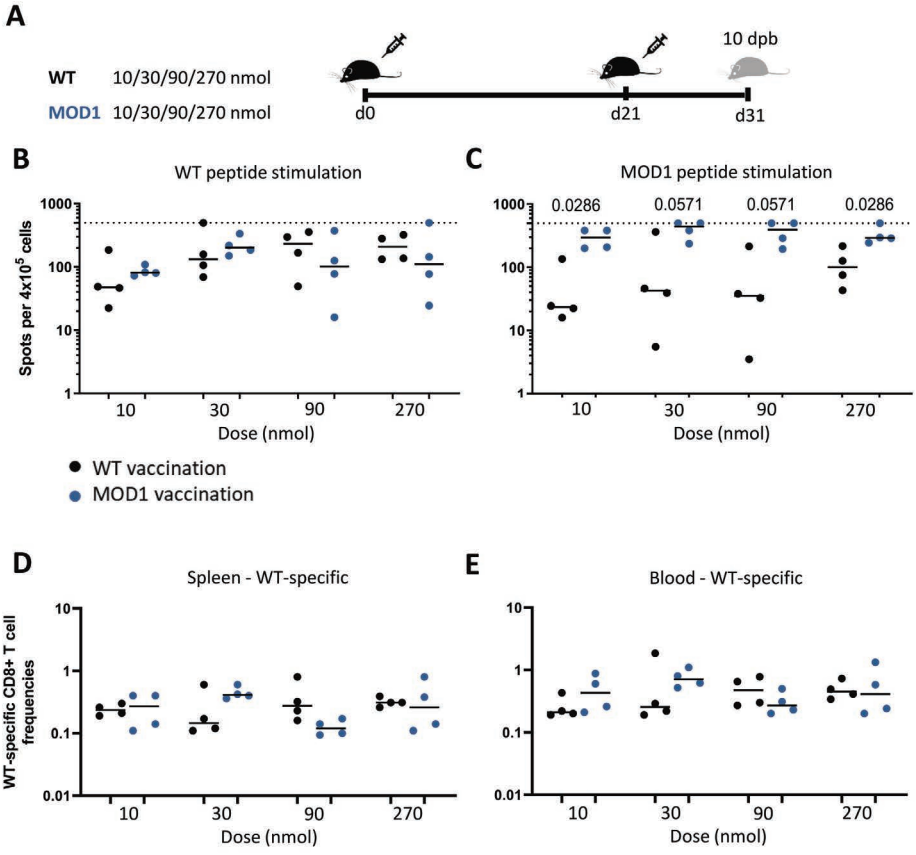

Figure 1: Dose-response experiment with WT and MOD1 peptide.
A) Study layout depicting the prime-boost strategy. Mice were primed at day 0 with peptide vaccination containing either WT peptide or MOD1 peptide with dose range 10-30-90-270 nmol and received the same treatment 21 days later (n=4 per treatment). Lymphocytes were isolated from spleen and blood 10 days post booster (dpd) vaccination. BC) Cellular responses were measured in IFNγ-ELISpot assays after vaccination and restimulation with B) WT peptide or C) MOD1 peptide. Horizontal dotted lines depict the upper limit of the assay. DE) WT-specific CD8⁺ T-cell frequencies were measured with the use of dextramers in D) spleen and E) blood of mice vaccinated with WT or MOD1 peptide. Results of individual mice are shown (points) with median (line). Mice vaccinated with WT are depicted in black, whereas mice vaccinated with MOD1 are depicted in dark blue. Differences between responses to WT and MOD1 are tested using Mann-Whitney U test.

Dissecting the T-cell response induced by the different CPLs

To compare the magnitude of the T-cell responses induced by the different peptide modifications and the WT peptide, we prime-boosted mice with 30 nmol of WT or one of the four CPLs (n=8 per treatment) (Table 1, Fig. 2A). Ten days after boosting, restimulation of splenocytes with the GILG-WT peptide in an IFNγ-ELISpot assay

showed that responses of CPL-vaccinated mice were comparable to those of mice vaccinated with WT (Fig. 2B). When stimulating the splenocytes with the peptide that was used for the vaccination, the IFNγ-response to MOD4 was significantly higher than the response to the WT peptide (Fig. 2C). The responses to the other CLPs were not significantly different from the response to WT, although MOD3 showed a trend to a higher response after homologous stimulation. The response after vaccination with the WT-peptide or CPLs was much lower compared to the response after virus infection (Fig. 2B+C). Although based on the results of our previous study we expected vaccination with all four CPLs to induce a higher IFNγ-response to the WT peptide than vaccination with the WT peptide itself [100], the current dataset (which has a higher power), shows that in fact vaccination with none of these CPLs induces a significantly increased IFNγ-response to the WT peptide.

To distinguish between T cells that recognize the WT peptide or only the CPLs, we combined a CPL- and WT peptide loaded dextramer staining to distinguish between $CD8^+$ T cells recognizing only the WT peptide (WT+), only the CPL (MOD+) or both (Double Positive, DP+) (Fig. 2D + Sup. Fig. 2A). WT-vaccinated mice, had a population of WT+ T cells, as well as a DP+ T-cell population, recognizing both the WT peptide and the CPL (Fig. 2D+E). Mice vaccinated with CPLs had both MOD+ and DP+ T-cell populations.

To investigate whether CPL-vaccination in general resulted in a higher T-cell response, we compared the total dextramer response (sum of WT+, DP+ and MOD+) induced by the different vaccinations. Vaccination with MOD1, 2 and 4 did not induce higher total T-cell frequencies compared to vaccination with WT peptide (Fig. 2E). The total dextramer+ T-cell response induced by MOD3-vaccination was significantly higher than the response induced by WT-vaccination ($p = 0.0281$, Sup. Fig. 2B). In line with the IFNγ-responses, T-cell frequencies measured by dextramer staining were significantly lower after peptide vaccination than after virus infection, both in terms of the WT-specific response and the total dextramer+ T-cell response (Sup. Fig. 2B). Importantly, for all four CPL vaccinations, we observed that the induced T-cell response consisted of a large fraction of T cells that only recognized the specific CPL, both in spleen (Fig. 2E) and in the blood (Sup. Fig. 2B). T cells recognizing only the CPLs and not the WT peptide are unlikely to contribute to T-cell protection upon IAV infection and are thus presumably non-relevant. To determine to which extent CPLs induced useful T cells, we calculated the ratio between the frequency of T cells recognizing both the WT peptide and the CPL (DP+) and the frequency of T cells recognizing only the CPL (MOD+).

Depending on the CPL, the amount of T cells recognizing the WT peptide was between 2 to 5 times smaller than the amount of T cells recognizing the CPL (Fig. 2F). Thus, although vaccination with some of the CPLs resulted in a higher IFNγ-response to the CPL and higher T-cell frequencies compared to WT-vaccination, the responses induced by CPLs consisted to a large extent of non-relevant T cells.

Recognition of naturally occurring peptide variants

Although the WT peptide is known to be relatively conserved, natural variants do occur. We wondered if CPL-vaccination could enhance the recognition of these natural variants when compared to vaccination with WT. We selected four different naturally occurring peptide variants for ex vivo restimulation, namely GILGFV**Y**TL, GILG**L**VFTL, G**M**LGFVFTL and GILGF**I**FTL, based on the highest frequency of occurrence [374]. T cells induced by vaccination with WT showed a comparable IFNγ-response against two other natural variants, G**M**LGFVFTL and GILGF**I**FTL, whereas GILGFV**Y**TL and GILG**L**VFTL were recognized poorly (Fig. 2G + Sup. Fig. 3). T cells induced by vaccination with any of the four CPLs also recognized GILGFV**Y**TL and GILG**L**VFTL relatively poorly. On the other hand, MOD3-vaccination resulted in higher responses against G**M**LGFVFT ($p = 0.0350$) compared to WT-vaccination, whereas vaccination with MOD4 resulted in higher responses against GILGF**I**FTL after restimulation ($p= 0.0499$) in comparison with WT-vaccination (Sup. Fig. 3). This suggests that some CPLs may enhance the recognition of naturally occurring GILGFVFTL viral variants.

Effect of peptide modification on the TCR repertoire of WT-specific T cells

We hypothesized that vaccination with CPLs would lead to a broader TCR repertoire diversity of WT-specific T cells, as extended peptide presentation allows for recognition by more T-cell clones. To investigate this, we sorted T cells recognizing the WT peptide and used Vβ sequencing to determine the WT specific TCR repertoire. The diversity of the TCR repertoire was calculated using the Simpson Diversity Index [225]. We observed relatively large within-group variation in the TCR diversity of the WT-specific T-cell pool, and no significant differences between mice vaccinated with WT peptide or with any of the four CPLs. However, the diversity of the TCR repertoire induced by MOD2 and MOD4-vaccination were both significantly lower compared to the TCR diversity induced by IAV infection (Fig. 3A). This was due to the presence of clonal expansions in MOD2 and MOD4-vaccinated mice, as the top five T-cell clones in these mice covered a significantly larger fraction of the induced T-cell pool than in IAV-infected mice (Fig. 3B).

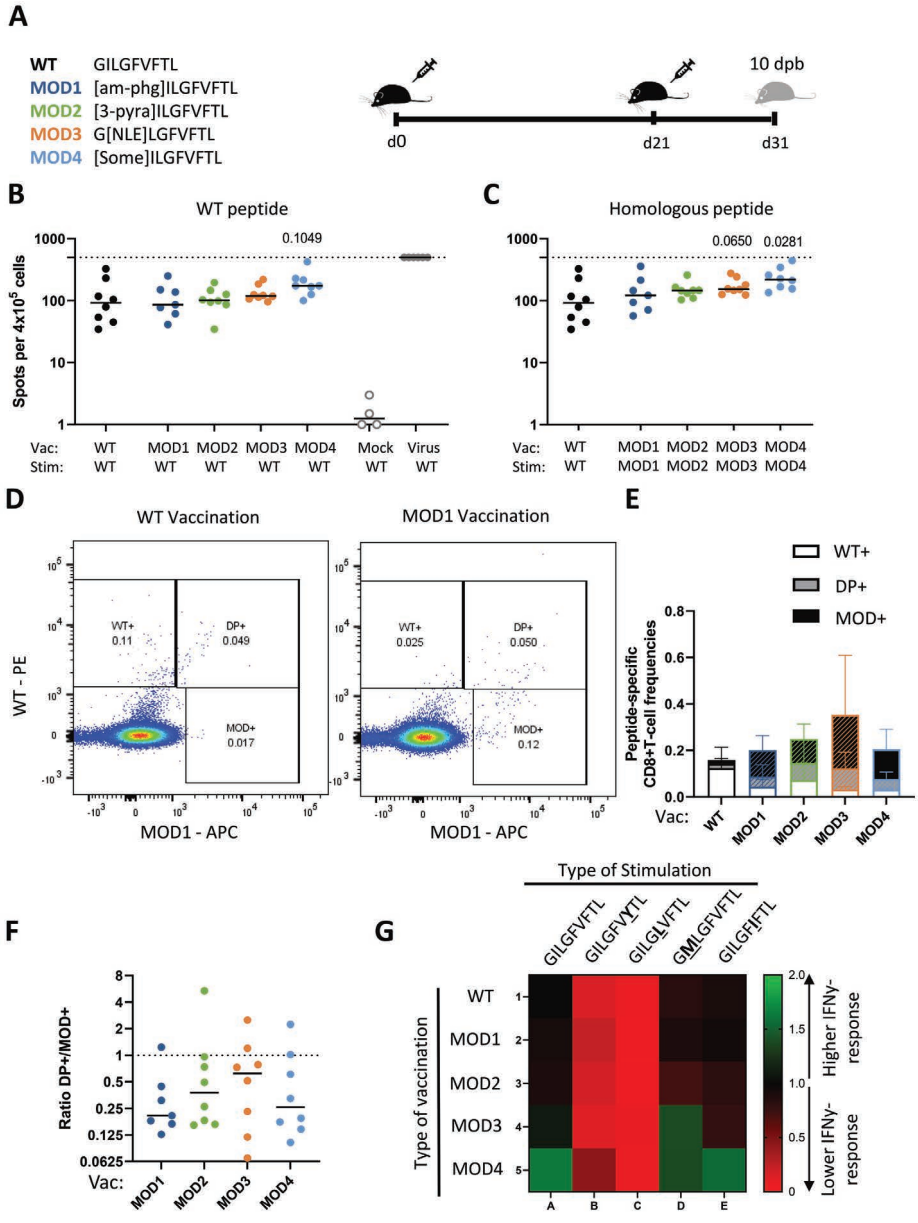

Figure 2: T-cell responses induced by CPL vaccination.
A) Study layout depicting the prime-boost strategy used to compare WT vaccination with the different CPLs (MOD1-4). Mice were primed with 30 nmol vaccine (either WT or one of four CPLs) and were boosted 21 days later. BC) Cellular responses were measured by IFNγ-ELISpot assay after restimulation of splenocytes with B) WT or C) homologous restimulation. Horizontal dotted lines depict the upper limit of the assay. D) Example of gating strategy in mice vaccinated with WT (left) and MOD1 (right) peptide using two different dextramers,

one loaded with WT and one loaded with MOD1. E) Overview of peptide-specific T-cell frequencies present in the WT+, DP+ and MOD+ gate. Stripes depict the specific T-cell populations that recognize the respective CPL (DP+ and MOD+) F) Ratio of number of T cells in the DP+ gate and number of T cells in the MOD+ gate after vaccination with one of the four CPLs. The higher the ratio, the larger the fraction of T cells recognizing the WT peptide. G) Heat-map of the IFNγ-response against 4 natural variants of the WT GILG peptide. Responses against WT by mice vaccinated with WT were used as reference response and set to 1 Data of individual mice are shown (points) with median (line). Mice vaccinated with WT are depicted in black. Mice vaccinated with CPL are indicated by the following colors: MOD1 – dark blue, MOD2 – green, MOD3 – orange, MOD4 – light blue. Differences between responses to WT and CPLs are tested using Mann-Whitney U test.

This difference was confirmed by a significantly lower TCR richness in MOD2 and MOD4 vaccinated mice when compared to WT-vaccinated mice and mice infected with IAV (Sup. Fig. 4A). The diversity of the WT-specific repertoire induced by WT-vaccination was very similar to the repertoire after natural infection, despite the large difference in WT specific T-cell frequencies. The similarity was reflected in a comparable TCR repertoire diversity, evenness and richness (Fig. 3A+B, Sup. Fig. 4A).

It has previously been shown that modifications in peptides can alter the 3D structure of peptide-MHC complexes, leading to the binding of different T-cell receptors [375]. Therefore, we investigated the overlap in sequences and characteristics of the TCR repertoires induced by WT and CPL-vaccination. There was not much overlap in TCR sequences between WT-vaccinated and CPL-vaccinated mice (Fig. 3C). Although at first sight this seems to suggest that CPL-vaccination induces a completely different TCR repertoire, in fact also the number of shared sequences between different WT-vaccinated mice (4 shared sequences) and between WT-vaccinated and IAV-infected mice (13, Fig. 3D) was low. Vaccination with MOD3 led to the highest number of shared TCR sequences with WT-vaccination (Fig. 3C).

Focusing on other characteristics of the TCR repertoire, like V and J usage and CDR3 length of the sequences, showed a skewing towards the usage of Vβ13-1 in the TCR repertoire of WT-vaccinated and IAV-infected mice. This bias seemed even more pronounced in mice vaccinated with MOD2, MOD3 and MOD4 (Fig. 3E), although these shifts were not significant. Within the Vβ13-1^{+} sequences, we observed skewing towards the use of the Jβ segments 2-7, 2-1 and 1-1 for all peptide vaccinations, as well as after IAV infection (Fig. 3F). It has previously been proposed that TCRs with a relatively long CDR3 region are more flexible and thereby more cross-reactive than TCRs with short CDR3 regions [376].

