Targeting and exploiting cytomegalovirus for vaccine development

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TARGETING AND EXPLOITING CYTOMEGALOVIRUS FOR VACCINE DEVELOPMENT

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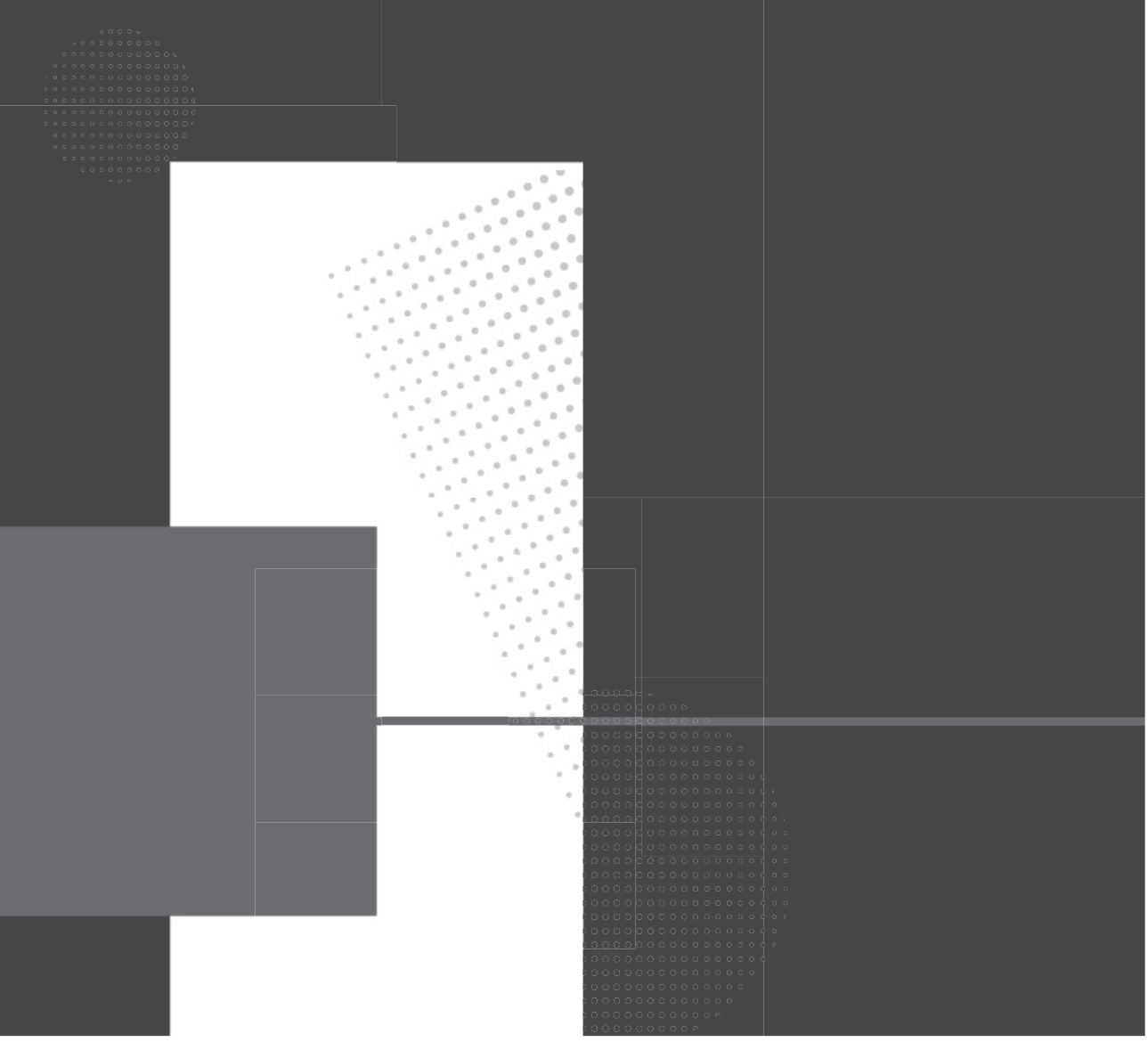
General introduction



GENERAL INTRODUCTION

Upon pathogen invasion, the innate and adaptive immune systems function in concert to evoke a pathogen specific immune response. Initially, pattern recognition receptors (PRRs) recognise pathogen-associated molecular patterns (PAMPs) and activate innate immune responses such as professional antigen presenting cells (APCs). Consequently, APCs (i.e. dendritic cells (DC), macrophages, B cells) instigate the induction of adaptive immune responses (cytotoxic T cells, helper T cells and antibodies) leading to pathogen clearance [1]. After pathogen eradication, immunological memory is formed and a rapid and more efficient adaptive immune response arises when encountering the same pathogen in the future. Immunological memory can be short- or long– lived, depending on the type and persistence of the pathogen.

Immunisation is the strategy of stimulating the host's defence with i.e. a dead or attenuated pathogen to establish protective or therapeutic immunity [2]. Vaccine development is a complex and multicomponent activity which requires comprehensive understanding of the host-pathogen interactions. Selection of the correct antigenic targets and delivery systems that will shape an effective vaccine-elicited immune response is a crucial step of the vaccine development process. The majority of the peptide- and protein-based vaccine platforms demonstrate low immunogenicity when adjuvants are lacking. Adjuvants act by providing innate and adaptive immune triggers and are currently used in several vaccine models [3-5]. The ultimate goal, is an affordable vaccine that generates strong and long-lasting immunity with the fewest possible side effects.

T CELLS

T cell activation

T cells (thymus-derived) and B cells (bone marrow-derived) are major cellular components of the adaptive immune response. T cell are crucial for mediating cellular immunity and B cells are primary responsible for humoral immunity. T cell activation occurs when a naïve T cell bearing a unique T cell receptor (TCR) encounters a DC presenting cognate antigen in the context of MHC molecules (***Signal 1***) [6]. In particular, peptides cleaved from endogenously produced proteins are presented by MHC class I molecules and subsequently recognized by T cell receptors of $CD8^+$ T cells leading into the induction of cytotoxic $CD8^+$ T cell responses. Extracellular proteins can be presented by MHC class II molecules to $CD4^+$ T cells leading to helper $CD4^+$ T cell induction [7]. Nevertheless, in some cases antigens from extracellular environment can be presented by APCs on MHC class I molecules and stimulate T- cell immunity, a process widely known as cross-presentation [8]. Priming of T cells requires approximately 16 to 20 hours of antigenic stimulation and activated T cells can be measured within 2 days.

Recruitment of $CD8^+$ T cells ends between 48-96 hours after antigenic challenge due to a feedback mechanisms that limits the duration of effective antigen presentation [9,10]. However, if the antigenic stimulation is less than 4-6 hour the programmed proliferation response is abortive.

Costimulatory signals (***Signal 2***) are considered essential for appropriate T cell activation. Mature DCs express several costimulatory ligands (i.e CD70, OX40L, , ICOSL, and the B7 molecules CD80 and CD86) which upon inflammatory conditions ligate to the equivalent costimulatory molecules (e.g. CD27, OX40, ICOS, CD28) expressed on T cells and strengthen T cell activation [11]. Certain inflammatory cytokines like IL-12 and type I interferons ***(Signal 3)*** play a crucial role in regulating adaptive immune responses and influence both primary and memory T cell development [12]. For generation of fully functional T cells concerted activation of all the three signals described above is required.

T cell expansion, contraction and memory formation

After virus encounter, T cell responses undergo through three phases ***i) expansion, ii) contraction*** and ***iii) memory development*** [13]. During the expansion phase, activated T cells clonally expand and acquire effector cell properties. In mice, the frequency of specific $CD8^+$ T cells can increase in size from 100-200 cells up to 1 x 10^7 cells during bacterial and viral infections [14,15]. Notably, the expression of interleukin-12 (IL-12) and interleukin-4 (IL-4) pro-inflammatory cytokines by innate immune cells (DCs) stimulates primary T cell activation and induction of Type 1 and Type 2 cytokine producing T cells. Activated $CD4^+$ and $CD8^+$ T cells acquire either a Type 1 or Type 2 cytokine profile. The cytokines produced by Type 1 T cells promote cell-mediated immunity and they include interferon- γ (IFN-γ), tumor necrosis factor (TNF) and interleukin-2 (IL-2) [16,17]. Type 2 T cells, secrete IL-4, IL-5, IL-6, IL-10 and IL-13 cytokines and promote B cell development and antibody responses [18]. Generally, cytokine production by $CD8^+$ T cells occurs in a rapid and transient, tightly regulated by antigenic contact [19]. In addition, $CD8^+$ T cells are also equipped with the cytolysis-inducing molecules perforin and granzyme and the apoptosis-inducing CD95 ligand (FasL/APO-1L) to execute antigen-bearing target cells [20].

$CD4^+$ T cell help is critical for proper priming of $CD8^+$ CTL cells. $CD4^+$ T cell help works by stimulating APCs through the CD40-CD40L pathway to prime $CD8^+$ T cell proliferation [21]. Although primary $CD8^+$ T cell responses to several infectious pathogens such as viruses and bacteria are generally unaffected by the absence of $CD4^+$ T cells, the memory and recall $CD8^+$ T cell response to these agents requires $CD4^+$ T cell help to initiate a second round of clonal expansion [22-24]. Moreover, a growing body of evidence indicates a capacity of $CD4^+$ T cells for potent cytolytic killing of virus infected cells [25-28].

After the expansion phase and termination of disease, more than 90% of the effector antigen-specific T cell pool is eliminated by programmed cell death (PCD) [29]. Several

factors are implicated in modulating this phase and retard or accelerate T cell contraction. For instance, IL-2 administration has been clearly shown to augment the magnitude and survival of the antigen-specific T cells, indicating that IL-2 signals are important for T cell expansion and maintenance [30,31]. However, expression of IFN-γ and cytolytic effector molecules such as perforin and granzyme have downsizing influence [32]. The role of TNF and CD95 on cells in the apoptosis of mature T cells is controversial [33-35]. Whether other factors also have contraction-modulating properties remains to be investigated. Down-regulation of activated T cells is of great importance for preventing immunopathology since excessive expansion or defective contraction of T cells (particularly CD8) results in severe or even fatal outcome [36].

After contraction, a small portion of antigen-specific T cells, termed memory T cells, remains. Memory T cell are endowed with the ability to generate a rapid and powerful immune response in case of re-infection with the same pathogen. In particular, extensive literature from animal models describes the superiority of memory T cells precursor frequency (>1000 fold) compared to naïve T cells which fosters induction of stronger and more plethoric immune responses during a secondary antigenic encounter. Memory T cells frequencies are stable over a long time [37]. Memory $CD8^+$ T cell homeostatic proliferation and long term survival is MHC interaction independent and is driven by the cytokines IL-7 and IL-15 [38]. Memory $CD4^+$ T cell replenishment is similar to $CD8^+$ T cells, whilst the need for IL-15 is less marked [39]. Remarkably, memory T cells show delayed secondary contraction and resistance to apoptosis through the upregulation of various anti-apoptotic molecules (e.g. BCL-2, BCL-XL) [40,41]. Memory T cells exhibit also better differentiation capacities and produce IFN-γ, RANTES, and the cytotoxic molecules perforin and granzyme B in larger quantities and more rapidly than naïve T cells [42-44].

Memory T cells can be categorized into effector-memory (T_{EM}), central memory (T_{CM}) or tissue-resident memory (T_{RM}) T cells based on the expression of certain homing and differentiation molecules [45]. T_{EM} can be distinguished based on the expression or lack of $CD62L^+CCR7^-$ surface molecules and the T_{CM} cells express $CD62L^+CCR7^+$ [46]. An important discrete difference between these two subsets is that T_{EM} cells predominantly circulate in non-lymphoid organs whereas T_{CM} cells preferentially localize in lymphoid tissues [47,48]. T_{RM} cells do not circulate but permanently exist in tissues.

CYTOMEGALOVIRUS INFECTION

Cytomegalovirus

Cytomegalovirus (CMV) belongs to the family of the β herpesviruses and it is also known as herpesvirus type 5. CMV has a long life cycle and has a genome size of over 230kb. The virus contains a double-stranded linear DNA enveloped by a proteinaceous matrix, which is packed into a lipid bilayer that contains viral glycoproteins. Transmission of the virus cannot occur through casual contact but via exposure to infected body fluids

(i.e blood, urine, saliva, breast milk, tears). As a result of productive virus replication immediate early, early and late viral proteins are synthesized [49]. After primary infection, the virus establishes lifelong latency within myeloid, endothelial and epithelial cell lineages. Cytomegalovirus replication is cell-type specific and depends on the stage of differentiation of the infected cells. Specifically, while monocytes repress transcription of the major immediate-early promoter and thus impede the production of new virions, macrophages and immature dendritic cells sustain productive infection [50-52]. During the lytic cycle of the infection, the virus replicates in diverse tissues (i.e spleen, liver and lungs) whereas the salivary glands are the primary site where viral replication is detected during latency [53,54].

The incidence of the CMV infection varies and its true sero-prevalence rate is difficult to determine due to virus latency and difficulty to be detected. Recent epidemiologic evidence suggests that approximately 60-80% of the human population is infected by the virus and 90% or more of the infected individuals acquire the virus till aged 40 years old age [55]. CMV infection usually is benign, with no clinical disease manifestations in immunocompetent individuals but it can reactivate and lead to severe complications in immunosuppressed and immunocompromised individuals, including allograft recipients and HIV patients. Moreover, CMV is currently the leading cause of congenital infections, and can cause long term neurological consequences such as hearing loss, visual impairment, and cognitive delays. Antiviral therapy for CMV exists, but it is accompanied with significant toxicity and prolonged treatment periods. The most commonly used antivirals for CMV disease include ganciclovir, valganciclovir, foscanet and cidofovir, all of which target to inhibit viral DNA amplification. At present there are no licensed vaccine-based countermeasures against CMV [56].

Vaccines against CMV seek to recapitulate or to improve the degree of protection bestowed by natural infection. In the last decades, many prophylactic and therapeutic vaccine strategies have been explored such as live attenuated viruses, human dense bodies, recombinant viral vectors, DNA, and subunit/adjuvant vaccines. The majority of those vaccines focused on the induction of broad neutralizing antibodies against the major envelope protein B (gB) or, more recently, against the gH pentamer complex [57]. Although some highly potent humoral-inducing vaccines have been developed, their main limitation is that they fail to provide long term protection. A series of studies on mice, non-human primates and humans provide evidence that vaccines against CMV should engage cellular T cell immunity. T-cell based vaccines designed to induce $CD4^+$ and/or $CD8^+$ T cell responses of sufficient magnitude, particular phenotype and functional traits that directly contribute to pathogen clearance, although not yet providing sterilizing immunity via cell mediated effector mechanisms is a rapidly expanding vaccination strategy that gains increasing attention [58-62]. Clinical phase I and II trials, incorporating humoral immune targets in combination with cellular immune targets, predominantly focus on the immediate early 1 protein (IE1) and protein 65 (pp65) as immunogens for cytotoxic T cells and gB as immunogen for neutralizing

antibodies. In these trials promising immune responses have been elicited. To this end, numerous novel vaccines modalities including replication-defective viral vectors and genetically disabled CMV are currently tested in experimental studies and preliminary results engender optimism that a successful vaccine will be developed [56,63].

T cell responses to cytomegalovirus

CMV-specific T cell responses exhibit unique features and follow an atypical kinetic profile. T cell responses to CMV dominate the memory T cell pool more than other (known) viruses. It is estimated that about 10% of the total circulating $CD4^+$ and $CD8^+$ memory T cell responses are CMV specific and these percentages may rise throughout the course of life [64]. The unique nature of CMV-specific T cell responses has been studied in depth using mouse CMV (MCMV) [54]. The majority of the MCMV-specific $CD8^+$ T cell responses that predominate during the acute infection phase follow the typical course, which is characterised by massive proliferation after antigen encounter, rapid contraction and long term maintenance at low levels [65]. These $CD8^+$ T cells are characterised by a conventional central memory phenotype ($CD127^+$, $CD62L^+$, $CD27^+$, $CD28^+$, KLRG-1^-, $CD44^-$ and IL-2^+) during the persistent phase of infection.

However, $CD8^+$ T cell responses to certain proteins do not contract but continue to intensify gradually over time and display an effector-memory phenotype ($CD127^-$, $CD62L^-$, $CD27^-$, $CD28^-$, KLRG-1^+, $CD44^+$ and IL-2^-). These T cells were termed inflationary to denote an ongoing rise in the response rate [66]. Inflation of memory CMV-specific $CD4^+$ T cells has also been observed [67]. Inflationary CMV-specific $CD8^+$ T cells with similar phenotypic features have been detected in humans and in primate models of CMV. Inflationary T cells often show restricted T cell receptor (TCR) usage, with many carrying T cell receptors specific for immunodominant CMV antigens. Despite their ongoing proliferation capacity, inflationary T cells retain their functionality and do not exhibit features of exhaustion as observed with certain other persistent viral infections.

For establishment of memory T cell inflation, repetitive antigen exposure is required, whilst systemic viral production does not seem to be a prerequisite to drive memory inflation. Also differences in the functional avidity for peptide-MHC complexes were not predictive whether epitopes elicit inflationary or non-inflationary T cell responses. Importantly, $CD4^+$ T cells, costimulatory molecules such as CD27 and OX40 and IL-2, IL-7, and IL-15 cytokines provide important signals for the maintenance of the inflationary T cell population but their precise mechanisms are under investigated [66,68,69] .

Animal models to study CMV pathogenesis and therapies

Treatments for CMV infections have been studied in murine, guinea pig, non-primate and primate models before being tested in humans. All models of CMV infection have significantly contributed to CMV research and they all contain a plethora of advantages and disadvantages.

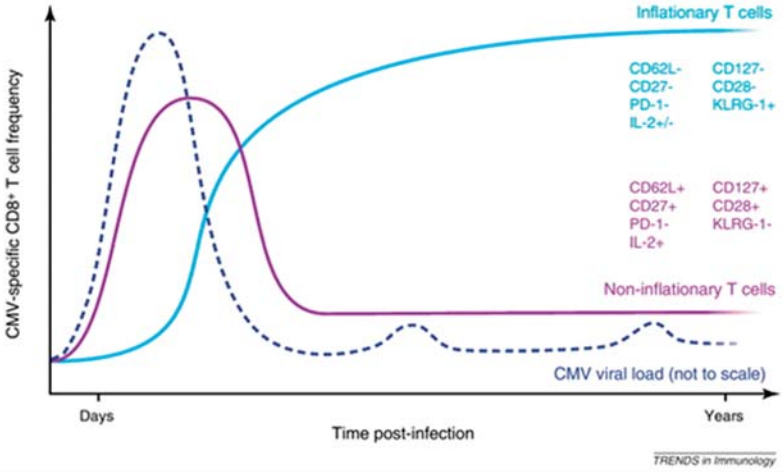


Figure 1. Longitudinal analysis of the CMV-specific T cell subsets. During CMV infection two types of T cells arise i) Conventional (non-inflationary) T cells which undergo expansion, contraction and development of stable memory pools and ii) Inflationary T cell pools which do not contract but gradually continue to accumulate in frequency throughout time. Phenotypic characteristics of inflationary and non-inflationary T cells are displayed. (Figure adapted from O'Hara, Weltens, Klenerman and Arens, Trends in Immunology, 2012; 33:84-90.)

Mice are widely used to study CMV immune functions and pathogenesis. MCMV infection resembles at a great extend HCMV biology. C57BL/6 mice alike humans are not considered particularly sensitive to CMV infection. Thus, C57BL/6 mice are a suitable model to study CMV immune responses and to investigate virus immune evasion mechanisms and new therapies in the context of immunocompetent settings. On the other hand, the BALB/c mouse strain lacks Ly49-associated NK cell functions and it is considerably susceptible to CMV infection [54]. Importantly, BALB/c mice infected with high dose MCMV (10^6 plaque-forming units) develop viremia rapidly (day 4-7 post infection) in several organs (e.g. liver, lungs, spleen, kidney) and direct disease manifestation that can even be lethal. Based on the aforementioned characteristics of MCMV infection in BALB/c mice, this mouse strain is particularly useful for studying CMV disease and testing potential antiviral treatments in the context of immunocompromised individuals. Whereas MCMV animal models recapitulate at most HCMV and there is increased availability of inbred mouse strains, a limitation of this model is that no vertical virus transmission can occur making MCMV a less suitable model to study congenital CMV infection [70,71].

Recently, ***humanised mice models*** have been developed for HCMV research. Humanised mice allow direct investigation of HCMV tropism, latency, reactivation and antiviral treatments. However, the generation of humanized mice requires technical modifications that restricts their use, and the development of immunity to HCMV is crippled [72].

Unlike mice, ***guinea pig*** models enable the study of congenital CMV infection. Specifically, guinea pig's placenta has a single trophoblast layer similar to humans which allows the virus to cross the placenta and infect the foetus. Vertical transmission rate in quinea pigs is approximately 40-60%, similar to what observed in the human settings. Notably, lytic guinea pig CMV (GPCMV) infection of immunocompetent guinea pigs leads to clinical disease symptoms such as splenomegaly, peripheral blood mononucleosis and self-limited viremia, analogous to what it is observed during HCMV infection. As with HCMV, the most important target of humoral immune response following GPCMV infection appears to be the gB homolog. This animal model has numerous advantages in terms of monitoring disease progression (i.e virologic endpoints) and evaluating therapeutic interventions. However, the usage of guinea pigs is less practical when it comes to large-scale vaccine testing, studies of the role of the immune system or experiments with (long term) follow up as compared to mice or rats [70].

Like mice also ***rats*** display human-like physiological responses to CMV infection. Importantly, almost all human genes noted to be associated with disease have known counterparts in the rat genome and extensive literature on rat CMV (RCMV) pathogenesis exists [73]. Furthermore, as with the guinea pig model RCMV can cross the placenta and infect the foetus, although the exact mechanism is still unclear. Recent studies suggest the potential use of the RCMV model to study CMV congenital transmission and vaccine development [74,75]. Hence, the rat model is significantly advanced compared to the guinea pig and mouse model for studying various aspects of CMV pathogenesis, therapeutic interventions and evaluating vaccine candidates for CMV congenital infection in humans. However, a major limitation in the study of RCMV is like for GPCMV the restricted availability of tools (e.g. monoclonal antibodies) to study the role of the immune system [70].

Finally, ***Rhesus macaques*** **infected with Rhesus** cytomegalovirus (RhCMV) is studied extensively. The RhCMV model showed a strong antigenic relationship with the HCMV and the chimpanzee CMV (CCMV) genome [76]. Some important limitations of the use of Rhesus macaques is the limited availability of inbred animals, the significantly higher cost for breeding and the requirement for specialised facilities and trained personnel compared to the rest available CMV animal models [77,78].

CMV AS A VACCINE VECTOR

Today numerous viral vectors are being exploited for potential clinical application against tumour malignancies and a broad range of infectious pathogens. Viral vaccine vectors are usually harmless, well tolerated viruses which carry an inserted antigen of choice. Viral vectors can be manufactured similarly to conventional vaccines and their safety prolife can be easily assessed, which makes them particularly attractive as vaccine platforms [79,80]. Modified vaccinia Ankara (MVA) and the adenovirus (Ad) vectors

are among the most popular and well investigated vectors which have shown very promising results [81-90]. The success of viral vector vaccines relies on their capacity to express antigens intracellularly, thereby inducing robust cellular immune responses in tissues, and in addition can carry proteins on their surface eliciting humoral (antibody-mediated) immunity. Early-phase trials have shown that targeted delivery via viral vector vaccines is feasible and ample opportunities for further improvement exist.

The majority of the viral vectors tested do not establish sufficient immunological memory, which may be related to their attenuated status [91]. Moreover, T cell responses to most viral vectors follow the conventional course which leads mostly to the formation of T_{CM} cells. Hence, upon antigen re-encounter, T_{CM} cells first undergo a phase of expansion and effector differentiation. In certain instances, this delay in the response may be detrimental for the host. Finally, immunological memory wanes over time and repetitive stimulation with the same viral vector or targeted antigen is required to maintain lifelong immunity [79,92,93]. All these crucial limitations led to the conceptualization of an alternative T cell based vaccine model that induces T_{EM} that are maintained for a long time. Unlike T_{CM} cells, T_{EM} cells are equipped with immediate cytotoxic function and localise in peripheral sites such as the mucus, one of the main portals for pathogens entry [94,95]. Hence, a vaccine with the ability to induce and maintain a large population of T_{EM} cells is expected to provide rapid effector activity and intercept pathogen replication at the most vulnerable phase of infection. T_{EM} -inducing vaccine vectors might overcome conventional viral vector vaccine limitations and establish long term efficacy.

CMV elicits a unique T cell repertoire containing both the conventional T_{CM} phenotype and the unconventional inflationary T_{EM} -like phenotype. The inflationary CMV T cells exhibit no signs of exhaustion and form a promising T cell subset that can be exploited in various vaccination settings [66,68,96,97]. Experimental studies with RhCMV vectors expressing SIV antigens have been conducted with very encouraging outcomes. Specifically, RhCMV-SIV vectors elicited and maintained robust and highly polyfunctional T_{EM} cell responses that were widely distributed at all potential SIV replication sites and recognized a broad range of MHC class I and II restricted epitopes. Remarkably, RhCMV-SIV vaccinated animals manifested an unprecedented long term pattern of protection or elite virus control without need for anamnestic stimulation [98-100].

However, the high seroprevalence rate of CMV among people raises concerns about the potential risk for tolerance to CMV-based vaccines due to pre-existing host immunity to the virus [101]. CMV has however the capacity to re-infect the same hosts [102]. Thus, the ability of CMV to re-infect encourages the use of CMV vector vaccines in CMV seropositive individuals as well as the potential for re-use of CMV vectors encoding the same or different target antigen in the same individuals. In addition, the ability of CMV to arrest and clear overtime residual SIV infection suggests that CMV vector based

vaccines can be used in therapeutic vaccine settings against HIV-1 or other chronic infectious diseases [103].

Finally, CMV-based vector vaccines could synergize with other vaccine platforms and potentially enhance overall vaccine efficacy. For instance, vaccines that are designed to elicit humoral immune responses could be combined and work together in a complementary fashion with CMV vectors designed to elicit robust cytotoxic T_{EM}-based cellular responses. Induction of acquisition blocking antibodies and highly effective long lasting T_{EM} responses could manage early and long term control of infections and cancer [103,104]. Important considerations in the use of wild-type human CMV vectors is the persistence and potential pathogenicity of the virus. These issues need to be actively evaluated in the upcoming first-in-human clinical studies for CMV vectors.

SYNTHETIC LONG PEPTIDE VACCINES

Vaccines consisting of multiple overlapping synthetic long peptides (SLPs) containing T cell epitopes constitute a very popular and promising prophylactic T cell-based immunization strategy. SLPs are chemically stable, can be easily synthesized, lack toxicity and oncogenic potential [105]. SLPs are usually designed as approximately 30-mer peptides overlapping by 10 to 15 amino acids. There is compelling evidence that the length of the peptide vaccine regulates the magnitude of the induced T cell responses [106,107]. Vaccination with exact MHC-binding peptides, usually 8-10 amino acids has been tested in many infectious diseases and following combination with adjuvants (i.e incomplete Freud adjuvant, montanide ISA-51) managed to protect against subsequent pathogen infection. However, a growing body of research evidence suggests that vaccination with short peptides is frequently not able to induce memory $CD8^+$ T cell responses and immunological tolerance to the immunizing antigens may occur [108-111]. Direct comparison of whole protein and short peptide vaccines with SLP vaccines has shown that the latter are cross-processed by intracellular routing leading to superior $CD8^+$ T cell stimulation [106,112]. Specifically, SLPs cannot bind directly to MHC class I molecule but are internalised and processed by specialised APCs (i.e DCs) before being presented to either $CD4^+$ or $CD8^+$ T cells. This process results in enhanced T cell activation and induction of more effective antigen-specific immune responses [107,113]. Hence, vaccination with SLPs is considered more efficient and an optimal approach for priming protective immunity compared to short peptides or protein vaccines.

Another important advantage of SLP vaccines is that they allow epitope selection *in vivo* based on the animal's or patient's own MHC profile. Additionally, multivalent SLP vaccines may facilitate simultaneous priming of T cells against multiple epitopes stimulating a broad immune response. Markedly, professional APCs can handle large pools of long peptides and are capable of properly exercising multiple MHC class I and

II peptide epitopes for presentation at the cell surface. Therefore, injection of pools of long peptides will lead at the induction of both $CD4^+$ and $CD8^+$ T cells, specific to all targeted epitopes, each of which can contribute to pathogen control. However, competition among SLPs may occur leading to lower responses.

Importantly, SLPs vaccines alone are poorly immunogenic and need to be combined with adjuvants or other immune modulators for sufficient innate immune stimulation and T cell induction. In several preclinical models, agonists of TLR3, TLR4, TLR7 and TLR9 significantly boosted the size and efficacy of virus/and or tumour-specific T cell responses when where combined with SLPs [114-116]. Similarly, in humans, the use of SLP vaccine formulations combined with adjuvants have managed to enhance not only the magnitude but also the activation status of the T cell response and provided promising benefits in preclinical and clinical cancer immunotherapy studies [117-121]. Although the capacity of the SLP vaccines to induce $CD8^+$ T cell responses is not $CD4^+$ T cell ''helper'' dependent, the presence of a helper peptide markedly improves the magnitude of the $CD8^+$ T cell response [122]. Furthermore, co-administration of agonistic antibodies (anti-OX40, anti-CD40, anti-4-1BB) *in vivo* to stimulate DC activation significantly improved the efficacy of the SLP vaccines in both mice and patients [116,123].

In conclusion, SLP vaccines has shown promising clinical results in a number of published and ongoing clinical trials. Combination of SLPs with other methods, such as nanomaterials and chemotherapy, or improved delivery systems is investigated and their clinical efficacy remains to be evaluated.

SCOPE AND OUTLINE OF THIS THESIS

The overarching research question studied within this thesis is whether the SLP vaccine modality can form an efficacious vaccination strategy against CMV infection.