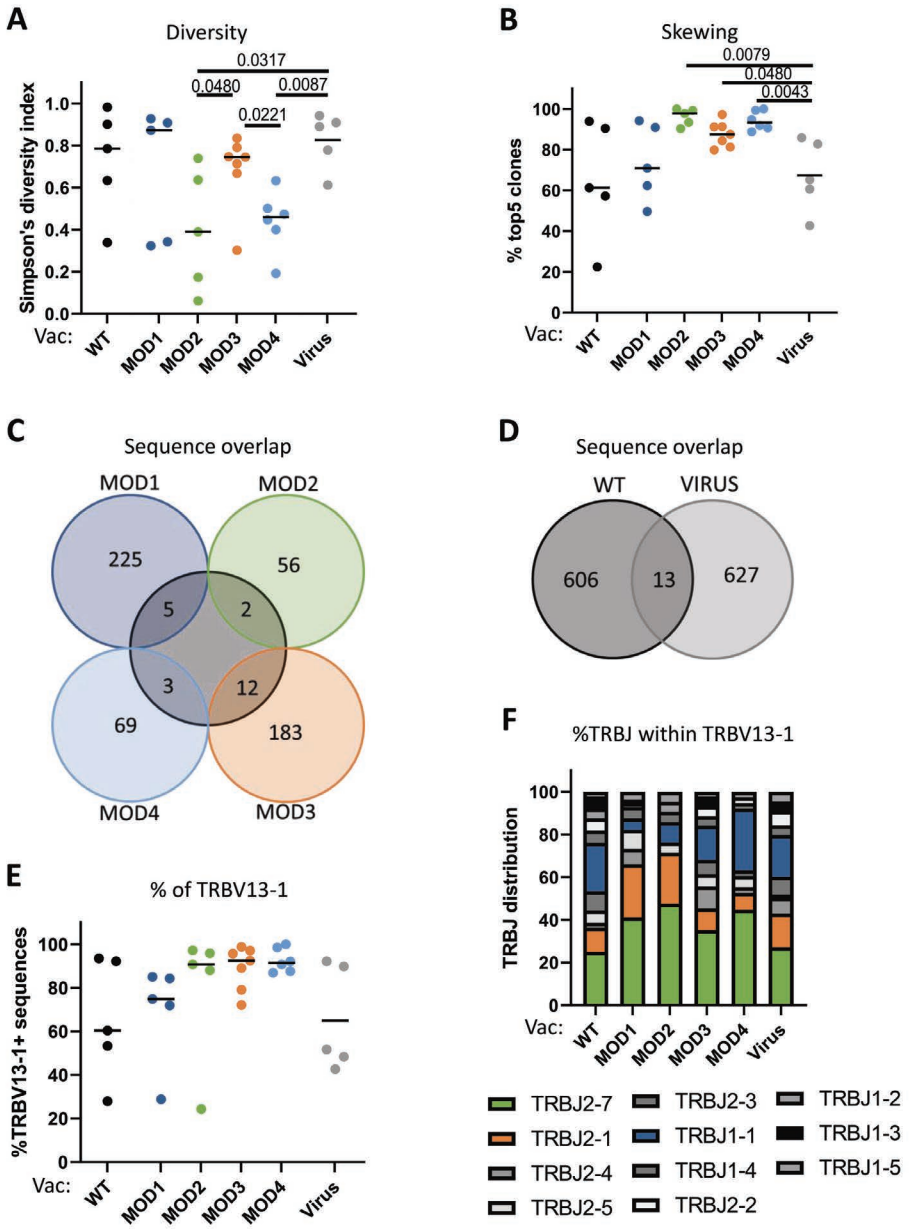

Figure 3: GILG-WT specific T-cell repertoire analyses after peptide vaccination.
A) Simpson's diversity index of the WT-specific TCR repertoire. B) Skewing of repertoire was calculated by the contribution of the top 5 largest clones per sample in percentages. C) Overview of the number of shared sequences between mice vaccinated with WT or one of the four CPLs or WT and the virus-infected mice (right). D) Overview of the percentage of sequences containing Vβ13.1 E) Overview of the different JB segments withing the Vβ13⁺ sequences. Data of individual mice are shown (points) with median (line). Differences between responses to WT and CPLs are tested using Mann-Whitney U test.

The TCR repertoire after WT-vaccination showed a bias towards a CDR3 length of 9 or 10 amino acids, while the WT-specific repertoires induced by CPLs showed a more prominent skewing towards one length (8 amino acids for MOD1 and MOD2, 10 amino acids for MOD3 and 9 amino acids for MOD4, Sup. Fig. 4B). Although MOD3 and MOD4 both induced higher IFNγ responses against some of the naturally occurring GILG variants than WT-vaccination (Fig. 2G), this was thus not associated with longer CDR3 regions of the responding T cells.

T-cell response prior to and after IAV-challenge in peptide-vaccinated mice

We next investigated the protective capacity of CPL-induced immunity in the early memory phase. We focused on MOD1 and MOD2-vaccination, as they were suggested to improve the T-cell response most strongly in our previous study [100]. For this purpose, we vaccinated mice with either WT, MOD1 or MOD2 peptide following the same prime-boost schedule as described earlier and subsequently challenged mice with IAV at day 54 (i.e. 33 dpb) (Fig. 4A). Half of the mice were sacrificed prior to IAV-challenge (day 51, 30 dpb) to study the early memory response present at the time of challenge. The other half of the mice were sacrificed 5 days post infection (dpi), i.e. at day 59, to analyze the immune response and the level of protection against IAV-challenge (Fig. 4A).

For all three peptide vaccinations, a clear contraction of the T-cell response was observed between day 31 and day 51 post booster vaccination, as measured by IFNγ-ELISpot after WT and homologous stimulation (Fig. 4BC). The contraction of the response was similar for mice vaccinated with WT, MOD1 or MOD2. Dextramer staining showed that also after contraction of the response, a large fraction of the T cells induced by vaccination with CPLs only recognized the modified peptide (Fig. 4D). For all three peptide vaccinations, the IFNγ-response against WT was higher post IAV-challenge (day 59) than before challenge (day 51, Fig. 4B). This was also observed after homologous restimulation of T cells from CPL-vaccinated mice (Fig. 4C). The IFNγ-response in mock-vaccinated mice also increased after IAV-challenge (Sup. Fig. 5B), but remained significantly lower than in WT and CPL-vaccinated mice, suggesting that the response in the vaccinated mice was a true memory response (Sup. Fig. 5C). Next we calculated the ratio of T cells recognizing both peptides (DP+) and cells recognizing only the modified peptide (MOD+) (Fig. 4D, Sup. Fig. 5A). Remarkably, the percentage of T cells recognizing the WT peptide seemed to increase after infection, however this was not significant ($p=0.07210$, Fig. 4D).

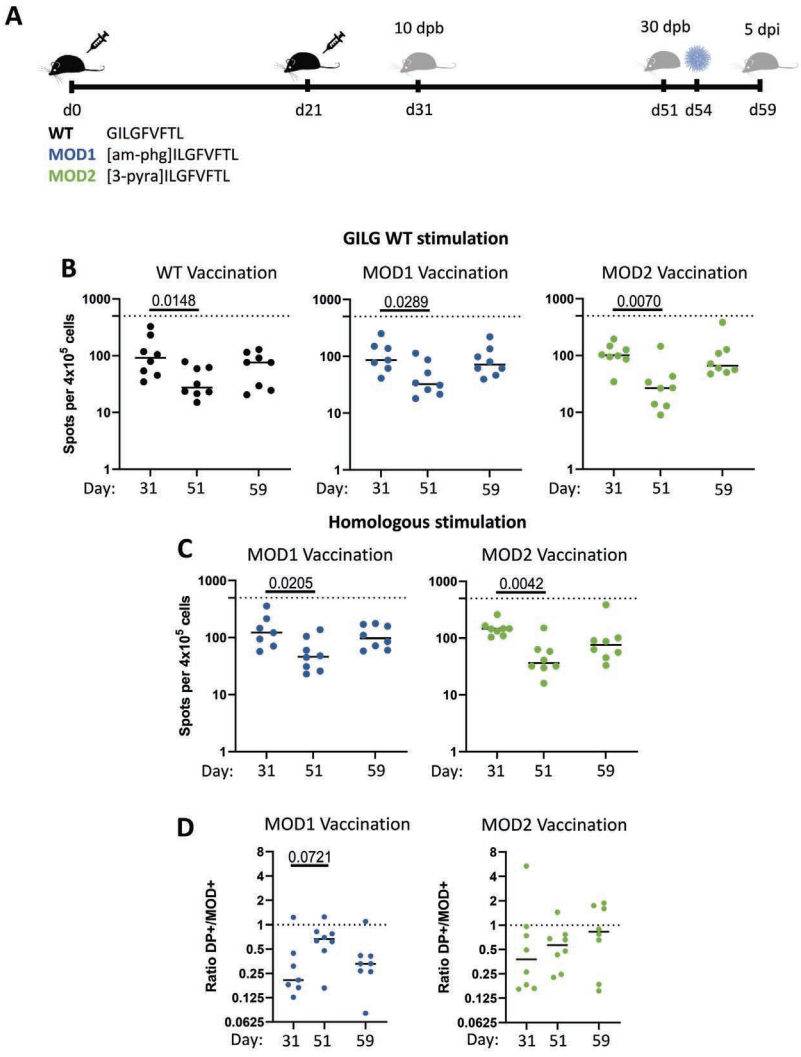

Figure 4: Memory response induced by peptide vaccination.
A) Study layout depicting the prime-boost strategy and subsequent IAV-challenge to compare WT vaccination with MOD1 or MOD2 vaccination. Mice were primed at day 0 with peptide vaccination (30 nmol) and received the same treatment 21 days later. At day 51 (30 days post booster (dpb)) half of the mice were sacrificed to study their memory T-cell responses. The other half received an IAV-challenge (PR8, 1x10^5 TCID50) at day 54 (31 dpb) and were sacrificed 5 days post infection (dpi, day 59). BC) Cellular responses were measured in an IFNγ-ELISpot after restimulation of splenocytes with B) WT or C) homologous restimulation with MOD1 or MOD2. D) Ratio of numbers of T cells in the DP+ and MOD+ gates after vaccination with MOD1 or MOD2. The higher the ratio, the larger the fraction of T cells recognizing the WT peptide. Mice vaccinated with GILG WT are depicted in black. Data of individual mice are shown (points) with median (line). Mice vaccinated with CPL are indicated by the following colors: MOD1 – dark blue, MOD2 – green. Horizontal dotted lines depict the upper limit of the assay. Vertical dotted lines depict the timing of the IAV-challenge. Difference between timepoints are tested using Mann-Whitney U test.

Unexpectedly, the fraction of T cells recognizing only the modified peptide increased after IAV-challenge of mice vaccinated with MOD1. This pattern was not observed in mice vaccinated with MOD2; in the latter mice, the ratio DP+/MOD+ cells remained close to one before and after IAV challenge.

Clinical disease and pathology after IAV-challenge

To assess whether peptide vaccination contributed to lower disease severity after IAV-challenge, i.e. whether it was associated with protection, we assessed clinical disease, pathology and virus replication after IAV-challenge in peptide-vaccinated mice. Mice vaccinated with WT or MOD1 showed a comparable relative weight loss after infection, which seemed a bit less than in mock-vaccinated mice (Fig. 5A). This might hint at some protection induced by WT and MOD1-vaccination, whereas the positive control mice, previously immunized with a virus infection, were completely protected. In contrast, MOD2-vaccinated mice showed the same relative weight loss as mock-vaccinated mice. An increase in relative lung weight (RLW, the ratio of the lung weight at termination to body weight at the day of challenge) resulting from edema and infiltrating leukocytes, is known to be a marker of disease in influenza infection [372]. Mice vaccinated with WT or MOD1 showed a comparable RLW at 5 dpi (Fig. 5B). The RLW in mice vaccinated with MOD2 and mock-vaccinated mice was significantly higher than in mice vaccinated with WT peptide. Surprisingly, the RLW of MOD2 mice appeared higher than mock-vaccinated mice although this was not significant. Virus titers were also significantly higher in the lungs of mice vaccinated with MOD2 than in mice vaccinated with WT peptide ($p=0.0003$, Fig. 5C) and again appeared higher than in mock-vaccinated mice, but the difference was not significant. Although mice vaccinated with MOD1 showed a comparable relative weight loss and RLW as mice vaccinated with WT (Fig. 5B), a significantly higher virus titer was observed in the lungs of MOD1-vaccinated mice ($p=0.0399$). Virus titers were absent and the RLW was not increased in the positive control. Together this suggests that vaccination with the CPLs investigated does not lead to better protection than vaccination with WT peptide and may even induce enhanced disease.

Next we assessed whether peptide vaccination could protect against lung pathology at the microscopic level. IAV Infection resulted in a mild to strong (peri) bronchitis, slight to mild (peri) bronchiolitis, slight to mild vasculitis and alveolitis in mice vaccinated with WT, MOD1, MOD2 or mock (Illustrated in Fig. 5D+E), however with different scores per treatment (Sup. Fig. 6). All mice were scored based on the pathology in different parts of the lungs, e.g. bronchi, bronchioli, blood vessels, interstitium and alveoli (Fig. 5D).

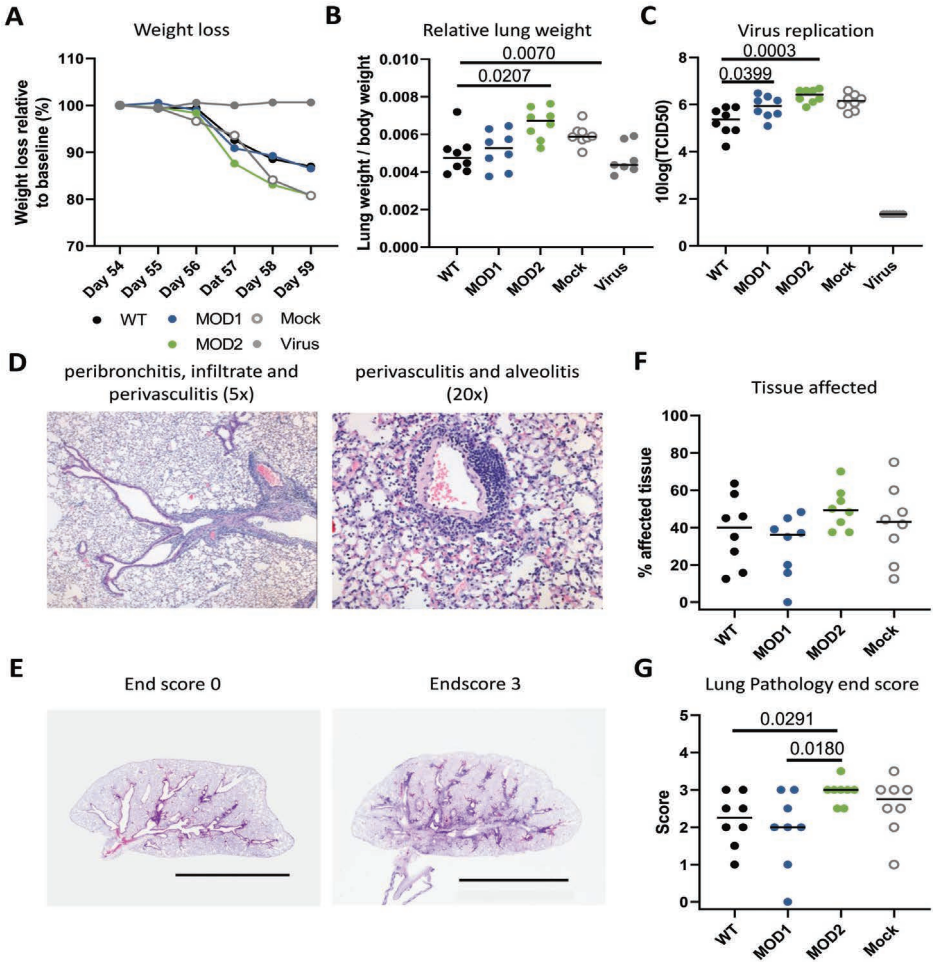

Figure 5: Clinical disease and pathology after IAV-challenge in peptide-vaccinated mice. *5 Days post IAV-challenge (PR8, 1x10^5 TCID50) mice were sacrificed to determine pathology. A) Weight loss in percentage from 1 until 5 days post infection (dpi). Weight at day 54 post booster was set at 100%. B) Lung/weight ratio, calculated by the weight of the right half of the lung, divided by the total body weight at 54 days post booster (dpb). C) Virus titers in the left part of the lung at 5 days post IAV challenge. D) Illustrations of the histopathology of the lung. Peribronchitis and infiltrate in the larger (not the smaller) bronchus and perivasculitis (left image, 5x magnification). Illustration of perivasculitis (score 3) and alveolitis (score 3, right image, 20x magnification). E) Overview of the histopathology of lungs with an end score of 0 (left image) and 3 (right image) illustrating damage and inflammation along the bronchi and bronchioli. Bar represents 0.5 mm. F) Percentage of lung-tissue affected per mice. G) End score (summarizing all the pathology scores) of the total histological damage based on scoring of all subcategories. Histopathology was performed using H&E staining. Pathology was scored on a scale of 0 to 5. Per mouse at least 8 microscopic field were scored, median score per mouse was used. Data of individual mice are shown (points) with median (line). Mice vaccinated with WT are depicted in black. Mice vaccinated with CPL are indicated by the following colors: MOD1 – dark blue, MOD2 – green. Mock vaccinated mice are depicted in grey (open), IAV-infected mice are depicted in grey (solid). Differences between responses to WT and CPLs are tested using Mann-Whitney U test.*

We found no significant differences between the different peptide vaccinations or mock-vaccination, in the percentage of affected tissue in the lungs (Fig. 5F). However, the end score (summarizing all the pathology scores) showed a significantly higher score (comparable to mock-vaccination) for the mice vaccinated with MOD2 compared to mice vaccinated with WT or MOD1 (Fig. 5G). This was primarily due to increased damage and inflammation in the bronchioli, two parameters very characteristic for influenza induced-lung pathology (Sup. Fig. 6). These parameters were even significantly higher in MOD2-vaccinated mice than in mock-vaccinated mice, which support the previous observed trends of an increased RLW and virus replication in MOD2 compared to mock-vaccinated mice (Sup. Fig. 6). Surprisingly, WT and MOD1-vaccination did not lead to a significantly decreased pathology in the lungs compared to mock-vaccinated mice, although some parameters were reduced (i.e. alveolitis in WT peptide mice) (Sup. Fig. 6). Together these data suggest that peptide vaccination -whether with CPLs or with WT peptide- does not lead to significantly enhanced protection against IAV infection. Mice vaccinated with MOD2 even showed increased pathology in the bronchioli after viral challenge compared to mock-vaccinated mice.