In **chapter 2**, the preventive efficacy of a SLP vaccine comprising MHC class I MCMV epitopes that exclusively elicit cytotoxic MHC class I T cell responses against lytic MCMV challenge was examined. The quality of the vaccine induced $CD8^+$ T cell responses was studied in depth and the vaccine efficacy was evaluated in two mouse strains of CMV infection (C57BL/6 and BALB/c mice). This study showed that the breadth of MHC class I SLP based vaccines determines the efficacy of the vaccine to significantly reduce the viral load.

Next in **chapter 3**, an effort was undertaken to optimize the efficacy of the MHC class I SLP based vaccine by the addition of $CD4^+$ T cell ''help'' and/or OX40 costimulation. SLPs containing MHC class II epitopes from 5 immunodominant MCMV antigens were tested individually or in combination with the MHC class I SLPs for possible prophylactic effect against lytic MCMV infection in C57BL/6 mice. In addition, OX40 signalling was enhanced to optimise vaccine-induced $CD4^+$ T cell responses and to enforce induction

of vigorous vaccine-specific $CD8^+$ T cell responses. Vaccination with both MHC class I and II SLPs in combination with OX40 costimulation led to superior protection and a strong reduction in viral titers after challenge. Subsequently in the appendix section of chapter 3, the therapeutic efficacy of the combinatorial MHC class I and II SLP vaccines was examined in the C57BL/6 mouse strain.

In **chapter 4**, studies were undertaken to discover novel HCMV T cell epitopes and enlarge the choice of antigens for potential HCMV T cell-based SLP vaccines. *In silico* methods using public immune epitope databases and *in vitro* screening of T cell reactivity against the indispensable for virus replication IE2 HCMV protein was performed in PBMCs obtained from healthy CMV seropositive and seronegative mid age volunteers. Highly immunogenic areas with MHC class I and II T cell epitopes were identified and could be used for vaccination purposes. These results were complemented with *in silico* screening for previously published epitopes to design the most promising MHC class I and II IE2-based CMV vaccine.

In **chapter 5,** the capacity of the CMV-based viral vector vaccines to protect and/or treat virus inducing cancer was tested. MCMV-based vaccine vectors expressing immunodominant HPV antigens in either inflationary or non-inflationary T cell epitope regions were generated. The diversity of the inflationary versus non-inflationary HPV specific T cell responses induced was thoroughly examined over time and the vaccine effectiveness was evaluated and showed that the level of pre-existing immunity determines the efficacy of MCMV-based vaccine vectors.

Chapter 6 provides a critical narrative overview of literature focused on the importance of T cell-based eliciting vaccines in counteracting chronic viral infections. The main determinants and mechanistic factors required to shape immunity and maximize prophylactic T cell vaccine efficacy were discussed.

Finally, a general discussion of the core research findings, the main strengths and weaknesses and fruitful future research directions are outlined in **chapter 7**. Important emphasis is also placed on whether the knowledge obtained from this thesis can be clinically utilised and translated into the design of more potent prophylactic T-cell based vaccines.

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Chapter 2

The breadth of synthetic long peptide vaccine-induced CD8$^+$ T cell responses determines the efficacy against mouse cytomegalovirus infection

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ABSTRACT

There is an ultimate need for efficacious vaccines against human cytomegalovirus (HCMV), which causes severe morbidity and mortality among neonates and immunocompromised individuals. In this study, we explored synthetic long peptide (SLP) vaccination as a platform modality to protect against mouse CMV (MCMV) infection in preclinical mouse models. In both C57BL/6 and BALB/c mouse strains, prime-booster vaccination with SLPs containing MHC class I restricted epitopes of MCMV resulted in the induction of strong and polyfunctional (i.e., IFN-γ^+, TNF^+, IL-2^+) $CD8^+$ T cell responses, equivalent in magnitude to those induced by the virus itself. SLP vaccination initially led to the formation of effector $CD8^+$ T cells ($KLRG1^{hi}$, $CD44^{hi}$, $CD127^{lo}$, $CD62L^{lo}$), which eventually converted to a mixed central and effector-memory T cell phenotype. Markedly, the magnitude of the SLP vaccine-induced $CD8^+$ T cell response was unrelated to the T cell functional avidity but correlated to the naive $CD8^+$ T cell precursor frequency of each epitope. Vaccination with single SLPs displayed various levels of long-term protection against acute MCMV infection, but superior protection occurred after vaccination with a combination of SLPs. This finding underlines the importance of the breadth of the vaccine-induced $CD8^+$ T cell response. Thus, SLP-based vaccines could be a potential strategy to prevent CMV-associated disease.

INTRODUCTION

Human cytomegalovirus (HCMV) contributes substantially to morbidity in immunocompromised individuals. Organ or hematopoietic stem cell transplant recipients, people infected with HIV and patients with lymphocytic leukaemia are particularly vulnerable to HCMV-associated disease [1]. Moreover, congenital HCMV infection of unborn and new born children can lead to severe and permanent neurological symptoms [2]. Although currently available antivirals for HCMV are able to decelerate viral progression, thereby reducing the odds for major side effects, they require prolonged treatment periods and are accompanied with significant toxicity. Adoptive transfer of HCMV-specific T cells is an alternative treatment modality but is costly and laborious. The apparent burden of HCMV-associated disease and the paucity of cost-effective measures without side-effects have led to major efforts to develop effective HCMV vaccines but unfortunately no licensed vaccines are currently available [3, 4].

There is accumulating evidence that effective control of persistent viral infections requires the induction of a balanced composition of polyfunctional T cell responses [5]. T cell immunity against CMV plays a critical role in controlling the primary viral infection and latency [6]. Whereas CMV-specific $CD4^+$ T cells are important during the primary infection phase, $CD8^+$ T cells are associated with greater benefits at the persistent infection phase and confer superior protection during reactivation and re-exposure [7-9]. Upon CMV infection, extra-ordinary large $CD8^+$ T cell responses of diverge phenotype arise. $CD8^+$ T cell response kinetics specific to most antigens follow the traditional course comprised by expansion after antigen encounter, rapid contraction, long-term maintenance at low levels and acquisition of a central-memory phenotype. Interestingly, $CD8^+$ T cell responses to certain CMV antigens do not dwindle post-infection but inflate and exhibit a polyfunctional effector-memory phenotype [10-13]. In immunocompromised hosts, the balance between CMV and cellular immunity is apparently underdeveloped or lost, and therefore instigating the development and/or restoration of the T cell compartment specific for CMV would be particularly informative.

The overarching aim of this study was to test a potential prophylactic vaccine platform against CMV based on synthetic long peptides (SLPs) containing immunodominant T cell epitopes. Previously, we reported that in therapeutic settings SLP-based vaccines can be successfully designed to stimulate effector and memory T cells against human papilloma virus-associated disease in mice and human [14-16]. As the efficacy of SLP-based vaccines is directly linked to the phenotypical and functional characteristics of the vaccine-induced $CD8^+$ T cell response, we rigorously evaluated SLP-induced T cell responses. MCMV-specific SLP vaccines, assessed in two different mouse strains (C57BL/6 and BALB/c mice), lead to strong polyfunctional T cell responses, and combined SLP vaccines targeting different antigens provide a successful vaccine modality to control MCMV infection.

RESULTS

Prime-boost vaccination with MHC class I-restricted SLPs leads to the induction of robust CD8$^+$ T cell responses

To assess the potential of SLP-based vaccines in eliciting protecting CD8$^+$ T cell responses against MCMV infection, we designed SLPs containing immunodominant MHC class I T cell epitopes from MCMV encoded proteins, and evaluated this vaccine platform in two different immunocompetent mouse strains with different susceptibility to MCMV; the C57BL/6 strain (MHC haplotype H-2^b) and the more MCMV-susceptible mouse strain BALB/c (MHC haplotype H-2^d) (S1 Table). C57BL/6 mice are less susceptible to MCMV infection compared to BALB/c mice because C57BL/6 mice express the NK cell-activating receptor Ly49H, which recognizes the MCMV protein m157 at the surface of infected cells [17-20].

Mice were vaccinated subcutaneously with SLPs along with the TLR9 ligand CpG as adjuvant. The SLP vaccine administration was well tolerated without adverse events. At day 7 after SLP immunization, epitope-specific CD8$^+$ T cell responses were detected in the blood but a booster vaccination was required for induction of vigorous CD8$^+$ T cell responses (Figure 1A and 1B). Prime-boosting with SLP vaccines induced very high frequencies of circulating CD8$^+$T cells against the noninflationary epitopes $M45_{985\text{-}993}$ and $M57_{816\text{-}824}$ in C57BL/6 mice, and were even higher than the percentages of the circulating MCMV-induced CD8$^+$ T cells at the peak of infection (day 7). Also the response against $m139_{419\text{-}426,}$ known to be non-inflationary during the early phase after MCMV and at later time points as inflationary, is strong. The response against the non-inflationary $M45_{507\text{-}515}$ epitope in BALB/c mice was even much higher in the SLP-vaccinated group as compared to the MCMV infected mice. The frequencies of the circulating CD8$^+$ T cells against the inflationary $M38_{316\text{-}323}$ and $IE3_{416\text{-}423}$ epitopes in C57BL/6 mice and the inflationary $m164_{257\text{-}265}$ and $IE1_{168\text{-}176}$ epitopes in BALB/c mice were comparable (Figure 1A and 1B).

SLP vaccines containing MHC class I epitopes may comprise unidentified class II epitopes and linear B cell epitopes leading to CD4$^+$ T cell and antibody responses. To exclude this possibility, we performed polychromatic intracellular cytokine staining with the SLPs and performed SLP-specific antibody ELISAs, respectively (S1 and S2 Figures). Neither MCMV-specific CD4$^+$ T cells nor peptide specific Abs were detected in these assays, indicating that the designed SLPs lead exclusively to antigen-specific CD8$^+$ T cell responses and that epitope-specific responses induced by SLP or MCMV can only be compared for CD8$^+$ T cells.

Longitudinal analysis of the antigen-specific CD8$^+$ T cell responses revealed that all SLP-induced T cell responses in both mice strains contracted gradually over time after the booster immunization (Figure 1B). Two months after the booster vaccination, the SLP-induced responses to most epitopes were still clearly detectable in blood. During MCMV infection, the epitope-specific CD8$^+$ T cell responses followed a different

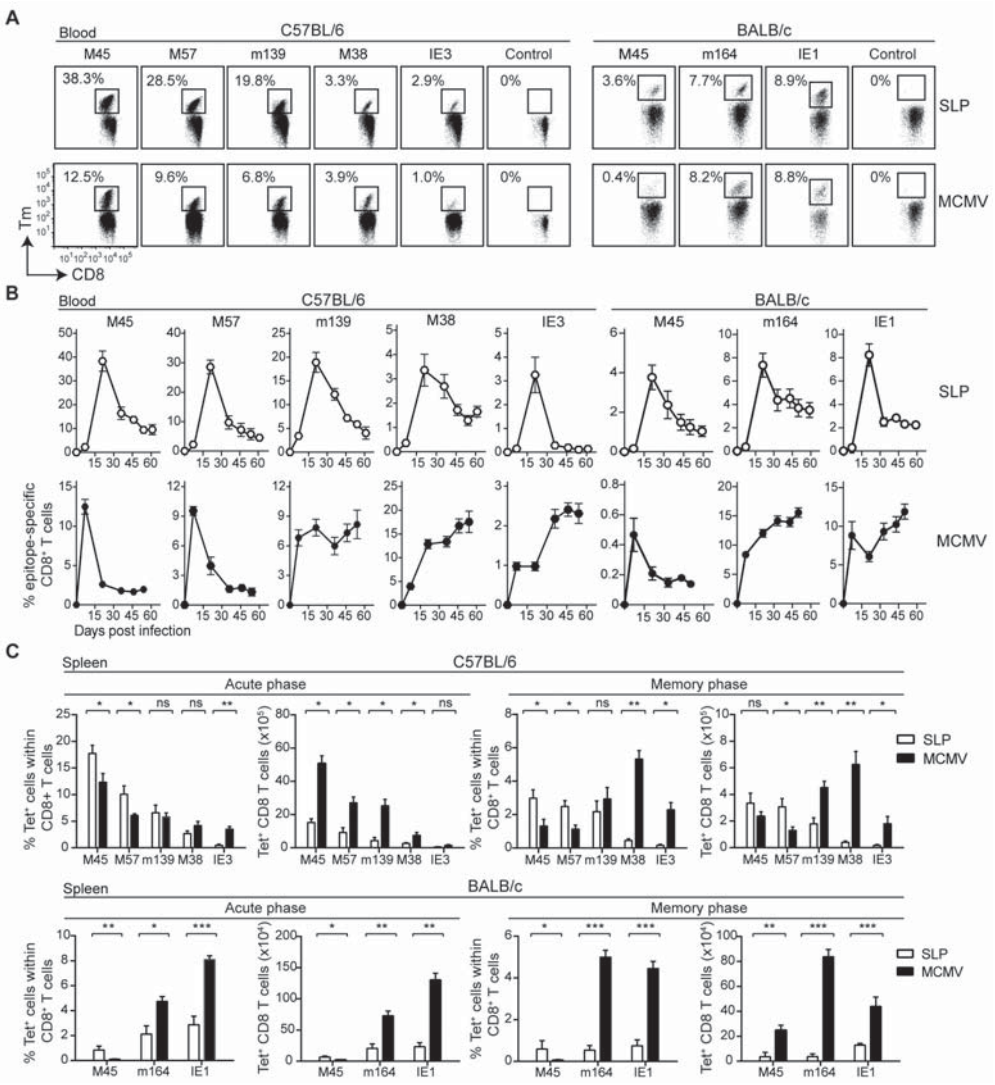

Figure 1. Prime-boost SLP vaccination provokes the induction of robust CD8⁺ T cell responses analogous to MCMV infection. (A) The magnitude of the CD8⁺ T cell responses specific to the indicated epitopes was determined in blood by MHC class I tetramer staining at day 7 post booster vaccination with SLPs and at day 7 post MCMV infection in C57BL/6 mice and in BALB/c mice. Representative flow cytometry plots show MHC class I tetramer (Tm) staining within the CD8⁺ T cell population. Numbers represent the percentage of Tm+ cells within the total CD8⁺ T cell population. **(B)** Longitudinal analysis of the epitope-specific CD8⁺ T cell responses induced by either SLP vaccination or MCMV infection in blood. Data represents mean values ± SEM (n=6 per group). **(C)** Percentages and total numbers of splenic SLP and MCMV-specific CD8⁺ T cells during the acute phase (at day 7 post booster vaccination and day 8 and after MCMV infection) and memory phase (at day 60 post booster vaccination and day 60 post MCMV infection) are shown. Data represents mean values + SEM (n=6 per group), and are representative of three independent experiments. *, P< 0.05; **, P<0.01; ***, P<0.001.

course, consistent with previous reports [10, 11]. T cell responses to the non-inflationary epitopes $M45_{985-993}$, $M57_{816-824}$, and $M45_{507-515}$ rapidly contracted after the peak response and were stably maintained in time while T cell responses to the epitopes $M38_{316-323}$, $m139_{419-426}$, $m164_{257-265}$, $IE1_{168-176}$ and $IE3_{416-423}$ inflated (Figure 1B). These data indicate that the context of epitope expression determines the kinetics of the T cell responses, which is uniform for diverse epitopes after SLP vaccination but in the case of MCMV infection this results in a dichotomy of responses related to the chronic nature of this infection.

At the peak after the booster SLP immunization (day 7-8), high frequencies of epitope-specific $CD8^+$ T cells, analogous to the responses elicited by MCMV virus were observed in the spleen (Figure 1C). However, in absolute numbers, MCMV infection led to a higher T cell magnitude compared to SLP vaccination, which can be attributed to virus-associated inflammation leading to splenomegaly. At the memory phase (day 60), MCMV-specific T cell responses to the non-inflationary epitopes were significantly lower than the equivalent SLP vaccine-induced responses (Figure 1C). The MCMV-induced $CD8^+$ T cell responses to the inflationary epitopes were of higher magnitude compared to those induced by SLP vaccination.

Taken together, these results show that prime-boost vaccination with SLP vaccines containing MHC class I MCMV epitopes elicit in mouse strains with different susceptibility to MCMV high percentages of effector and memory $CD8^+$ T cells that contract gradually in time.

The T cell precursor frequency determines the magnitude of SLP vaccine-induced $CD8^+$ T cell responses

Next, we aimed to dissect the underlying mechanisms of the relatively low responses to some of the SLPs (i.e. M38 and IE3 in C57BL/6; M45 in BALB/c) compared to the other. First, we endeavoured to alter the SLP sequences by altering the C-terminal cleavage, which may improve the immunogenicity (S3 Figure). However, the altered $M38_{316-323}$ SLP did not exhibit a significant improvement in the SLP-induced T cell response whilst the altered SLP containing the $IE3_{416-423}$ epitope elicited responses were actually reduced.

Then we questioned if the differences in the magnitude of the T cell responses triggered by the various single SLP vaccines might be related to the functional avidity of the T cells, which is determined by the affinity of the peptide for MHC and the TCR affinity for the peptide-MHC complex (Figure 2A and 2B). The SLPs elicited T cells with different levels of functional avidity but no correlation was found with the strength of the $CD8^+$ T cell response. Moreover, in both C57BL/6 and BALB/c mice the functional avidity of the T cells, elicited either by SLP vaccines or MCMV infection, were remarkably similar and remained stable in time as they were similar during the acute and memory phase of response. Thus, differences in TCR affinity are not involved in the observed difference in the magnitude of the T cell responses.

The data presented above illustrated that factors other than peptide-MHC/TCR affinity are implicated in shaping the strength of SLP-induced T cell responses. Recently,

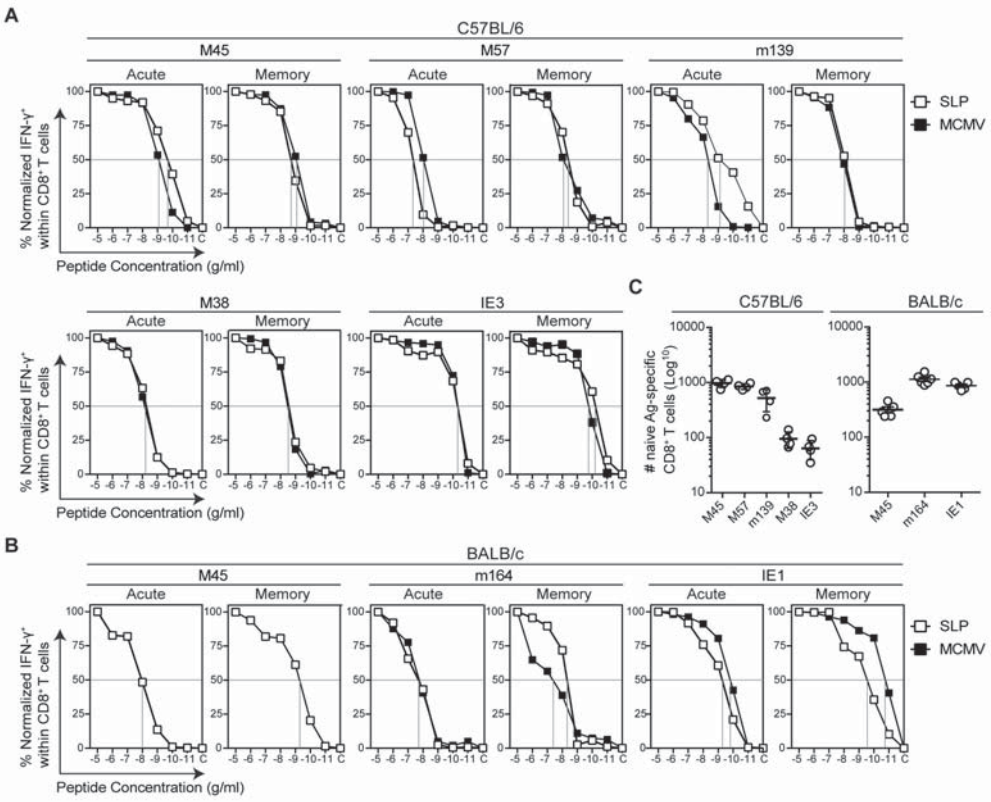

Figure 2. The T cell precursor frequency but not the functional avidity of the SLP vaccine-induced CD8⁺ T cells predicts the magnitude of the response. (A) Splenocytes from SLP vaccinated (n=4-5 mice per epitope) and MCMV infected C57BL/6 mice (n=4-5 mice) were isolated during the acute (day 8 post booster SLP vaccination and MCMV infection) and memory (day 60 post booster SLP vaccination and MCMV infection) phase and were restimulated with various peptide concentrations in the presence of brefeldin A. The percentage of IFN-γ producing CD8⁺ T cells was measured and normalized to the response at the highest peptide concentration. **(B)** Similar description as in (A) for experiments performed with BALB/c mice. Note that the $M45_{507\text{-}515}$ CD8⁺ T cell response during MCMV infection was too low to obtain accurate results. Shown are the functional avidity curves and are representative of at least 2 independent experiments. Data represents mean values. **(C)** Absolute numbers of epitope specific T cell precursors present in spleen and lymph nodes of naive C57BL/6 and BALB/c mice were determined by tetramer staining. Each symbol represents an individual mouse. Data represents mean values ± SEM (n = 4-6 per group). Experiments were performed twice with similar outcome.

it was shown that the precursor frequency of naive T cell populations can predict the immunodominance hierarchy of viral epitope specific CD8⁺ T cell responses [21]. To test whether the precursor frequency is predictive for the magnitude of SLP-induced T cell responses we determined the precursor frequency of all the epitopes included in this study in naive C57BL/6 and BALB/c mice (Figure 2C). In C57BL/6 mice, the precursor frequencies for the $M45_{985\text{-}993}$ and $M57_{816\text{-}824}$ epitopes were among the highest followed

by the precursor frequencies to the $m139_{419-426}$ epitope. The lowest precursor frequencies were detected to the $M38_{316-323}$ and $IE3_{416-423}$ epitopes, confirming a previous report [22]. In BALB/c mice, the highest precursor frequencies were observed for the $m164_{257-265}$ and $IE1_{168-176}$ epitopes whereas the frequency of $M45_{507-515}$ specific T cells was lower (Figure 2C). Markedly, the average level of the precursor frequency of each epitope-specific $CD8^+$ T cell population was proportional to the expansion of the antigen-specific populations found in mice following either SLP immunization or MCMV infection. Together, these results indicate that naive precursor frequencies rather than TCR avidity determine the magnitude of SLP vaccine-mediated $CD8^+$ T cell responses.

Phenotypical and functional characteristics of SLP-induced $CD8^+$ T cells

To assess the phenotypical and functional quality of MCMV-specific $CD8^+$ T cells induced by either the SLPs or the virus, we determined the formation of the diverse T cell subsets that develop after antigenic challenge. Early after the booster, SLP vaccination resulted in the induction of a highly activated $CD8^+$ T cell subset exhibiting an effector-like phenotype ($CD62L^{lo}$, $CD44^{hi}$, $CD127^{lo}$, $KLRG1^{hi}$), which completely resembled the MCMV-specific T cell phenotype during the acute phase of the infection (Fig 3A and 3B). In the memory phase, both SLP- and MCMV-induced T cell phenotypic traits diverged (Figure 3C and D). All SLP-induced $CD8^+$ T cells exhibited a fairly mixed phenotype sharing features of both central-memory T cells ($CD62L^{hi}$, $CD44^{lo}$, $CD127^{hi}$, $KLRG1^{lo}$), effector-memory T cells ($KLRG1^{hi}$, $CD44^{hi}$, $CD127^{lo}$, $CD62L^{lo}$) but also an intermediate phenotype (i.e. $KLRG1^{hi}$, $CD127^{hi}$). As expected, during MCMV infection, the non-inflationary $M45_{985-993,}$ $M45_{507-515}$ and $M57_{816-824}$-specific $CD8^+$ T cells gained a predominant central memory-like phenotype while the inflationary $M38_{316-323}$, $m139_{419-426}$, $IE3_{416-423}$, $m164_{257-265}$ and $IE1_{168-176}$-specific T cells appeared mostly effector-memory like.

To assess the cytokine profiles of the SLP-induced $CD8^+$ T cells, we performed intracellular cytokine staining for IFN-γ, TNF and IL-2 and compared these to MCMV-induced T cells. At the peak response after booster vaccination, SLP-induced T cells consisted mainly of single IFN-γ and double IFN-γ/TNF producing populations (Figure 4A and S4 Figure). The cytokine producing traits of the MCMV-induced effector $CD8^+$ T cells matched in general with the SLP-elicited T cells. Except relatively more single IFN-γ producing $CD8^+$ T cells after MCMV infection compared to SLP vaccination were found in the T cell populations reactive to the epitopes $IE3_{416-423}$, $IE1_{168-176}$, $M45_{507-515}$ and $m164_{257-265.}$

At the memory phase, the SLP-specific $CD8^+$ T cells gained the ability to co-produce the three cytokines, at the expense of single cytokine producing cells (Figure 4B and S4 Figure). This gain in triple cytokine production (IFN-γ/TNF/IL-2) during MCMV infection is mainly observed in the non-inflationary $CD8^+$ T cells. Both during the acute and memory phase, the percentage of the total $CD8^+$ T cell population producing IFN-γ, either in case of SLP vaccination or MCMV infection, corresponded to the percentage of MHC class I tetramers, indicating full differentiation of the elicited T cells.

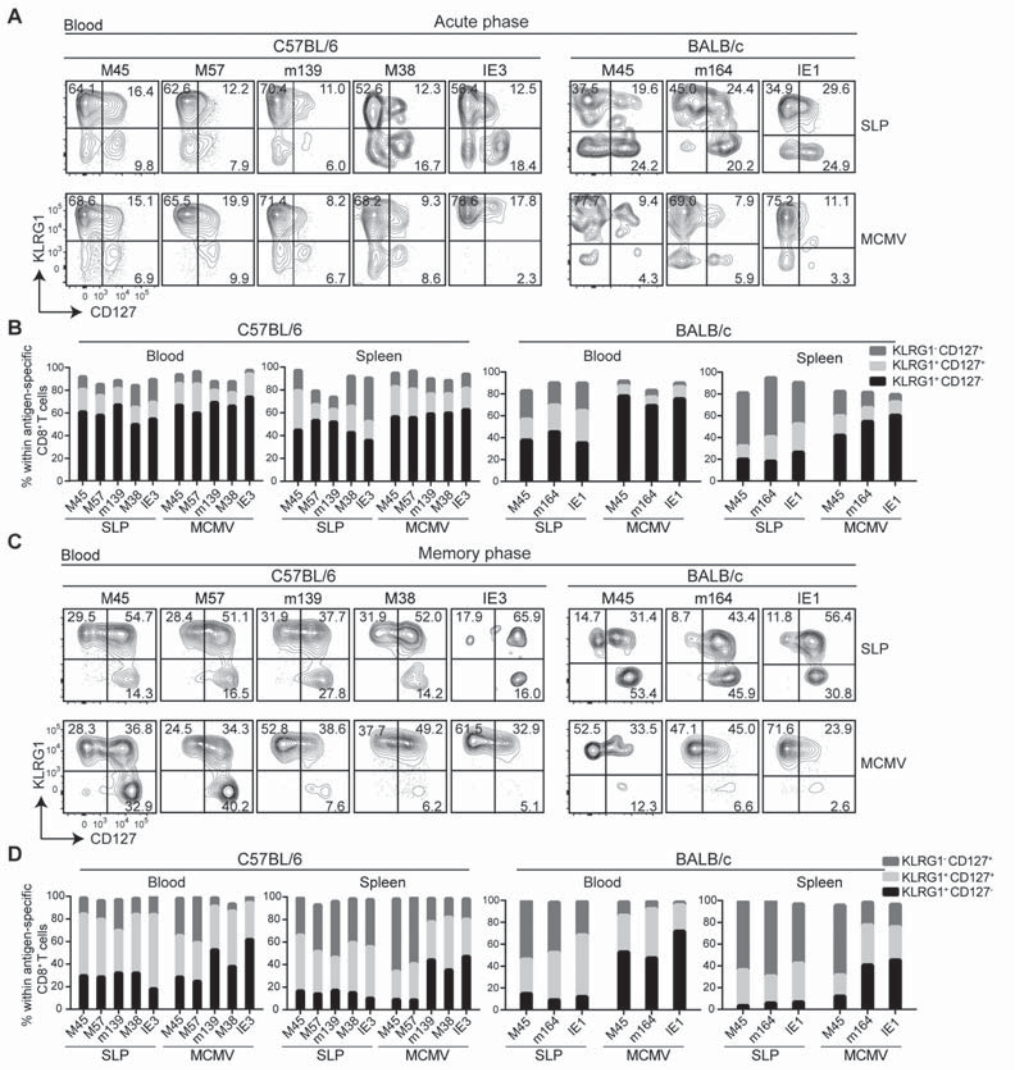

Figure 3. Phenotypic heterogeneity of SLP vaccine-induced CD8⁺ T cells. (A) Peripheral blood from either SLP immunized (day 7 after booster peptide vaccination) or MCMV infected C57BL/6 and BALB/c mice (day 7 post infection) were stained with MHC class I tetramers and for cell surface molecules. Representative plots show CD127 versus KLRG1 expression on tetramer-positive CD8⁺ T cells. **(B)** Cell-surface characteristics of tetramer-positive CD8⁺ T cells in blood and spleen at day 8 post booster SLP vaccination and day 8 after MCMV infection. **(C)** Peripheral blood from either SLP immunized (day 60 after booster peptide vaccination) or MCMV infected C57BL/6 and BALB/c mice (day 60 post infection) were stained with MHC class I tetramers and for cell surface molecules. Representative plots show CD127 versus KLRG1 expression on tetramer-positive CD8⁺ T cells. **(D)** Cell-surface characteristics of antigen-specific CD8⁺ T cells in blood and spleen at day 60 post booster SLP vaccination and day 8 after MCMV infection. Data represents mean values, and are representative of three independent experiments (n=6 per group).

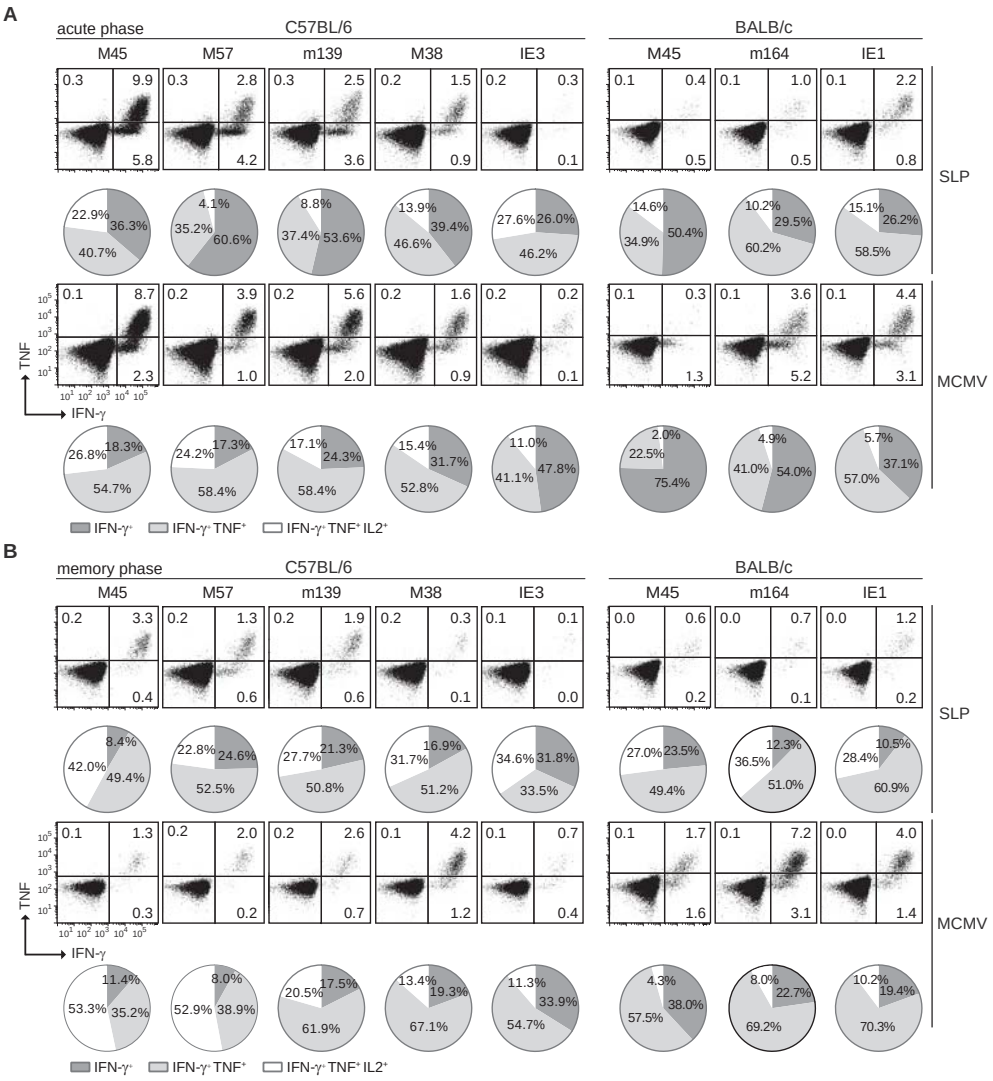

Figure 4. SLP vaccination elicits polyfunctional CD8⁺ T cells. Following SLP vaccination or MCMV infection the cytokine polyfunctionality of splenic CD8⁺ T cells was determined after peptide restimulation. Representative plots show IFN-γ versus TNF production at **(A)** day 8 (acute phase) and **(B)** day 60 (memory phase) post booster vaccination and post MCMV infection. Pie charts depict the percentages of the single (IFN-γ), double (IFN-γ/TNF) and triple (IFN-γ/TNF/IL-2) cytokine producers of each antigen-specific T cell population upon peptide stimulation. Data represents mean values, and are representative of three independent experiments (n=4-5 per group). Statistics of the results depicted in these pie charts are reported in S4 Fig.

A hallmark of memory T cells is the ability to undergo secondary expansion upon antigenic challenge [23]. To assess this property of vaccine-induced memory T cells, we performed adoptive transfer experiments in which congenically marked (CD45.1⁺)

memory $M45_{985-993}$ and $m139_{419-426}$-specific $CD8^+$ T cells from SLP vaccinated and MCMV infected mice were isolated and transferred into naive recipient mice, which were subsequently challenged with MCMV (Figure 5). SLP-induced $M45_{985-993}$ and $m139_{419-426}$-specific T cells expanded; albeit to a lesser extend as compared to the MCMV-induced (Figure 5). The MCMV-elicited $M45_{985-993}$-specific T cells exhibited, corresponding to their central-memory phenotype, a superior capacity in expansion as compared to the MCMV-elicited $m139_{419-426}$-specific T cells with an effector-memory phenotype. Of note, the expansion of the SLP-induced M45-specific T cells was comparable to the m139-specific T cells induced by MCMV, although the phenotype of SLP-induced cells were more central-memory like. This indicates that the instruction that T cells receive in different settings can result in cells with a different expansion potential despite a seemingly similar phenotype based on markers for central/effector memory cells. All together we conclude that SLP-based vaccines induce a heterogeneous pool of memory T cells with a secondary expansion potential that is somewhat lower as compared to memory T cells elicited by virulent virus.

Combinatorial SLP vaccination confers superior efficacy against CMV infection

The various SLP vaccine formulations were evaluated for their capacity to confer protection against MCMV challenge (at day 60 after booster vaccination). In C57BL/6

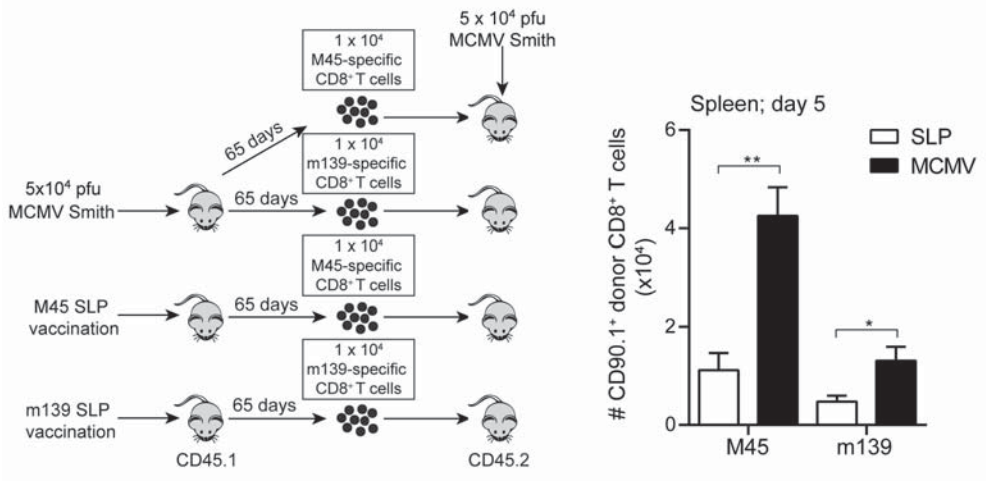

Figure 5. Secondary expansion potential of SLP-induced $CD8^+$ T cells upon MCMV challenge. $M45_{985-993}$ and $m139_{419-426}$ epitope specific $CD8^+$ T cells were isolated from the spleen at day 60 after booster vaccination and infection of SLP vaccinated and MCMV infected CD45.1 mice. 1×10^4 antigen-specific $CD8^+$ T cells were adoptively transferred into naive C57BL/6 (CD45.2) recipient mice. Recipient mice were subsequently infected i.p. with 5×10^4 PFU MCMV-Smith. The total numbers of the donor derived $M45_{985-993}$ and $m139_{419-426}$ $CD8^+$ T cells were determined in the spleen at day 5 post challenge. Data represent mean values + SEM (n=5). Experiments were performed twice with similar outcome. *, $P<0.05$; **, $P<0.01$.

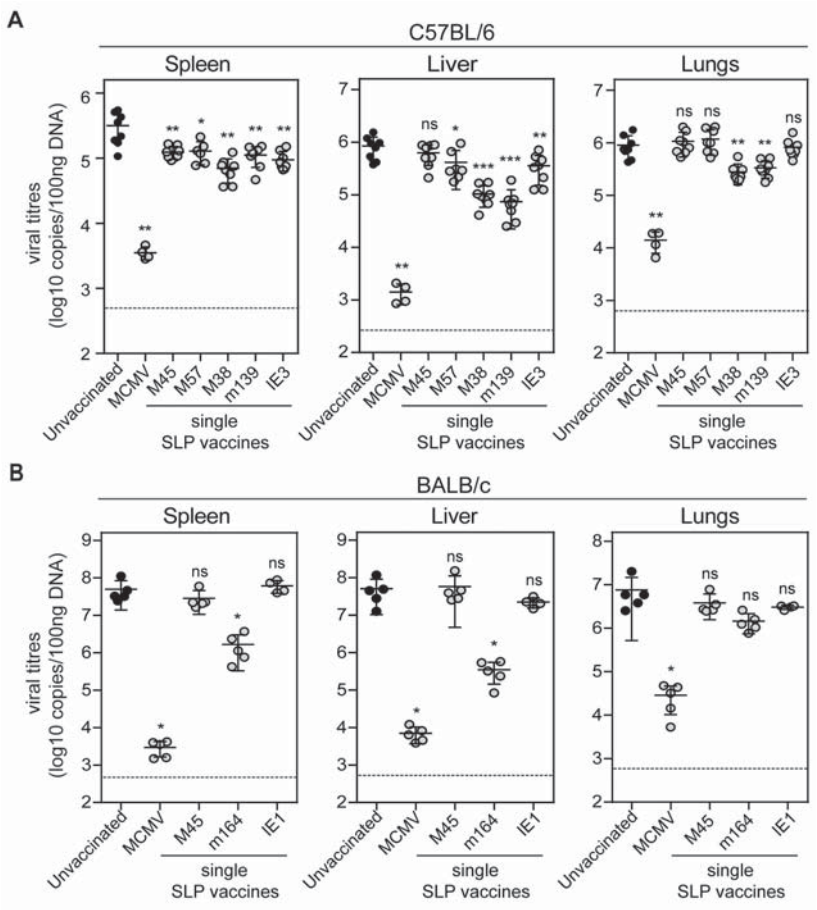

Figure 6. Efficacy of single SLP vaccines against acute MCMV infection. Unvaccinated (naive), SLP vaccinated and MCMV infected C57BL/6 and BALB/c mice were challenged at day 60 post vaccination/infection with 5×10^4 PFU and 5×10^3 PFU salivary gland-derived MCMV Smith, respectively. At day 5 post challenge, spleen, liver, and lungs were isolated and the viral genome copies were determined by qPCR. The viral titres of individual **(A)** C57BL/6 and **(B)** BALB/c mice are depicted (n=5-8 per group). Mean ± SEM is indicated. Dashed line represents the detection limit as measured in naive mice. Experiments were performed twice with similar outcome. *, P< 0.05; **, P<0.01; ***, P<0.001; ns, not significant.

mice, the viral load of unvaccinated (naive) mice challenged with MCMV was found to be significantly higher in spleen, liver and lungs, when compared to the viral load of MCMV re-challenged mice that successfully controlled a previous MCMV infection, indicating that pre-existing immunity to MCMV can clearly reduce the viral load upon re-infection (Figure 6A). All the different SLP vaccines resulted in a reduction in viral load in the spleen compared to unvaccinated mice, albeit less effective when compared to MCMV infected mice. Mice vaccinated with the SLPs containing the $M38_{316\text{-}323}$ and $m139_{419\text{-}426}$ epitopes display a significant reduction in viral titres in the liver and lungs.

Also, the $M57_{816-824}$ and $IE3_{416-423}$ epitope containing SLPs were capable in reducing the viral replication in the liver after MCMV challenge, albeit to a lesser extent (Figure 6A).

Re-challenge of MCMV infected BALB/c mice resulted in substantial protection of the $m164_{257-265}$ epitope containing SLP vaccine in spleen and liver (Figure 6B). The $M45_{507-515}$ and $IE1_{168-176}$ epitope containing SLPs however did not induce protective immunity in vaccinated mice. These results indicate that certain SLPs but not all have the potency to elicit protective immunity against virus challenge, and that this protection is not necessarily correlating to the size of the SLP-induced $CD8^+$ T cell response.

Since vaccination with the $m139_{419-426}$ and $M38_{316-323}$ epitope containing SLPs was accompanied with some reduction of the viral load, we examined in C57BL/6 mice whether vaccination with these two, or even more, SLPs combined were able to exceed the protection efficacy of single SLP immunization. Strong and long-lived peptide-specific $CD8^+$ T cell responses were measured in mice vaccinated with the mixture of the m139 SLP plus the M38 SLP and with a mixture of all 5 SLP vaccines (Figure 7A). Notably, the T cell response against each peptide epitope with the combined SLP vaccines was lower as compared to single SLP vaccination (except for the m139-specific response), indicating that competition among antigen-specific $CD8^+$ T cell populations can occur in multivalent vaccines. Especially, altered were the responses to the epitopes in M57 and IE3 because these were not boosted (Figure 7A). Such competition among T cells during boosting has also been observed after viral infection [24]. The kinetics of the combined SLP vaccine-induced T cell responses were found similar to single SLP vaccines, and the phenotype (Figure 7B) and the cytokine polyfunctionality of the T cells as well (S5 Figure).

At day 60 post booster vaccination, mice were challenged with MCMV and 5 days later viral titres were measured in different organ tissues. The efficacy of the combined SLPs to protect upon acute MCMV challenge was remarkably improved compared to the single SLP vaccines, as all mice that received a mixed SLP vaccine exhibited significant reduction in the viral load, especially in the liver (Figure 7C), suggesting that the breadth of the response or the magnitude of the total anti-viral response is important. Remarkably, the combination of the m139 SLP with the M38 SLP was as efficacious as the combination with all 5 SLPs. To assess if superior viral control was related to the breadth of the response, we adoptively transferred 1×10^4 m139 SLP-induced $CD8^+$ T cells, 1×10^4 M38 SLP-induced $CD8^+$ T cells, or an equal total number of a pool of both m139 (0.5×10^4) and M38 (0.5×10^4) SLP-induced $CD8^+$ T cells in naive recipient mice (Figure 7D). The transfer of SLP-induced $CD8^+$ T cell populations with a dual specificity resulted in a significant reduction in viral titres, while the transfers of equal amounts of T cells with single specificity did not. Thus, combinations of at least two distinct SLP vaccines have an increased potency to protect compared to single SLP vaccines, indicating that the breadth of the vaccine-induced $CD8^+$ T cell responses plays a crucial role in anti-viral immunity. We conclude that vaccination with single SLPs can

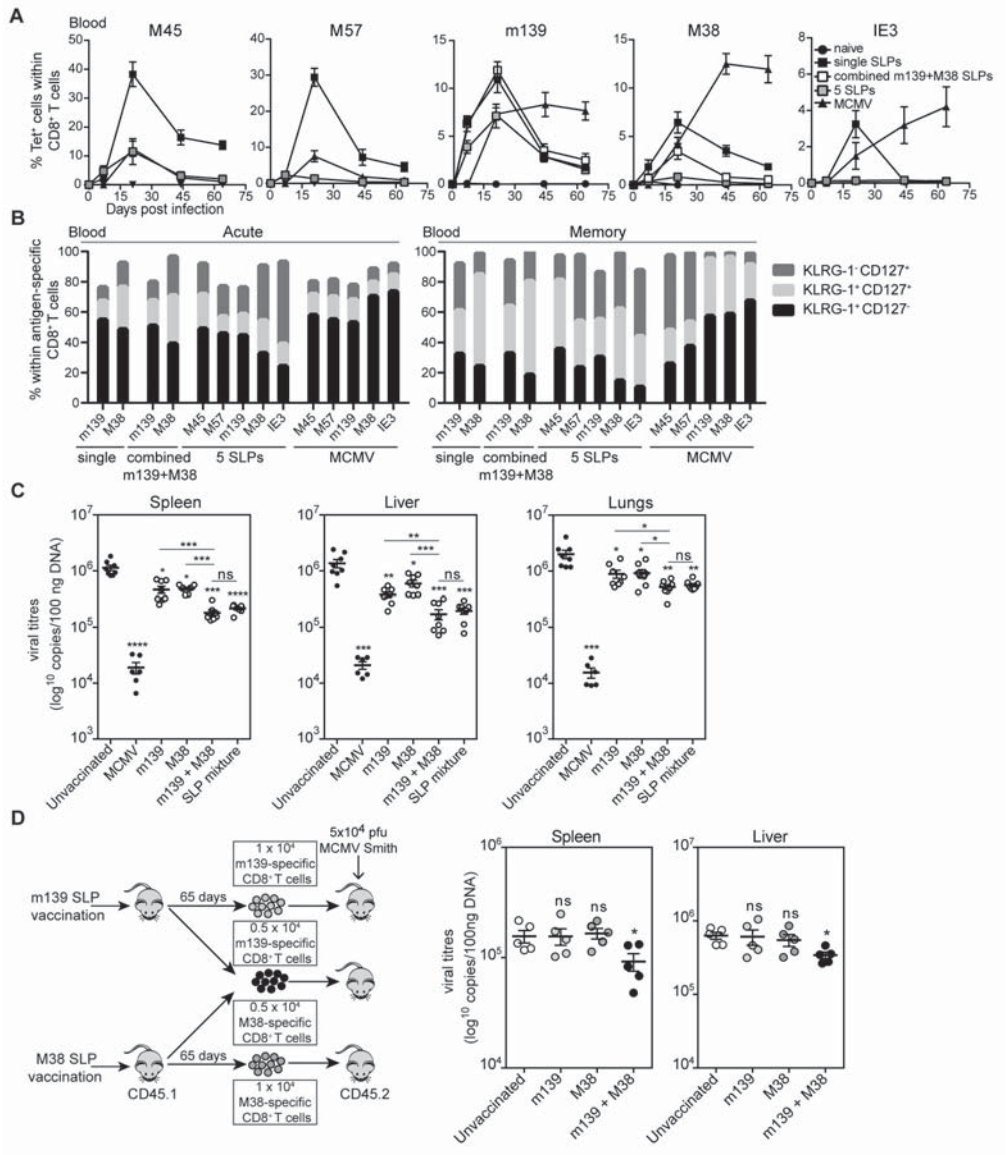

Figure 7. Enhanced efficacy against CMV infection by combinatorial use of distinct SLP vaccines. C57BL/6 mice were vaccinated with single m139 and M38 SLPs, a combination of these SLPs, and a mixture of five different (M45, M57, m139, M38, IE3) SLPs a mixture of five different SLPs. **(A)** Kinetics of the antigen-specific $CD8^+$ T cells in the blood at day 7 post booster vaccination. Data shown are mean values ± SEM (n=8) **(B)** Phenotypic profile of the (combinatorial) SLP vaccine-induced $CD8^+$ T cells in blood at day 7 (acute) and day 60 (memory) post booster vaccination. Data represent mean values (n=8). Experiment was performed twice with similar outcome. **(C)** Unvaccinated (naive), (combined) SLP vaccinated and MCMV infected C57BL/6 mice were challenged 65 days post booster-vaccination/infection with 5×10^4 PFU salivary gland-derived MCMV Smith. At day 5 post challenge, liver, lungs and spleen were isolated and the viral genome copies were determined by qPCR. The viral titres of individual mice are depicted (n=6-8 per group).

(D) Splenic antigen-specific $CD8^+$ T cells were sorted at day 60 post booster-vaccination of single $m139_{419-426}$ or $M38_{316-323}$ SLP vaccinated CD45.1 mice. 1×10^4 $m139_{419-426}$, 1×10^4 $M38_{316-323}$ or 0.5×10^4 $m139_{419-426}$ plus 0.5×10^4 $M38_{316-323}$ $CD8^+$ T cells were adoptively transferred into naive CD45.2 recipient mice. Subsequently, recipient mice were infected i.p. with 5×10^4 PFU MCMV-Smith. At day 5 post infection spleen and liver were isolated and the viral genome copies were determined by qPCR. The viral titres of individual mice are depicted (n=6 per group). Mean ± SEM is also shown. The detection limit was below 1000 genome copies as measured in naive mice. Experiments were performed twice with similar outcome. Statistical difference is indicated (*, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001; ns, not significant) as compared to the unvaccinated group unless otherwise indicated.

be applied as a prophylactic vaccine strategy against CMV infection, but vaccination with combinations of different SLPs serve as a superior vaccine technology platform against viral challenge.

DISCUSSION

In this study, we report that SLP-based vaccines are an effective modality against CMV infection. In a prime-boost vaccine regimen, SLPs containing single MCMV epitopes are highly immunogenic in both C57BL/6 and BALB/c mice, and generate long-lasting polyfunctional $CD8^+$ T cell responses. Our study revealed three key findings. First, the magnitude and phenotype of the SLP-induced T cell responses initially resemble those evoked by a real viral infection. Second, the magnitude of the SLP-induced T cell response strongly correlated to the naive T cell precursor frequency, and third the protection against viral infection by SLP-induced memory $CD8^+$ T cells was most pronounced when vaccination was performed with combinations of distinct SLPs leading to an increased breadth of the antigen-specific T cell response.

In the last decades many vaccine strategies such as attenuated virus, DNA constructs, protein, and virally vectored vaccines targeting HCMV have been developed [3, 4]. The focus of most of these vaccines was to generate protective antibodies. Our finding that SLP-based vaccines that solely provoke $CD8^+$ T cell responses are efficacious suggests that the design of more efficient vaccines against CMV should incorporate the induction of $CD8^+$ T cell immunity. Although we observed some epitope competition among SLP vaccine-induced $CD8^+$ T cell responses, we anticipate that inclusion of $CD4^+$ T cell and B cell epitopes will further improve the vaccine efficacy given that $CD4^+$ T cells and antibodies have also antiviral actions against CMV. Moreover, SLP-based vaccines allow further refinement by different prime-booster regimens and by combinations with adjuvants, immunomodulatory antibodies or other vaccine platforms [25]. Conceivably, this will positively impact the phenotype and effectivity of the vaccine-induced T cells.

As to date, the high $CD8^+$ T cells responses elicited with the SLP vaccines encoding MCMV epitopes have not been observed before with other SLPs including those containing epitopes of human papilloma virus (HPV) [14], lymphocytic choriomeningitis virus (LCMV) [26], influenza [27] or model antigens [28]. This may be explained by the relatively high precursor frequency of T cells responding to some of the MCMV epitopes. Our study indicates that it is of interest for T cell-based vaccines to determine the antigen-specific T cell precursor frequencies as these correlate to the magnitude of the vaccine-induced antigen-specific response, allowing the selection of epitopes generating the most robust responses. This knowledge can be very useful for development of vaccines that are based on selection of epitopes. Nevertheless, the magnitude of the vaccine-induced T cell response appears not necessarily to correlate to protective immunity but seems to depend also on the specificity. For example, in C57BL/6 mice, the large vaccine-elicited responses to the $M45_{985–993}$ and $M57_{816-824}$ epitopes do not provide as good protection as the seemingly lower response to the $M38_{316–323}$ epitope. Similarly, in BALB/c mice the m164 SLP confers immunity in liver and spleen whereas the $IE1_{168-176}$ epitope containing SLP, which is analogous in magnitude, does not show protective effects. Previous studies using short peptide or DNA vaccination also reported that the strength of the vaccine-induced IE1-specific $CD8^+$ T cell response does not necessarily correlate to protection [29-31], suggesting that the quality of the vaccine-induced T cell is more decisive.

Dissimilarities in transcription of viral genes [32], which may even vary in different tissues, as well as the efficiency of peptide processing and presentation at the cell surface, may also be implicated in the differential efficacy of the T cell response to each particular epitope to confer resistance to MCMV. In this respect, it is of interest to note that SLP vaccines containing "inflationary" epitopes (i.e., M38 and m139) elicit better protection as compared to the non-inflationary epitopes. This may relate to differences in the presentation of the inflationary epitopes as compared to the non-inflationary epitopes by infected cells and/or by (cross-presenting) APCs.

An important requirement for memory inflation is chronic antigenic exposure [12]. The fact that SLPs do not elicit inflation suggests that SLPs are broken down in such a manner that epitopes are not presented over a long period of time as occurs during persistent CMV infection. Other factors important for memory inflation during CMV infection, such as dependence on certain T cell costimulatory interactions (e.g., CD27-CD70 [33]), are likely also not in place at late time points post SLP vaccination. In addition, a characteristic feature of inflationary T cells is their predominant effector-memory like phenotype. The SLP vaccine-induced T cells are not mostly effector-memory like, as may be expected because of the apparent absence of memory inflation. Although the expansion of the SLP-induced $CD8^+$ T cells seems to be somewhat negatively influenced as compared to virus-induced T cells, it remains to be determined whether protection on a per-cell basis is influenced as well. Nevertheless, the SLP-induced T cells were well capable to reduce the viral load upon viral challenge,

especially when a mixture of distinct SLPs was used for vaccination. The somewhat lesser expansion potential of the SLP-induced T cells might relate to some of the differences in the phenotype of the SLP and MCMV-elicited T cells. Although the effector T cells induced by either SLP boost vaccination or MCMV infection had an analogous phenotype (KLRG1^{hi}, CD44^{hi}, CD127^{low}, CD62L^{low}, IL2$^{+/-}$) and cytokine profile, the memory T cells elicited by SLPs displayed a mixed profile of effector-memory (KLRG1^{hi}, CD127^{lo}), central-memory (KLRG1^{lo}, CD127^{hi}) and double-positive T cells (KLRG1^{hi}, CD127^{hi}). In contrast, MCMV infection induces a more polarized phenotype: either a central-memory phenotype (mainly non-inflationary responses) or an effector-memory phenotype (mainly inflationary responses). Whether a lack of CD4^{+} T cell helper signals [34] or a lack of virus-associated inflammatory signals [26] is responsible for the observed SLP vaccine-associated phenotype and secondary expansion potential remains to be examined in future studies.

We showed that the efficacy of SLP vaccines to protect against MCMV is primarily driven by the breadth of the CD8^{+} T cell responses rather than the magnitude of the individual SLP vaccine-induced T cell responses. A possible explanation is that viral infected cells are to a certain degree resistant to CD8^{+} T cell mediated killing due to sophisticated immune evasion mechanisms including downmodulation of MHC class I molecules and prevention of apoptosis [35-37]. Accordingly, it has been estimated that one effector CD8^{+} T cell kills only 2-16 MCMV-infected cells per day and the probability of death of infected cells increases for those contacted by more than two CTLs, which is indicative of CTL cooperation [38]. Our study suggests that multiple encounters with cytotoxic CD8^{+} T cells with different specificity result in more effective killing of infected cells.

Overall, this study provided evidence that SLP-based vaccines eliciting memory CD8^{+} T cell responses have protective effects against acute MCMV infection with respect to lowering the viral load in tissues. These promising results highlight the need for additional studies to elucidate the role of vaccine-induced T cells against CMV and other persistent viral infections.

MATERIALS AND METHODS

Mice

C57BL/6 mice and BALB/c mice were purchased from Charles River Laboratories (L'Arbresle, France). CD45.1 (Ly5.1) congenic mice on a C57BL/6 background were obtained from The Jackson Laboratory. Mice were maintained under specific-pathogen-free conditions at the Central Animal Facility of Leiden University Medical Center (LUMC), and were aged 8-10 weeks at the beginning of each experiment. The mice did not undergo any immunosuppressive treatments and were fully immunocompetent.

Ethics Statement

All animal experimental protocols were approved by the LUMC Animal Experiments Ethical Committee in accordance with the Dutch Experiments on Animals Act and the Council of Europe (numbers 13156 and 14187).

Virus production, infections and determination of viral load

MCMV virus stocks were prepared from salivary glands of BALB/c mice infected with MCMV-Smith (American Type Culture Collection (ATCC)). The viral titres of the produced virus stocks were determined by viral plaque assays with 3T3 mouse embryonic fibroblasts (MEFs) (ATCC). Age- and gender-matched C57BL/6 mice were infected with 5×10^4 PFU MCMV, and age- and gender-matched BALB/c mice with 5×10^3 PFU MCMV. Viruses were administered intraperitoneally (i.p) in a total volume of 400 ml in PBS. 65 days post-booster vaccination or infection, SLP vaccinated or MCMV infected mice were (re)-challenged with 5×10^4 PFU MCMV. Determination of viral load was performed by real-time PCR as described previously [39].

Peptides synthesis and vaccination

Short (9-10 aa) and long (20-21 aa) peptides containing MHC class I-restricted T cell epitopes from MCMV encoded proteins in C57BL/6 and BALB/c mice were produced at the peptide facility of the LUMC (peptide sequences are described in S1 Table). The purity of the synthesized peptides (75-90%) was determined by HPLC and the molecular weight by mass spectrometry. Synthetic long peptide (SLP) vaccinations were administered subcutaneously (s.c.) at the tail base by delivery of 50 mg SLP and 20 mg CpG (ODN 1826, InvivoGen) dissolved in PBS in a total volume of 50 ml. Booster SLP vaccinations were provided after 2 weeks. Vaccination with a mixture of SLPs was done with 50 mg of each SLP and 20 mg CpG.

Flow cytometry

Cell surface and intracellular cytokine stainings of splenocytes and blood lymphocytes were performed as described [40]. For examination of intracellular cytokine production,

single cell suspensions were stimulated with short peptides for 5 h in the presence of brefeldin A or with long peptides for 8 h of which the last 6 h in presence of brefeldin A (Golgiplug; BD Pharmingen). MHC class I tetramers specific for the following MCMV epitopes: $M45_{985–993}$, $M57_{816–824}$, $m139_{419-426}$, $M38_{316–323}$, and $IE3_{416–423}$ in C57BL/6 mice and $M45_{507–515}$, $m164_{257-265}$ and $IE1_{168-176}$ in BALB/c mice were produced as reported [41]. Fluorochrome-conjugated mAbs were purchased from BD Biosciences, Biolegend or eBioscience. Flow cytometry gating strategies are shown in S6 Fig. Samples were acquired with the LSRFortessa cytometer (BD Biosciences) and analysed with FlowJo-V10 software (Tree star).

T cell functional avidity assay

A peptide dose-response titration was performed to determine and compare the TCR avidity of the $CD8^+$ T cells induced after SLP vaccination and MCMV infection at the acute and memory phase. In brief, spleenocytes were stimulated with various concentrations of short peptide in presence of 2 μg/ml brefeldin A for 5 h at 37°C. Subsequently, cell surface staining and an intracellular IFN-γ staining were performed. Responses were analysed using the same approach as described above.