DISCUSSION

We investigated how vaccination with CPLs of the immunodominant conserved influenza GILG epitope influences the induced antigen-specific T-cell response in comparison to vaccination with the WT peptide. Although all four CPLs we used were previously shown to have increased binding affinity to the HLA-A2 complex [100], we here show that vaccination with none of these CPLs leads to a larger T-cell response to the WT peptide. In fact, the majority of the induced T cells in CPL-vaccinated mice only recognized the CPL and not the WT peptide. Nevertheless, some of the CPLs showed enhanced recognition of naturally occurring viral GILGFVFTL variants. Furthermore, the T-cell repertoire directed against the WT peptide showed larger clonal expansions of only a few clones when induced by MOD2 or MOD4-vaccination compared to WT-vaccination. CPL vaccination showed a comparable contraction of the T-cell response in the memory phase as WT-vaccination, and did not lead to improved protection against IAV infection when compared to WT-vaccination. Vaccination with one of the CPLs even seemed to result in enhanced disease after IAV infection. Thus, CPLs induced a large number of "non-relevant" T cells, which failed to recognize the WT peptide, which might play a role in the enhanced pathology observed after IAV-challenge.

In this study, we chose to vaccinate with MHC-binding enhanced versions of the immuno-dominant GILGFVFTL peptide. This peptide seemed an ideal vaccine candidate as the GILG peptide is very conserved between IAV strains, most likely because mutations in the peptide lead to functional constraints for the virus [27]. The fact that almost all HLA-A2$^+$ individuals have T cells specific for this peptide suggests the importance of this peptide in the CD8$^+$ T-cell response against IAV infection. Also in HLA-A2 transgenic mice, the response against GILG was found to be dominant [377]. Here, we nevertheless found that vaccination with CPLs of GILG provided minor to no improvement in the cellular response against the GILG WT peptide. An explanation could be that it is harder to improve an already immunodominant response than a subdominant response. On the other hand, the responses induced by all peptide vaccinations were nowhere near the magnitude of the response induced by viral infection, suggesting that there should still be room for improvement. Nevertheless, we found that vaccination with WT and MOD1 provided better protection against IAV-challenge than mock-vaccination, based on weight loss and the relative lung weight. Thus, our data suggest that vaccination with a single peptide (WT or MOD1) could already lead to some level of protection. On the other hand, we also observed a significant increase in damage and inflammation of the bronchioli in MOD2-vaccinated mice compared to mock-vaccinated mice. These aberrations are typical for an influenza infection and their enhancement supports the increased trend in the relative lung weight, which is a non-biased measurement of cellular infiltration and edema formation. Altogether, this indicates that vaccination with CPLs can also lead to enhanced pathology after infection and that this aspects should be carefully considered when peptide vaccine strategies are further pursued.

It could be that vaccination with CPLs of subdominant peptides is in fact a more promising path. In line with this, we previously observed that the IFNγ-responses against the subdominant FMYSDFHFI and NMLSTVLGV peptides were improved more significantly by the introduction of non-proteogenic modifications than the response to GILG [100]. However, as the T-cell responses induced by WT FMYSDFHFI and WT NMLSTVLGV vaccination were low or non-detectable, modifications of these peptides were less suited for in depth immunological analysis, for which comparisons between WT and CPL induced T-cell responses are required. It would nevertheless be interesting to investigate the potential effect of vaccination with a combination of CPLs, targeting different peptides of the virus, as this may lead to a broader T-cell response [378], more comparable to the response induced by virus infection.

All CPLs that were investigated were modified at, or close to, the anchor residues of the GILGFVFTL peptide, which are important for binding to the MHC molecule (P1 or P2), with the underlying idea that the exposed part of the peptide that is recognized by the T-cell receptor would be left intact. We therefore expected that most T cells induced by CPL-vaccination would still be able to recognize the WT peptide. This was not the case, however. Based on dextramer staining we found that for all four CPLs, the majority of the induced T cells recognized only the modified peptide and not the WT peptide. We speculate that this may be due to a different positioning of the peptide in the HLA complex, resulting in a distinct presentation of the central part of the peptide. This phenomenon has indeed been shown for other peptides, for which one substitution at an anchor residue dramatically altered the conformation of the peptide-MHC complex [375] or the conformation of the TCR contact sides [379]. It remains to be investigated whether the induction of a large fraction of non-relevant T cells is characteristic for the GILG peptide or whether it also occurs when modifying other influenza epitopes. It has been suggested that the GILG peptide is a featureless "plain vanilla" peptide, meaning that it lacks prominent side chains pointing towards the T-cell receptor when presented by HLA-A2 [175, 380]. Therefore, there may be fewer ways to bind the peptide and thus only a few TCRs may be sufficiently suited to bind the peptide-MHC complex. This may lead to a highly conserved and skewed T-cell repertoire recognizing this peptide-MHC complex in humans [144, 173, 176]. Therefore it could be possible that modifying the GILG peptide has even greater effects, as it may disrupt the "plain vanilla" binding, resulting in the possibility of more different TCRs to recognize the peptide-MHC complex, compared to peptides that are already presented in a different way.

All four CPLs investigated here induced a large fraction of non-relevant T cells that did not recognize the WT epitope. It would be interesting to further investigate the consequences of the induction of these non-relevant T cells and to study whether they play a role in the observed increased lung pathology in mice vaccinated with MOD2. It has recently been suggested that chemical modification directly alters the immunogenicity of a peptide and could thereby lead to the activation of potentially autoreactive T cells via molecular mimicry of endogenous ligands [381]. Whether this also played a role in the increased lung pathology observed in MOD2-vaccinated mice remains to be investigated.

To understand if a single amino acid substitution leading to enhanced HLA binding can influence the T cells recognizing the peptide, we also performed T-cell repertoire analyses. If anything, our data suggest that the enhanced MHC binding

affinity of the tested CPLs led to more skewing of the repertoire towards Vβ13-1, and not to a broader repertoire as we hypothesized. Although one approach to enhance protection could be the selection of a superior T-cell clone [382], this may not be preferable in the protection against IAV. It is generally thought that a broader TCR repertoire is preferable in protection against mutating viruses, as it increases the chance that T cells are present that can also recognize escape variants of the WT peptide, as has been observed for several other viral infections [46-48]. In this light, the increased skewing of the T-cell repertoire against the WT peptide that we observed after vaccination with some of the CPLs may not be preferable.

The highly skewed and public GILG WT-specific TCR repertoire in humans mostly consists of TCRs expressing TRBV19, TRAV27 and an RS motif in the CDR3β region [173, 383]. In our mouse model, we also observed a strong skewing in the Vβ sequences, however towards TRBV13-1 (homologous to human TRBV10) and not towards TRBV19. We did not observe a dominant motif in the CDR3 regions of the TRBV13-1^{+} T-cell sequences. The mouse-model we used consists of a transgenic HLA-A2 molecule of which the α-3-domain is mouse-specific. This transgenic HLA-A2 molecule could play a role in the differences observed, as also the contact points of the MHC-molecule may play an important role in the TRBV19 skewing observed in humans.

After the IAV-challenge, we expected to see a shift towards the recognition of the WT peptide. Very much to our surprise, in mice vaccinated with MOD1, the fraction of T cells recognizing the WT peptide (useful T cells) decreased. As we only measured these frequencies in the spleen, it is tempting to speculate that in mice vaccinated with MOD1, the T cells that specifically recognized the WT peptide were present at the site of infection. This would be in line with the lower pathology observed in the MOD1-vaccinated mice. Further research on the presence of antigen-specific T cells in the lung would be needed to clarify this.

As influenza A virus infection remains a worldwide health threat, the development of new vaccination strategies is essential. There is a clear need for a more universal vaccine inducing protection against the ever-changing seasonal and potentially pandemic influenza viruses. A strategy that efficiently induces a cellular response against conserved epitopes holds great promise. Although peptide-based vaccinations have the potential to fill this gap, we show that the strategy to enhance MHC-binding by chemically altering the peptides to improve the immune response also has its limitations. More research is needed into different

aspects of peptide vaccination in the battle against infections, including defining the right target-peptide, determining the effect on the diversity of the induced TCR repertoire and monitoring the induction of non-relevant T cells as an off-target immune response with possible unwanted side effects.

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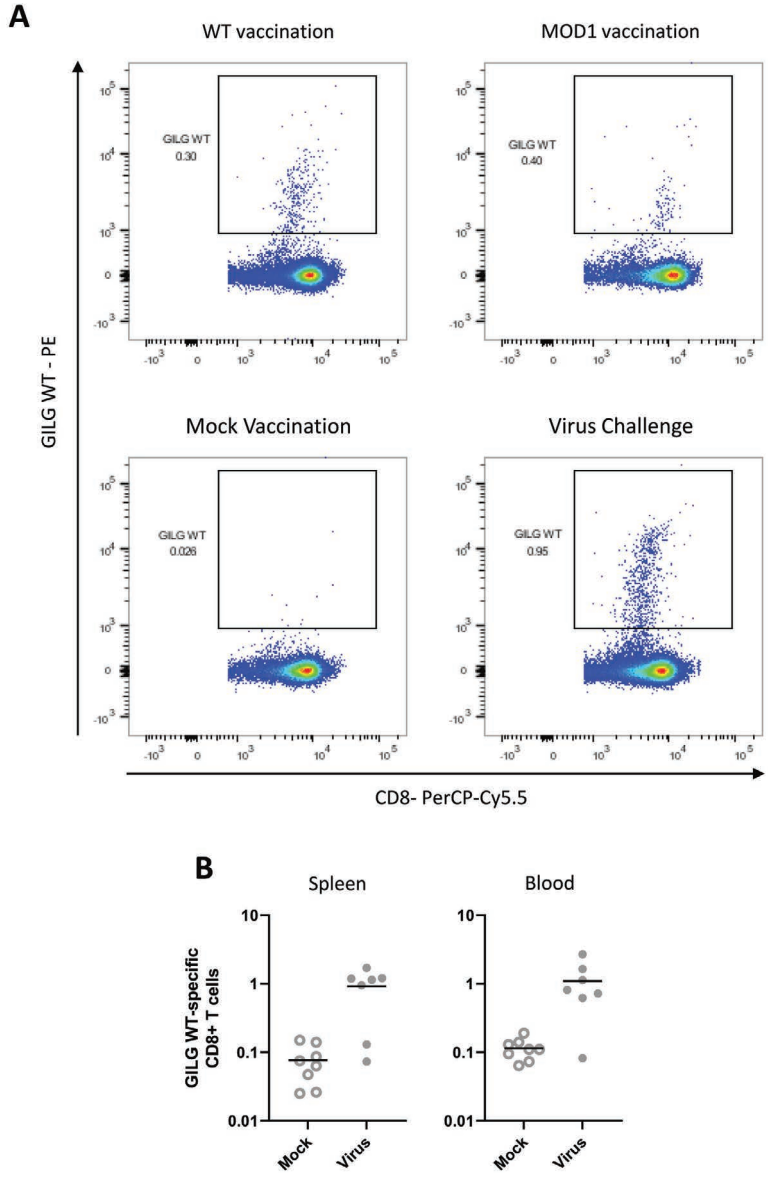
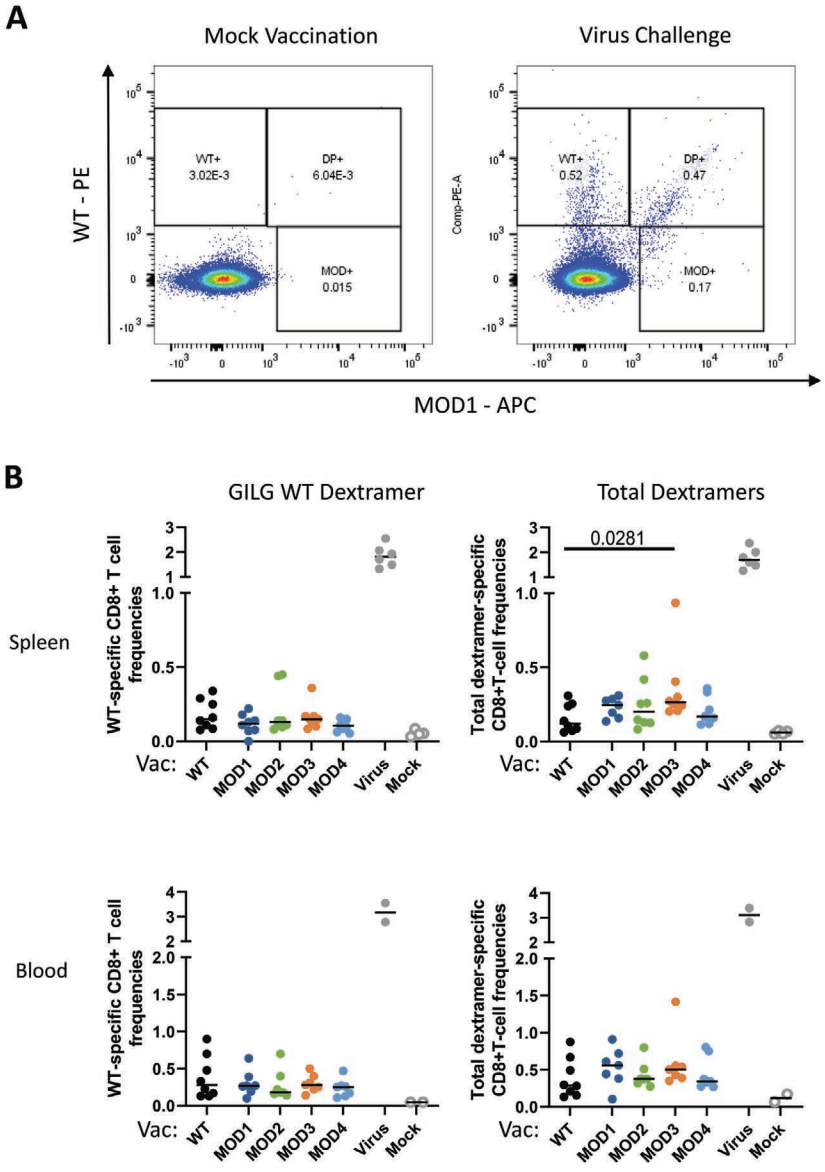
SUPPLEMENTALS

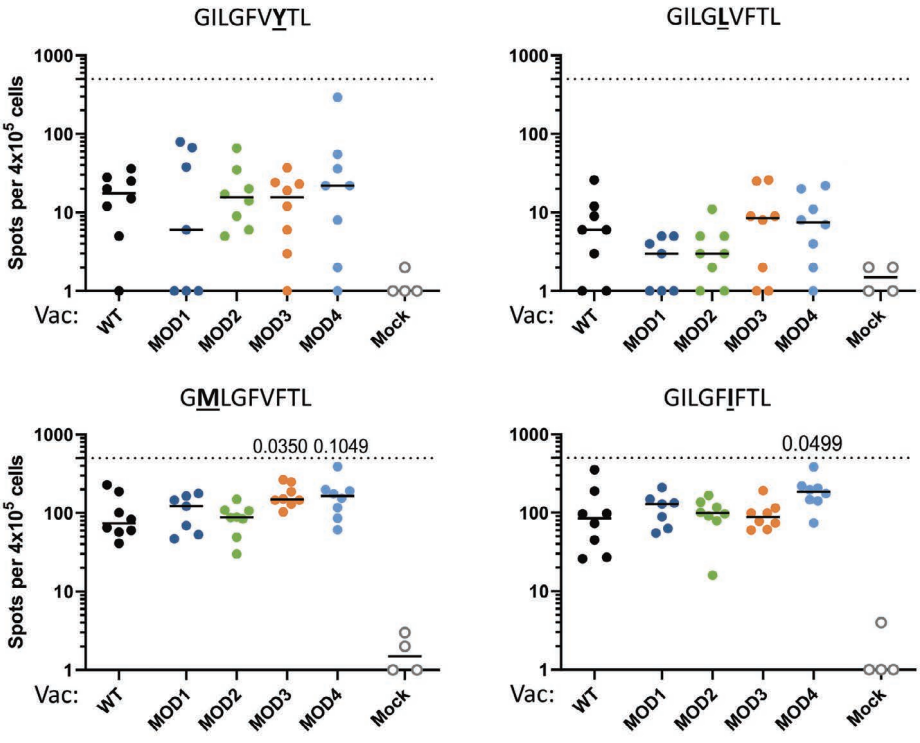


Supplementary Figure 1: Control Dextramer-staining.
WT-positive CD8⁺ T cell frequencies were measured by flow cytometry using WT-peptide loaded dextramers. A) Gating of WT-positive CD8⁺ T cells in mice vaccinated with 10 nmol of WT or MOD1 peptide and the negative (mock vaccinated mice) and positive control (IAV-infection) as measured by flow cytometry. B) WT-specific CD8⁺ T cell frequencies in mock-vaccinated and virus-infected mice. Data of individual mice are shown (points) with median (line),



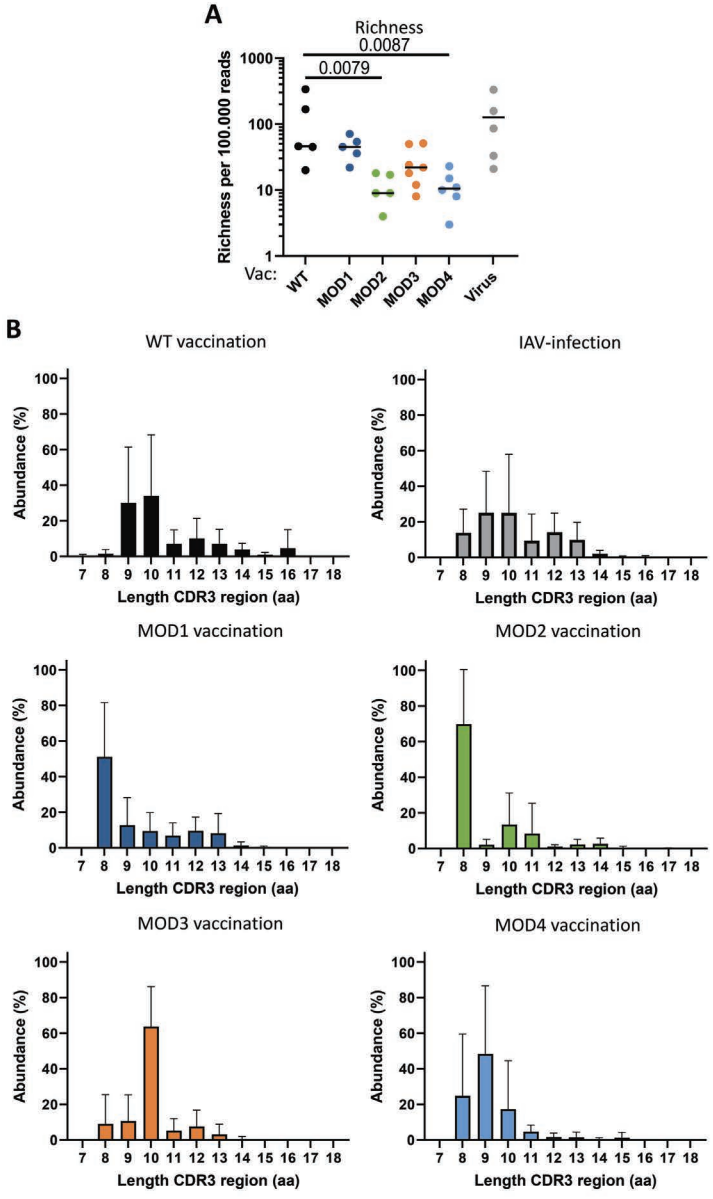
Supplementary Figure 2: Control dextramer-staining and Dextramer+ frequencies after CPL vaccination.