Antibody detection by ELISA

Blood was collected from the retro-orbital plexus and after brief centrifugation, sera were obtained and stored at −20°C. Specific immunoglobulin levels in serum were measured by ELISA as described [39]. Briefly, Nunc-Immuno Maxisorp plates (Fisher Scientific) were coated either with 2 μg/ml SLPs or with MCMV-Smith in bicarbonate buffer, and after blocking with skim milk powder (*Fluka BioChemika*) diluted sera were added. Next, plates were incubated with HRP-conjugated antibodies (SouthernBiotech) to detect different Ab isotypes. Plates were developed with TMB substrate (Sigma Aldrich) and the colour reaction was stopped by addition of 1M H_2SO_4. To serve as a positive control, a peptide from the M2 protein (eM2) of influenza A virus with identified ability to induce antibodies and corresponding serum was used. Optical density was read at 450 nm (OD_{450}) using a Microplate reader (Model 680, Bio-Rad).

Determination of the T cell precursor frequency

To determine the endogenous naive precursor frequency of MCMV-specific $CD8^+$ T cell populations in C57BL/6 and BALB/c mice, enrichment assays of antigen-specific $CD8^+$ T cells were performed as described [42]. In short, single cell suspensions were generated from pooled spleen and lymph nodes (mesenteric, inguinal, cervical, axillary, and brachial) of individual mice. Cells were stained with PE and APC-labelled MHC class I tetramers for 0.5 h at RT, then washed, labelled with anti-PE and anti-APC microbeads (Miltenyi Biotec), and passed over a magnetized LS column (Miltenyi Biotec). The tetramer-enriched fractions were stained with fluorochrome labelled Abs

against CD3 (clone 500A2), CD4 (clone L3T4), CD8 (clone 53-6.7) for 30 min at 4°C, and subsequently analysed. Samples were acquired with the LSRFortessa cytometer (BD Biosciences).

Adoptive transfers

The expansion capacity and vaccine efficacy of SLP vaccine and/or MCMV-induced antigen-specific $CD8^+$ T cells was determined by adoptive transfers. Splenic $CD8^+$ T cells from chronically (day 60) infected and SLP vaccinated $CD45.1^+$ mice were enriched with magnetic sorting using the $CD8^+$ T cell isolation kit in accordance with the manufacturer's protocol (Miltenyi Biotec). Next, cells were stained with MHC class I tetramers and with fluorochrome labelled antibodies against CD3 and CD8. Tetramer positive $CD8^+$ T cells were sorted using a FACSAria II Cell Sorter (BD Biosciences) and 1×10^4 $tetramer^+$ $CD8^+$ T cells were transferred (retro-orbital in a total volume of 200µl in PBS) into naive $CD45.2^+$ C57BL/6 recipients. Recipients were subsequently (2 h later) infected with 5×10^4 PFU MCMV. At day 5 post viral challenge the viral titres were determined by qPCR and the number of donor-specific $CD8^+$ T cells by flow cytometry.

Statistical analyses

Statistical significance was assessed with Student's t-test or ANOVA using GraphPad Prism software (GraphPad Software Inc., USA). The level of statistical significance was set at $P<0.05$.

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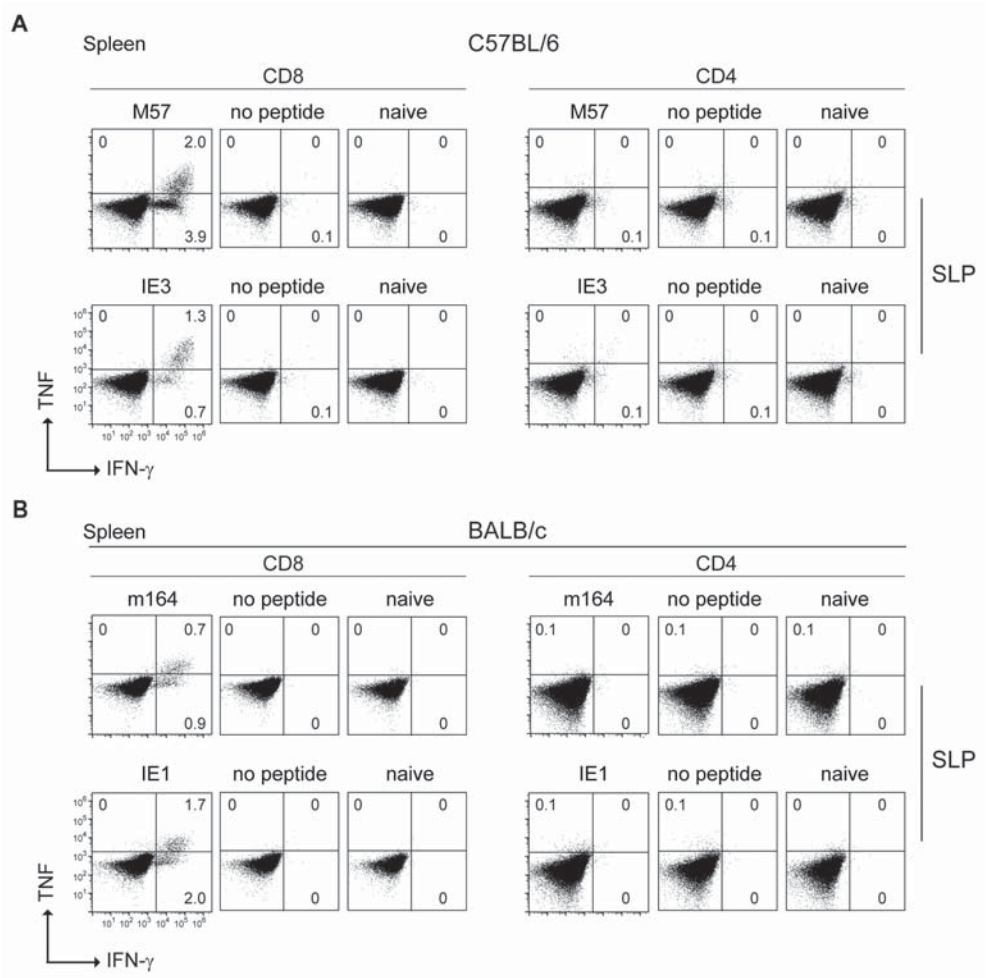
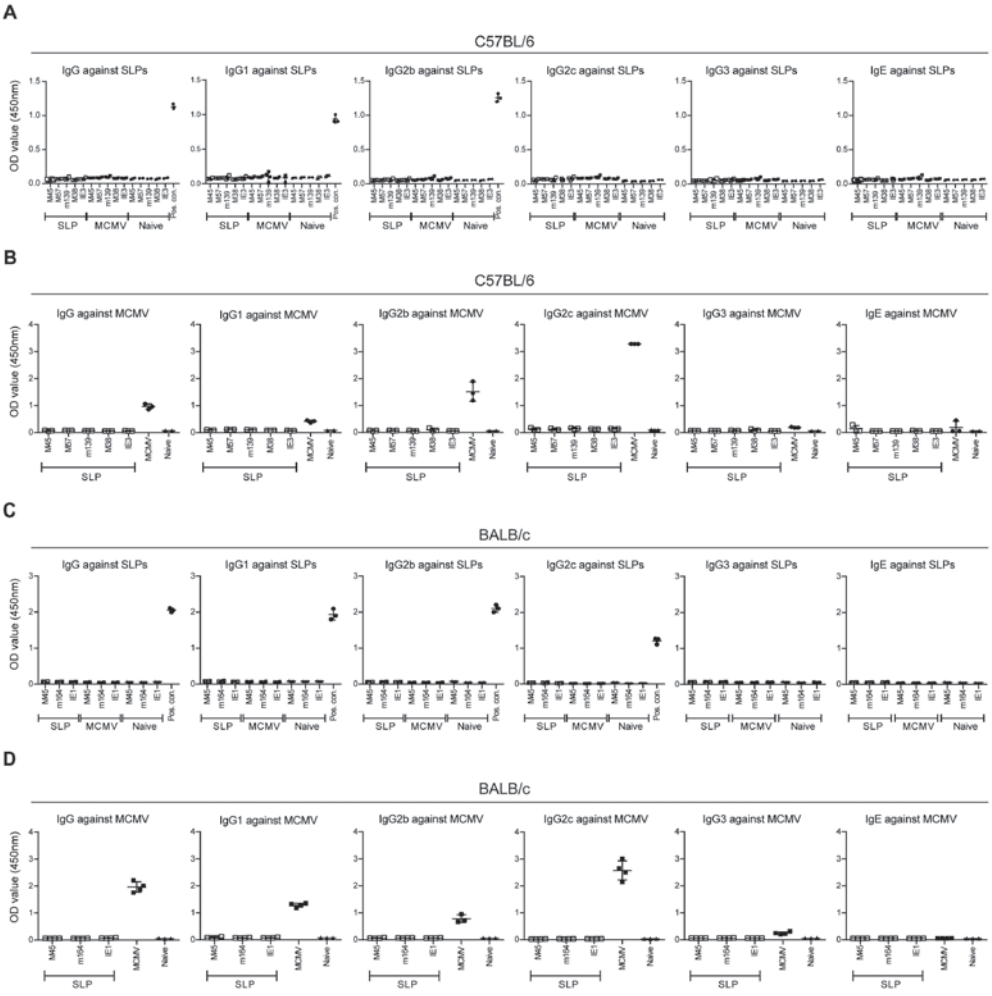
Supplementary Table 1. MCMV peptide-specific CD8 T cell epitopes.

Mouse strain	Protein (ORF)	SLP sequence *	Epitope residues (aa)	MHC allele - restriction	Stage of expression	Inflation ?	Human CMV Homologue
C57BL/6	M45	REDVVK**HGIRNASFI**TGCSA	985-993	H-2-D^{b}	Early gene	No	UL45
C57BL/6	M57	FPACGL**SCLEFWQRV**LQNS	816-824	H-2-K^{b}	Early gene	No	UL57
C57BL/6	m139	VVLVGARG**TVYGFCLL**SND	419-426	H-2-K^{b}	Early gene	Yes	US22
C57BL/6	M38	VTLI**SSPPMFRV**PVNPVPGG	316-323	H-2K^{b}	Early gene	Yes	UL38
C57BL/6	Altered M38	EGPPMPMTVTLI**SSPPMFRV**	316-323	H-2K^{b}	Early gene	Yes	UL38
C57BL/6	IE3	DKSRKYPA**RALEYKNL**PFR	416-423	H-2-K^{b}	Immediate early gene	Yes	IE2
C57BL/6	Altered IE3	KKCREDKSRKYPA**RALEYKNL**	416-423	H-2-K^{b}	Immediate early gene	Yes	IE2
BALB/c	M45	RITER**VGPALGRGL**YSTVV	507-515	H-2-D^{d}	Early gene	No	UL45
BALB/c	m164	RTWGAD**AGPPRYSRI**FWAV	257-265	H-2-D^{d}	Early gene	Yes	-
BALB/c	IE1/ pp89	GRLMYDM**YPHFMPTNL**GPSEK	168-176	H-2-L^{d}	Immediate early gene	Yes	IE1

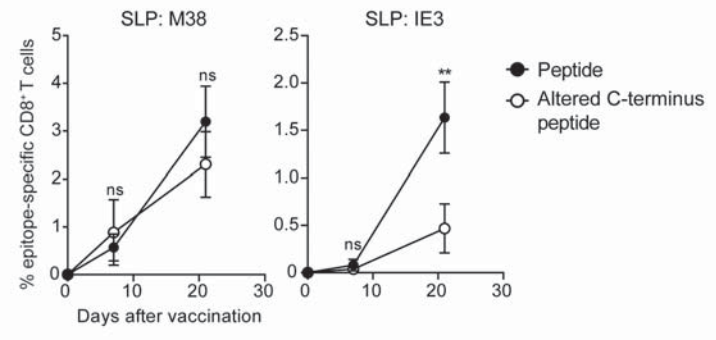
*Bold underlined amino acid (aa) residues within the SLP sequence indicate the CTL epitope.



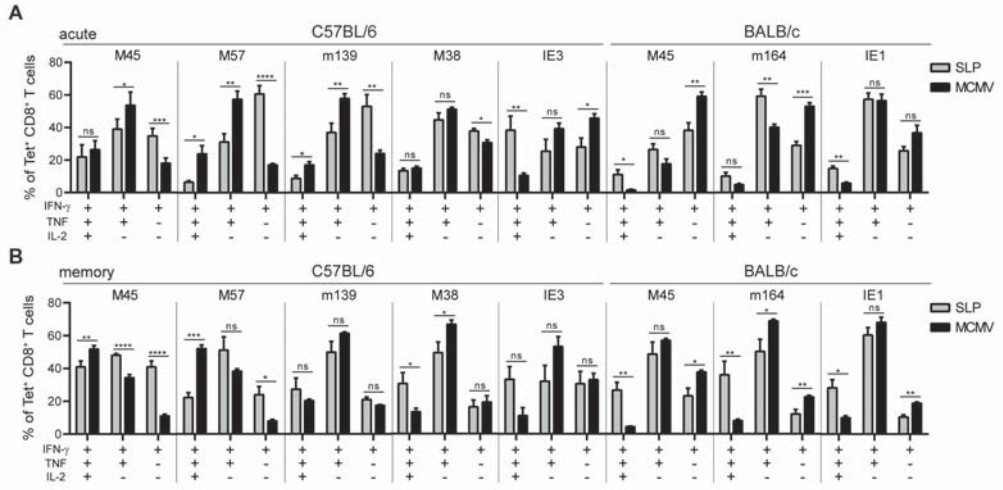
Supplementary Figure 1. MHC class I SLP vaccines do not elicit activation of CD4⁺ T cells. **(A)** C57BL/6 and (B) BALB/c mice were vaccinated SLPs, and at day 8 following vaccination the intracellular cytokine production by CD8⁺ and CD4⁺ T cells was determined after restimulation with long peptide. Representative plots show IFN-γ versus TNF production of CD8⁺ and CD4⁺ T cells of **(A)** $M57_{816\text{-}824}$ and $IE3_{416\text{-}423}$ epitope containing SLP vaccinated C57BL/6 mice, and of **(B)** $m164_{257\text{-}265}$ and $IE1_{168\text{-}176}$ epitope containing SLP-vaccinated BALB/c mice. No peptide controls and naive mice were used as negative controls. IFN-γ and TNF reactivity was only observed in CD8⁺ T cells. Similar data were observed for the other SLPs (i.e., M45, m139 and M38 SLPs in C57BL/6 mice and M45 SLP in BALB/c mice). Experiments were performed twice with similar outcome (n=3-4 mice per group).



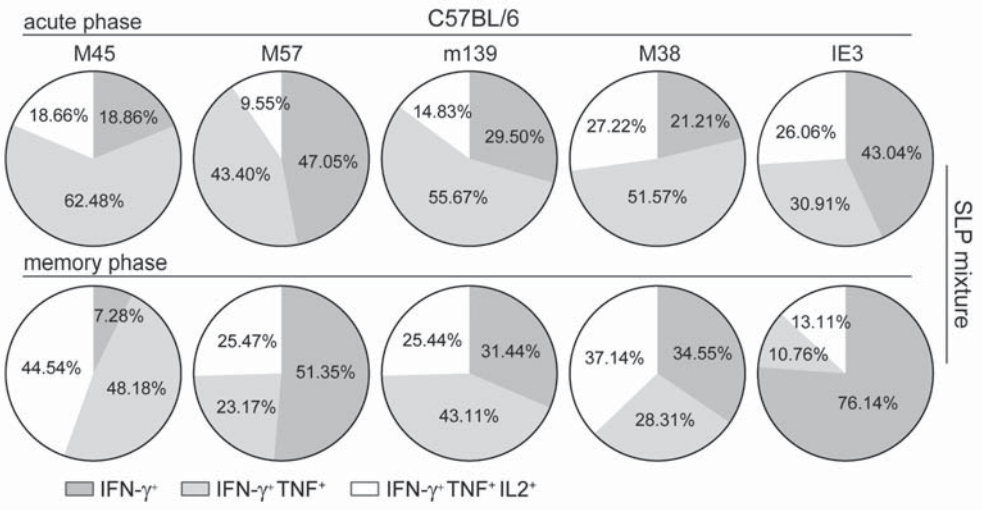
Supplementary Figure 2. Vaccination with MHC class I restricted SLPs does not lead to the induction of peptide-specific Abs. At day 60 post booster SLP vaccination or MCMV infection serum samples were obtained from C57BL/6 and BALB/c mice. ELISA plates were coated with either SLP (**A, C**) or MCMV (**B, D**) and detection of peptide specific Ab (including IgG, various IgG subtypes, and IgE) in the serum was assessed. To serve as a positive control, a peptide with known ability to induce antibodies and corresponding serum was used. Optical density values were measured at 450nm. Each symbol represents an individual mouse. Mean value ± SEM (n=3-4 mice per group) is indicated. Experiments were performed twice with similar outcome.



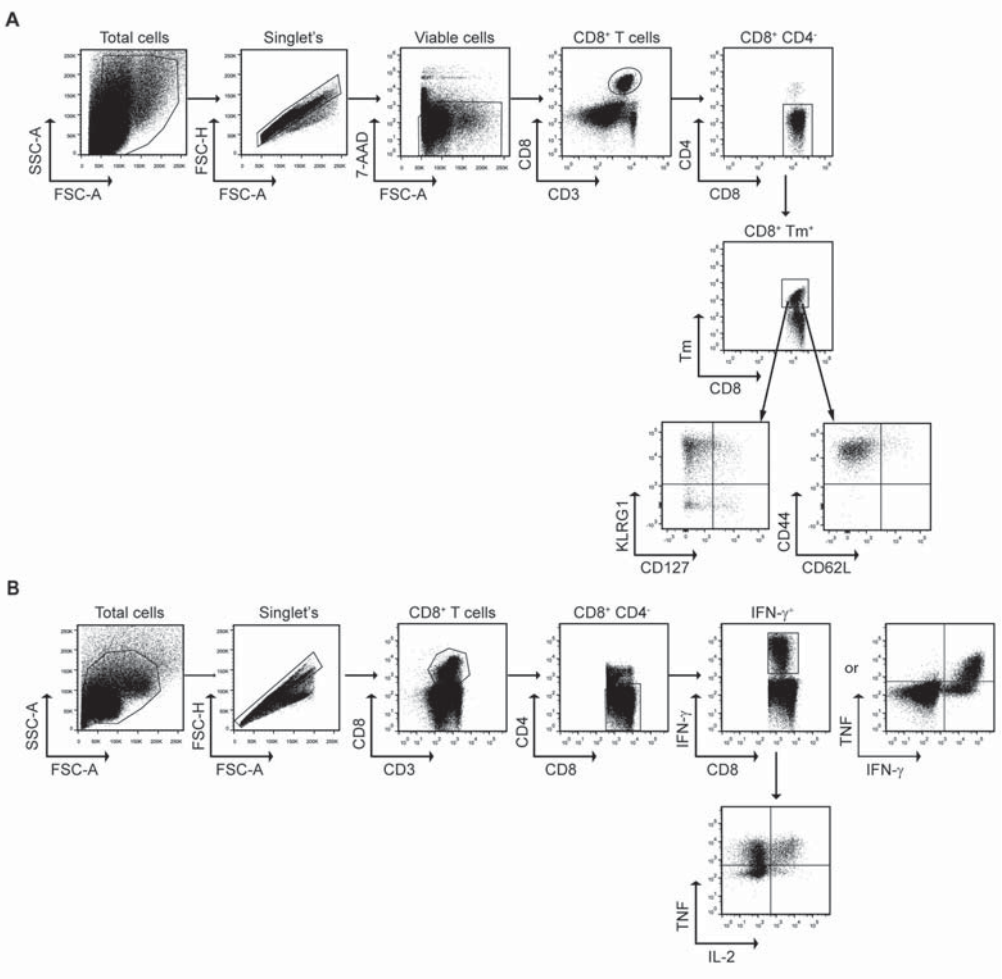
Supplementary Figure 3. Epitope alteration at the C-terminus did not improve the immunogenicity. Alterations of the C-terminus sequence residues of the $M38_{316-323}$ and $IE3_{416-423}$ epitope containing SLPs was performed and their capacity to induce epitope-specific $CD8^+$ T cell responses was tested and compared to the unaltered SLPs. MHC class I tetramer staining on blood was performed to assess the immunogenicity of the $M38_{316-323}$ and $IE3_{416-423}$ peptides on C57BL/6 mice (n=4). Data represent mean values ± SEM (n=4 mice/group). *, P<0.05; **, P<0.01; ns, not significant.



Supplementary Figure 4. SLP vaccination elicits polyfunctional $CD8^+$ T cells. Statistical analysis of the results depicted in the pie charts of Fig. 4. The cytokine polyfunctionality of the splenic $CD8^+$ T cells elicited after SLP vaccination or MCMV infection was determined after 5 hours *in vitro* restimulation with short peptides. Graphs show the percentages of the single (IFN-γ), double (IFN-γ/TNF) and triple (IFN-γ/TNF/IL-2) cytokine producers of each antigen-specific T cell population upon peptide stimulation. Data represents mean values + SEM, and are representative of three independent experiments (n = 4-5 per group). *, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001; ns, not significant.



Supplementary Figure 5. Analysis of the magnitude and functionality of the multivalent SLP vaccine-induced CD8$^+$ T cells. C57BL/6 mice were prime-boost vaccinated with a mixture of 5 SLPs. The cytokine production capacity of the splenic SLP vaccine-induced CD8$^+$ T cells was examined by intracellular cytokine staining at day 8 (acute phase) and 60 (memory phase) after booster vaccination. Pie charts depict the percentages of the single (IFN-γ), double (IFN-γ/TNF) and triple (IFN-γ/TNF/IL-2) cytokine producers of each antigen-specific T cell population after 5 hours *in vitro* restimulation with short peptide. Data represents mean values, and are representative of three independent experiments (n = 6 mice per group).



Supplementary Figure 6. Flow cytometry gating strategy. (A) Representative plots show the gating strategy to detect MHC class I tetramer positive cells in blood and spleen. In sequential gating, cells were first gated on lymphocytes (forward scatter vs. side scatter), then singlets (FSC-A vs. FSC-H), followed by viability (FSC-A vs. 7-AAD). Next, live cells were analysed for the expression of CD3 and CD8 while cells positive for CD4 were excluded. MHC class I tetramer positive cells (CD8 vs. Tm) were analysed for the expression of KLRG1, CD44, CD127 and CD62L surface markers. **(B)** Gating strategy for intracellular cytokine staining. Utilizing a sequential gating analysis, cells were initially gated on lymphocytes (FCS-A vs. SCS-A) and singlets (FCS-A vs. FSC-H). Next, cells were analysed for the expression of CD3 and CD8 while cells positive for CD4 were excluded. IFN-γ producing $CD8^+$ T cells were further analysed for expression of TNF and IL-2.

Chapter 3

Enforced OX40 stimulation empowers booster vaccines to induce effective CD4$^+$ and CD8$^+$ T cell responses against mouse cytomegalovirus infection

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ABSTRACT

There is an imperative need for effective preventive vaccines against human cytomegalovirus (HCMV) as it poses a significant threat to the immunologically immature, causing congenital disease, and to the immune compromised including transplant recipients. In this study we examined the efficacy of synthetic long peptides (SLPs) as a $CD4^+$ and $CD8^+$ T cell-eliciting preventive vaccine approach against mouse CMV (MCMV) infection. In addition, the use of agonistic OX40 antibodies to enhance vaccine efficacy was explored. Immunocompetent C57BL/6 mice were vaccinated in a prime-boost vaccination regiment with SLPs comprising various MHC class I and II-restricted peptide epitopes of MCMV-encoded antigens. Enforced OX40 stimulation resulted in superior MCMV-specific $CD4^+$ as $CD8^+$ T cell responses when applied during booster SLP vaccination. Vaccination with a mixture of SLPs containing MHC class II epitopes and OX40 agonistic antibodies resulted in a moderate reduction of the viral titers after challenge with lytic MCMV infection. Markedly, the combination of SLP vaccines containing both MHC class I and II epitopes plus OX40 activation during booster vaccination resulted in polyfunctional (i.e., IFN-γ^+, TNF^+, IL-2^+) $CD4^+$ and $CD8^+$ T cell responses that were even higher in magnitude when compared to those induced by the virus, and this resulted in the best containment of virus dissemination. Our results show that the induction of strong T cell responses can be a fundamental component in the design of vaccines against persistent viral infections.

INTRODUCTION

It is estimated that 60-80% of people worldwide are infected by the prototypic β-herpesvirus human cytomegalovirus (HCMV). CMV establishes low level viral persistence within immunocompetent hosts without clinical symptoms. However, it can cause life-threatening disease in the immunological immature (unborn babies and newborns) and immunocompromised individuals (e.g. bone marrow and organ transplant recipients) [1,2]. Although new antiviral drugs against CMV are in clinical development, the most commonly used agents display toxicity. Importantly, no licensed prophylactic or therapeutic vaccines exist for CMV at present. Consequently, there is an imperative need to identify potent vaccine modalities to prevent HCMV infection [3-5].

$CD4^+$ and $CD8^+$ T cell responses play a critical role in controlling CMV infection in both mouse and human. While $CD4^+$ T cells seem to be more crucial in the early phase after infection, $CD8^+$ T cells are imperative during latency and harbor superior protective properties upon re-challenge [6-9]. Moreover, adoptive transfer approaches established the pivotal role of CMV-specific $CD4^+$ and $CD8^+$ T cells in orchestrating virus replication control [10-12]. During CMV infection, $CD4^+$ and $CD8^+$ T cell responses either follow the traditional course comprised by massive expansion followed by rapid contraction and maintenance at low levels or instead do not undergo contraction but remain at high frequency or even expand gradually. The latter has been described as memory T cell inflation, and has been observed for a restricted set of immunodominant CMV antigens [13-16]. Memory inflation is thought to occur due to low-level persistent antigenic priming and requires certain costimulatory receptor-ligand pairs of which CD27-CD70 and OX40-OX40L interactions are important [17,18]. Phenotypically inflationary T cells exhibit effector-like properties without signs of exhaustion [14,16,19]. The comparable nature of the T cell response to MCMV and HCMV is also found for B cell and NK cell responses, and is likely related to the similarities of both viruses in tropism, pathology and the establishment of latent infection that reactivates upon immunosuppression [20,21].

Recently, we demonstrated that synthetic long peptide (SLP) vaccines, designed to exclusively induce MHC class I-restricted $CD8^+$ T cells, were able to elicit robust and polyfunctional T cell responses that led to reduced MCMV replication in C57BL/6 and BALB/c mouse strains after challenge [22]. However, live MCMV vaccines were more efficient which prompted us to further improve the SLP vaccine efficacy. Since $CD4^+$ helper T cells promote long-term maintenance of memory $CD8^+$ T cells, also during MCMV infection [23,24], and display direct anti-viral capabilities [12,25], the induction of $CD4^+$ T cells may improve the efficacy of the SLP vaccine. Here we analyzed the potency of SLP vaccines inducing MCMV-specific $CD4^+$ T cells, either alone or in conjunction with SLPs eliciting MCMV-specific $CD8^+$ T cells. Enforced OX40 signaling was used to enhance the expansion of both $CD4^+$ and $CD8^+$ T cell subsets. We show that combined

administration of SLPs eliciting $CD4^+$ and $CD8^+$ T cells and OX40 stimulation during booster vaccination leads to a startling increase of both the T cell magnitude and polyfunctionality, ultimately leading to efficient control of lytic MCMV infection.

RESULTS

Prime/boost vaccination with SLPs inducing MCMV-specific $CD4^+$ T cell responses

MHC class II epitopes from MCMV encoded proteins have previously been identified by us in the C57BL/6 mouse strain (MHC haplotype $H\text{-}2^b$) [11], and five immunogenic epitopes (i.e., $m18_{872–886}$, $M25_{409–423,}$ $m139_{560–574,}$ $m142_{24–38}$ and $m09_{133–147}$) were selected for the SLP vaccine platform (Table 1 in Supplementary Material). Initially, the potential of single SLP-based vaccines in eliciting $CD4^+$ T cell responses was assessed in a prime/boost vaccination setting (2 weeks apart) with the TLR9 ligand CpG as adjuvant. At day 8 after the first SLP vaccination, $CD4^+$ T cell responses were not detected by polychromatic intracellular cytokine staining (data not shown) but they became detectable in the spleen at day 8 after the booster SLP vaccination (Figure 1A). However, these responses were relatively low when compared to SLP-induced $CD8^+$ T cell responses [22]. Analysis of the cytokine profile revealed the presence of single IFN-γ, double IFN-γ/TNF and triple IFN-γ/TNF/IL-2 cytokine producing $CD4^+$ T cell populations (Figure 1B).

For clinical applications multiple SLPs need to be combined in order to deal with MHC heterogeneity. Moreover, the breadth of SLP vaccines is important for the efficacy [22]. Hence, mice were vaccinated with a mixture of the 5 SLPs and the $CD4^+$ T cell response for each individual peptide epitope was measured. $CD4^+$ T cell reactivity to all MHC class II epitopes was detected (Figure 1C), albeit that the response to each individual peptide was lower when compared to single SLP vaccination, suggesting that competition among $CD4^+$ T cell peptide epitopes occurs in multivalent vaccines. Moreover, in comparison to the $CD4^+$ T cell response observed after MCMV infection, vaccination with a mixture of MHC class II SLPs resulted in lower numbers of MCMV-specific T cells (Figure 1C). Nevertheless, the cytokine polyfunctionality of the SLP-elicited $CD4^+$ T cells was augmented compared to MCMV-induced $CD4^+$ T cells (Figure 1D). Taken together, these results show that prime/boost vaccination with a mixture of MHC class II epitope-containing SLPs elicits polyfunctional MCMV-specific $CD4^+$ T cell responses, but in magnitude these are lower as compared to those induced by the virus itself.

Enforced OX40 triggering during booster vaccination shows superior induction of SLP-elicited $CD4^+$ T cell responses

Next, we attempted to augment the magnitude of the SLP-induced $CD4^+$ T cell responses. As OX40-mediated signals are important for enhancing $CD4^+$ T cell expansion and survival [26], we decided to use an agonistic OX40 antibody that provides *in vivo* OX40 stimulation. First, we investigated the scheduling of the agonistic OX40 antibody

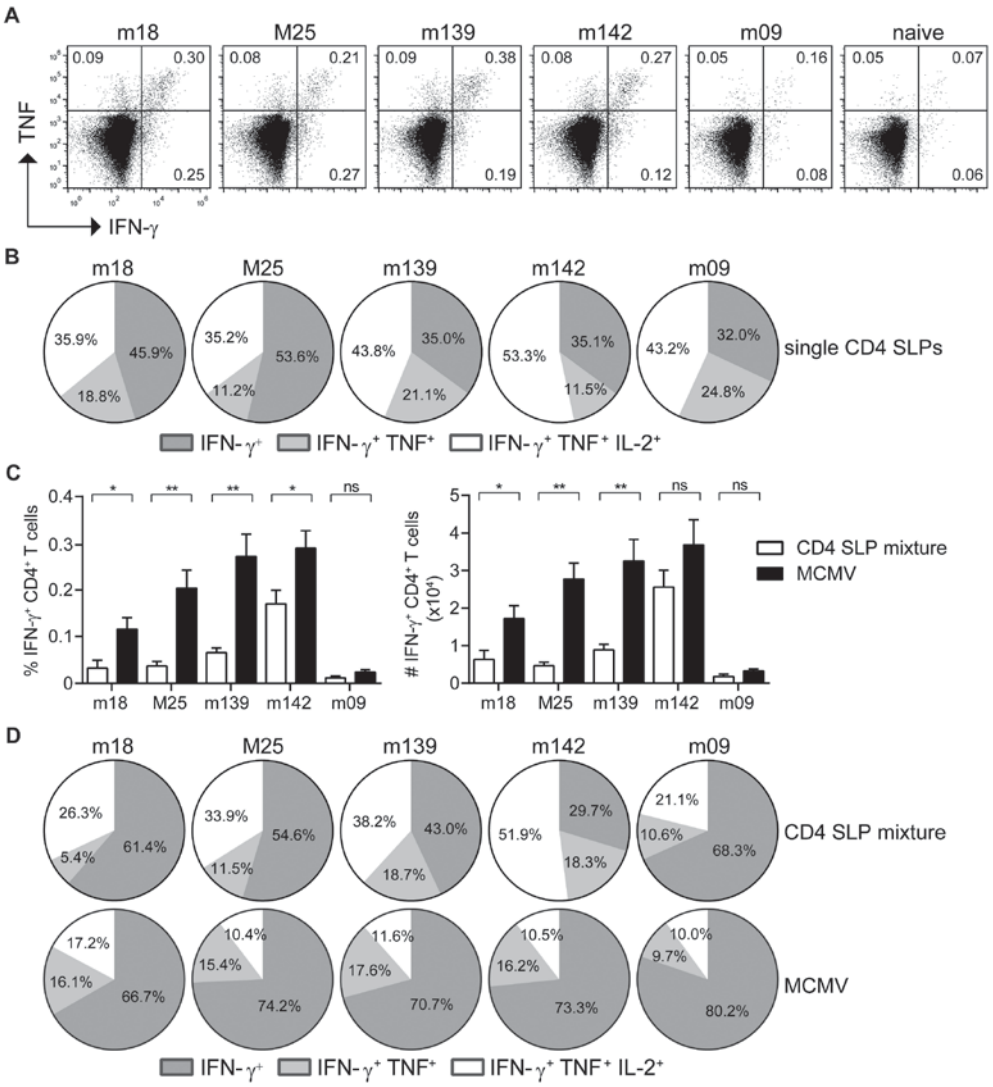

Figure 1. Prime-boost SLP vaccination with combined MHC class II SLPs provokes the induction of strong and polyfunctional CD4⁺ T cell responses. (A) At day 8 post booster vaccination with single SLPs containing MHC class II epitopes, the magnitude of the CD4⁺ T cell responses specific to the indicated epitopes was determined by intracellular cytokine staining after restimulation with peptide. Representative plots show percentages of IFN-γ versus TNF cytokine production of the vaccine-elicited CD4⁺ T cells. **(B)** Pie charts depict the percentages of the single (IFN-γ), double (IFN-γ/TNF) and triple (IFN-γ/TNF/IL-2) cytokine producers of each antigen-specific CD4⁺ T cell population at day 8 post booster vaccination with single MHC class II SLPs. **(C)** Percentages (left) and total numbers (right) of splenic SLP and MCMV-specific IFN-γ⁺ CD4⁺ T cells at day 8 post booster vaccination with a mixture of all 5 MHC class II SLPs are shown. **(D)** Pie charts depict the percentages of the single (IFN-γ), double (IFN-γ/TNF) and triple (IFN-γ/TNF/IL-2) cytokine producers of each antigen-specific CD4⁺ T cell population at day 8 after booster vaccination with a mixture of all 5 MHC class II SLPs. Data represent mean values, and are representative of 3 independent experiments (n=4-5 per group). *, P< 0.05; **, P< 0.01; ns, not significant.

administration (i.e. during priming only, during booster only or during priming and booster) in order to obtain the most optimal $CD4^+$ T cell stimulation (Figure 2A). The magnitude of the T cell response elicited by the SLP containing the $M25_{409–423}$ epitope was measured 8 days post booster vaccination in the spleen. OX40 stimulation clearly increased the magnitude of the $M25_{409–423}$-specific $CD4^+$ T cell responses, and remarkably this was most prominent when the mice received agonistic OX40 antibody during the booster vaccination only (Figures 2B, C). Markedly, a >100-fold increase in IFN-γ^+ $CD4^+$ T cells was observed when compared to SLP vaccination without enforced OX40 stimulation, whereas the response was 17-fold and 5-fold higher than in mice receiving OX40 antibody during priming only or during both priming and booster vaccination, respectively (Figure 2C). In addition, there was a striking gain in cytokine polyfunctionality when agonistic OX40 antibody was provided during booster vaccination only (Figures 2D-F). Compared to SLP vaccination, the increase in absolute numbers of triple IFN-γ/TNF/IL-2 producers was even >200 fold (Figure 2E).

Next, we examined whether the strong increase in MCMV-specific $CD4^+$ T cells by administration of agonistic OX40 antibody during booster vaccination was also evident in case of a mixture of SLPs. Clearly, at day 8 post booster vaccination a strong increase in both percentages and absolute numbers of the peptide-specific IFN-γ^+ $CD4^+$ T cells was observed for all epitopes in the mixture (Figure 3A). In addition, administration of OX40 antibody dramatically improved the cytokine polyfunctional traits (Figure 3B). Most profoundly, OX40 stimulation augmented the IL-2 production capacity of the vaccine-induced $CD4^+$ T cells (Figure 3B, C). Together, these results demonstrate that activation of the OX40 axis during booster SLP vaccination leads to superior $CD4^+$ T cell expansion and induction of cytokine polyfunctionality.

Having established a powerful means to augment SLP vaccines containing MHC class II epitopes, we tested if the used SLPs may comprise unidentified class I epitopes and/or linear B cell epitopes leading to $CD8^+$ T cells and antibody responses, respectively. However, intracellular cytokine staining did not reveal any induction of MCMV-specific $CD8^+$ T cells and SLP-specific antibody ELISAs were negative (Figures S1A, B in Supplementary Material). Furthermore, increased percentages of activated NK cells were also not detected after SLP vaccination (Figure S1C in Supplementary Material), indicating that the intended MHC class II epitope-containing SLPs with enforced OX40 stimulation exclusively activate antigen-specific $CD4^+$ T cell responses.

Provision of OX40 stimulation during booster vaccination also advances SLP-induced $CD8^+$ T cell responses

To improve our previously reported $CD8^+$ T cell eliciting SLP vaccine modality [22], we here envisaged to combine both MHC class I and II epitope containing SLPs. We therefore, also analysed the impact of OX40 engagement on vaccine-induced $CD8^+$ T cells in a similar scheduling experiment using a SLP exclusively containing the $CD8^+$ T cell peptide epitope $M57_{816-824}$ (Figure S2A in Supplementary Material). Consistent

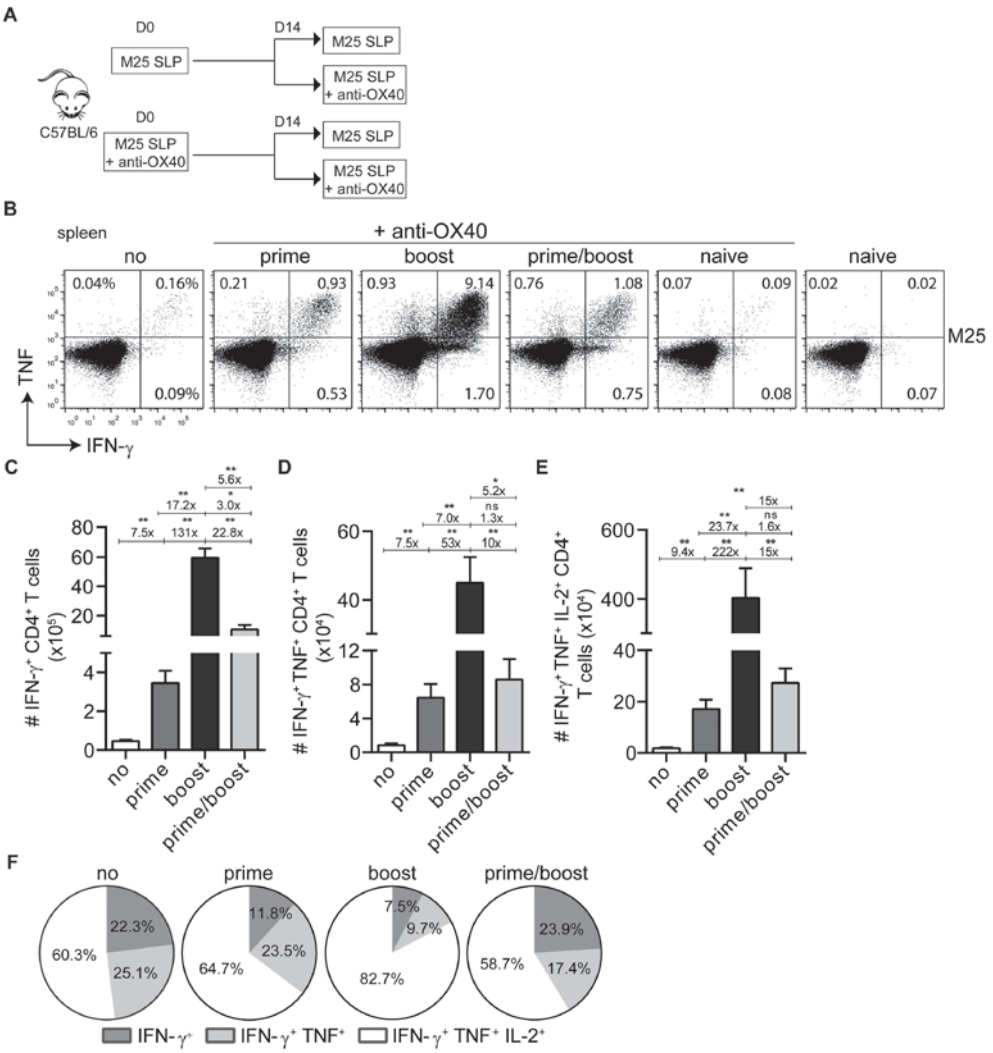

Figure 2. Activation of the OX40 axis during booster vaccination with a single MHC class II SLP vaccine propels increment of the vaccine-induced CD4⁺ T cell response. (A) Scheme of the experimental procedure and the vaccination timeline. Wild type C57BL/6 mice were vaccinated (i) s.c with $M25_{409–423}$ MHC class II SLP alone or (ii) with $M25_{409–423}$ MHC class II SLP (s.c.) along with anti-OX40 mAb (i.p.). 2 weeks after prime vaccination mice from group (i) and (ii) were divided into two groups respectively and a booster immunization was administered. Half mice received only the $M25_{409–423}$ SLP and the other half were injected anti-OX40 mAb in addition to the $M25_{409–423}$ SLP. **(B)** The total size of the splenic $M25_{409–423}$ SLP vaccine induced CD4⁺ T cells from each group was measured by intracellular cytokine staining. Representative plots depict percentages of IFN-γ versus TNF cytokine producing CD4⁺ T cell populations at day 8 post booster vaccination. **(C)** Total numbers of splenic IFN-γ⁺ producing $M25_{409–423}$ antigen-specific CD4⁺ T cells at day 8 post booster SLP vaccination and differential anti-OX40 mAb treatment are shown. **(D)** Total double (IFN-γ/TNF) and **(E)** triple (IFN-γ/TNF/IL-2) cytokine producers of $M25_{409–423}$ vaccine-specific CD4⁺ T cells measured in spleen at day 8 post booster vaccination. Fold differences among each population

are also depicted **(F)** Pie charts show the percentages of the single (IFN-γ), double (IFN-γ/TNF) and triple (IFN-γ/TNF/IL-2) cytokine producers of each M25409–423-specific CD4+ T cell population upon vaccination with M25409–423 SLP and anti-OX40 mAb. Data represent mean values, and are representative of three independent experiments (n=5-6 per group). *, P< 0.05; **, P< 0.01; ns, not significant.

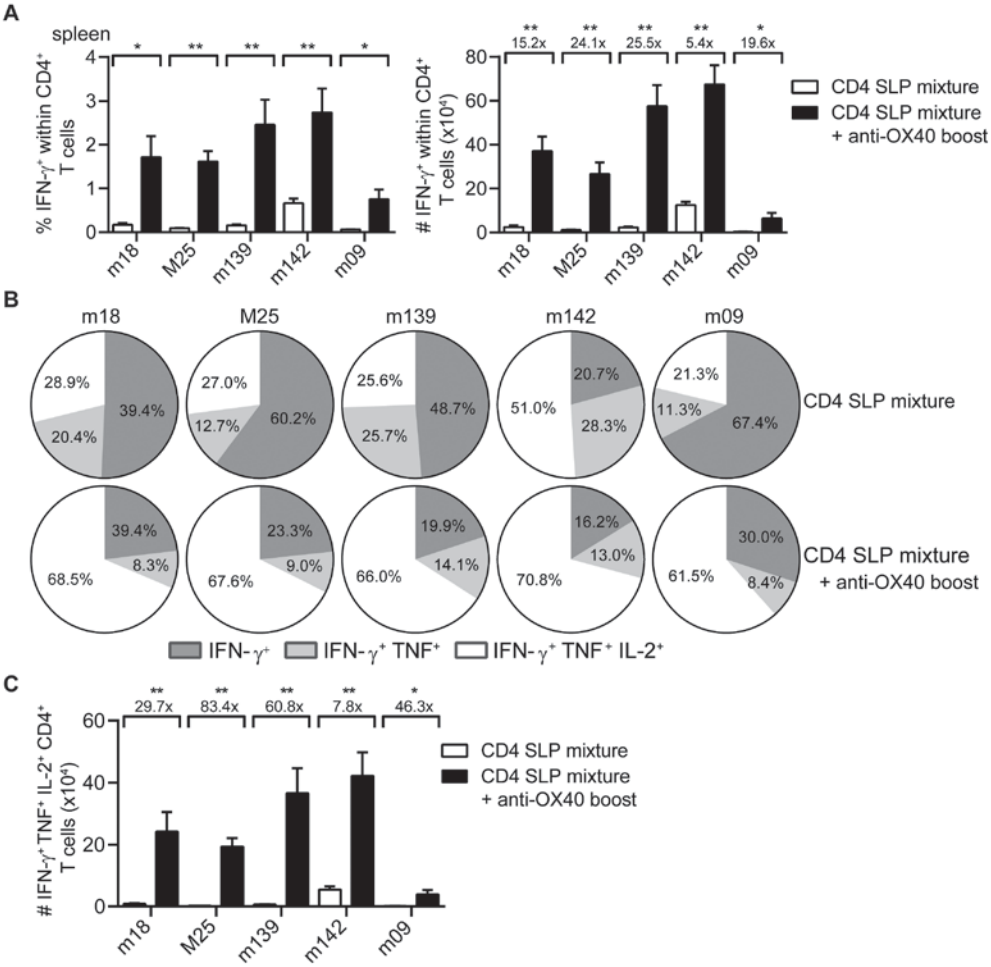

Figure 3. OX40 activation during booster vaccination with combinatorial MHC class II SLP vaccines leads to the induction of robust and polyfunctional CD4+ T cell responses. (A) Percentages (left) and total numbers (right) of the splenic epitope-specific IFN-γ+ CD4+ T cell responses elicited at day 8 post booster vaccination with a mixture of SLPs containing MHC class II epitopes alone (CD4 SLP mixture; white bars) or with SLPs containing MHC class II epitopes and anti-OX40 mAb (CD4 SLP mixture + anti-OX40 boost; black bars). **(B)** Pie charts show the percentages of the single (IFN-γ), double (IFN-γ/TNF) and triple (IFN-γ/TNF/IL-2) cytokine producers of each antigen-specific CD4+ T cell population at day 8 post booster vaccination with a mixture of MHC class II SLPs alone or with MHC class II SLPs plus anti-OX40 mAb. **(C)** Total triple (IFN-γ/TNF/IL-2) cytokine producers of each antigen-specific CD4+ T cell population were

measured in spleen at day 8 post booster vaccination with a mixture of MHC class II SLPs and anti-OX40 mAb or with SLPs alone. Fold difference is indicated. Data represent mean values, and are representative of 3 independent experiments (n=6 per group). *, P< 0.05; **, P< 0.01.

with the results described for $CD4^+$ T cells, mice that were vaccinated and treated with agonistic OX40 antibody during booster vaccination displayed the strongest SLP-induced $CD8^+$ T cell response in both blood and spleen (Figures S2B-F in Supplementary Material). The number of SLP-induced $CD8^+$ T cells, determined either by IFN-γ reactivity or by MHC class I tetramer binding, at the peak after the boost were similar, indicating that OX40 stimulation during SLP vaccination (provided either during priming, during booster or during prime/boost) induces functional (non-exhausted) $CD8^+$ T cells (Figures S2C,D in Supplementary Material). The cell-surface phenotype based on KLRG1 and CD127 of the vaccine-induced $CD8^+$ T cells at the peak of the response after the booster was comparable (Figure S2G in Supplementary Material).

Next, the impact of OX40 stimulation during booster vaccination was evaluated for a mixture of MHC class I epitope-containing SLPs. Longitudinal analysis of the SLP-specific $CD8^+$ T cell response revealed that OX40 stimulation during booster vaccination clearly amplifies the expansion of all SLP vaccine-induced $CD8^+$ T cells measured in blood and spleen (Figures 4A, B), although this effect was relatively lower when compared to that seen for $CD4^+$ T cells. At the peak of the response, 8 days after booster SLP vaccination, the magnitude of the IFN-γ^+CD8^+ T cell response specific for each MHC class I-restricted epitope was again proportional to the MHC class I tetramer response (data not shown). However, the polyfunctional cytokine profile was improved upon OX40 stimulation (Figures 4C, D). In particular OX40 stimulation augmented the percentages and absolute number of the triple cytokine producers in particular (Figures 4C, D).

To gain insight into the mechanisms underlying the apparent impact of OX40 stimulation during booster vaccination, we examined the expression of the pro-apoptotic protein BCL-2, a known target of OX40 triggering and implicated in T cell survival [27]. BCL-2 expression was up-regulated by the vaccine-specific $CD8^+$ T cells when OX40 stimulation was provided during booster vaccination compared to no OX40 stimulation (Figure 4E). Moreover, when agonistic OX40 antibody was administered during both primary and booster vaccination, BCL-2 expression was down-regulated. These results indicate that stimulation of OX40 can bolster vaccine-induced $CD8^+$ T cell expansion through a BCL-2 dependent mechanism, if this stimulation is correctly scheduled.

Over time, the SLP-induced $CD8^+$ T cell responses contracted, yet the OX40 boosted epitope-specific $CD8^+$ T cell responses to M38, M45 and M57 were maintained at higher levels (Figures 5A). At the memory phase (60 days after booster vaccination), there were higher percentages and numbers of triple (IFN-γ/TNF/IL-2) cytokine producing vaccine-

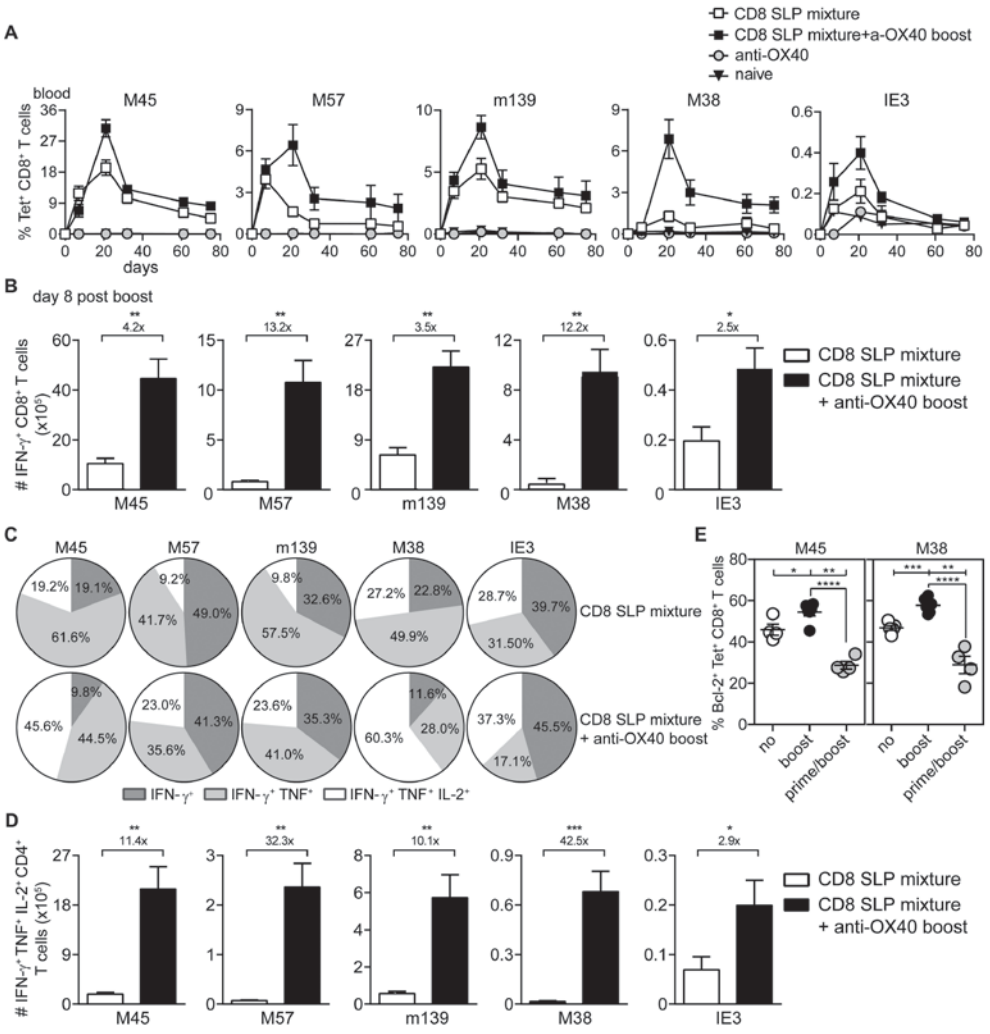

Figure 4. OX40 ligation during booster vaccination with a mixture of MHC class I-epitope containing SLPs leads to the induction of strong and polyfunctional CD8⁺ T cell responses. **(A)** Longitudinal analysis of the epitope-specific CD8⁺ T cell responses in blood induced by combinatorial MHC class I epitope containing SLP vaccines without (CD8 SLP mixture) and with anti-OX40 mAb administration (given only during booster vaccination) (CD8 SLP mixture + a-OX40 boost). Data represents mean values ± SEM (n=12 per group). **(B)** Total IFN- γ⁺ cytokine producing CD8⁺ T cells for each antigen-specific population detected in spleen at day 8 post booster vaccination with CD8 SLP mixture (white bars) or with CD8 SLP mixture + anti-OX40 boost (black bars). **(C)** Pie charts show the percentages of the single (IFN-γ), double (IFN-γ/TNF) and triple (IFN-γ/TNF/IL-2) specific cytokine producers of the antigen-specific CD8⁺ T cell populations at day 8 post booster vaccination. **(D)** Total numbers of IFN-γ/TNF/L-2 cytokine producing CD8⁺ T cells for each antigen-specific population detected in spleen at day 8 post booster vaccination. **(E)** At day 8 after booster vaccination with combined MHC class I SLPs and/or anti-OX40 mAb (during booster or during both prime/boost), the BCL-2 protein expression was measured within the antigen specific CD8⁺ T cells in spleen by flow cytometry. Fold changes between groups are ▸

depicted. Data represents mean values + SEM (n=6 mice per group), and are representative of 3 independent experiments. *, P< 0.05; **, P< 0.01; ***, P< 0.001; ****, P< 0.0001.

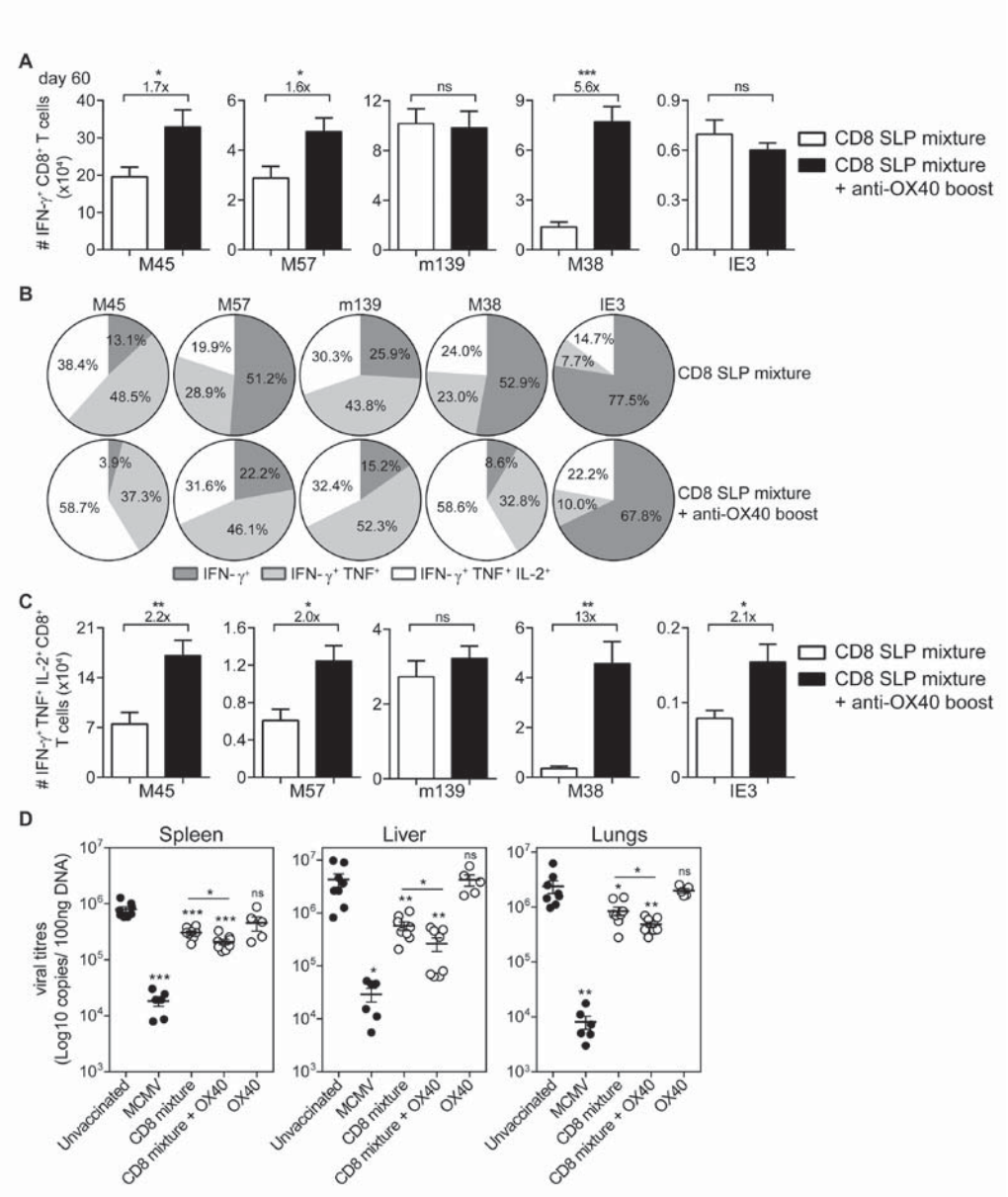

Figure 5. Agonistic OX40 mAb administration during booster vaccination leads to improved memory SLP-elicited CD8⁺ T cell responses. (A) Total single IFN-γ⁺ cytokine producing CD8⁺ T cells for each antigen-specific population detected in spleen at day 60 post booster vaccination with combinatorial MHC class I SLPs and anti-OX40 mAb (provided during booster vaccination) (CD8 SLP mixture + anti-OX40 boost) or with MHC class I SLPs alone (CD8 SLP mixture). Fold changes between groups are depicted. Data represents mean values + SEM (n=6 per group). **(B)** Pie charts show the percentages of the single (IFN-γ), double (IFN-γ/TNF) and triple (IFN-γ/

TNF/IL-2) cytokine producers of each antigen-specific $CD8^+$ T cell population in spleen at day 60 post booster vaccination with a mixture of MHC class I SLPs and anti-OX40 mAb (administered only during booster vaccination) or with MHC class I SLPs alone. **(C)** Total triple IFN-γ^+/TNF^+/IL-2^+ producing $CD8^+$ T cells for each antigen-specific population detected in spleen at day 60 post booster vaccination. Fold changes between groups are depicted. Data represents mean values + SEM (n=6 per group). **(D)** Different groups of mice (unvaccinated (naive), MCMV infected (live virus vaccine), vaccinated with a mixture of all 5 MHC class I SLPs, vaccinated with a mixture of all 5 MHC class I SLPs and anti-OX40 mAb (given only during booster vaccination) or treated with a-OX40 mAb only) were challenged at day 60 post vaccination/infection with 5×10^4 PFU salivary gland-derived MCMV Smith. At day 5 post challenge spleen, liver and lungs were harvested and the viral genome copies were determined by qPCR. The viral titers of individual mice are depicted (n=5-8 mice per group). Mean ± SEM is indicated. The detection limit was below 1000 genome copies as measured in naive mice. Experiments were performed twice with similar outcome. *, P< 0.05; **, P< 0.01; ***, P< 0.001; ns, not significant as compared to the unvaccinated group unless otherwise indicated.

induced $CD8^+$ T cells in mice that received agonistic OX40 antibody during booster vaccination (except for $m139_{419\text{-}426}$) (Figures 5B, C).