A) Example of gating strategy in mice vaccinated with mock (negative control) and after virus challenge (positive control), using a combination of two different dextramers, one loaded with WT and one loaded with MOD1. B) CD8⁺ T-cell frequencies recognizing WT only (WT+) or both WT and CPL (DP+) as measured in spleen and blood. Mice vaccinated with GILG WT are depicted in black. Data of individual mice are shown (points) with median (line). Mice vaccinated with CPL are indicated by the following colors: MOD1 – dark blue, MOD2 – green, MOD3 – orange, MOD4 – light blue. Differences between responses to WT and CPLs are tested using Mann-Whitney U test.



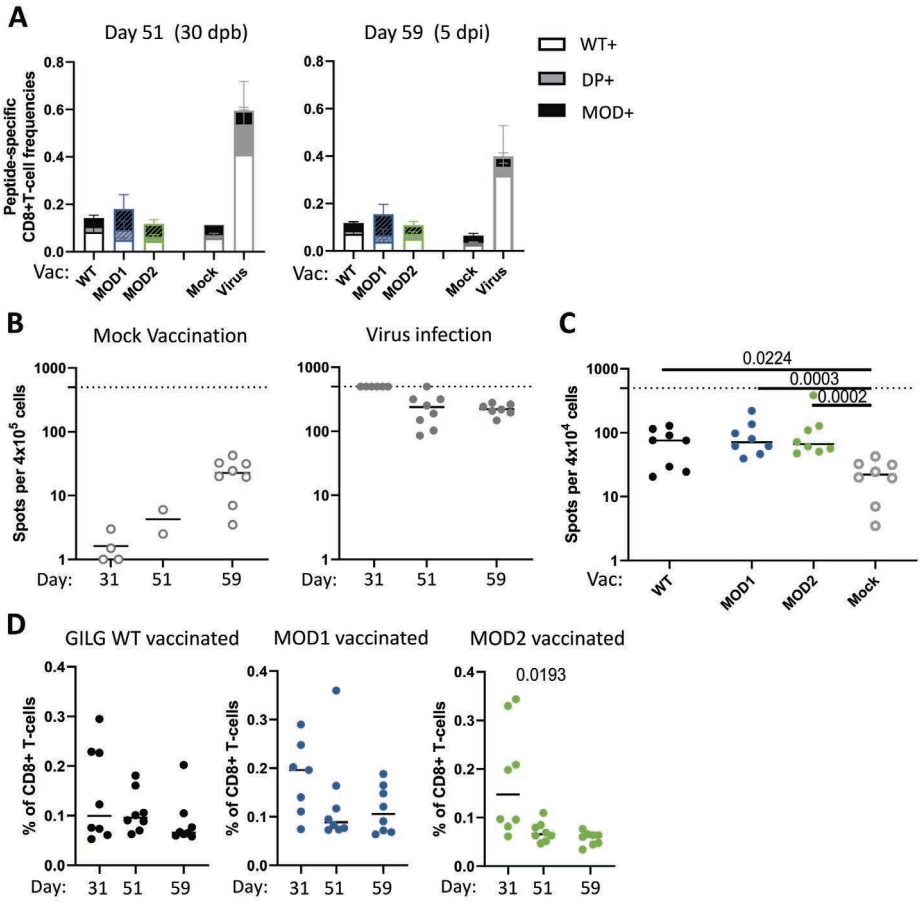
Supplementary Figure 3: IFNγ-response against natural variants of the GILG epitope after CPL vaccination.

IFNγ-responses after restimulation of splenocytes with natural variants of GILG WT as measured in IFNγ-ELISpot. Data of individual mice are shown (points) with median (line). Mice vaccinated with CPL are indicated by the following colors: MOD1 – dark blue, MOD2 – green, MOD3 – orange, MOD4 – light blue. Differences between responses to WT and CPLs were tested using the Mann-Whitney U test.

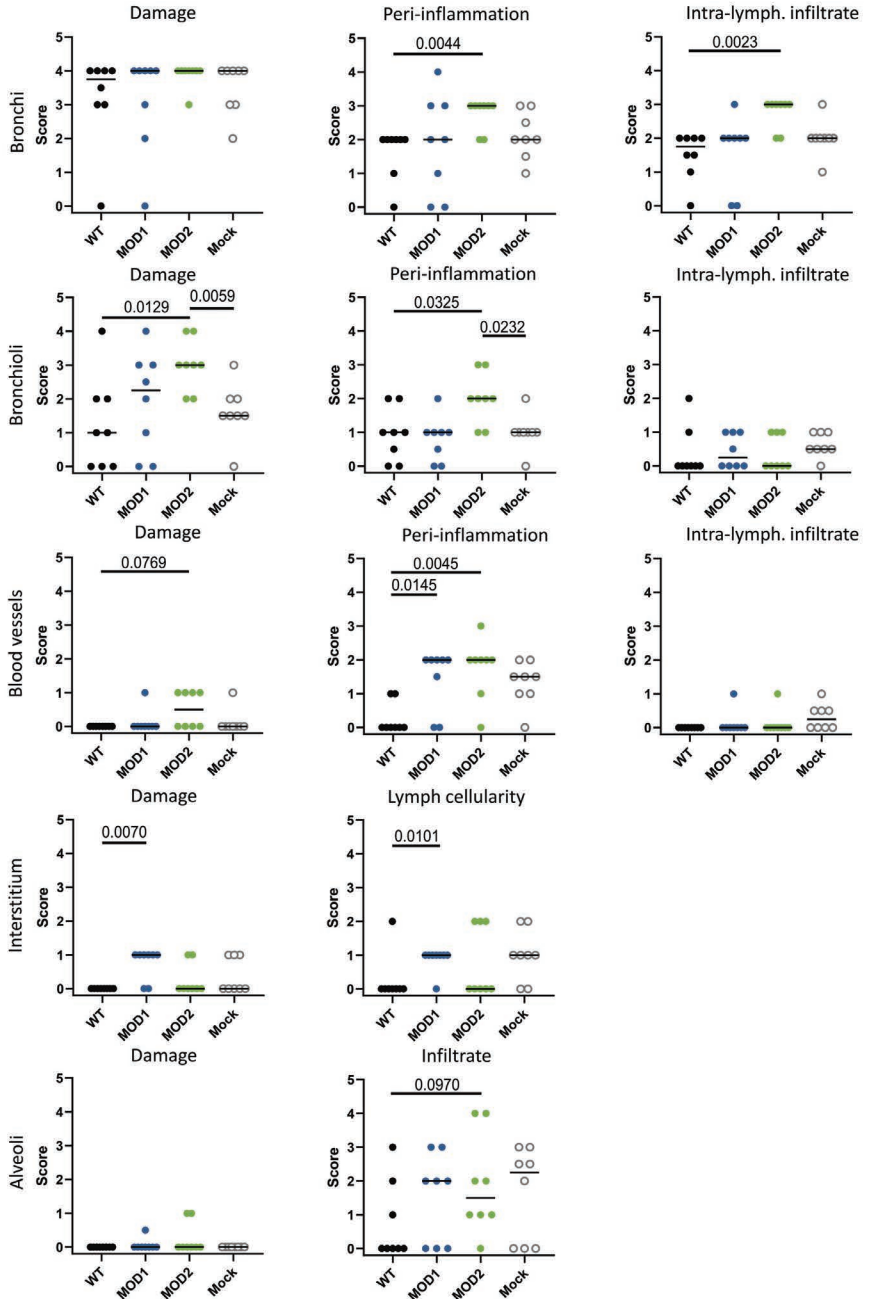


Supplementary Figure 4: Richness and CDR3 length of the WT specific TCR repertoire after peptide vaccination.

A) Richness of the WT-specific T-cell repertoire after vaccination with WT peptide or CPLs and after influenza infection. Richness of the WT-specific T-cell repertoire calculated by iteratively sampling (100.000 times), normalized to 100.000 reads per sample. B) Distribution of the CDR3 length (in frequency) of the TCR sequences of the GILG-WT specific T-cell repertoire in the different treatment groups. Data of individual mice are shown (points) with median (line). Differences between responses to WT and CPLs are tested using Mann-Whitney U test.



Supplementary Figure 5: T-cell memory response after peptide vaccination.
A) Overview of dextramer+ T cells present in the WT+, DP+ and MOD+ gate. B) Cellular responses against WT in mock vaccinated mice (negative control) and after virus challenge (positive control). C) Comparison of IFNγ-responses at day 59 in mice vaccinated with WT, MOD1 or MOD2 with mock vaccinated mice after restimulation of splenocytes. D) Frequency of WT-specific CD8⁺ T cells of mice vaccinated with WT, MOD1 or MOD2 at different days post vaccination and after IAV-challenge. Data of individual mice are shown (points) with median (line). Mice vaccinated with WT are depicted in black. Mice vaccinated with CPL are indicated by the following colors: MOD1 – dark blue, MOD2 – green. Differences between responses to WT and CPLs are tested using Mann-Whitney U test.

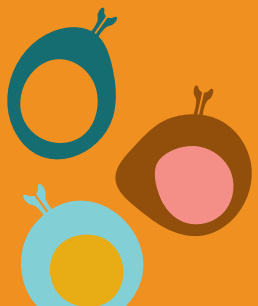


Supplementary Figure 6: Histopathology scores in different parts of the lungs after IAV-challenge in peptide-vaccinated mice.

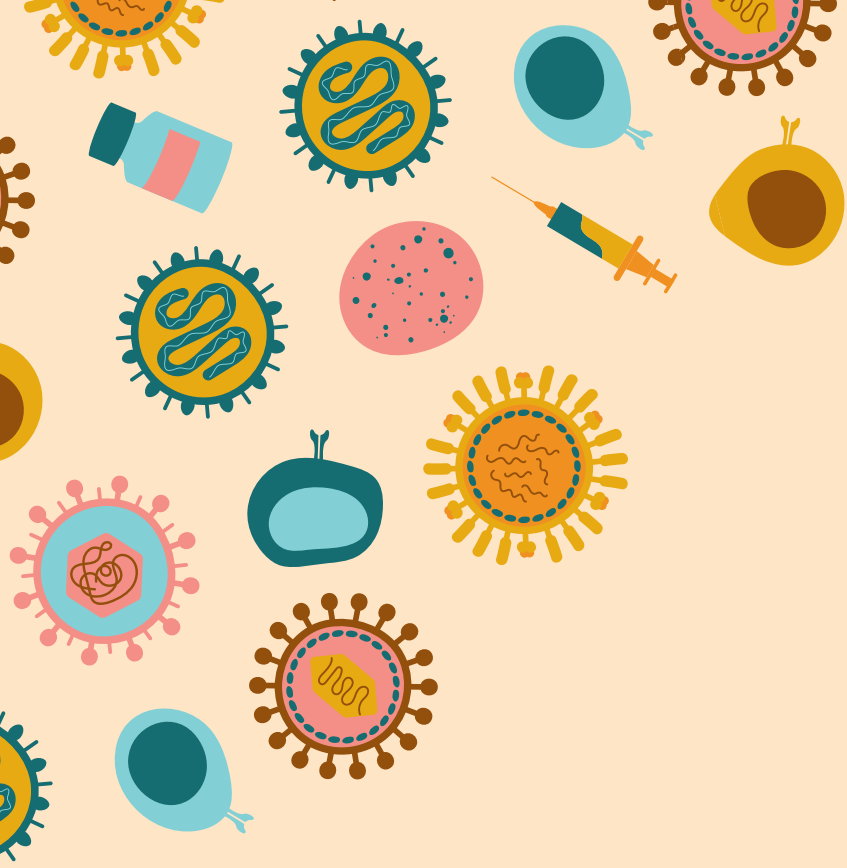
Overview of the different histopathology scores in bronchi, bronchiole, blood vessels, interstitium and alveoli. Pathology was scored on a scale from 0 (no aberrations) to 5 (severe damage). Per mouse at least 8 microscopic fields were scored; median scores per

mouse were plotted. Data of individual mice are shown (points) with median (line). Mice vaccinated with WT are depicted in black. Mice vaccinated with CPL are indicated by the following colors: MOD1 – dark blue, MOD2 – green. Mock vaccinated mice are depicted by open grey symbols; IAV-infected mice are depicted by solid grey symbols. Differences between responses to WT and CPLs are tested using Mann-Whitney U test.

DISCUSSION



General discussion and future perspectives



T CELLS & VACCINATION

Role of T cells in the protection against viral infections

The importance of antibodies and their role in preventing infections is beyond question [282]. The vast majority of vaccines that are nowadays used, are based on the induction of a protective humoral response [282, 384]. Therefore, the height of the antibody response is often used as a measure for vaccine immunogenicity. Antibodies are able to eliminate virus particles via neutralization or opsonization before the virus particles will infect host cells. This can result in long-term protective immunity, but only as long as the pathogen is not able to escape the induced antibody response. If a virus mutates and other variants emerge, the induced antibodies may not recognize the virus anymore.

Once a virus particle is able to bypass the humoral immune response, infection of the host's cells can occur. Some viruses have more "strategies" to escape (vaccine-induced) humoral immune responses than others, and are more successful in establishing an infection in the host's cells. Protection against these viruses may therefore rely more heavily on the cellular immune response [385, 386]. Influenza A virus (IAV), for example, is known to undergo antigenic drift, in which the surface-proteins (targeted by the vaccine) mutate [326, 327]. This way, the influenza virus can escape recognition of influenza-specific antibodies. As IAV-vaccination induces mostly a humoral immune response [387], this antigenic drift is part of the reason why a yearly update of IAV-vaccination is needed. The cellular response on the other hand, may play an important role in providing heterosubtypic immunity against influenza through recognition of more conserved epitopes of internal proteins of the virus [143, 388]. Also against relatively stable chronic viruses, such as Epstein Barr virus (EBV) [389, 390] and cytomegalovirus (CMV) [391, 392], the cellular response plays a significant role. These chronic herpesviruses are able to hide within host cells and establish chronic infection. Although our immune system is not able to clear these chronic viruses, T cells continuously help to keep these infections under control. The role of T cells against other viruses, including mumps virus (MuV), is less clear. The measles, mumps and rubella (MMR)-vaccination is a prime example of vaccination where the read-out focuses on antibody levels [293, 393], however significant T-cell responses can be observed after infection [299, 394] and vaccination [287, 297, 395]. As in the past decades, waning of the vaccine-induced response and the circulation of different strains has led to outbreaks in the human population, there may be a significant role for $CD8^+$ T cells in the (renewed) protection against MuV. As the T-cell response plays an important role

in protection against viral infections, there is an increasing interest in the induction of T cells through vaccination [396-398].

The protective level of T-cell responses

For most viral infections, correlates of protection have been determined based on the antibody response. Antibody titers can be measured relatively easily, and are frequently used to determine vaccine immunogenicity. The T-cell response on the other hand, is largely understudied as a correlate of protection [399]. It is unknown which number or frequency of virus-specific T cells is needed to result in a protective T-cell response, or whether in fact, specific functional features of the T-cell response dictate protection [385]. Therefore, it is important to characterize both quantity and quality of T cells needed for protection [16, 398]. Qualitative aspects of the T-cell response involve T-cell phenotype, functionality and level of diversity and cross-reactivity [400]. For example, T-cell responses against human immunodeficiency virus (HIV), a virus with a particularly high mutation rate, it is of importance that T cells also recognize antigenic variants [401]. To enable this, a diverse virus-specific T-cell receptor repertoire is required.