We also tested the impact of the in vivo OX40 stimulation on the secondary expansion potential, a hallmark of memory T cells. We performed adoptive transfer experiments in which congenically marked ($CD45.1^+$) memory $CD8^+$ T cells from SLP vaccinated mice were isolated and transferred into naïve recipient mice, which were subsequently challenged with MCMV (Figure S3A in Supplementary Material). Overall, the SLP-induced memory $CD8^+$ T cells isolated from mice that also received OX40 stimulation during booster vaccination expanded better compared to the vaccine only group (Figure S3B, C in Supplementary Material).

Subsequently, we examined whether OX40 stimulation is able to further improve the prophylactic efficacy of the combined MHC class I SLP vaccine. At day 60 post booster vaccination, mice were virally challenged and the titres were quantified in spleen, liver and lungs. Similar to our previous study [22], vaccination with a mixture of MHC class I SLPs resulted in reduction of the virus titres (Figure 5D). Mice that received OX40 stimulation during the booster showed increased potency to control the virus compared to mice that received no extra stimulation of OX40 (Figure 5D). All together, these data suggest that enforced OX40 stimulation during booster SLP vaccination does not only impact the expansion of activated $CD8^+$ T cells but also has a long-lasting constructive influence on the magnitude and the functional profile of the vaccine-elicited $CD8^+$ T cells leading to more effective viral control.

SLP vaccines inducing both $CD4^+$ and $CD8^+$ T cells confer superior protection against MCMV infection

To evaluate if the provision of $CD4^+$ T cells would benefit the $CD8^+$ T cell response, C57BL/6 mice were vaccinated with a mixture of either all 5 MHC class I epitope-

containing SLPs or with a mixture of all 5 MHC class I and all 5 class II epitope-containing SLPs (Table 1 in Supplementary Material). Agonistic OX40 mAb was provided during the boost only. The $CD8^+$ T cell response after the prime vaccination was higher when the SLP vaccine contained a mixture of MHC class I and II peptide epitopes (Figure 6A and Figure S4A in Supplementary Material), stressing the importance of $CD4^+$ T cell help during the priming. Also, the phenotype of the vaccine-induced $CD8^+$ T cells after prime vaccination revealed slightly more effector-type ($KLRG\text{-}1^+CD127^-$) $CD8^+$ T cells when $CD4^+$ T cell help was provided (Figure S4B Supplementary Material), whereas the polyfunctionality of the vaccine-induced $CD8^+$ T cells remained unchanged (data not shown). However, at day 7/8 after booster SLP vaccination, the effect of $CD4^+$ T cell help on the magnitude of the vaccine-induced $CD8^+$ T cell response was no longer detectable in blood (Figure 6A) and spleen (Figure 6B). At this time-point, also no difference in the $CD8^+$ T cell cytokine polyfunctionality and phenotype was found (Figures 6C, D). Furthermore, the size and polyfunctionality of the $CD4^+$ T cells responses induced at the peak response after combined MHC class I and II SLP vaccination was similar as vaccination with MHC class II SLPs only (Figures S5A, B in Supplementary Material). Taken together, vaccination with a mixture of MHC class I and II epitope-containing SLPs resulted in enhanced primary $CD8^+$ T cell expansion compared to vaccination with class I epitope-containing SLPs only, but no additional effects of the helper T cells were observed after the enforced OX40 stimulation provided during booster vaccination.

Ultimately, we analysed the prophylactic efficacy of the SLP-induced MCMV-specific $CD4^+$ and $CD8^+$ T cells to control viral replication. At day 60 post booster SLP vaccination, mice were infected with MCMV and 5 days later the viral titers were quantified. The viral load of unvaccinated (naive) mice challenged with MCMV was found significantly higher in spleen, liver and lungs compared to the viral load of mice that received earlier a virulent virus as a vaccine. This result suggests that pre-existing immunity to MCMV can inhibit virus replication during subsequent infection (Figure 7). Furthermore, the viral titers of mice that had received the virulent virus-based vaccine were similar to those measured during chronic MCMV infection, indicating that this is the maximum of immune control that can be achieved. Mice vaccinated with the mixture of SLPs containing MHC class II epitopes plus OX40 triggering during booster vaccination displayed significant reduction in viral load in all tested organs (Figure 7), indicating that the SLP-induced MCMV-specific $CD4^+$ T cells display direct antiviral properties. Vaccination with the mixture of MHC class I SLPs plus anti-OX40 during booster resulted in a more efficient reduction of viral load in all organs. Strikingly, mice that received a mixture of MHC class I and II epitope-containing SLPs and OX40 stimulation during booster vaccination displayed the strongest reduction in viral load (Figure 7). Notably, the SLP vaccine-induced reduction in viral load in the spleen and liver was almost as effective as to what is observed after vaccination with virulent virus. Thus, SLP vaccines comprising a mixture of MHC class I and II SLPs has the highest protection potency

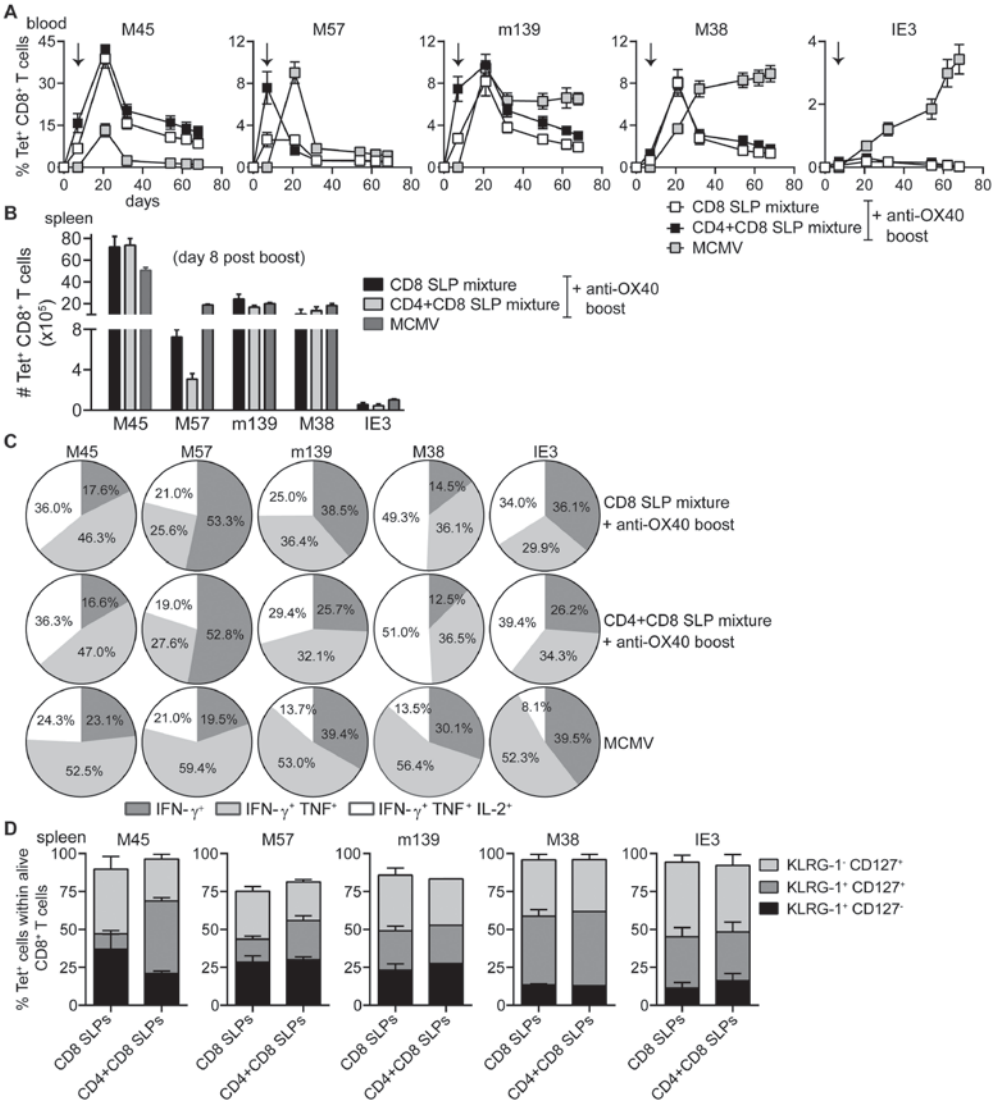

Figure 6. Combinatorial MHC class I and class II epitope-containing SLP vaccines and OX40 agonistic mAb during booster vaccination leads to robust induction of both CD4⁺ and CD8⁺ T cell responses. C57BL/6 mice were vaccinated s.c. with a combination of all MHC class I epitope-containing SLPs (CD8 SLP) or with a combination of all MHC class I and MHC class II-epitope containing SLPs (CD4 + CD8 SLP). In both groups agonistic OX40 mAb was administered i.p. during booster vaccination. **(A)** Kinetics of the antigen-specific CD8⁺ T cells measured by MHC class I tetramer staining in blood. Data shown are mean values ± SEM (n=18 mice/group) **(B)** Total MHC class I tetramer-specific CD8⁺ T cells induced by combinatorial MHC class I or MHC class I plus MHC class II SLP vaccination compared to MCMV infection at day 8 post booster vaccination in spleen. Data represent mean values (n=5 mice/group). **(C)** The cytokine production capacity of the splenic SLP vaccine-induced CD8⁺ T cells was examined by intracellular cytokine staining at day 8 after booster vaccination. Pie charts depict the percentages of the single (IFN-γ), double ▸

(IFN-γ/TNF) and triple (IFN-γ/TNF/IL-2) cytokine producers of each antigen-specific $CD8^+$ T cell populations at day 8 post booster vaccination. **(D)** Phenotypic analysis of the combinatorial SLP vaccine-induced $CD8^+$ T cells in spleen at day 8 post booster vaccination. Data represent mean values (n=5 mice/group) and are representative of three independent experiments.

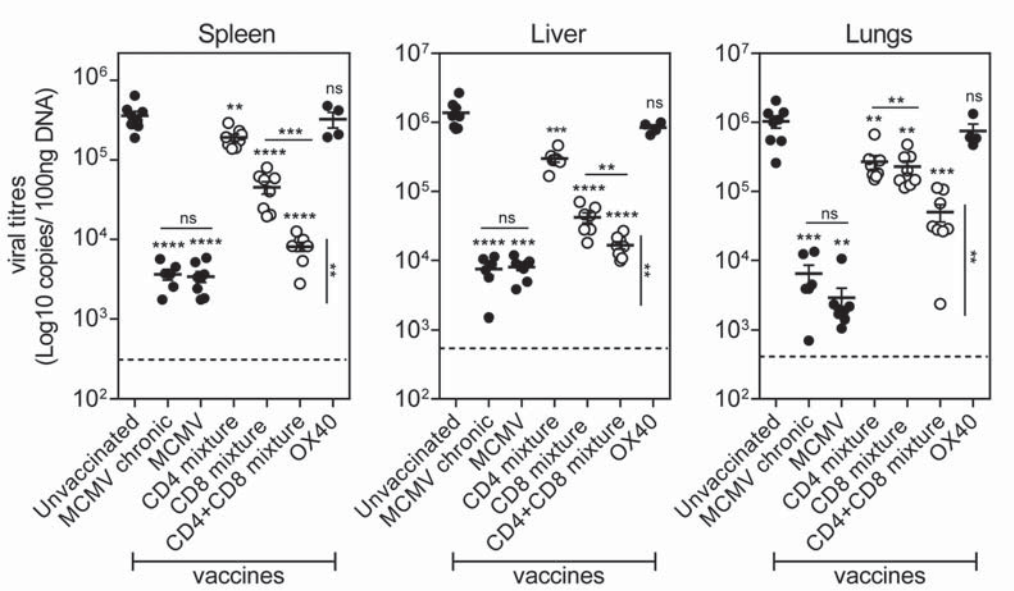

Figure 7. Prime-boost vaccination with combinatorial MHC class I and class II epitope-containing SLPs exhibits increased potency to protect against lytic MCMV infection. Unvaccinated (naive), MCMV (live-virus vaccine), combined MHC class II SLPs (CD4 mixture), combined MHC class I SLPs (CD8 mixture), combined MHC class I and II SLPs (CD4+CD8 mixture) and anti-OX40 mAb only treated C57BL/6 mice were challenged 60 days post booster-vaccination/ infection with 5×10^4 PFU salivary gland-derived MCMV Smith. At day 5 post challenge, liver, lungs and spleen were harvested and the viral genome copies were quantified by qPCR. To further evaluate the efficacy of the vaccines, the viral titers of chronically infected (MCMV chronic/day 60) mice were also measured. The viral titers of individual mice are depicted (n=4-8 per group). Statistical significance between the unvaccinated group and the rest of the groups is indicated with asterisks above each group. A statistical comparison between the CD4 + CD8 mixture group and the MCMV (live virus vaccine) and MCMV chronic group was also performed (vertical statistical bar). For both comparisons the statistical difference was found the same (**, P< 0.01). The detection limit was below 1000 genome copies as measured in naive mice. Experiment was performed twice with similar outcome. Statistical difference is indicated **, P< 0.01; ***, P< 0.001; ****, P< 0.0001; ns, not significant as compared to the unvaccinated group unless otherwise indicated.

compared to similar vaccines that elicit merely MCMV-specific $CD4^+$ or $CD8^+$ T cell responses. All together, we conclude that OX40 activation during booster vaccination empowers SLP-induced memory $CD4^+$ and $CD8^+$ T cells to efficiently counteract lytic MCMV infection.

DISCUSSION

In this study, we show that SLP-based vaccine strategies eliciting vigorous polyfunctional $CD4^+$ and $CD8^+$ T cell responses are highly effective against MCMV infection. While SLP-based vaccines that evoke solely $CD8^+$ T cell responses display already efficacy against lytic MCMV infection [22], vaccination with a mixture of various immunodominant MHC class II and MHC class I MCMV epitopes and the combination with enforced OX40 stimulation results in superior vaccine efficacy. Former explored CMV vaccines focused mainly on the induction of neutralizing antibodies and thus far did not show substantial efficacy [3-5]. Our finding that SLP-based vaccines that merely provoke $CD4^+$ and $CD8^+$ T cell responses are almost as efficient as a virulent vaccine suggests that the inclusion of T cell stimulating antigens should facilitate the design of more efficient vaccines against CMV.

Signals through the OX40 costimulatory receptor are known to regulate expansion and survival of both $CD4^+$ and $CD8^+$ T cells after antigen encounter [26,28]. Consistent with this, OX40 had a robust effect on the magnitude of the effector SLP vaccine-induced $CD4^+$ and $CD8^+$ T cells and on their capacity to induce "Th1" cytokine responses (especially IL-2). This effect of OX40 costimulatory signals was stronger when agonistic antibody to OX40 was provided during booster SLP vaccination. We hypothesize that primed T cells more rapidly and stronger upregulated OX40 leading to greater benefit during enforced OX40 stimulation. OX40 ligation seems to work better on $CD4^+$ T cells as compared to $CD8^+$ T cells, which may be related to the higher expression of OX40 on $CD4^+$ T cells [29,30]. Due to its capacity to regulate both $CD4^+$ and $CD8^+$ T cells, OX40 is a promising candidate in immunotherapy of chronic viral infections and cancer [28]. In this study we did not detect toxicity of OX40 agonistic antibodies but a better understanding of potential side-effects is required.

$CD4^+$ T cell responses in CMV infection have been long known as important contributors to control primary infection [9,31]. In MCMV infection, $CD4^+$ T cells direct the quality and persistence of inflationary $CD8^+$ T cells and B cell responses [23,24,32]. Here we show that vaccine-induced $CD4^+$ T cells solely can confer moderate protection against acute MCMV infection. These results are consistent with other reports showing $CD4^+$ T cell effectivity against MCMV and HCMV [9,12,25,33-35]. Furthermore, we observed that addition of $CD4^+$ T cell help during vaccination with MHC class I SLP vaccines promoted priming of naïve $CD8^+$ T cells. After booster vaccination in settings wherein OX40 costimulation was enhanced, no additional effects of CD4 help on the magnitude and phenotype of the $CD8^+$ T cells were observed, suggesting that

enforced OX40 stimulation during booster vaccination may replace the need for CD4 help signals. On this basis, the improved prophylactic vaccine efficacy of the combined MHC class I and II SLP vaccines appears to be more additive rather than synergistic.

Another interesting observation was that the vaccine efficacy was somewhat better in liver and spleen than in lungs. A possible explanation for this discrepancy is that the SLP-induced $CD8^+$ T cells are better capable to control the virus (lowering the viral titers) in the spleen and liver than the SLP-induced $CD4^+$ T cells. A marked difference in the efficacy of SLP-induced $CD4^+$ and $CD8^+$ T cells as observed in liver and spleen is not found in the lungs, and can thus be dictated by the tissue environment. Differences in site-specific control of MCMV-specific $CD4^+$ and $CD8^+$ T cells is known to be especially important in the salivary glands but for other tissues this may be important as well [36]. Nevertheless, the combined SLP vaccines inducing both $CD4^+$ and $CD8^+$ T cell responses clearly improved the efficacy of the vaccine in all organ tissues. In this respect it is of interest to note that SLP-based vaccines can be further refined by different prime-booster regimens, inclusion of B cell epitopes and by combinations with adjuvants, immunomodulatory antibodies or other vaccine platforms [37].

This study provided evidence that SLP-based vaccines eliciting broad $CD4^+$ and $CD8^+$ T cell responses can effectively control lytic MCMV infection without contribution by humoral responses. The use of OX40 as an adjuvant for MCMV peptide immunization strongly bolstered the development of effective $CD4^+$ and $CD8^+$ T cells. Future studies to examine the ability of the SLP vaccine-induced T cells and OX40 costimulation to boost immune responses in immunocompromised settings of CMV infection or other chronic viral infections are strongly encouraged by these promising findings. Taken together, our data highlight the importance of designing CMV vaccines that elicit effective $CD4^+$ and $CD8^+$ T cell responses.

MATERIALS AND METHODS

Mice

Wild-type female C57BL/6 mice (8-10 weeks) were purchased from Charles River Laboratories (L'Arbresle, France) and Ly5.1 (SJL; CD45.1) congenic mice on a C57BL/6 genetic background were obtained from The Jackson Laboratory. Mice were bred and housed under specific-pathogen-free (SPF) conditions at the Central Animal Facility of Leiden University Medical Center (LUMC). Experimental procedures were approved by the LUMC Animal Experiments Ethical Committee and conducted according to the Dutch Experiments on Animals Act and the Council of Europe (#13156 and #14187).

Viral infections

Virus stocks were prepared from salivary glands of BALB/c mice infected with MCMV-Smith (*American Type Culture Collection (*ATCC)). The viral titres of the produced virus

stocks were determined by viral plaque assays with *mouse NIH-3T3 Embryonic Fibroblasts (ATCC)*. C57BL/6 mice were infected intraperitoneally (i.p) with 5×10^4 PFU MCMV in 400 µl of PBS. 60 days post-booster vaccination or infection, mice were (re)-challenged with 5×10^4 PFU MCMV. Viral loads in spleen, liver and lungs were determined by real-time PCR at day 5 post challenge as described previously [38]. Due to differences in peak viral replication, the viral load in the salivary glands was not measured.

Peptides and vaccination

Short (9-10 aa) and long (20-21 aa) peptides containing MHC class I-restricted T cell epitopes [22] and 15 aa long peptides containing MHC class II epitopes [39] were produced at the GMP-peptide facility of the LUMC. The purity (75-90%) of the synthesized peptides was determined by HPLC and the molecular weight by mass spectrometry. All peptide sequences used in this study are listed and described in S1 Table in Supplementary Material. Both single and mixed synthetic long peptide (SLP) vaccines were administered subcutaneously (s.c) at the tail base by delivery of 50 µg of each SLP and 20 µg CpG (ODN 1826, InvivoGen) in a total volume of 50 µl in PBS. Booster SLP vaccinations were provided after 2 weeks. At the indicated times (during prime and/or booster vaccination), mice were injected i.p. with 150 µg agonistic OX40 mAb (clone OX86) dissolved in 150 µl of PBS. All SLP vaccine administrations were well tolerated without adverse events and signs of hypersensitivity.

Flow cytometry

To evaluate $CD4^+$ and $CD8^+$ T cell responses, cell surface and intracellular cytokine staining in splenocytes and blood lymphocytes were performed as previously described [40]. In brief, to determine the cytokine production capacity, single-cell suspensions from spleens were stimulated with short MHC class I peptides (2 µg/ml) for 5 h in the presence of brefeldin A (Golgiplug; BD Pharmingen) or with long MHC class II peptides (5 µg/ml) for 8 h of which the last 6 h in presence of brefeldin A. MHC class I tetramers specific for the $M45_{985–993}$, $M57_{816–824}$, $m139_{419-426}$, $M38_{316–323}$, and $IE3_{416–423}$ MCMV epitopes were used. Fluorochrome-conjugated mAbs were obtained from BD Biosciences, Biolegend or eBioscience. Flow cytometry gating strategies are shown in S6 Fig. All data were acquired on a LSRFortessa cytometer (BD Biosciences) and analysed with FlowJo-V10 software (Tree star).

Antibody detection by ELISA

Total IgG, IgG_{2b}, IgG_{2c}, IgG_3, IgE and IgA concentrations were determined by ELISA in serum samples as previously described [38]. Briefly, Nunc-Immuno Maxisorp plates (Fisher Scientific) were coated overnight with 2 µg/ml MHC class II SLPs in bicarbonate buffer, and after blocking (skim milk powder, Fluka BioChemika) sera from mice i) chronically infected, ii) long term vaccinated with MHC class II SLP vaccines and anti-OX40 mAb treated (i.p. during booster vaccination only) or iii) uninfected were added. Next, plates

were incubated with various HRP-conjugated antibodies (SouthernBiotech) to detect different immunoglobulin isotypes. Plates were developed with TMB substrate (Sigma Aldrich) and the colour reaction was stopped by the addition of 1 M H_2SO_4. To serve as a positive control, a peptide from the M2 protein (eM2) of influenza A virus with known ability to induce antibodies and corresponding serum was used. Optical density was read at 450 nm (OD_{450}) using a Microplate reader (Model 680, Bio-Rad).

Adoptive T cell transfers

The secondary expansion potential of the SLP vaccine-induced antigen-specific $CD8^+$ T cells receiving agonistic OX40 mAb only during booster vaccination was determined by adoptive transfers. Splenic memory (day 65) $CD8^+$ T cells from SLP vaccinated $CD45.1^+$ congenic mice were negatively enriched with magnetic sorting using the $CD8^+$ T cell isolation kit (Miltenyi Biotec). 2×10^6 total $CD8^+$ T cells were retro-orbitally injected (in a total volume of 200 µl in PBS) into naive $CD45.2^+$ recipients. Recipient mice were rested for 2 hours and concomitantly infected with 5×10^4 PFU MCMV. Subsequently, in order to quantify the number of the vaccine-antigen specific $CD8^+$ T cells that was transferred, a representative amount of cells was stained with MHC class I tetramers and with fluorochrome labelled antibodies against CD44, CD3, CD4 and CD8. The number of the donor's vaccine-specific T cells transferred was ranging between 8×10^3- 2.5×10^4 cells for the group that did not receive OX40 mAb and between 1.8×10^4- 4.5×10^4 cells for the OX40 mAb boosted group. 6 days later the number of vaccine-specific $CD8^+$ T cells of the donor was measured (based on the expression of the CD45.1 marker) by flow cytometry, and the fold expansion was calculated.

Statistical analyses

Statistics were calculated using the unpaired Student's t-test or ANOVA in GraphPad Prism software (GraphPad Software Inc., USA). *p < 00.5, **p < 0.01, ***p < 0.001, and ****p <0.00001.

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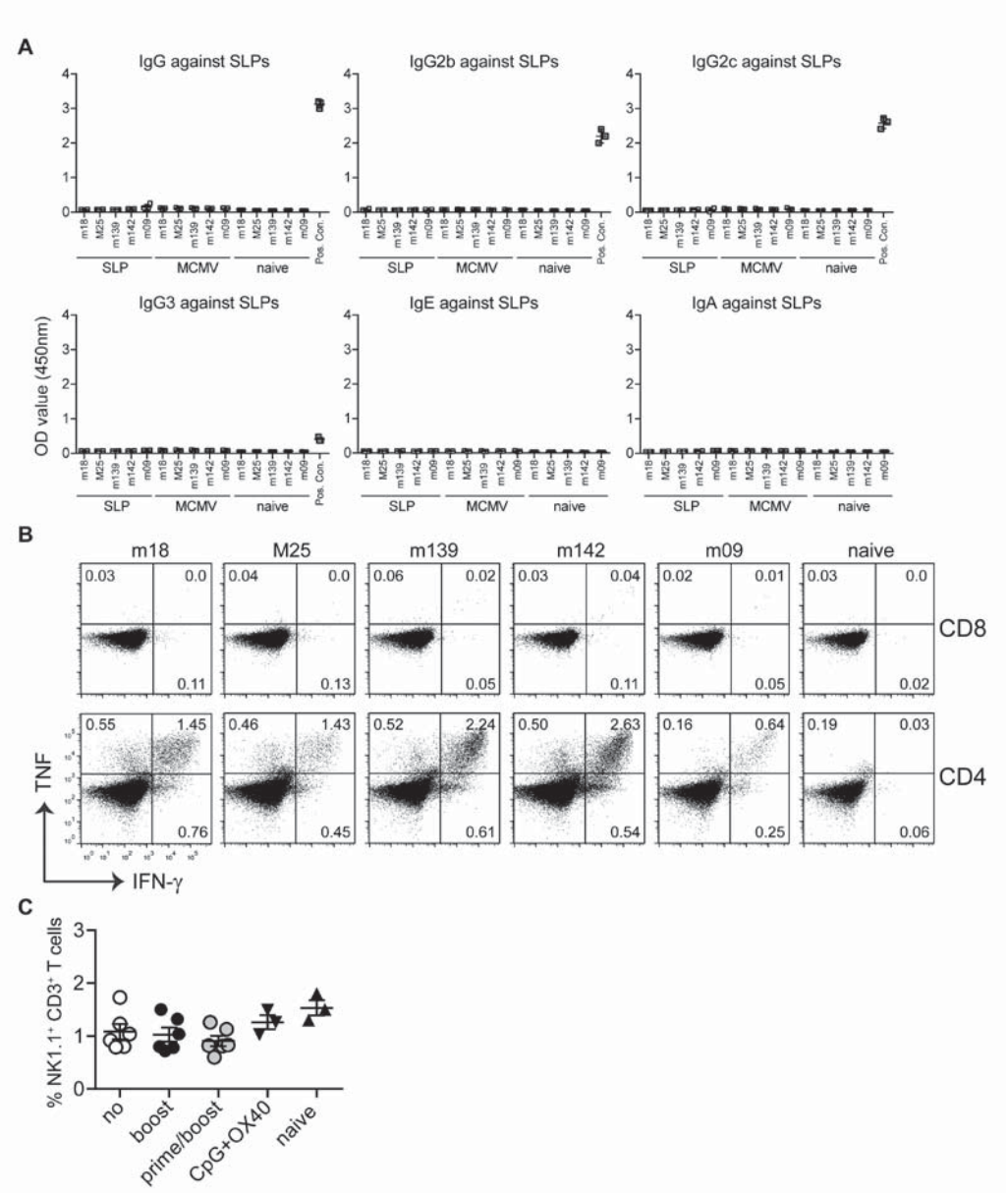
Supplementary Table 1. Depiction of the MHC class I and II peptide epitopes and SLPs used in this study.