Role of T-cell repertoire in vaccination

The diversity of the virus-specific TCR repertoire is an important correlate of protection against viral infections [117]. The higher the diversity of the TCR repertoire, the higher the chance that our immune system can recognize virus-derived epitopes [40]. With a more diverse repertoire, the chance of a TCR with a high affinity for a particular epitope to be present is also higher [44]. In addition, a broader repertoire could lead to better protection against viral mutations, as the chance of a T cell being present that will recognize the mutated version (escape variant) of the virus-derived epitope is higher [46-48]. Therefore, measures of TCR diversity could aid in defining the quality of T-cell responses against viruses and could support novel vaccine design [99]. Before we are able to use TCR repertoire information to optimize T-cell responses against infections through new vaccine designs, a deeper understanding of the composition and diversity of the TCR repertoire is required.

Specifically, we identified the following knowledge gaps:

1. *How long is antigen-specific TCR diversity maintained?* Studies into the maintenance of the antigen-specific TCR repertoire hold important information on the ideal moment of vaccination.
2. *How do (new) antigen challenges shape the antigen-specific TCR repertoire?* This information may give important insight in the effect of booster vaccinations,

but may also hold clues on whether certain people could be more at risk for certain infections than others.

3. *How diverse does the antigen-specific TCR repertoire need to be?* It remains unknown whether there is a minimal diversity of the TCR repertoire needed for optimal protection, whether and how to establish this level of diversity.

In this thesis, we have performed several studies to fill in (parts of) these knowledge gaps (see Fig. 1 for an overview). The key findings of these studies are summarized in Box 1. Here we discuss the insights from these studies for the above knowledge gaps and how this could influence vaccine strategies or identify individuals at risk based on their TCR repertoire. In the second part of this Discussion, we will go further into the challenges in TCR repertoire research.

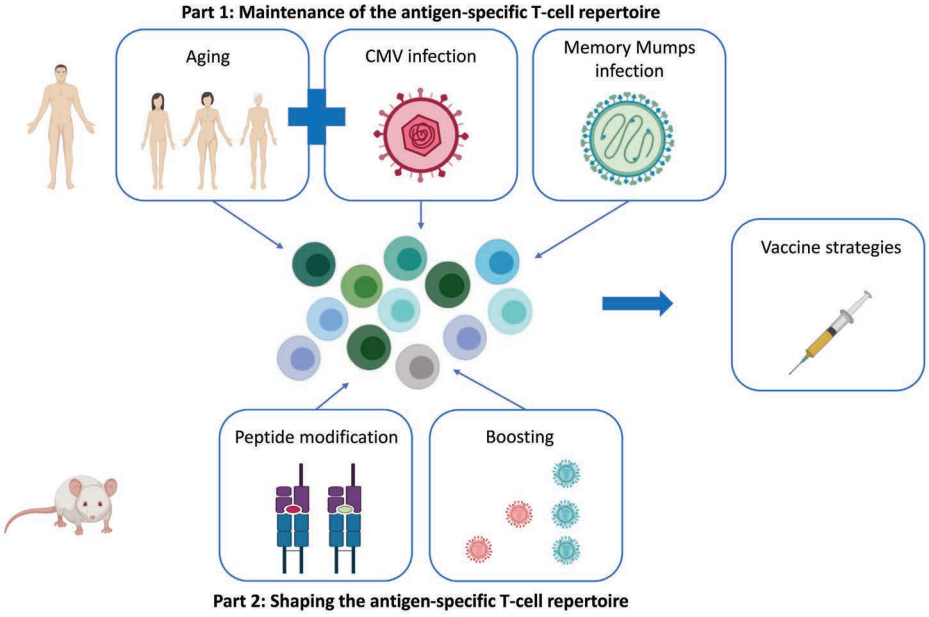


Figure 1: Overview of this thesis.
In this thesis, we studied the effect of age and CMV-infection on the EBV-specific TCR repertoire and followed the MuV-specific TCR repertoire after infection in humans to get insight in how the antigen-specific T-cell repertoire is maintained. In mice, we investigated how the IAV-specific TCR repertoire is shaped by previous infections and peptide vaccinations. Key findings of these studies are summarized in Box 1 and may play a role in the design of new vaccination strategies.

Box 1: Key findings of this thesis

In this thesis, we have focused on the CD8⁺ T-cell response against different viruses, namely cytomegalovirus (CMV), Epstein-Barr virus (EBV), mumps virus (MuV) and influenza A virus (IAV). We show that:

- EBV-specific and CMV-specific TCR diversity decrease with age (Chapters 3+4)
- CMV-positive individuals have a lower EBV-specific TCR diversity than CMV-negative individuals (Chapter 4).
- There are no significant differences between CMV⁺ and CMV- individuals in the IAV-specific T-cell response, as well as the IAV-specific TCR repertoire, after IAV-infection (Chapter 5).
- The MuV-specific TCR repertoire remains stable between 1.5 and 9 months post MuV-infection (Chapter 6).
- MuV-specific T-cell responses post MuV-infection do not differ between unvaccinated and previously MMR-vaccinated individuals (Chapter 6).
- Boosting of pre-existing IAV-specific T cells leads to a decrease in IAV-specific TCR diversity, due to expansion of dominant clones (Chapter 7).
- Time between current and previous IAV-infection does not influence the IAV-specific T-cell response and repertoire (Chapter 7)
- Vaccination with modified IAV-specific CD8⁺ T-cell peptides with enhanced HLA-A2 binding affinity leads to a skewed T-cell repertoire including many non-relevant T cells (Chapter 8).
- Modified IAV-specific peptide vaccination can enhance pathology after IAV-challenge (Chapter 8).

PART 1: KNOWLEGDE GAPS IN TCR REPERTOIRE

HOW LONG IS ANTIGEN-SPECIFIC TCR REPERTOIRE DIVERSITY MAINTAINED?

It is generally assumed that the diversity of the TCR repertoire decreases with age, and that this decrease plays a role in the weakened cellular response against infections and vaccinations at older age [19, 132]. Indeed, for the naive T-cell pool it has been shown that with age both the number of T cells and their TCR diversity decrease [133, 134, 136]. When a new antigen is encountered at older age, this limited naive T-cell diversity will presumably influence the newly formed antigen-specific T-cell repertoire in the memory pool [402, 403]. To improve protection of older adults, vaccination at an earlier age could be considered in order to ensure the induction of T cells from a more diverse naive T-cell repertoire, leading to a more diverse repertoire in the memory pool at older age [404]. However, the chance of success of this strategy would to a large extent depend on the capacity to maintain the antigen-specific T-cell repertoire in the memory pool up until old age [41].

1.1: Maintenance of TCR diversity in chronic infections

In **Chapter 3**, we show that most literature suggests that the CMV-specific and EBV-specific TCR repertoires are stable for at least a couple of years. However, these observations are mostly based on the persistence of dominant clones. In **Chapter 4**, we dived further into the effect of age on the CMV- and EBV-specific TCR repertoires, but with a focus on the total diversity of these antigen-specific T-cell repertoires instead of only the dominant clones. We observed a clear decrease in diversity of both the CMV-specific and the EBV-specific TCR repertoire with age. We hypothesize that the observed loss of diversity is most likely due to persistence of the dominant clones and a loss of lower frequency clonotypes over time, which could reconcile our findings with those described in **Chapter 3**. In order to confirm the loss in diversity of the CMV-specific and EBV-specific T-cell repertoires with age and the conservation of the most dominant clones, a more extensive longitudinal study should be performed.

1.2 Maintenance of TCR diversity in acute infections

The number of studies that have been addressing the maintenance of the antigen-specific TCR repertoire against acute viruses is very limited. The main reason for this is perhaps that maintenance of specific T cells after acute infection is quite difficult to measure. The frequencies of many antigen-specific T cells decline soon after infection and become undetectable within a short time after infection, if they are not boosted [405]. To be able to study the maintenance of an antigen-specific T-cell response after acute infection, Yellow Fever (YF)-vaccination is often used as a model. This live attenuated vaccine is thought to be one of the most effective vaccines currently available for the induction of T cells and a YF-specific memory T-cell response can still be measured decades after vaccination. However, data on the dynamics of the T-cell repertoire against YF over a longer period of time remain limited and we can only build on the study of Minervina et al. [210]. This study indicated a remarkable maintenance of YF-specific T cells for 30 years, but also a loss of clones, as the magnitude of the response and the number of expanded clones after a YF-booster were significantly lower than after primary YF-vaccination [210]. In vivo deuterium labeling of $CD8^+$ T cells induced by YF vaccination showed that after the expansion phase, the memory $CD8^+$ T cells turned to a relatively quiescent state in which they divided less than once a year [406]. Together with their open chromatin profile at effector genes, this may explain why these cells were still ready to respond even decades after vaccination.

In **Chapter 3** we describe some studies focusing on the IAV-specific TCR repertoire that reported a loss of dominant T-cell clones with age. It remains to be elucidated

whether this loss of antigen-specific T-cell diversity is merely due to time, or whether there is a role for IAV re-infection (see Q2). In **Chapter 6** we followed the MuV-specific T-cell response in both vaccinated and unvaccinated individuals for 1.5 until 36 months after MuV-infection. We observed that the frequency of the antigen-specific T-cell response decreased significantly between 1.5 and 9 months after infection and was even lower at 18 and 36 months. Nevertheless, based on the maintenance of the dominant clones, the antigen-specific TCR repertoire was relatively stable between 1.5 and 9 months post MuV-infection, and was maintained in both vaccinated and unvaccinated individuals.

As the goal of vaccination is to induce long-term memory [16, 407], it is important to unravel which T cells are maintained after infection and why. However, up till now this remains a mystery. Could it be a stochastic process or is the fate of every T cell determined? As mentioned earlier, longitudinal studies are needed to obtain these insights. However, to answer these questions we have to look further than the TCR repertoire. Combining TCR repertoire analyses with for example in vivo deuterium labeling and chromatin immunoprecipitation sequencing (ChIP-seq) would not only identify T cells based on their specificity, but would also give information about their lifespan as well as their functional fate.

HOW DO (NEW) ANTIGEN CHALLENGES SHAPE THE ANTIGEN-SPECIFIC TCR REPERTOIRE?

The TCR repertoire in the memory pool is constantly undergoing changes due to the encounter of new virus infections or vaccinations, but also due to antigenic re-encounters or reactivation of chronic infections [407]. All these different antigenic stimulations together shape the TCR repertoire.

2.1 Repeated antigen-stimulation

Almost every vaccination needs to be administered in two or multiple doses in order to boost the immune response to a protective level [16, 397]. Antigenic boosting is also an effective way to restore a waning response, especially for the humoral response. Boosting of humoral responses leads (amongst other processes) to re-entering of B cells into the germinal centers (GC) of secondary lymphoid organs, where memory B cells undergo affinity maturation. During the GC reaction, B cells are formed with slightly different B-cell receptors (BCRs), potentially with increased affinity for the re-encountered virus [408]. This phenomenon, also known as affinity maturation, is thought to underlie the improved recognition of SARS-CoV-2 variants after COVID-19 boosters [409].

For T cells, boosting -whether via vaccination or via virus re-encounter- may not necessarily lead to a more favorable outcome. Although boosting of T cells does lead to a quick increase in the magnitude of the T-cell response [405, 410], repeated antigen-stimulation may affect the functioning of antigen-specific T cells, as well as their TCR repertoire [315]. In the case of persistent antigen stimulation, as seen during chronic infections and cancer, T cells can go into a state of "exhaustion", in which they show a loss of function [303]. Studies focusing on repeated antigen stimulations via infection or vaccination have shown that boosting $CD8^+$ T cells repeatedly can result in a decrease in their cytolytic potential, cytokine production and proliferate capacity , and may thus ultimately also result in T-cell exhaustion [366, 411, 412]. Thus, in contrast to the improved antibody response observed after boosting thanks to affinity maturation, extensive boosting of T cells may in fact lead to dysfunctional T cells [48].

In **Chapter 3** we have already described how repeated antigenic stimulation may influence the TCR repertoire. Although some studies have reported a loss of dominant (high affinity) clones upon repeated stimulation [177, 187, 413] (as observed for the IAV-specific TCR repertoire [176, 179]), others found evidence for their expansion [184, 185] (as observed for the CMV-specific TCR repertoire). These differences [414] may in part be explained by factors such as the dose [400, 415] or duration [412] of antigen stimulation. This may be influenced by characteristics of the virus, e.g. whether it leads to a chronic/persistent or an acute infection, or state of senescence of the responding T cells [186], or by factors related to the immune response, e.g. the expression of various co-stimulatory or inhibitory receptors, and may be linked to the virus, e.g. chronic stimulation or acute stimulation. The large-scale and repeated SARS-CoV-2 vaccinations that have been given in the past year, which have also induced T-cell responses [416, 417], offer an excellent opportunity to investigate the impact of repeated antigen encounter on the diversity of the TCR repertoire and its functional characteristics, in a relatively short timeframe within humans.

2.2 Separating infection history and aging

As described earlier, at older age a lower IAV-specific T cell response is typically observed, which is due to a decrease in IAV-specific T-cell numbers and a loss of dominant public T-cell clones (**Chapter 3**). Impaired T-cell responses against IAV and other respiratory infections in older adults are often interpreted as a direct effect of age [20, 418], while in fact they may be due to the infection history, e.g. multiple antigenic stimulations. It is estimated that humans are infected with IAV every 5-10 years [168, 360]. This raises the question whether the lower T-cell

responses at older age are truly due to aging, or partly due to a different/longer infection history.

Measuring the effect of infection history in humans is challenging, as it is directly associated with age. Therefore, in **Chapter 7**, we switched to a mouse model. We show evidence for lower IAV-specific T-cell diversity in mice after a secondary infection, when compared to the response after primary infection, due to a higher prevalence of the most dominant clones in the repertoire. This specific change in the repertoire was observed after one antigen booster. It would be interesting to study the effect of additional antigen stimulations. Will this process eventually lead to exhaustion of the stimulated T cells, and thus a loss of the most dominant, high affinity T-cell clones? And could the "age effect" observed for the IAV-specific T-cell repertoire in fact be explained by repeated antigenic stimulation?

2.3 Influence of CMV on the antigen-specific TCR repertoire

Not only homologous antigen stimulations may influence the antigen-specific T-cell response and TCR repertoire, even heterologous infections may have an influence, as described in **Chapter 3**. This could be due to cross-reactivity of T-cell clones [71, 205], but may also occur when totally different viruses trigger the immune system [182]. Especially the effect of CMV on the memory T-cell pool has been the focus of many studies. CMV-infection leads to high frequencies of CMV-specific T cells in the blood leading to an enlarged total memory T-cell pool in CMV^+ individuals [35, 82]. The presence of these high frequencies of CMV-specific T cells is thought to have a negative influence on other, non-CMV-specific T cells in the memory pool [150]. The idea behind this is that CMV-specific T cells strongly compete for proliferation and survival factors and thereby partially outcompete other antigen-specific T cells.[200, 201]. Studies supporting this hypothesis are mostly based on mouse experiments [203, 220], while in humans this effect is less clear. In CMV-positive humans a decrease in the diversity of the memory T-cell pool has been observed, but whether the presence of large CMV-specific clonal expansions affects other antigen-specific T cells has remained unclear.

At first sight, the data on the EBV-specific TCR repertoire in **Chapter 4** suggest that CMV-infection has a negative effect on the TCR repertoire diversity of other, non-CMV-specific T cells present in the memory pool, as we observed a decreased EBV-specific TCR diversity in CMV^+ individuals. Very much to our surprise, however, within the group of CMV^+ individuals, we observed a positive association between CMV-specific and EBV-specific T-cell frequencies. A possible explanation for this positive association is that both CMV and EBV may reactivate as a result of the

same trigger (or even reactivate each other), as is observed in transplant patients. Furthermore, these reactivations, and thus antigenic stimulations, may then lead to an increase in EBV-specific clonal expansions, and thus a lower diversity. Therefore, the negative effect on the EBV repertoire, may be caused by CMV, but by a trigger that underlies both changes in CMV and EBV latency.

The data presented in **Chapter 5** suggest that due to their large numbers, CMV-specific T cells tend to "overshadow" other T cell specificities that are present in the TCR repertoire. We show that, despite the lower frequencies of IAV-specific T cells in CMV^+ compared to CMV^- individuals, the response against a new IAV infection is not hampered in CMV^+ individuals. Although the data on TCR repertoire diversity in this study are limited to only 4 donors and a minimal amount of TCR sequences, we observed no clear differences in the IAV-specific TCR repertoires of CMV^- and CMV^+ individuals. In line with this, we found that severity of disease was not increased in CMV^+ individuals, suggesting no or limited clinical impact of CMV-infection on the influenza-specific protective response.

In conclusion, although CMV has long been thought to negatively influence the non-CMV-specific TCR response, the data from **Chapter 4** and **5** do not fully support this hypothesis. In the field of CMV-research, there is increasing evidence that CMV is not only negatively affecting the T-cell population. The effect of CMV on the TCR diversity of non-CMV-specific memory T cells in humans need to be studied in more detail. Due to its chronic nature, EBV-specific T cells might not be representative for other non-CMV-specific T cells, and the IAV-specific TCR repertoire data need to be extended. More insights into why MCMV infection in mice is supposedly detrimental may also give insight into why in humans such a negative effect has not been observed. Such differences could be related to the relatively high dose of MCMV inoculum used in mice , due to differences in infection order between mice and men, e.g. first IAV and then CMV, or due the repeated antigen exposure with IAV in humans. The latter two explanations would be interesting to test in a mouse model. So far, the data suggest that CMV-seropositivity does not necessarily influence the T-cell response against other viruses and consequently. This would suggest that CMV-seropositivity may not be an important factor to take into account when vaccinating with T-cell based vaccines.

HOW DIVERSE DOES THE ANTIGEN-SPECIFIC TCR REPERTOIRE NEED TO BE?

So far, we have focused on whether the diversity of the antigen-specific repertoire is preserved over time or after infection and interpreted a more diverse repertoire as a more favorable outcome. Indeed, several studies have shown that a more diverse (antigen-specific) T-cell repertoire is linked to a more favorable outcome of disease [52, 419, 420]. However, it remains unknown what is the minimum level of diversity that is needed to provide protection against infection. This information is of importance when aiming to identify individuals at risk for severe infection, based on information from their TCR repertoire.

Roughly speaking, T-cell repertoire diversity is based on two parameters: the different varieties of TCRs (richness) and the distribution of these TCRs (evenness) over the repertoire [12, 13]. The naive T-cell pool contains most repertoire diversity, with an enormous richness of different TCRs, all present in relatively low frequencies [65, 66]. The naive T-cell pool is estimated to be at least 100 times more diverse than the memory pool [65]. The premise that a high level of diversity in the naive T-cell pool leads to better protection against new antigen encounters seems logical. However, could it be that for the antigen-specific repertoire, maximum diversity may not always be best?

3.1 Could a skewed repertoire lead to better protection?

In the antigen-specific TCR repertoire, significant levels of skewing are often observed [52, 84, 421]. This means that only a few TCRs are dominating the response against a virus, leading to a relatively low diversity of the virus-specific repertoire. The cause for a more skewed repertoire can be due to, amongst other reasons, repeated antigen stimulations, the presentation of the epitope [422], the level of cross reactivity of the TCRs [48], but also characteristics of the virus itself, including stability. The more genetically stable the virus is, the less diverse (or cross-reactive) the responding TCR repertoire needs to be, as there is no need to recognize mutated versions of the virus.

Although no direct link has been found between a more skewed antigen-specific TCR repertoire and a more favorable outcome, some associations in this direction have been observed. As described in **Chapter 3**, the findings about the IAV-specific TCR repertoire are mostly based on the response against the A2-GILG epitope. Although IAV is a relatively rapidly mutating virus, this mostly holds for its surface proteins. Its $CD8^+$ T-cell epitopes, in contrast, which are mostly present

in internal proteins, are thought to be relatively conserved. The A2-GILG-specific repertoire in younger individuals is highly skewed towards TCRs containing the Vβ-segment TRBV19 and an RS motif in their CDR3 region [173, 176]. The dominant TCRs containing these characteristics are lost with age, leading to a richer and more private IAV-specific repertoire in older adults. This could mean that the diminished IAV-specific response observed with age [19], is actually associated with a less skewed IAV-specific TCR repertoire, due to the loss of dominant public clones. As shown in **Chapter 4** and in several other studies [81, 82, 86], the CMV-specific and EBV-specific TCR repertoires are also skewed, like the IAV-specific T-cell repertoire, but become even more skewed, and thus less diverse, with age. This phenomenon is especially pronounced in the CMV-specific TCR repertoire [83, 414]. The expansion of the most dominant T-cell clones over time is thought to be due to the high affinity of the TCR for its epitope and may thus be favorable for the T-cell response [83]. Also in **Chapter 7**, the abundance of the most dominant T-cell clone in the IAV-specific TCR repertoire was shown to increase after a second infection compared to a primary infection, which was linked to a higher (memory) T-cell response. Thus, a more skewed antigen-specific TCR repertoire may actually be more optimal based on functional properties, as long as the targeted epitope is not subject to mutations.

3.2 How specific does the antigen-specific TCR repertoire need to be?

TCRs are highly specific for virus-derived epitopes to prevent binding of TCRs to self-epitopes [43]. Although self-reactive T cells are also present in healthy humans and mice, they are normally contained. Uncontained self-reactive T cells can cause pathology, as occurs in several autoimmune diseases. Also in the context of viral infections like RSV [423] and IAV [424],[424] immunopathology caused by T cells is observed, however the exact factors that play a role in this have not been determined. In **Chapter 8**, we observed that the mice vaccinated with modified (MOD) IAV peptides suffered significantly more after IAV infection than mice that were vaccinated with the WT-peptide or the placebo. Although we did not determine the mechanisms behind the enhanced pathology, based on the TCR repertoire, there were two factors that could have played a role: 1) the presence of large dominant specific T cell clones, as the TCR repertoire against the WT epitope of mice vaccinated with the MOD-peptide was highly skewed, or 2) the presence of a large fraction of non-relevant T cells. These "non-relevant" T cells were induced by a modified peptide, which was structurally very similar to the WT epitope. In fact, there was only one amino acid difference between the WT and the modified peptide, which could mean that more T cells responded against the IAV-infection, leading to enhanced pathology. Future studies are needed to understand why

some small epitope modifications may lead to enhanced pathology, while others do not, and to determine whether modified peptide vaccination could potentially be used to induce a protective and nonpathological T-cell response.

3.3 An extra level of diversity

As already mentioned before, T-cell cross-reactivity is an important feature that increases the diversity of the T-cell repertoire, as it may lead to increased recognition of viral escape variants. Another level of diversity, which is often ignored, is functional diversity of the T-cell response. Functional diversity is based on the differences in phenotype of the antigen-specific T cells and the corresponding differences in the immune response, based on proliferation capacity, cytokine secretion or homing potential [40]. Functional diversity cannot be measured using standard TCR sequencing methods, unless it is combined with RNA-seq analysis. Especially the transcriptional profiles obtained in single cell analyses may give valuable information about the functional capacities of a T-cell clone. The functional diversity of the TCR repertoire is also of importance for the antigen-specific T-cell response, as it indicates which effector functions will be used to battle the virus. This can differ even for T cells with the same TCR, as overlapping TCRs have been found in different memory subsets [425]. Therefore, it would be ideal to also take this level of diversity into account when designing and evaluating new T-cell inducing vaccine strategies.

PART 2: CHALLENGES IN TCR REPERTOIRE RESEARCH

There are still some challenges to be tackled before TCR-sequencing can effectively be used in the design of new vaccination strategies or in diagnostic or clinical settings. These challenges include the following technical issues:

1. There are still large discrepancies between TCR sequencing methods, which hamper the comparability of data between studies.
2. It remains unknown how well the $CD8^+$ T-cell response against a single epitope represents the response against the whole virus.
3. It is not known whether the TCR repertoire in the circulation is representative of that in the tissues.

DISCREPANCIES BETWEEN TCR SEQUENCING METHODS

There are several methods to prepare a T-cell population for TCR repertoire sequencing, which all have their advantages and disadvantages. Although the specific method that is used can influence and bias the sequencing data, no golden

standard for TCR sequencing has been defined yet [117]. Also in this thesis, two different methods have been used to perform TCR sequencing. The basis of the two methods is comparable, as both are based on the SMART technique [119, 121].

For the first part of this thesis on human samples (**Chapters 4-6**), we used a method based on the protocol of Mamedov et al [119], while for the second part (**Chapters 7 and 8**), we switched to a commercially available kit. The protocol of Mamedov et al. has been designed for bulk sequencing and incorporates unique molecular identifiers (UMIs) during cDNA synthesis. We chose for bulk sequencing because a large number of samples had to be sequenced. The number of cells in each sample was very limited however, and as a result we had to optimize the protocol of Mamedov et al to be able to get a signal from these low numbers of cells. Unfortunately, the low cell yields resulted in contamination during the sequencing protocol. We noticed a lot of false-positive cases of shared TCRs, not only between individuals for the same antigen-specificity (e.g. CMV-specific), but also between different antigen-specificities (e.g. CMV-specific and EBV-specific). This overlap was only observed when samples were sequenced within the same run. We were able to perform an extensive clean-up of the data, thanks to the incorporation of the UMI information (see the clean-up process described in the supplementary data of **Chapter 4**), so the sequence data of the samples with the largest cell input could be analyzed. In order to compare samples of different sizes, the total number of clones analyzed per sample was downsized to 20. With hindsight, knowing that the samples would in the end be down-sampled to such low numbers of clones, single cell sequencing would have been a better option for these projects.

For the analyses of **Chapters 7** and **8**, we wanted to switch to a commercially available kit, as it gave us more and a higher quality of sequencing reads. However, for this kit, UMIs were unfortunately not available.

Knowing the importance of UMIs, we therefore investigated how, in our hands, the use of UMIs would influence the results. This was done by analyzing data obtained with the protocol of Mamedov et al. with or without the use of the UMIs. Fig. 2 shows the data for 3 representative mouse sample. Analyzing the data based on the UMIs did not result in significant changes in the clonal distribution and we thus used the commercial kit in the mice experiments. However, when we compared the data obtained with the protocol of Mamedov et al. with the data obtained with the commercial kit, we observed some disturbingly large differences between samples prepared using the two different methods (Fig. 2). Within some of the samples,

there was a totally different clonal distribution, in the sense that frequencies of shared clones differed dramatically, or dominant clones detected with one of the methods were not detected with the other. The diversity measures of the samples were also very different between the methods. For example, according to data obtained with the Mamedov protocol, the TCR repertoire of mouse 43 was more diverse than that of mouse 34. Comparing the TCR diversity of these two mice based on the commercial kit yielded the opposite conclusion. As within every study we always used one method and thus treated every sample the same, we therefore hope our conclusions are not purely the result of our method choice.

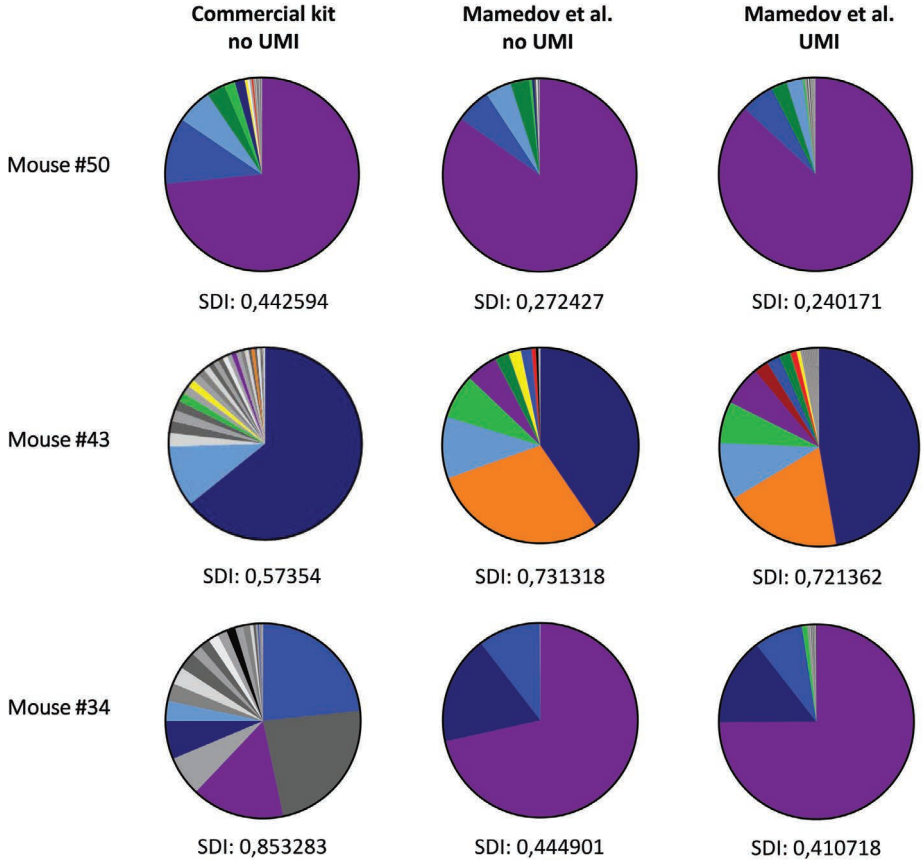


Figure 2: Characterization of the antigen-specific TCR repertoire in mice, using different methods.
T-cell repertoire of (ASN-specific) CD8⁺ T cells of 3 representative mice are shown. Per mouse, 3 pies are shown, which each depict the repertoire analyzed via a different method. From left to right: Commercial kit (without UMIs), the Mamedov protocol without the use of UMIs, and the Mamedov protocol with UMIs. Colors represent shared TCRβ sequences within mice. Note that the colors do not represent shared sequences between mice. Grey scales depict unique TCRβ sequences. SDI = Simpson's diversity index.

Similar differences between methods have also been observed by others [112, 426]. For example, in the study of Bareness et al, 9 different TCR kits were compared, and large differences were observed in accuracy and in intra- and inter-sample reproducibility between methods [112]. The amount of starting material can also have a large influence on the detection of rare clones and thus on the estimated diversity of the repertoire. In addition, the data analysis method can lead to differences between studies [9, 225]. Several different pipelines have been used to analyze TCR sequencing data [9], however an even larger problem are the manually set thresholds that are set, which can influence the outcome [117]. This could also be observed in **Chapter 4-6**, where we saw that our end results were influenced by how strictly the UMI correction was performed. In our case, we checked that our qualitative conclusions were not dependent on the level of UMI correction, but such checks are not always performed.

The fact that the outcome and thus conclusions of TCR repertoire data can be so dependent on the method used is very disturbing. These differences between techniques may not only lead to inconsistent information, but also raises the question whether different TCR repertoire studies can at all be compared. In general, one would expect that at least the most dominant clones would be identified, regardless of the method used. Indeed, shared antigen-specific TCR sequences have been observed in different individuals, sequenced with different methods in different studies. It is nevertheless important that the field works towards a more standardized method and analysis of TCR sequencing data. The implementation of controls, such as duplicates of a sample or a reference sample, would already give insight in whether the frequencies of different T-cell clones are reliable [427]. Furthermore, we still believe that the use of UMIs is highly recommendable, because it increases the accuracy of the data, as exemplified by the data clean-up that we could perform in the earlier chapters. Unfortunately, the use of UMIs may come at the cost of sensitivity, as the efficacy of the cDNA synthesis decreases with the length of the template switch oligo nucleotide (Chapter 2). A more standardized TCR sequencing method becomes even more important, if in the future, TCR sequencing will be used in diagnostic and clinical settings [117], and to optimize vaccine strategies.

HOW WELL DOES THE CD8+ T-CELL RESPONSE AGAINST A SINGLE EPITOPE REPRESENT THE RESPONSE AGAINST THE WHOLE VIRUS?

In most antigen-specific T-cell repertoire studies, TCR diversity is based on the response to a single viral epitope. Especially when focusing on immunodominant epitopes, it is assumed that the responses against these epitopes include a large part of the total T-cell response against these viruses and are representative for the total virus-specific response. However, different peptides are presented differently to TCRs, as a result of differences in e.g. the concentration or the duration of peptide presentation on the cell surface. This may lead to large differences in the diversity of the responding TCR repertoire.

In **Chapter 6** the MuV-specific T-cell response against three different MuV-specific epitopes was studied. Based on earlier studies, all three epitopes seemed to be immunodominant, although a significant difference in the frequencies of reactive T cells against the three epitopes was observed. Although the three epitopes differed based on presentation density and binding strength to the HLA-A2 molecule, this did not correlate with the height of the MuV-specific T-cell response. In addition, we observed no significant differences between these three epitopes in terms of the phenotype, dynamics and TCR diversity of the T-cell response after MuV-infection.

In **Chapter 7**, four different IAV-specific epitopes were analyzed. Again, the dynamics of the antigen-specific T-cell response differed clearly between the four epitopes, both after the priming and booster infection. After the priming infection, the immunodominant epitopes (ASN and SSL) induced higher antigen-specific IFNγ-responses than the subdominant epitopes (SSY, MGL). Even for the two immunodominant epitopes (ASN and SSL), the responses differed in terms of magnitude and phenotype after the priming infection, and this difference became even more pronounced after the second infection. We only performed TCR repertoire analyses for ASN-specific T cells, but other studies have compared the ASN-specific and SSL-specific T-cell repertoires and showed that the SSL specific T-cell repertoire is much more private and diverse than the ASN-specific repertoire [193, 194, 351]. Thus, although in some cases the response against one epitope may indeed be representative for the total virus-specific response, this may not always be the case.

Differences between T-cell response against epitopes from the same virus have been suggested to be most pronounced for chronic viruses. For example, cells infected with EBV or CMV express different epitopes during the lytic and latent phase of infection. The immunodominant CMV- and EBV-epitopes studied in **Chapter 4**, A2-NLV (CMV, pp65-protein) and A2-GLC (EBV, BMLF1-protein), are both expressed in the lytic phase of infection. The response and clonal expansions against lytic epitopes are often more dominant than the response against epitopes expressed in the latent phase [428, 429]. This is even more pronounced in the response against murine CMV [430]. Thus, the response and TCR repertoire against epitopes expressed in the lytic phase may not be representative for the epitopes presented during the latent phase of infection.

Does this imply that we need to study the T-cell response to all relevant epitopes when studying the virus-specific T-cell response? At least, insight into the response against a diverse set of important epitopes would be preferable. Unfortunately, however, not all epitopes may be known or induce sufficiently large T-cell responses, and it remains unclear which responses are most important. Recently, studies have tested whether antigen-specific TCR repertoire dynamics after infection or vaccination can be measured in the bulk memory T-cell pool [98, 210, 431, 432]. Such studies make use of clonal expansions occurring in response to the infection or vaccination, which are large enough to be detected in the total memory pool. It has been suggested that the magnitude of the T-cell response against an infection or vaccination could be measured using TCR sequencing, even without prior knowledge of the epitopes the response is directed to [210, 431]. Although this method is still in its infancy, theoretically it captures the total virus-specific T-cell response and not only the response to identified epitopes.