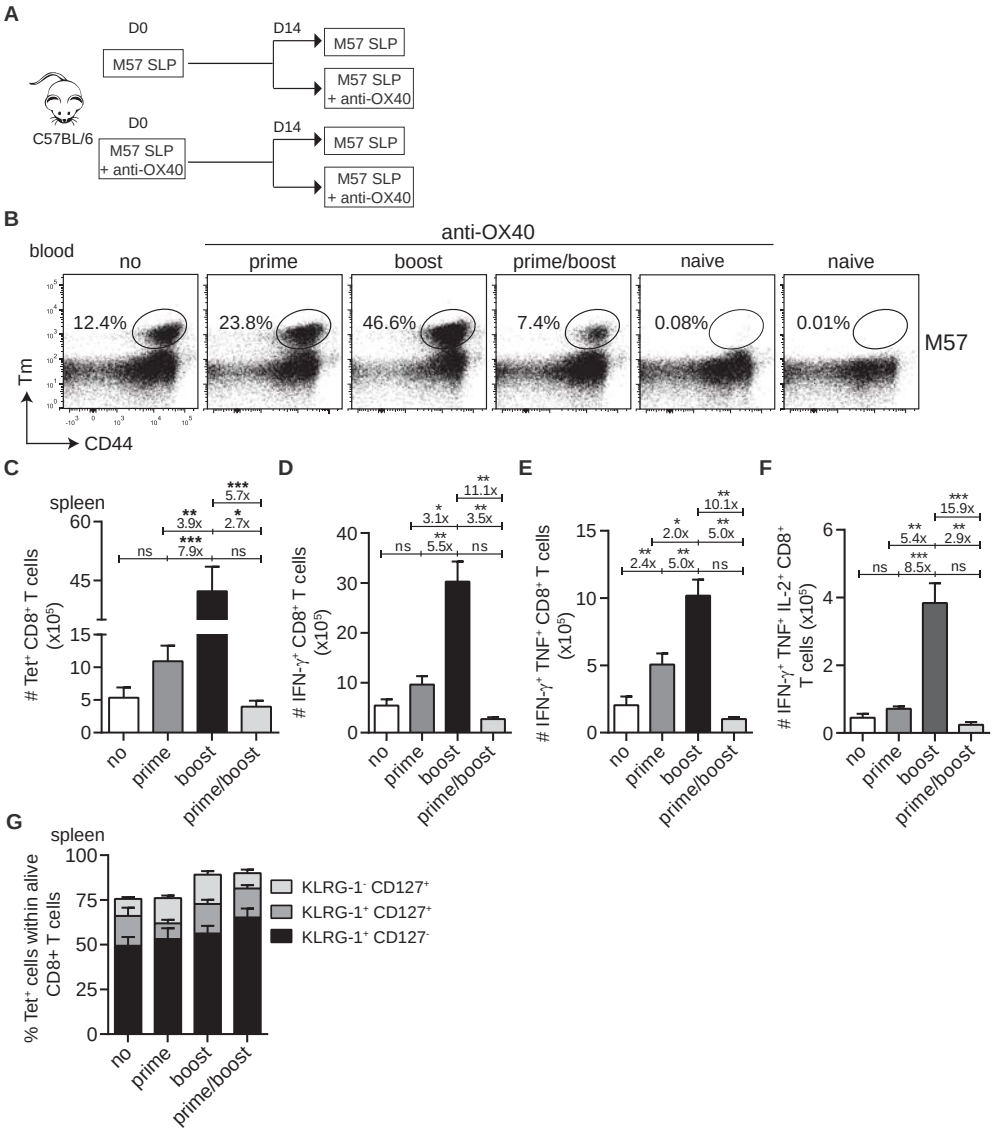
Mouse strain	Protein (ORF)	SLP sequence *	Epitope residues (aa)	MHC allele - restriction	Stage of expression	Inflation?	Human CMV Homologue
C57BL/6	m18	**NERAKSPAAMTAEDE**	872–886	I-A^b	Immediate early gene	No	
C57BL/6	M25	**NHLYETPISATAMVI**	409–423	I-A^b	UL25 family homologue,	No	UL25 (GF1)
C57BL/6	m139	**TRPYRYPRVCDASLS**	560–574	I-A^b	tegument proteinUS22 family homologue	No	US22
C57BL/6	m142	**RSRYLTAAAVTAVLQ**	24–38	I-A^b	US22 family homologue,	No	US26
C57BL/6	m09	**GYLYIYPSAGNSFDL**	133–147	I-A^b	Glycoprotein family,	Yes	
C57BL/6	M45	REDVVK**HGIRNASFI**TGCSA	985-993	H-2-D^b	Early gene	No	UL45
C57BL/6	M57	FPACGL**SCLEFWQRV**LQNS	816-824	H-2-K^b	Early gene	No	UL57
C57BL/6	m139	VVLVGARG**TVYGFCLL**SND	419-426	H-2-K^b	Early gene	Yes	US22
C57BL/6	M38	VTLI**SSPPMFRV**PVNPVPGG	316-323	H-2K^b	Early gene	Yes	UL38
C57BL/6	IE3	DKSRKYPA**RALEYKNL**PFR	416-423	H-2-K^b	Immediate early gene	Yes	IE2

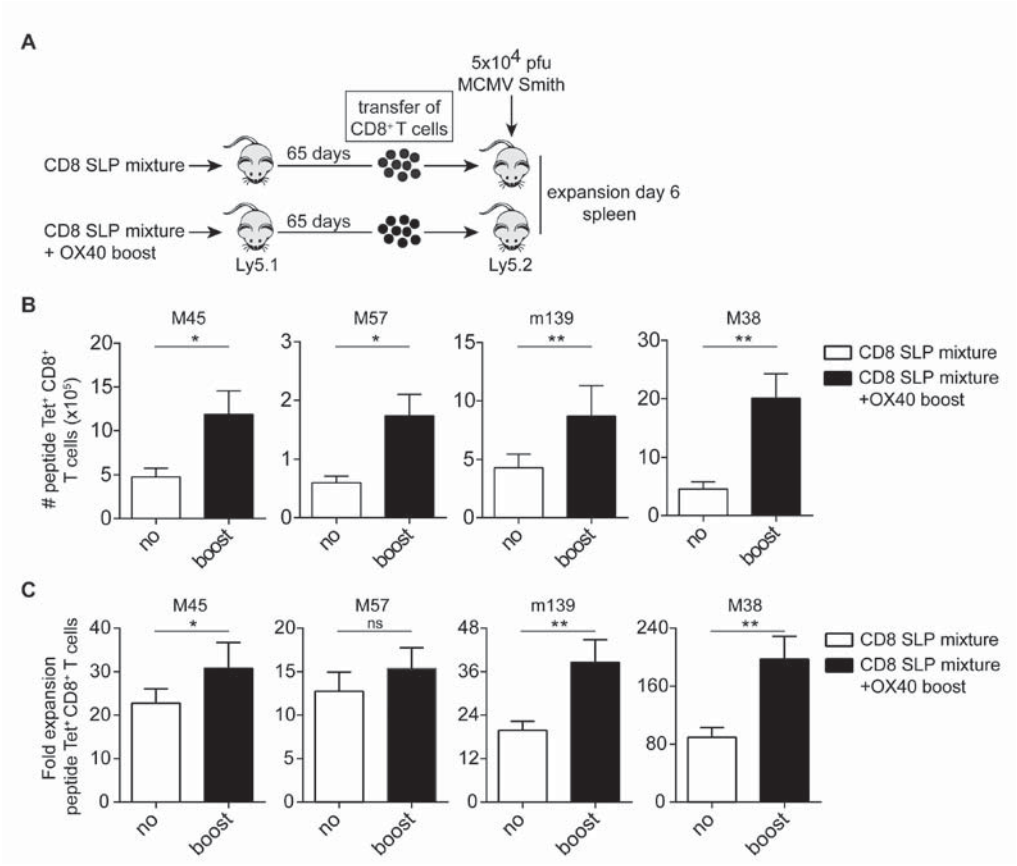
* Bold underlined amino acid (aa) residues within the SLP sequence indicate the CTL epitope.

Supplementary Figure 1. MHC class II SLP vaccines do not induce peptide-specific Abs and do not activate $CD8^+$ T cells and NK cells. **(A)** At day 60 post booster MHC class II SLP vaccination and anti-OX40 mAb administration (i.p. during booster vaccination only) or MCMV infection serum samples were obtained from C57BL/6 mice. ELISA plates were coated with SLP and detection of peptide specificAb (including IgG, various IgG subtypes, IgE and IgA) in the serum was assessed. To serve as a positive control, a peptide with known ability to induce antibodies and corresponding serum was used. Optical density values were measured at 450 nm. Each symbol represents an individual mouse. Mean value ± SEM (n=3-4 mice per group) is indicated. **(B)** At day 8 following booster MHC class II SLP vaccination and a-OX40 mAb administration the intracellular cytokine production by $CD8^+$ and $CD4^+$ T cells was determined after restimulation with long peptide (5µg/ml) for 8 h and of which the last 6 h in presence of brefeldin A at 37°C. Representative plots show percentages of IFN-γ versus TNF production of $CD8^+$ and $CD4^+$ T cells of each peptide specific population in spleen. No peptide stimulation and naive mice were used as negative controls. IFN-γ and TNF reactivity was only observed in $CD4^+$ T cells. Experiments were performed twice with similar outcome (n=5 mice per group). **(C)** Potential NK cell activation following vaccination with combinatorial MHC class II SLP vaccines and different doses of anti-OX40 mAb administration was investigated. At day 7 after booster vaccination cell surface staining for NK cell marker NK1.1 was performed in blood. No NK cell proliferation was triggered by the vaccines. Each symbol represents an individual mouse. Mean value ± SEM (n=3-6 mice per group) is indicated.

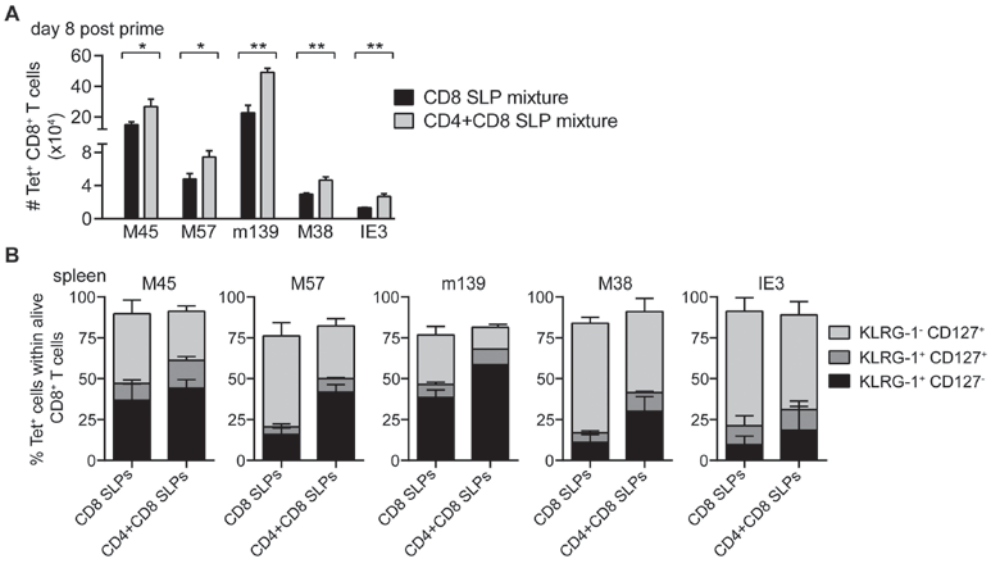

A
IgG against SLPs
IgG2b against SLPs
IgG2c against SLPs
IgG3 against SLPs
IgE against SLPs
IgA against SLPs
OD value (450nm)
SLP
MCMV
naive
Pos. Con.
B
m18
M25
m139
m142
m09
naive
CD8
CD4
TNF
IFN-γ
C
% NK1.1+ CD3+ T cells
no
boost
prime/boost
CpG+OX40
naive

Supplementary Figure 2. Agonistic OX40 mAb administration during booster MHC class II-restricted SLP vaccination provokes accrual of the vaccine-induced CD4$^+$ T cell responses. (A) Scheme of the experimental procedure and the vaccination timeline. Wild type C57BL/6 mice were vaccinated (i) s.c with $M57_{816\text{-}824}$ MHC class I SLP alone or (ii) with $M57_{816\text{-}824}$ MHC class I SLP (s.c.) along with anti-OX40 mAb (i.p.). 2 weeks after prime vaccination, mice from group (i) and (ii) were divided into two groups respectively and a booster immunization was administered. Half mice received only $M57_{816\text{-}824}$ SLP and the other half were injected anti-OX40 mAb in addition to M57816-824 SLP. (B) Percentages and **(C)** total size of the $M57_{816\text{-}824}$ SLP vaccine induced CD8$^+$ T cells from each group was measured by tetramer staining in blood and spleen at day 7 and 8 after booster vaccination respectively. (D) Total single (IFN-γ^+), (E) double (IFN-γ^+TNF$^+$) and **(F)** triple (IFN-γ^+TNF$^+$IL-2$^+$) cytokine producers of vaccine-antigen-specific CD8$^+$ T cell populations measured in spleen at day 8 post booster vaccination with $M57_{816\text{-}824}$ SLP and different doses of anti-OX40 mAb. Fold differences among each population is shown **(E)** Phenotypic profile of the SLP vaccine-induced CD8$^+$ T cells in spleen at day 8 post booster vaccination. Data represent mean values, and are representative of three independent experiments (n=5 mice per group). *, P< 0.05; **, P< 0.01; ***, P< 0.001; ns, not significant.

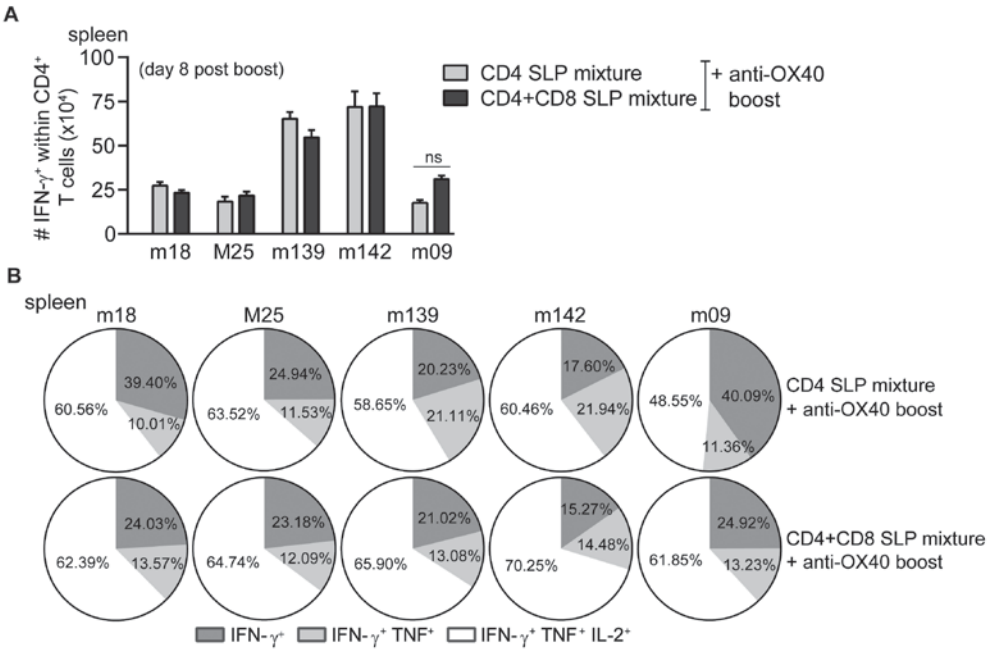

A
D0
M57 SLP
D14
M57 SLP
M57 SLP + anti-OX40
C57BL/6
D0
M57 SLP + anti-OX40
D14
M57 SLP
M57 SLP + anti-OX40
B
blood
anti-OX40
no
prime
boost
prime/boost
naive
naive
12.4%
23.8%
46.6%
7.4%
0.08%
0.01%
M57
Tm
CD44
C
spleen
Tet+ CD8+ T cells (x10^5)
D
IFN-γ+ CD8+ T cells (x10^5)
E
IFN-γ+ TNF+ CD8+ T cells (x10^5)
F
IFN-γ+ TNF+ IL-2+ CD8+ T cells (x10^5)
G
spleen
% Tet+ cells within alive CD8+ T cells
KLRG-1- CD127+
KLRG-1+ CD127+
KLRG-1+ CD127-
no
prime
boost
prime/boost



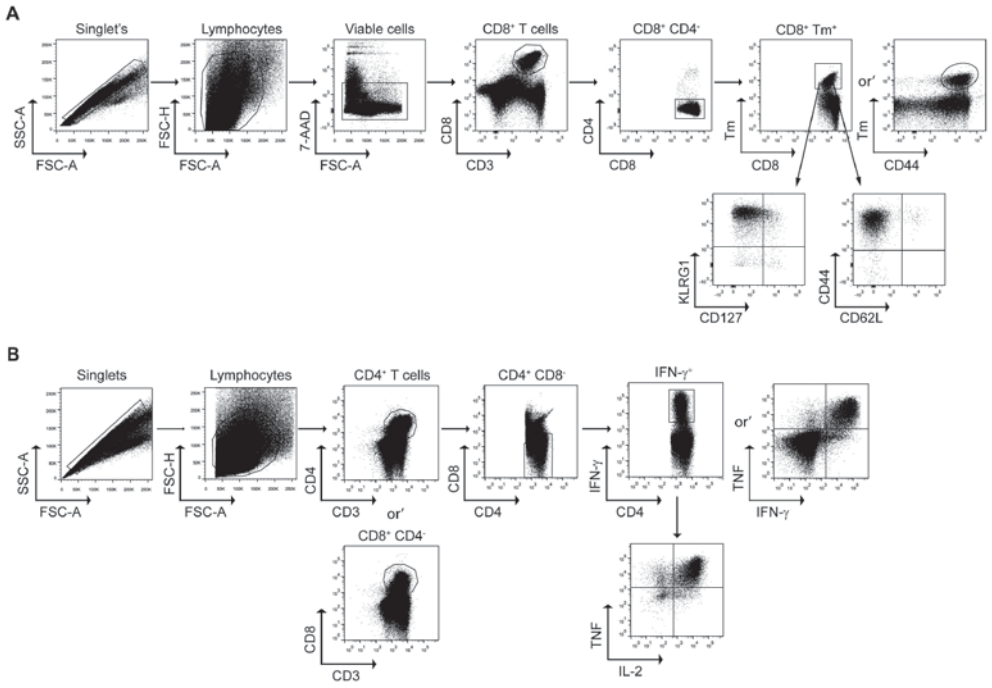
Supplementary Figure 3. OX40 activation during booster combinatorial MHC class I SLP vaccination improves the secondary expansion potential of the memory vaccine-elicited CD8$^+$ T cells. (A) Scheme of the experimental setup and the vaccination schedule followed. Briefly, CD45.1$^+$ congenic mice were vaccinated s.c. in a prime boost vaccination schedule with a mixture of all 5 MHC class I SLPs solely or along with anti-OX40 mAb (one dose administered only during booster vaccination). 65 days post booster vaccination splenic CD8$^+$ T cells were negatively enriched and 2×10^6 total CD8$^+$ T cells were retro-orbitally transferred into naive C57BL/6 recipient mice. Recipient mice were subsequently challenged (i.p.) with 5 × 104 PFU MCMV and 6 days later the expansion capacity of the donor's CD45.1$^+$ Tet$^+$ CD8$^+$ T cells was measured by tetramer staining in spleen. **(B)** Fold expansion of the donor's antigen-specific CD8$^+$ T cells transferred was measured in spleen at day 6 after viruschallenge. Data represents mean values + SEM, and are representative of 2 independent experiments (n= 9 mice per group). *, P< 0.05; **, P< 0.01; ns, not significant.



Supplementary Figure 4. Inclusion of CD4⁺ T cell responses during prime vaccination with combinatorial MHC class I SLP vaccines improves the magnitude of the vaccine-induced CD8⁺ T cells. C57BL/6 mice were vaccinated s.c. with a mixture of all MHC class I SLPs or with a combination of all MHC class I and II SLPs (S1 Table). **(A)** Graph shows total numbers of the tetramer epitope-specific CD8⁺ T cells responsesdetected in spleen at day 8 post prime vaccination. **(B)** Phenotypic traits of the vaccine antigen-specific CD8⁺ T cells in spleen at day 8 post prime vaccination. Note that anti-OX40 mAb was not injected during prime vaccination. Data represents mean values (n=5 mice per group), and are representative of 3 independent experiments. *, P< 0.05; **, P< 0.01.



Supplementary Figure 5. The magnitude and polyfunctionality of the CD4⁺ T cell responses elicited by booster combinatorial MHC class II SLP vaccination is not altered by the presence of CD8⁺ T cell responses. (A) Total size of antigen-specific CD4⁺ T cell population measured in spleen at day 8 post booster combinatorial MHC class II SLP vaccination (light grey bars) or a mixture of both MHC class II and MHC class I SLPs (back bars). Anti-OX40 mAb was administered in both groups during booster vaccination. **(B)** Cytokine profile of the vaccine induced CD4⁺ T cells in spleen at day 8 post booster vaccination. Same group description as above. Data represents mean values (n=5 mice per group), and are representative of 3 independent experiments. ns, not significant.



Supplementary Figure 6. Flow cytometry gating strategy. (A) Representative plots show the gating strategy to detect MHC class I tetramer positive cells in blood and spleen. In sequential gating, cells were first gated for singlets (FSC-A vs. SSC-A), then for lymphocytes (FSC-A vs. FSC-H), followed by viability (FSC-A vs. 7-AAD). Next, live cells were analysed for the expression of CD3 and CD8 while cells positive for CD4 were excluded. MHC class I tetramer positive cells (CD8 vs. Tm) were analysed for the expression of KLRG1, CD44, CD127 and CD62L surface markers. **(B)** Gating strategy for intracellular cytokine staining to detect $CD4^+$ and $CD8^+$ vaccine elicited T cell responses. Utilizing a sequential gating analysis, cells were initially gated for singlet's (FSC-A vs. SSC-A) and then for lymphocytes (FSC-A vs. FSC-H). Next, cells were analysed for the expression of CD3 and CD4 while cells positive for CD8 were excluded. IFN-γ producing $CD4^+$ T cells were further analysed for expression of TNF and IL-2. A similar gating strategy was followed to identify $CD8^+$ T cell responses.

Appendix to chapter 3

ASSESSING THERAPEUTIC SLP VACCINATION TO TREAT PERSISTENT MCMV INFECTION

A therapeutic CMV vaccine may be of clinical benefit in immunocompromised individuals that are otherwise unable to cope with CMV infection. Evidently in congenital infection, allogeneic hematopoietic stem-cell transplantation (allo-HCT), solid organ transplantation, but also in other immunocompromised settings the host immune system is frequently found to be unable to control CMV infection leading to severe morbidity and mortality. A therapeutic CMV vaccination strategy that is able to elevate (pre-existing) immunity to CMV in presence of an ongoing CMV infection, may potentially also reduce CMV-associated morbidity . A plethora of studies performed in MCMV have confirmed the importance of T cell-mediated immunity in resolving acute infection and limiting latent virus reactivation. $CD8^+$ T cells appeared to be able to block virus spread and $CD4^+$ T cells were crucial for virus clearance from the salivary grands [1-4]. Despite significant progress in the area of prophylactic CMV vaccines as evidenced by the number of current candidate vaccines [5-8], only a very limited number of studies with a primary focus on therapeutic CMV vaccination have been conducted [8-11].

In Chapters 2 and 3 prophylactic vaccination with a combination of MHC class I and/or MHC class II SLPs containing immunodominant epitopes managed to reduce viral titers upon primary MCMV infection. Administration of anti-OX40 agonistic antibodies further improved the vaccine efficacy and a remarkable reduction in the viral titers was observed in the organs tested (spleen, liver, lungs). Guided by these promising results, the therapeutic potential of the SLP vaccination platform during established MCMV infection was investigated. The experimental procedure followed is presented in **Figure 1A**. Briefly, immunocompetent C57BL/6 mice were infected with 5×10^4 PFU salivary gland-derived MCMV Smith strain, and 2 weeks after infection a prime-boost SLP vaccination with 2 weeks interval was provided. Candidate vaccines tested included a combination of all the MHC class I SLPs, a combination of all MHC class II SLPs and a mixture of both **(Chapter 3, Table S1 in Supplementary Material)**. Agonistic OX40 mAb was provided during the boost in all tested groups.

Since the ability of a vaccine to enhance immunogenicity of self-antigen T cell responses has been associated with control of viremia [11,12], the capacity of the SLP vaccines to activate the immune system and boost virus induced $CD8^+$ T cell responses was evaluated by MHC class I tetramer assays in blood 10 days after the booster vaccination **(Figure 1B).** The non-inflationary MCMV M45-specific $CD8^+$ T cell response was markedly amplified following immunization with a mixture of MHC class I and II SLPs. However, no significant difference in the magnitude of the post-vaccination m139, M38 and IE3 inflationary MCMV epitope-specific $CD8^+$ T cells responses compared to the control group (unvaccinated) was observed. Consistent with previous reports, viral titers in spleen and liver tissues during the persistent phase of MCMV infection (here day 39 post infection) were found severely reduced, although still clearly detectable in

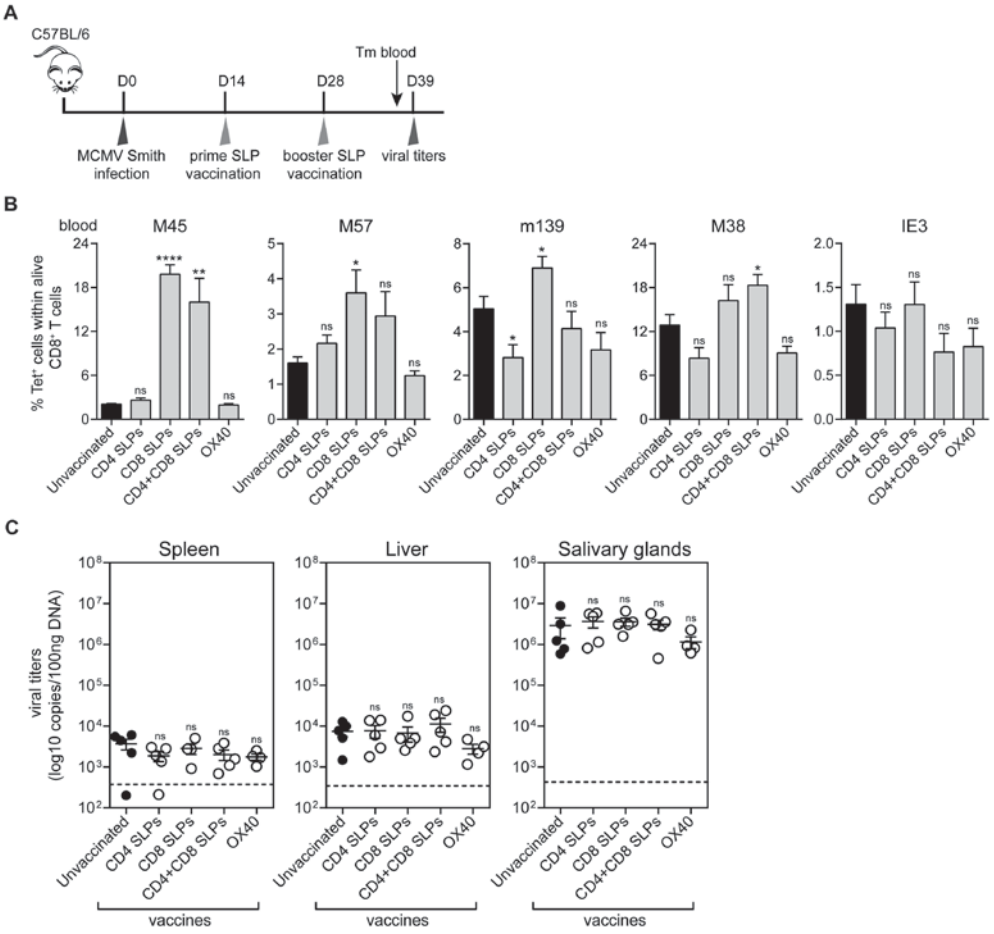


Figure 1. Combination of MHC class I and II epitope-containing SLP vaccines and OX40 agonistic mAb stimulation during booster vaccination exhibits no therapeutic efficacy against established MCMV infection. (A) C56BL/6 mice were infected (i.p.) with 5 x 10^4 PFU salivary gland-derived MCMV Smith strain. At day 14 post infection mice were divided in several groups and subsequently vaccinated (s.c. in the tail) in a prime-boost vaccination schedule with a combination of all i) MHC class I epitope-containing SLPs (CD8 SLPs), ii) MHC class II epitope-containing SLPs (CD4 SLPs) and iii) with both MHC class I and II epitope-containing SLPs (CD4 + CD8 SLPs). In all groups, agonistic anti-OX40 mAb (150 µg/ml) was provided (i.p.) during the booster vaccination. In parallel, unvaccinated (naïve) and anti-OX40 mAb only treated C57BL/6 mice were also challenged to serve as controls. **(B)** Percentages of virus epitope-specific $CD8^+$ T cell responses measured in blood at day 10 post booster vaccination. **(C)** At day 11 post boost mice were sacrificed and the viral genome copies were measured in spleen, liver and salivary glands by qPCR. The viral titers of individual mice are depicted (n = 4–5 mice per group). Dash lined indicate the detection limit as measured in naive mice. Experiment was performed twice with similar outcome. Statistical difference is indicated *P < 0.1; **P < 0.01; ****P < 0.0001; ns, not significant as compared to the unvaccinated group.

all tested groups (**Figure 1C**). In contrast, high viral titers were measured in the salivary glands, the major site of CMV persistence and replication. Interestingly, for all tested vaccine combinations no difference in the viral titers was observed indicating that none of the candidate SLP vaccines is potent to control persistent MCMV infection.

A possible explanation for this finding is that the viral infection already activated and boosted CMV-specific T cells to a level that could not be increased anymore by the tested SLP vaccines. The response that was most strongly enhanced was against the non-inflationary M45 $CD8^+$ T cell epitope. The observation that the increase in this response did not translate into reduced viral titers was to be expected since we previously showed that vaccine-induced M45-specific $CD8^+$ T cells exhibited minor cytotoxic function and prophylactic vaccine efficacy [13]. Viral $CD8^+$ T cell responses against the m139 and M38 inflationary epitopes were previously found by us to be the most efficacious in prophylactic settings [13], yet these were only slightly boosted following therapeutic vaccination. Amplifying these inflationary responses may be key to show therapeutic efficacy. This however raises a significant challenge as the inflationary T cells may already be maximally tickled by the (reactivated) virus. A particularly informative future direction is to stringently test whether the SLP vaccines increase only the virus-preexisting T cell responses or are also able to prime a new repertoire of anti-viral T cell responses. Moreover, it is of interest to test the efficacy of the SLP vaccines in immunocompromised settings (i.e irradiation or chemotherapy-induced myelosuppression). A therapeutic CMV vaccine that will succeed to boost the host's antiviral T cell immunity in e.g. allo-HCT settings has a great potential to complement or even replace costly T cell adoptive transfer treatments and high toxicity antiretroviral therapies currently used to contain CMV spread.

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Chapter 4

The spontaneous response to Human Cytomegalovirus immediate early protein 2 is focused on 5 highly immunogenic regions and predominantly comprises polyfunctional type 1 CD4$^+$ T cells

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ABSTRACT

Human Cytomegalovirus (HCMV) is an omnipresent pathogen that is associated with increased morbidity and mortality of immunocompromised individuals. Studies of T-cell immunity to HCMV primarily reflect anti-HCMV pp65 or immediate early (IE) antigen 1 (IE1) activity. Recent evidence highlights the importance of the major immediate-early 2 (IE2) protein, which is expressed early after HCMV infection and reactivation, for triggering the lytic cycle of HCMV infection. In this study, we assessed IE2 HCMV T-cell responses in the peripheral blood of 15 HCMV-seropositive and 6 HCMV-seronegative healthy donors using IE2 synthetic long peptide (SLP) pools and cytokine flow cytometry. The response was dominated by $CD4^+$ T cells as IE2-specific $CD8^+$ T-cell reactivity was measured in only 3 donors. Surprisingly, an IE2 HCMV specific T-cell response was detected in 3 out of 6 donors who were screened negative for HCMV antibody. Most of the donors recognised chiefly the $IE2_{351-434}$ residues, revealing a remarkably immunogenic area of the protein. Numerous HLA class I- and II-restricted IE2 T-cell epitopes were identified, which significantly advances the existing evidence. Functional characterization of the IE2 T-cell responses uncovered 5 highly immunogenic IE2 SLPs, which induced polyfunctional Th1 cytokine (IFN-γ^+/ TNFα^+/ IL-2^+) responses. The testing of these 5 highly immunogenic IE2 SLPs as potent candidates for T-cell based vaccine platforms aiming to inhibit CMV infection by targeting the expression of IE genes is warranted.