The two studies of Minervina et al. provide beautiful examples of how TCR repertoire sequencing could be used as a readout for vaccine efficacy. However, as one study is based on SARS-CoV-2 infection [431], while the other one uses YF vaccination as a model [210], both studies have a model that induces a high T-cell response, making the detection of these clonal expansions easier. Unfortunately, T-cell responses induced by most vaccinations are not of such high magnitude as seen for YF-vaccination. It remains to be tested whether also the more subtle changes in the memory T-cell pool induced by vaccination can be detected. Another problem that we need to overcome is how to determine whether the observed clonal expansions are indeed antigen-specific, or whether they are the result of bystander effects. Although the study investigating the TCR repertoire after SARS-CoV-2 infection nicely confirmed the specificity of the clonal expansions

based on overlaps with other known SARS-CoV-2-specific TCR sequences [431], for most viruses this information is not yet available.

IS THE TCR REPERTOIRE IN THE CIRCULATION COMPARABLE TO THAT IN THE TISSUES?

In **Chapters 4-6** we focused on the antigen-specific TCR repertoire in humans. For this, we only looked at T cells derived from the blood, since blood is relatively easy to isolate. However, blood only contains 2-3% of an individual's T cells, meaning that most T cells are actually residing elsewhere, such as in lymphoid tissues (bone marrow, spleen, lymph nodes), at mucosal sites (lung, intestines) or in the skin [433]. Recently, a distinct fraction of memory T cells has been discovered in the tissues, called tissue-resident memory T cells (TRM cells) [434, 435]. These TRM cells do not recirculate, but stay at the site of infection to provide a rapid response to re-infection [356]. They have been suggested to be in a relatively quiescent state and to remain in the tissue for prolonged periods of time [436]. The T-cell repertoire induced at the site of infection may thus be more stable than that observed in the circulation.

A few studies have been able to compare TCR repertoires in blood and tissues in humans. Clonal analysis of naive T cells in the spleen and lymph nodes revealed no overlaps between the different lymphoid sites, regardless of age [241]. This may not come as a surprise, as 1) the naive repertoire is extremely diverse, and thus even sampling twice from one compartment in an individual yields little to no overlap between the two samples, and 2) naive T cells are not yet primed at a certain location and continuously migrate between secondary lymphoid organs via blood and lymph [437]. On the other hand, clonal analysis of memory T cells in lymphoid tissue, lung and blood showed inconclusive results. Central memory T cells in blood and thoracic duct lymph did not only have comparable T-cell frequencies, but also the clonotypes at these sites turned out to be largely overlapping [240]. In contrast, the effector memory subset was less overlapping between blood and thoracic duct lymph. PD-1^{+} CD8^{+} T cells from 2 different inflamed joints of a juvenile idiopathic arthritis patient also showed a clear overlap of TCR sequences between the two joints [438]. Zooming in on the antigen-specific repertoire suggests that there is a clear overlap between tissues. Poon et al. showed that both IAV- and CMV-specific T cells localize in different tissues, e.g. blood, bone marrow, lungs and lymph nodes. In a substantial part of the donors the IAV-specific response was especially high in the lungs, while CMV-specific frequencies were highest in the bone marrow [439]. However, the repertoire characteristics (TRBV-usage and shared

clones) indicated that the antigen-specific repertoires were consistent across the different tissues. Differences in TRBV-usage were based on donor and virus, but not on tissue [439]. The same was concluded by Sant et al, who investigated the IAV-specific TCR repertoire in lung, spleen and LN of humans [179]. Pizolla et al, who compared the TCR repertoire of different populations (based on CD69 and CD103 expression) in the lung[440]. $CD103^{+}CD69^{+}$ cells, which are thought to represent TRM cells, showed a large overlap of clonotypes and comparable diversity with CD103-$CD69^{+}$ and CD103-CD69- T cells, which are assumed to be mostly circulating cells. This repertoire sharing was especially observed for high frequency clonotypes. This was confirmed by a mouse experiment in which large overlaps were found in the IAV-specific TCR repertoires in spleen and BAL [193]. Thus, the TCR repertoire of the memory pool is suggested to show significant overlaps between tissues, although this could change over time as TRM cells have been proposed to live longer than circulating T cells.

Maintenance of the TCR repertoire in the tissues

Several studies have suggested that TCR repertoire diversity in tissues is maintained for longer periods of time than in blood, which would be in line with the relative longevity of TRM cells. Koguchi-Yoshioka et al. showed a reduction in TCR repertoire diversity, T-cell density and cytokine production of total T cells in the blood of older adults as compared to younger individuals [441]. In contrast, T-cell repertoire diversity, T-cell density and cytokine production of T cells in the skin were suggested to be maintained with age, as they showed comparable results between older and younger adults. Based on data on the diversity of different memory subsets in lymphoid organs, lung and blood in three individuals collected over five decades, Miron et al. also suggested that tissue-specific memory TCR repertoire diversity is maintained with age [425]. There was an increase in clonality (leading to decreased diversity) in the blood with age, but not in the other tissues.

Due to their important role in the protective response, TRM cells are also subject in the development of new vaccination strategies. For example, in the case of IAV infection, the site of infection is the lungs/respiratory tract. Targeting lung TRM cells may lead to a quicker response during virus encounter and enhance protection. Although TRM cells have been suggested to be in a quiescent stage, TRM cells in the lungs of mice were actually shown to disappear after approximately 7 months [331, 436]. More information is needed about the T cell repertoire and its maintenance in different tissues, in order to determine whether administering vaccines locally could have a positive effect on the protective T cell repertoire.

FUTURE ROLE OF TCR REPERTOIRE IN VACCINATION STRATEGIES

Current diagnostics and vaccine strategies do not yet take TCR diversity into account and using the TCR repertoire as a biomarker is still in its infancy. This is because there is still a lot to discover about the dynamics of the TCR repertoire, like correlations between findings and clinical outcomes. Especially due to the highly private and complex information hidden in each individual's TCR repertoire, we are not yet able to translate this information to new vaccine strategies. However, the highly private information hidden in the repertoire of different individuals also provides an opportunity, as the TCR repertoire can be seen as the ultimate personalized biomarker [442].

Recently, some promising steps have been made in extracting information from the TCR repertoire. One study has shown that it is possible to predict the specificity or HLA-background of individuals based on their TCR sequences [95]. Additionally, Dupic et al. recently reported that even genetically identical individuals can be identified based on the TCR repertoire information of a few thousand T cells.

The information hidden in the TCR repertoire could show us which individuals are at risk for certain infections, or who is in need for a (booster) vaccination [99]. With this information, more personalized vaccine programs could be designed. One of these strategies could be early vaccination of individuals in which the diversity of the naive T-cell pool is starting to decrease, to make sure they still have sufficient diversity in their T-cell pool to be able to trigger a diverse antigen-specific T-cell repertoire. Such personalized strategies could also be used for specific subgroups of the population, based on common characteristics like sex or HLA-background.

Currently, one of the focusses of the TCR repertoire field is to identify which TCRs are specific for which antigen. So far, this is mostly based on deep learning approaches and finding motifs, based on TCRs with known antigen specificity [96, 97]. The next step would be to determine which of these antigen-specific T cells play the most important functional role in protection against infection, and can hence act like "super-TCRs". With this information at hand, vaccination strategies could become more personalized and hence more optimized, if such important TCRs could be added to the repertoire, via peptide vaccination or possibly even via transfer of genetically modified T cells. To get a better understanding of which T cells play the most important roles, it could be useful to compare antigen-specific

repertoires from individuals who are at risk for disease with those from individuals who are protected.

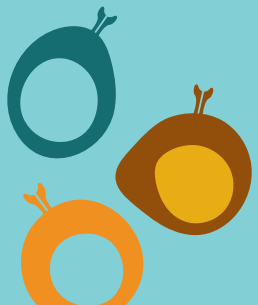
CONCLUDING REMARKS

Sequencing the immune repertoire holds great promise for more personalized vaccine strategies. However, we still have a long way to go and before we can apply it in the real world, we first have to unravel the dynamics of T-cell repertoire diversity over time. In this thesis, we have provided insights in the maintenance and shaping of the antigen-specific TCR repertoire and have discussed how this knowledge could be used in the development of new vaccine strategies to protect older adults. This thesis also highlights the complexity of the TCR-sequencing field and some of the technical challenges that have to be overcome before we are able to use TCR repertoires at large scale in diagnostic or clinical settings.

The efforts made in the last decades and the generation of large databases of TCR sequences have laid the basis for a possible future role of TCR repertoire sequencing in predicting health and disease. Our next challenge will be to obtain a better understanding of why certain TCRs are maintained and what their role is in the protective viral response. From there on, one could test how we could modify and enhance the protective features of the TCR repertoire.


9

APPENDIX



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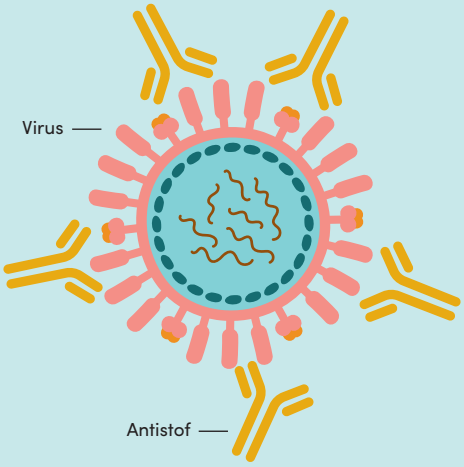
NEDERLANDSE SAMENVATTING

Het immuunsysteem

Gedurende het leven komt de mens constant in aanraking met verschillende ziekteverwekkers, waaronder virussen. Hoewel virussen in vergelijking tot de mens vrij simpel in elkaar zitten, kan een virusinfectie veel schade aanrichten en in sommige gevallen zelfs leiden tot de dood. Om ons te beschermen tegen zo'n "simpel" virus, beschikt ons lichaam over een complex afweersysteem. Het afweersysteem bestaat uit verschillende type witte bloedcellen die allemaal op een andere manier zorgen dat het virus wordt opgeruimd. Daarnaast onthoudt het afweersysteem informatie over het virus, zodat als je een tweede keer geïnfecteerd wordt met hetzelfde virus, je vaak minder of helemaal niet meer ziek wordt. Bij het onthouden van virussen spelen vooral B-cellen en T-cellen een belangrijke rol.

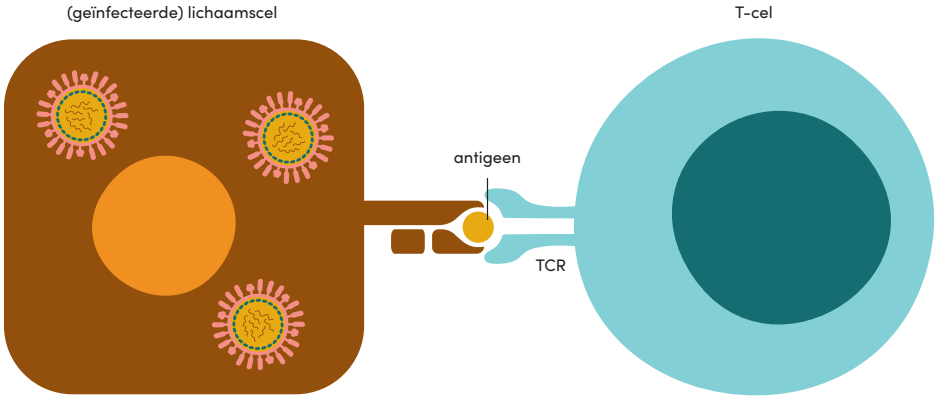
B-cellen produceren Y-vormige eiwitten, beter bekend als antistoffen, die aan de buitenkant van een virusdeeltje binden (zie figuur 1 voor de schematische weergave van antistoffen die een virus neutraliseren). Binding door antistoffen biedt op verschillende manieren bescherming, waarvan de meest bekende neutralisatie is (zie Figuur 1 voor de schematische weergave van antistoffen die een virus neutraliseren). Dit betekent dat er zoveel antilichamen aan het virusdeeltje binden, dat het virus niet meer in staat is om lichaamscellen te infecteren.



Figuur 1: *Schematische weergave van antistoffen die een virusdeeltje binden.*

Als een virus niet volledig geneutraliseerd wordt, kan het lichaamscellen infecteren en zich zo vermenigvuldigen. Dit is het moment dat T-cellen in actie komen, de cellen die centraal staan in dit proefschrift. Als een cel geïnfecteerd is met een

virus, presenteert de cel aan de buitenkant kleine stukjes van het virus, die we antigenen noemen. T-cellen kunnen deze antigenen herkennen met behulp van hun T-cel receptor (TCR) en zullen vervolgens de geïnfecteerde cel, met het virus erin opruimen (zie figuur 2 voor een schematische weergave van een T-cel die een antigen herkent).

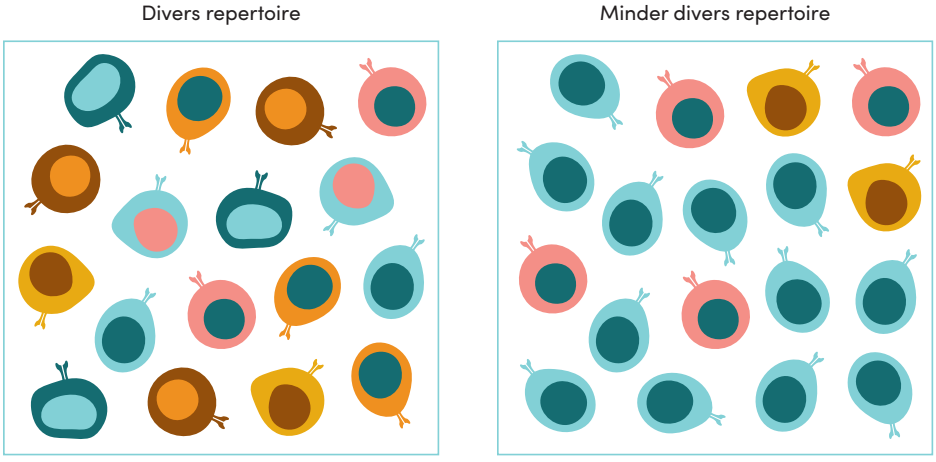


Figuur 2: *Schematische weergave van een T-cel die via zijn T-cel receptor een antigeen herkent dat gepresenteerd wordt aan de buitenkant van een virusgeïnfecteerde lichaamscel.*

De eerste keer dat B- en T-cellen een virus herkennen, veranderen ze van naïeve cellen in geheugencellen. Geheugencellen zijn veel sneller in staat om te reageren als ze hetzelfde virus nog een keer zien, omdat ze in groteren getale aanwezig zijn, en omdat ze - in tegenstelling tot naïeve cellen - direct in actie komen om te reageren zodra het virus het lichaam weer binnenkomt. Van dit principe wordt gebruik gemaakt wanneer je gevaccineerd wordt: door middel van een vaccinatie, die vaak een stukje van het echte virus bevat of een verzwakte vorm is van het virus, wordt er gezorgd dat naïeve B- en T-cellen die het virus kunnen herkennen geheugencellen worden. Wanneer het echte virus vervolgens je lichaam infecteert, zal het immuunsysteem direct reageren en het virus opruimen, nog voordat je (ernstig) ziek wordt. Ondanks de belangrijke rol van T-cellen in de immuunreactie tegen virusinfecties, wordt meestal alleen gekeken naar antistoffen om te bepalen hoe goed iemand beschermd is tegen een virus. Dit komt onder andere doordat het moeilijker is om T-cellen te meten dan antistoffen, maar ook doordat het niet geheel duidelijk is hoeveel en welke T-cellen nodig zijn voor een effectieve T-cel reactie tegen een virus.

Het T-cel repertoire

Een belangrijk onderdeel van de T-cel reactie waar de laatste jaren steeds meer onderzoek naar wordt gedaan is de T-cel diversiteit. Iedere T-cel heeft een unieke T-cel receptor (TCR) waarmee het specifieke antigenen kan herkennen. Om alle verschillende antigenen, en dus virussen te herkennen, beschikt ieder individu over een enorm aantal verschillende T-cellen, met verschillende TCRs. Dit wordt ook wel het T-cel repertoire genoemd. Dit T-cel repertoire verschilt per individu, en bepaalt welke antigenen herkend worden en welke immuunreactie tegen een virus wordt gemaakt. Er wordt geschat dat een mens tussen de 10^6 en 10^{11} verschillende TCRs in het lichaam heeft om alle mogelijke virussen te herkennen. Over het algemeen heerst het idee dat hoe diverser het T-cel repertoire is, hoe groter de kans is dat een virus herkend wordt als het een lichaam infecteert. Figuur 3 geeft een weergave van een divers en een minder divers T-cel repertoire.



Figuur 3: *Overzicht van een divers (links) en minder divers (rechts) T-cel repertoire. Diversiteit wordt aangegeven door verschillende kleuren T-cellen. Een divers T-cel repertoire bevat T-cellen met veel verschillende kleursamenstellingen en een minder divers T-cel repertoire bevat een groot aantal dezelfde (dominante) T-cellen.*

Het T-cel repertoire is continu onderhevig aan veranderingen. Een belangrijke factor die van invloed is op de diversiteit van het T-cel repertoire is leeftijd. Dit komt aan de ene kant doordat de thymus, het orgaan waar nieuwe (naïeve) T-cellen worden aangemaakt, steeds minder functioneel wordt en aan de andere kant doordat T-cellen niet eeuwig overleven. Infecties en vaccinaties hebben ook invloed op het T-cel repertoire, met name het geheugen T-cel repertoire, omdat ze ervoor zorgen dat bepaalde T-cellen toenemen in aantal of dat er nieuwe T-cellen aan het immunologisch geheugen worden toegevoegd. Welke

T-cellen zich bevinden in het geheugen T-cel repertoire geeft dus informatie over welke virusinfecties en vaccinaties iemand heeft doorgemaakt. Door te bepalen hoe divers dit T-cel repertoire is, zou je in theorie kunnen afleiden hoe goed iemands afweerreactie is tegen een bepaald virus en hoe goed een persoon dus beschermd is.

De focus van dit proefschrift

De informatie opgeslagen in het T-cel repertoire zou dus in principe gebruikt kunnen worden om mensen die risico lopen om ernstig ziek te worden te identificeren en extra bescherming te bieden. Toch zijn er nog een heleboel vragen die beantwoord moeten worden over de dynamiek van het T-cel repertoire en wat dit betekent voor de bescherming tegen virussen voordat dit mogelijk is in de praktijk. Daarom hebben we in dit proefschrift gefocust op het in kaart brengen van het antigeen-specifieke T-cel repertoire. Dit is het deel van het T-cel repertoire dat een bepaald antigeen van een virus kan herkennen. Door naar het antigeen-specifieke T-cel repertoire te kijken, is het mogelijk de afweerreactie tegen een bepaald virus te bestuderen. Het antigeen-specifieke T-cel repertoire kan worden onderzocht door T-cellen uit het bloed te halen en vervolgens van hun TCRs de DNA-sequentie te bepalen. Op deze manier hebben we onderzocht hoe het T-cel repertoire verandert onder invloed van veroudering, (herhaaldelijke) infecties en vaccinaties.

Dit proefschrift is opgebouwd uit twee delen. In het eerste deel is gekeken hoe stabiel het T-cel repertoire blijft over de tijd. In het tweede deel van dit proefschrift is gekeken hoe het antigeen-specifieke T-cel repertoire gevormd wordt door (her) infectie en vaccinatie.

Deel 1: Behoud van diversiteit in het T-cel repertoire

Met de leeftijd treden er veranderingen op in het afweersysteem, die gevolgen kunnen hebben voor hoe goed een persoon reageert op virussen die het lichaam binnenkomen. Met name bij T-cellen worden grote veranderingen gezien met het ouder worden. Zo zijn er in ouderen minder naïeve T-cellen om nieuwe virussen te herkennen en geheugencellen te vormen. Hierdoor zijn ouderen gevoeliger voor bepaalde infectieziektes, maar werken vaccinaties ook vaak minder goed. Eén van de manieren om mensen op latere leeftijd te beschermen tegen infecties, zou kunnen zijn hen eerder te vaccineren, nog voordat de diversiteit van hun immuunsysteem achteruitgaat. Hierdoor is de kans groter dat de juiste naïeve T-cellen nog aanwezig zijn om geheugencellen te vormen. Voor zo'n alternatieve

vaccinatiestrategie is het van wezenlijk belang dat de gevormde geheugencellen wel behouden blijven tot op latere leeftijd.

In **hoofdstuk 3** wordt een overzicht gegeven van wat er tot op heden bekend is over het behoud van geheugen T-cellen over de tijd. Hiervoor is specifiek gekeken naar het deel van het T-cel repertoire dat de chronische herpesvirussen cytomegalovirus (CMV) en Epstein-Barr virus (EBV), en het influenza A virus (griepvirus) herkent. De studies omschreven in dit hoofdstuk laten zien dat het antigeen-specifieke T-cel repertoire tegen CMV en EBV een aantal jaar stabiel blijft. Dit is voornamelijk gebaseerd op de aanwezigheid van antigeenspecifieke T-cellen die in de grootste aantallen aanwezig zijn, ook wel de dominante T-cellen genoemd. Het antigeen-specifieke T-cel repertoire tegen het influenza A virus daarentegen, lijkt over de tijd juist deze dominante T-cellen te verliezen.

Naast veroudering lijkt voor alle drie deze virussen ook de infectiegeschiedenis een belangrijke rol te spelen in het behoud van het T-cel repertoire. Het chronische karakter van CMV en EBV betekent dat de virussen nooit echt uit het lichaam worden opgeruimd, en dat ze om de zoveel tijd opnieuw lichaamscellen zullen proberen te infecteren. In het geval van influenza A virus, wordt ieder mens meerdere keren in zijn leven opnieuw besmet. Voor alle drie de virussen geldt dus dat de geheugen T-cellen telkens opnieuw in actie moeten komen, wat leidt tot grote veranderingen in het antigeen-specifieke T-cel repertoire. Aan de ene kant laten verschillende studies zien dat de meest dominante T-cellen toenemen in aantal door herhaaldelijke infectie (zoals wordt gezien bij CMV), waarschijnlijk doordat dit de T-cellen zijn die de belangrijkste rol spelen in de immuunreactie en de virusantigenen het beste herkennen. Aan de andere kant zijn er studies die juist het verlies van dominante T-cellen laten zien (zoals gezien bij influenza A virus), waarbij gedacht wordt dat de meest dominante T-cellen uiteindelijk afsterven omdat ze telkens in actie moeten komen.

Om het allemaal nog complexer te maken is er ook nog een rol voor “heterologe” infecties weggelegd. Dit betekent dat infectie met het ene virus invloed heeft op het antigeen-specifieke T-cel repertoire tegen een ander virus. Het virus waarvoor de meest opvallende effecten op T-cellen tegen andere virussen zijn gevonden is cytomegalovirus (CMV). Het immuunsysteem van dragers van CMV vertoont meer verschijnselen van veroudering dan het immuunsysteem van mensen zonder CMV. Dit komt onder meer door het extreem hoge aantal geheugen T-cellen die kunnen reageren op CMV. Er wordt gedacht dat deze hoge aantallen CMV-specifieke T-cellen andere T-cellen (negatief) beïnvloeden,

doordat er competitie is voor groei- en overlevingsfactoren. Het T-cel repertoire en de beschermende afweerreactie tegen andere virussen is daardoor minder goed in mensen met CMV vergeleken met mensen zonder CMV. In **hoofdstuk 3** hebben wij ook verschillende studies beschreven die de invloed van CMV-infectie op het T-cel repertoire bestuderen. Al met al benadrukt dit hoofdstuk dat er nog veel onduidelijkheid is over de veranderingen in het antigeen-specifieke T-cel repertoire over de tijd en onder invloed van CMV-infectie. Voor ons is dit de reden om in hoofdstuk 4 en 5 verder uit te zoeken wat de rol is van veroudering en CMV-infectie op de afweerreactie en het antigeen-specifieke T-cel repertoire.

In **hoofdstuk 4** wordt verder ingegaan op hoe het CMV- en EBV-specifieke T-cel repertoire veranderen door veroudering en hoe co-infectie met CMV het EBV-specifieke T-cel repertoire beïnvloedt. Omdat het lastig is een individu 20 jaar lang te volgen tijdens een PhD-traject van 5 jaar, is ervoor gekozen het antigeen-specifieke T-cel repertoire van jongvolwassenen te vergelijken met dat van ouderen. We zien dat het uiterlijk van CMV- en EBV-specifieke T-cellen anders is in oudere individuen vergeleken met jongvolwassenen, en dat de diversiteit van het antigeen-specifieke T-cel repertoire vermindert met de leeftijd. Daarnaast laten we zien dat co-infectie met CMV leidt tot een lagere diversiteit van het EBV-specifieke T-cel repertoire. Wat hier precies het mechanisme achter is blijft onduidelijk, maar aan de hand van de data lijkt het er niet op dat CMV-specifieke T-cellen concurreren met EBV-specifieke T-cellen, aangezien we juist een positieve associatie vinden tussen het percentage CMV- en EBV-specifieke T-cellen in een persoon.

Om verder in te gaan op het effect van co-infectie met CMV, wordt er in **hoofdstuk 5** bestudeerd hoe CMV de immuunreactie tegen influenza A virusinfectie beïnvloedt. We vinden weer dat vooral in ouderen een effect van CMV-infectie aanwezig is, wat in het geval van influenza A virus leidt tot een lager percentage geheugen T-cellen die het influenza A virus herkennen. We vergelijken in deze studie daarom de afweerreactie tegen het influenza A virus in ouderen die wel of niet drager zijn van CMV. Opvallend is dat we “in rust”, dus als er geen actieve influenza A virusinfectie is, grote verschillen wat betreft aantal en uiterlijk zien in de influenza A virus-specifieke T-cellen tussen ouderen die wel of niet drager zijn van CMV. Deze verschillen zijn echter verdwenen op het moment dat we kijken naar de influenza A virus-specifieke T-cellen tijdens influenza A virusinfectie. Dit wordt zowel gezien voor de hoogte van de T-cel reactie, als voor de samenstelling van het T-cel repertoire.

In **hoofdstuk 6** gooien we het over een andere boeg, door te kijken naar de T-cel reactie tegen het bofvirus. In het geval van het bofvirus is er tot dusver het meest bekend over de antilichaam reactie na vaccinatie, terwijl de rol van T-cellen in de bescherming tegen het bofvirus minder duidelijk is. Hoewel het grootste deel van de Nederlandse bevolking gevaccineerd is tegen het bofvirus, treden er toch nog infecties met bof op, met name onder studenten, ook als ze volledig gevaccineerd zijn tegen het bofvirus. In Hoofdstuk 6 analyseren we de T-cel reactie in jongvolwassenen na infectie met het bofvirus. Anderhalve maand na infectie zien we een grote hoeveelheid bof-specifieke T-cellen in het bloed. Deze aantallen worden significant minder over de tijd, maar blijven wel detecteerbaar. Als we vervolgens het bof-specifieke T-cel repertoire bekijken 1.5 maand en 9 maanden na infectie, valt op dat dit T-cel repertoire stabiel blijft, ondanks de afname in het aantal bof-specifieke T-cellen. Wat daarnaast opvalt, is dat de bof-specifieke T-cel reactie in individuen die gevaccineerd zijn met het BMR vaccin, niet verschilt van dat in ongevaccineerde individuen. Het blijft dus onduidelijk uit dit onderzoek wat de rol is van bof-specifieke geheugen T-cellen na vaccinatie in de bescherming tegen bofinfectie.

Deel 2: Het T-cel repertoire vormen

In het tweede deel van dit proefschrift bekijken we hoe het T-cel repertoire zich vormt onder invloed van infecties en vaccinaties. In de hoofdstukken 7 en 8 maken we gebruik van experimenten in muizen. Voor veel immunologisch onderzoek is het helaas (nog) niet mogelijk om vervangende systemen te gebruiken waardoor proefdieren noodzakelijk blijven. Dit komt doordat het immuunsysteem zo complex is en het belangrijk is de T-celreactie in dit complexe systeem te bestuderen.

Zoals beschreven in hoofdstuk 3, is het niet duidelijk hoe herhaaldelijke infectie het T-cel repertoire beïnvloedt. Eén van de mogelijke redenen voor de verschillende uitkomsten die zijn gerapporteerd in de literatuur is een verschil in tijd tussen de eerste en de tweede infectie. Daarom bestuderen wij in **hoofdstuk 7** hoe de tijd tussen infecties en vaccinaties invloed kan hebben op de geheugen T-cellen die gevormd worden. Hiervoor bestuderen we hoe influenza virus-specifieke T-cellen reageren tijdens influenza infectie in 12 maanden oude muizen met een verschillende infectiegeschiedenis. De muizen hebben ofwel nooit eerder, ofwel 3 maanden of 6 maanden eerder een infectie met influenza doorgemaakt. In deze studie zien we dat het hebben van een infectiegeschiedenis leidt tot expansie van de dominante virus-specifieke T-cellen bij infectie op 12 maanden, vergeleken met muizen die geen eerdere infectie hebben doorgemaakt. Er is geen verschil

tussen de T-cel reactie of het T-cel repertoire van de muizen die 3 maanden of 6 maanden eerder geïnfecteerd zijn met het influenza A virus.

Tot slot, kijken we in **hoofdstuk 8** hoe de geheugen T-cellen kunnen worden beïnvloed door vaccinatie om een betere bescherming te bieden tegen infecties. Dit doen we door muizen te vaccineren met antigenen die heel erg lijken op een influenza A virus antigeen, maar een klein beetje zijn aangepast. Deze aangepaste antigenen worden langer aan T-cellen gepresenteerd dan het normale antigeen, waardoor er hypothetisch een sterkere T-cel reactie kan ontstaan. Helaas blijkt uit deze studie dat deze sterkere T-cel reactie voornamelijk bestaat uit veel geheugen T-cellen die het normale antigeen – en dus ook het influenza virus – niet meer herkennen. De T-cellen die wel op het normale antigeen reageren, lijken een minder divers T-cel repertoire te hebben. Daarnaast blijkt dat vaccinatie met één van de aangepaste antigenen juist een negatief effect heeft op het ziektebeeld tijdens een volgende infectie met influenza A virus. Deze strategie van vaccinatie met aangepaste antigenen lijkt dus helaas niet altijd voor betere bescherming tegen influenza A virus infectie te zorgen.

Implicaties voor vaccinatiestrategieën

In dit proefschrift hebben we meer inzicht gekregen in wat wel en wat niet werkt om de juiste T-cellen aan te zetten en te behouden. Maar als er iets is wat dit proefschrift laat zien is het wel dat de complexiteit van het T-cel repertoire nog lang niet is ontrafeld. Dit betekent ook dat we nog een lange weg te gaan hebben, voordat het T-cel repertoire kan worden ingezet als diagnostisch hulpmiddel. Dat deze weg nog lang is, komt deels doordat we niet in staat zijn om alle informatie in het T-cel repertoire goed af te lezen, maar ook doordat het niet duidelijk is wat alle informatie betekent.

Toch is het veld de afgelopen jaren hard vooruitgegaan. Zo zijn er studies die hebben laten zien dat de genetische achtergrond van mensen te herleiden is uit hun T-cel repertoire en is men zelfs in staat om tweelingen te identificeren gebaseerd op het T-cel repertoire. Daarnaast heeft een aantal studies, gebaseerd op het T-cel repertoire, laten zien tegen welke virussen er al geheugencellen waren gevormd. Dit zijn zeker stappen in de goede richting om uiteindelijk het T-cel repertoire in te kunnen zetten in de diagnostiek, en uiteindelijk individuen te identificeren die belang hebben bij bepaalde vaccinaties.

CURRICULUM VITAE

Josien Lanfermeijer was born on July 14, 1991 in Haren, Groningen, the Netherlands. She finished her secondary school at the Zernike College in Haren in 2009. Subsequently she started her bachelor studies Biomedical Sciences at Utrecht University. After two years, she took a gap year to become a board member of the study association M.B.V. Mebiose and attended a summer course in Regenerative Medicine at the Utrecht Summer School. She would remain active during her bachelor and master studies as a student representative at the faculty council and the I&I education committee.

In 2013, she enrolled in the master program Infection and Immunity, also at the University of Utrecht. As part of the master's degree, she started with an internship at the department of Medical Microbiology at the University Medical Centre in Utrecht under the supervision of prof. dr Emmanuel Wiertz and dr. Rutger Luteijn. Here she studied the influence of TAP1 and TAP2 gene polymorphisms on TAP functioning. It was here where her interest in the interaction between the immune system and viral infections was ignited.

Before continuing with her second research internship she performed an internship to learn about the education and communication of science at NEMO science museum under the supervision of Hiljanne van der Meer and Elles van Vegchel. For her second scientific internship she wanted to experience science at a company and went to biotech company Genmab, Utrecht the Netherlands. Here she studied the influence of Fc:Fc enhancement and mono- and bivalent binding antibodies on ADCC under the supervision of dr. Frank Beurskens and dr. Patrick Engelberts.

In September 2016, she started as a PhD candidate at the department of Immune Mechanisms of the National Institute for Public Health and the Environment (RIVM) in collaboration with the Center for Translational Immunology of the University Medical Center. Her supervisors were prof. dr. Debbie van Baarle, prof. dr. José Borghans and dr. Jorgen de Jonge. Her research focused on determinants of immunodominance and diversity of the immune repertoire. The results obtained during her PhD are described in this thesis.

Since February this year Josien works as a medical science liaison at AstraZeneca at the department of Vaccines & Immune therapies.

Next to her study and work, Josien always had an urge to interfere, arrange and organize activities for others. The free time she has is filled with (a lot of) sports, like swimming, water polo and mountain biking, as well as singing and enjoying life with her family and friends. But she is the happiest spending time in nature with her great loves, Maarten and Siep.

Josien and her dog Siep,
August 30, 2021

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The memory CD8+ T-cell response in mice is not influenced by time since previous infection.

[†]These authors have contributed equally to this work and share first authorship

[$]These authors have contributed equally to this work and share last authorship

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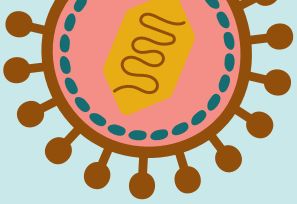

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Master has given Dobby a sock.
Master has presented Dobby with clothes.

Dobby is free.

Dobby in Harry Potter and the Chamber of Secrets (Film)