INTRODUCTION

The β-herpesvirus Human Cytomegalovirus (HCMV) is a widespread pathogen that infects a large proportion of the population worldwide. After primary infection, the virus establishes lifelong latency within its host. HCMV infection is usually benign, with no clinical disease manifestations in immunocompetent individuals but it can lead to severe complications in susceptible individuals including newborns, allograft recipients, and HIV patients [1-4].

Despite extensive research, a licensed vaccine intervention to treat or prevent CMV infection remains elusive [5,6]. Studies in patients concur on the importance of T cells for establishment of immunity and long-lasting memory against HCMV [7-11] Recently, T-cell based vaccines designed to induce $CD4^+$ and/or $CD8^+$ T-cell responses have received significant research attention. Evidence from various models of mouse CMV infection (MCMV) suggests that vaccine-induced T-cell responses may successfully control CMV replication without the aid of antibodies [12-14].

Many of the HCMV proteins are recognized by $CD4^+$ and $CD8^+$ T cells in healthy HCMV-seropositive subjects [15]. While T cells most frequently target the antigens pp65 and immediate-early 1 (IE1), the dominance and magnitude of the T-cell response to IE1 is associated with protection from HCMV-induced disease [16,17]. Interestingly, IE1 but also the major immediate-early 2 (IE2) protein are abundantly expressed and play a key role in initiating lytic cycle virus gene expression and replication [18]. Deletion of the IE1 gene partially inhibits viral DNA replication [19], whereas IE2 gene is indispensable for expression of early lytic genes and virus replication [20,21]. This makes the IE antigens promising HCMV immune targets for vaccine approaches that aim to attack viral infection at an early phase and prevent wide dissemination [22]. While vaccines against IE1 are already tested [23-26], less is known about the immune response to IE2. Characterization of IE2-specific T-cell immunity in protected healthy individuals will reveal its immunogenicity and may foster the development of vaccines targeting indispensable proteins of CMV.

In this study, we aimed to identify novel HLA class I- and II-restricted IE2 T-cell epitopes. We mined the Immune Epitope Database (IEDB) to record previously identified IE2 epitopes and examined IE2-specific T-cell reactivity, directly *ex-vivo*, in the peripheral blood of 15 HCMV seropositive and 6 seronegative healthy donors, using overlapping synthetic long peptides (SLPs) covering the entire IE2 amino acid sequence. Interestingly, IE2-specific T-cell reactivity could be detected directly *ex-vivo* in more than half of all screened individuals. The response was dominated by $CD4^+$ T cells and IE2-specific $CD8^+$ T-cell reactivity was found in only 3 donors. A number of previously identified but also new T-cell epitopes were found. Among all immunogenic SLPs there were five, which were frequently recognized among healthy donors, containing epitopes recognized by $CD4^+$ T cells and/or $CD8^+$ T cells, and considered to be useful in vaccine development.

RESULTS

Ex vivo detection of CD4$^+$ and CD8$^+$ T-cell responses against various HCMV IE2 peptide pools

To identify new HCMV IE2 MHC class I and MHC class II restricted T-cell epitopes a traditional high-throughput and well established screening strategy using overlapping SLPs was performed [27]. Importantly, the use of overlapping SLPs accelerates prediction of the exact peptide sequence recognized when a positive response is monitored. Synoptic illustration of the approach is presented in Figure 1.

A cohort of 21 healthy human volunteers (15 HCMV-seropositive and 6 HCMV-seronegative), aged between 34-65 years, were recruited irrespective of gender, ethnic and educational background criteria (Table 1). To capture virus-primed T-cell reactivity, the directly *ex vivo* detectable IE2-specific cellular response against each of the peptide pools, was evaluated based on the percentage of antigen-specific IFN-γ and TNFα producing CD4$^+$ and CD8$^+$ T cells.

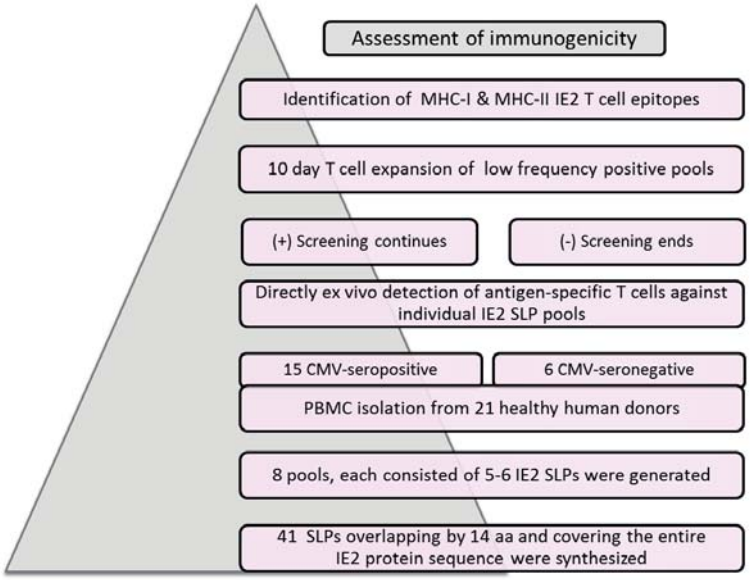

Figure. 1. Overview of the screening strategy to discover IE2-specific T cell epitopes. Forty-one '28-mer SLPs overlapping by 14 aa and covering the whole IE2 HCMV protein were synthesized. Eight pools each consisted of 5-6 SLPs. PBMCs from 21 healthy adult human individuals of which 15 were HCMV-seropositive and 6 HCMV-seronegative were screened by *ex vivo* ICS for direct recognition of IE2 SLP pools. Next, T cells that recognized peptide pools were cultured *in vitro* for 10 days and subsequently expanded T cells were analysed for IE2-specific CD4$^+$ and CD8$^+$ T cells using ICS and flow cytometric analysis. Positive responses against individual SLPs were confirmed and IE2-specific MHC class I and II epitopes were identified. A set of 5 IE2 peptides that were frequently recognized by CMV seropositive healthy donors was identified.

Table 1. Donor demographic characteristics and summary of the total pp65 and IE2 pool responses measured by *ex vivo* ICS. 21 healthy human volunteers were tested for $CD4^+$ and $CD8^+$ T-cell recognition of HCMV pp65 and IE2 SLP pools. Donor's demographic characteristics including the donor's number, age, gender (male = M/female = F) and HCMV status are depicted. HLA-A/B/C and HLA-DR/DQ/DP restrictions of each donor is indicated when available. The *ex vivo* positive (+) or negative (-) $CD4^+$ and/or $CD8^+$ T-cell responses against the IE2 and pp65 SLP pools are shown for each donor.

				Ex vivo T cell response		Class I			Class II	
Donor #	**Age**	**Gender**	**CMV status**	**pp65 CD4/CD8**	**IE2 CD4/CD8**	**HLA-A**	**HLA-B**	**HLA-C**	**HLA-DR**	**HLA-DQ**
1	65	M	+	+/+	+/+	2, 11	55, 35	3, 4	1, 15	5, 6
2	56	M	+	+/-	+/+	2, 3	44, 6	3, -		
3	62	M	+	-/+	+/-	2, 3	27, 35	2, 4	1, 4	
4	48	M	+	-/-	-/-	2, 3	7, 35	4, 7	1, 15	1
5	53	F	+	-/-	+/-	2, 3	7, 35	4, 7		
6	46	F	+	+/+	+/-					
7	64	F	+	+/+	+/-	1, 2	44, 56			
8	51	F	+	+/-	+/-					
9	56	F	+	-/-	-/-	2, 29	7, 44	7	4, 7	2, 8
10	51	M	+	+/+	+/+					
11	42	M	+	+/+	+/-					
12	44	F	+	+/+	+/-	2, 24	62, 38	3	4, 14	5, 3
13	45	M	+	-/-	-/-					
14	61	M	+	-/+	-/-					
15	55	F	+	-/-	+/-	2, 3	44, 62	3	4, 7	2, 3
16	54	M	-	-/-	+/-	31, 68	51, 39		11, 14	5, 7
17	34	M	-	-/-	+/-					
18	54	M	-	-/-	-/-	1, 3	39, 35	4		
19	53	F	-	-/-	-/-					
20	46	M	-	-/-	+/-	23, 68	51, 44	4		
21	58	M	-	-/-	-/-	2, 24	14, 14	6		

Interestingly, IE2-specific $CD4^+$ T-cell responses were detected in 11 of the 15 HCMV-seropositive donors and IE2-specific $CD8^+$ T cell responses were detected in 3 of these donors (Table 1, Figures 2A and B). Surprisingly, IE2-specific $CD4^+$ T-cell reactivity was also detected in 3 HCMV-seronegative donors (donor 16, 17 and 20). Peptide pool 6 ($IE2_{351-434}$) was the most frequently recognized immunogenic region (9/21 positive responders), followed by pool 1 ($IE2_{1-84}$) and pool 7 ($IE2_{421-504}$). Peptide pools 3, 4 and 5 ($IE2_{141-364}$) were barely recognized (Figures 2A and B). Notably, the magnitude of the *ex vivo* detected response was low and a phenotypic analysis was difficult.

Typical examples of HCMV-seropositive subjects (donor 1 and 6) and HCMV-seronegative subjects (donor 16) who displayed IFN-γ^+/TNFα^+ $CD4^+$ T-cell reactivity upon stimulation with IE2 peptide pools are provided in Figure 3.

In parallel, the response to the pp65 peptide pool was measured as this allowed us to compare and interpret the magnitude and impact of the IE2 T-cell immune response during HCMV infection. Although the level of the responses fluctuated among donors, most of the HCMV seropositive donors showed $CD4^+$ T-cell reactivity against both antigens (Table 1, Figure 2 and Figure 4A). The size of the pp65 $CD4^+$ T-cell responses, however, did not exhibit a direct correlation with the equivalent IE2 peptide pool responses (Figure 4B). Furthermore, while IE2-specific $CD8^+$ T-cell reactivity was infrequently detected, the pp65 SLP pool was recognized by $CD8^+$ T cells in 8 out of 15 seropositive donors and none of the seronegative donors (Figure 4C, Table 1 and Figure 2B).

Taken together, *ex-vivo* immune reactivity testing showed that the HCMV IE2 protein has a high propensity to activate $CD4^+$ T cells comparable to pp65. Especially, the amino acid sequence covered by the IE2 peptide pool 6 is recognized by the majority of the donors responding to IE2, revealing a remarkably immunogenic area of the protein. In contrast to pp65, the IE2 protein does not efficiently stimulate *ex vivo* detectable $CD8^+$ T-cell reactivity.

Identification of MHC class I- and II- restricted IE2 T-cell epitopes

Responding donor PBMCs were stimulated with each of the individual peptides present in the pool to confirm the *ex vivo* detected IE2-specific T-cell responses and to identify the peptides recognized in each of the recognized IE2 peptide pools. The responding T cells were first expanded for 10 days *in vitro*, then stimulated with each individual

Figure. 2. *Ex vivo* IFN-γ^+/TNFα^+ $CD4^+$ (A) and $CD8^+$ (B) T-cell responses measured in each donor following stimulation with different peptide pools. Donor's identification number (ID) and HCMV status are depicted. Responses to the pp65 and IE2 pools are also shown. Unstimulated samples (negative) response measured present the background response. Cellular cytokine responses >2 fold higher than the background responses were considered positive (light grey) and the peptide response was further evaluated. Positive $CD4^+$ T-cell responses were measured in 14 donors and positive $CD8^+$ T-cell responses in 3 donors.

A

Ex vivo IFN-γ⁺/TNFα⁺ cytokine producing CD4⁺ T cell responses

Donor #	pool 1	pool 2	pool 3	pool 4	pool 5	pool 6	pool 7	pool 8	negative (%)	pp65 pool	IE2 pool	CMV status
1									0.0034			+
2									0.0062			+
3									0.0053			+
4									0.0041			+
5									0.0008			+
6									0.0009			+
7									0.0019			+
8									0.0031			+
9									0.0008			+
10									0.0027			+
11									0.0013			+
12									0.0004			+
13									0.0041			+
14									0.0471			+
15									0.0009			+
16									0.0017			-
17									0.0007			-
18									0.0212			-
19									0.0046			-
20									0.0431			-
21									0.0562			-

<2 >2 >4 >10

B

Ex vivo IFN-γ⁺/TNFα⁺ cytokine producing CD8⁺ T cell responses

Donor #	pool 1	pool 2	pool 3	pool 4	pool 5	pool 6	pool 7	pool 8	negative (%)	pp65 pool	IE2 pool	CMV status
1									0.0096			+
2									0.0091			+
3									0.0009			+
4									0.0022			+
5									0.0025			+
6									0.0068			+
7									0.0022			+
8									0.0043			+
9									0			+
10									0.0086			+
11									0.0102			+
12									0.0201			+
13									0.0191			+
14									0.0101			+
15									0			+
16									0			-
17									0			-
18									0			-
19									0			-
20									0.0102			-
21									0.0051			-

<2 >2 >4 >10

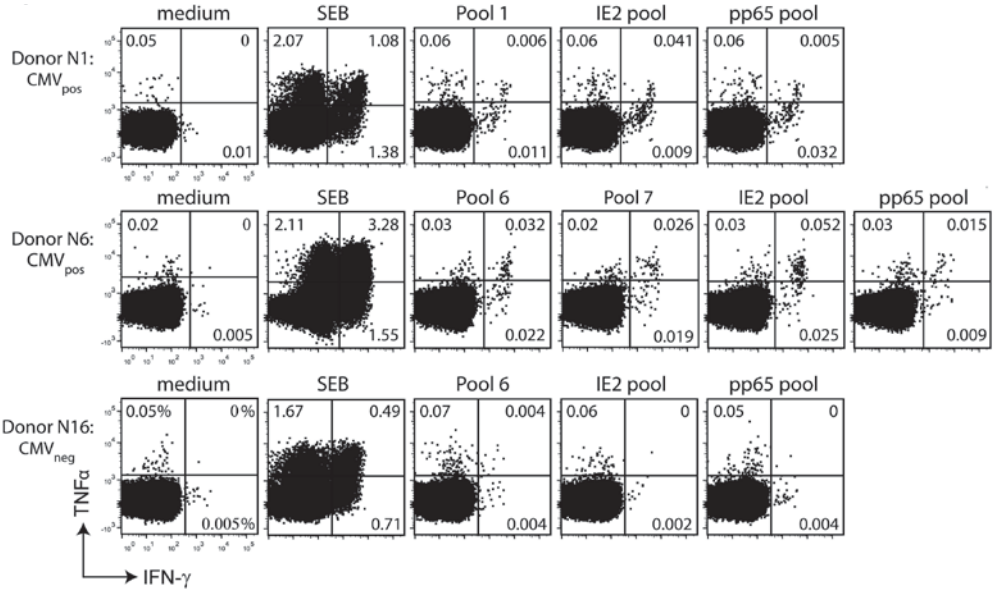

Figure. 3. CD4⁺ T-cell recognition of IE2 SLP pools in HCMV-seronegative and seropositive subjects. Donor's PBMCs were stimulated with 8 different IE2 peptide pools (each pool consists of 5-6 SLPs) and T-cell reactivity was measured by ICS assay and analysed by flow cytometry. Typical examples of *ex vivo* IFN-γ⁺/ TNFα⁺ CD4⁺ T-cell reactivity captured in HCMV-seropositive (donor 1 and 6) and HCMV-seronegative individuals (donor 16) are displayed. The background response was measured in unstimulated PBMCs (medium) and SEB served as positive control. The x-axis of the fluorescent intensity plots indicates percentages of IFN-γ and the y-axis TNFα cytokine production within CD4⁺ T cells.

SLP followed by polychromatic ICS. Peptide-specific CD4⁺ and CD8⁺ T-cell reactivity was detected in 11 and 3 donors, respectively, confirming the *ex vivo* measured responses. Representative examples of robust reactivity detected in the peptide-expanded PBMC of HCMV-seropositive and seronegative donor's T cells are depicted in Figure 5. Few donors displayed reactivity to one SLP but most of them reacted to 2 or more SLPs with donor 6 showing a CD4⁺ T-cell response to 13 different SLPs (Supplementary Table 2).

In the end, the *in vitro* culture revealed many more IE2-specific T-cell epitopes than appreciated from the *ex vivo* ICS where IE2 peptide pools were tested. A summary of all CD4⁺ and CD8⁺ T-cell responses measured against individual SLPs is provided in Tables 2 and 3, respectively.

The Immune Epitope Database (IEDB) site was used to search for all known MHC class I and MHC class II T-cells epitopes of the IE1 (UL123) and IE2 (UL122) HCMV viral proteins till June 2016. A chart depicting the exact position and HLA restriction of all the previously identified IE1 and IE2 T-cell epitopes (including the T-cell epitopes identified in this study) is given in Supplementary Fig. 2. Importantly, we were able to confirm most of the previously reported MHC class I- and class II-restricted IE2 specific

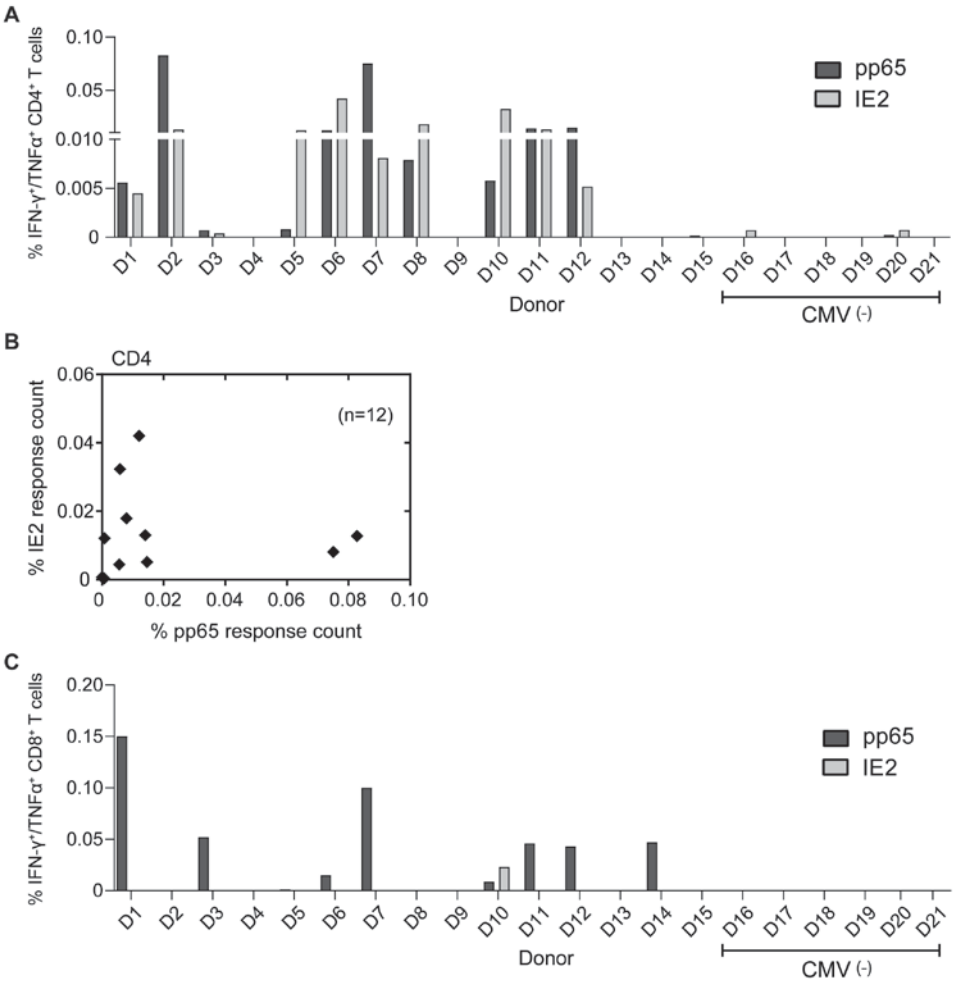

Figure. 4. Characterization of the total IE2 and pp65 HCMV-specific CD4⁺ and CD8⁺ T-cell responses measured in HCMV-seropositive and seronegative subjects. Donor PBMCs were screened by *ex vivo* ICS for direct detection of IE2 and pp65 pool specific CD4⁺ and CD8⁺ T-cell reactivity. In total 21 donors were tested, of whom 15 were HCMV-seropositive and 6 HCMV-seronegative **(A)** Percentages of the total pp65 and IE2 pool IFN-γ⁺/ TNFα⁺ CD4⁺ T-cell responses detected across the whole population sample are shown. **(B)** No correlation between the magnitude of the pp65 and IE2 pool CD4⁺ T-cell responses was observed in responder donors (n=12 and $p > 0.055$). **(C)** Frequency of IFN-γ⁺/ TNFα⁺ cytokine producing CD8⁺ T-cell responses measured *ex vivo* against the pp65 and IE2 peptide pools I (n=21). CD8⁺ T-cell reactivity against the IE2 peptide pool was detected only in donor 10, whereas pp65 specific CD8⁺ T-cell reactivity was measured in 8 out of 15 HCMV-seropositive donors.

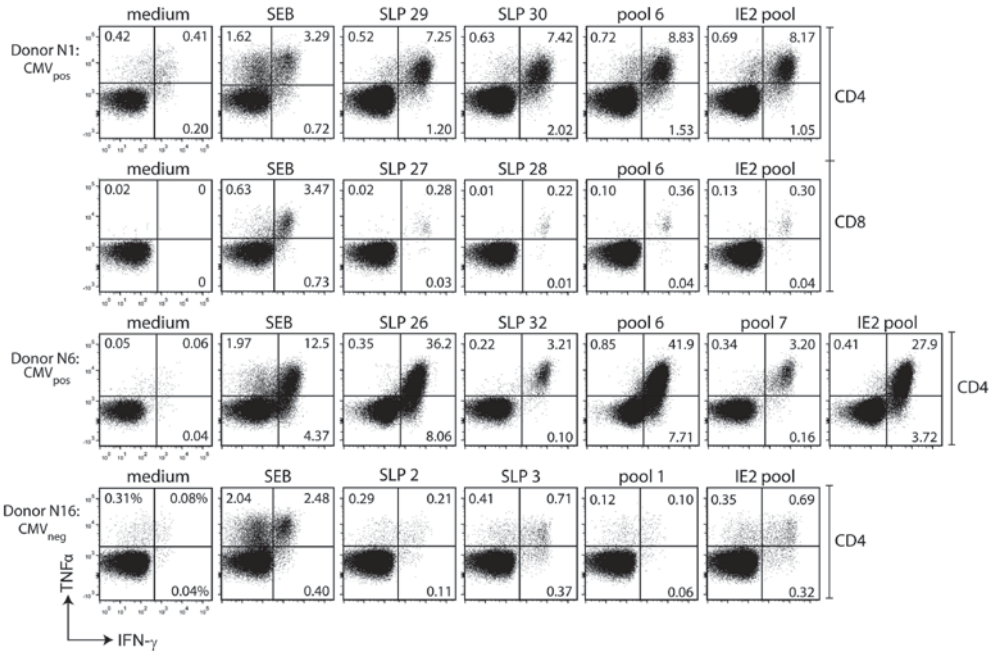

Figure. 5. CD4⁺ and CD8⁺ T-cell epitope identification in HCMV-seropositive and seronegative subjects. *Ex vivo* positive peptide pools for CD4⁺ and CD8⁺ T-cell reactivity PBMCs were expanded following 10 days *in vitro* culture and subsequently stimulated with each SLP contained in the relevant pools. Characteristic plots of T-cell epitope validation ICS results are shown for 2 HCMV-seropositive individuals (donor 1 and 6) and a HCMV-seronegative donor (donor 16). For donor 1, CD4⁺ and CD8⁺ T-cell reactivity to SLPs 29 and SLP 30 was measured indicating the presence of MHC class I and II epitopes. Responses to individual SLPs, peptide pools and the IE2 pool are depicted. As a negative control, unstimulated PBMCs (medium) were used and as a positive control SEB. The x-axis of the fluorescent intensity plots indicates percentages of IFN-γ and the y-axis TNFα cytokine production within CD4⁺ or CD8⁺ T-cell populations.

T-cell epitopes [28-37], but we also identified a total of 6 new CD4⁺ T-cell epitopes and 1 new CD8⁺ T-cell epitope.

Polyfunctional cytokine profiling of T-cell responses against the most immunogenic IE2 epitopes

The number of individuals responding to a peptide is a reflection of its immunogenicity. From the 41 individual IE2 SLPs there were 5 of which a unique sequence is present that was recognized by the CD4⁺ T cells (SLPs #3, 26, 28, 30 and 32) or CD8⁺ T cells (SLP #27/28) of the majority of tested donors (peptide *sequences marked in bold in* Table 2 and 3). As the functional traits of the T-cell response responding to such a peptide is an important determinant for its potential protective efficacy against HCMV infection, the cytokine profile of all the IE2-specific T-cell epitopes was examined. All selected peptide epitopes elicited a polyfunctional cytokine response as indicated by the percentage of T cells

Table 2. Summary of all identified IE2-specific CD4⁺ T-cell epitopes. CD4⁺ T-cell responses of individual IE2 pools (i.e. pool 1= p1) and the corresponding numbers of SLPs in each pool are depicted. SLP amino acid residues and sequence are also presented. The number of the responder donors and the frequency of the IFN-γ-producing CD4⁺ T cells measured in individual donors by ICS assay are displayed. Epitopes which have been described for the first time in this study are shown in red and all previously reported epitopes are shown in black.

SLP #		Peptide	Sequence	Responders/ donors tested	Responding donor	ICS (%)	Identified epitope	Identified epitope sequence	Reference
p1	1	$IE2_{1-14}$	MESSAKRKMDPDNPDEGPSSKVPRPETP	1/5	16	0.083			This paper
2		$IE2_{15-42}$	DEGPSSKVPRPETPVTKATTFLQTMLRK	1/5	16	0.13			This paper
3		$IE2_{29-56}$	**VTKATTFLQTMLRKEVNSQLSLGDPLFP**	4/5	2	0.57			This paper
					11	0.13			
					12	0.091			
					16	0.81			
4		$IE2_{43-70}$	EVNSQLSLGDPLFPELAEESLKTFEQVT	1/5	16	0.12			This paper
5		$IE2_{57-84}$	ELAEESLKTFEQVTEDCNENPEKDVLAE	1/5	16	0.06			This paper
p2	6	$IE2_{71-98}$	EDCNENPEKDVLAELGDILAQAVNHAGI	1/3	10	0.48	$IE2_{86-100}$	GDILAQAVNHAGIDS	(30), this paper
7		$IE2_{85-112}$	LGDILAQAVNHAGIDSSSTGPTLTTHSC	1/3	10	0.56	$IE_{286-100}$	GDILAQAVNHAGIDS	(30), this paper
10		$IE2_{127-154}$	VAVTNTPLPGASATPELSPRKKPRKTTR	1/3	12	0.15			This paper
p3	11	$IE2_{141-168}$	PELSPRKKPRKTTRPFKVIIKPPVPPAP	1/2	11	0.18			(30), this paper
12		$IE2_{154-182}$	PFKVIIKPPVPPAPIMLPLIKQEDIKPE	1/2	11	0.13			(30), this paper
p6	26	$IE2_{351-378}$	**TPNVQTRRGRVKIDEVSRMFRNTNRSLE**	4/8	1	0.50	$IE2_{356-370}$	TRRGRVKIDEVSRMF	(30), this paper
					5	0.16			
					6	42.82			
					20	0.12			
27		$IE2_{365-392}$	EVSRMFRNTNRSLEYKNLPFTIPSMHQV	4/8	1	0.54	$IE2_{383-397}$	PFTIPSMHQVLDEAI	(30), this paper
					5	0.10			
					6	16.4			
					10	0.32			
28		$IE2_{379-406}$	**YKNLPFTIPSMHQVLDEAIKACKTMQVN**	8/8	1	0.91	$IE2_{383-397}$	PFTIPSMHQVLDEAI	(30), this paper

Table 2. (continued)

SLP #	Peptide	Sequence	Responders/ donors tested	Responding donor	ICS (%)	Identified epitope	Identified epitope sequence	Reference
				5	1.01			
				6	13.9			
				7	0.31			
				10	1.12			
				15	1.62			
				17	0.78			
				20	0.10			
29	$IE2_{393-420}$	LDEAIKACKTMQVNNKGIQIIYTRNHEV	6/8	1	7.54	$IE2_{408-422}$	KGIQIIYTRNHEVKS	(30), this paper
				5	6.69	$IE2_{412-420}$	IIYTRNHEV	
				6	6.18			
				10	10.62			
				16	0.015			
				20	0.10			
30	$IE2_{407-434}$	**NKGIQIIYTRNHEVKSEVDAVRCRLGTM**	6/8	1	8.17	$IE2_{408-422}$	KGIQIIYTRNHEVKS	(30), this paper
				5	5.13	$IE2_{412-420}$	IIYTRNHEV	
				6	3.13			
				10	14.2			
				17	0.74			
				20	0.12			
p7 31	$IE2_{421-448}$	KSEVDAVRCRLGTMCNLALSTPFLMEHT	2/5	6	1.95	$IE_{2438-452}$	ALSTPFLMEHTMPVT	(30), this paper
				10	0.17			
32	$IE2_{435-462}$	**CNLALSTPFLMEHTMPVTHPPEVAQRTA**	4/5	3	0.23	$IE2_{438-452}$	ALSTPFLMEHTMPVT	(30), this paper
				6	3.19	$IE2_{443-457}$	FLMEHTMPVTHPPEV	
				7	0.27			
				10	6.98			

Table 2. (continued)

SLP #	Peptide	Sequence	Responders/ donors tested	Responding donor	ICS (%)	Identified epitope	Identified epitope sequence	Reference
33	$IE2_{449-476}$	MPVTHPPEVAQRTADACNEGVKAAWSLK	1/5	10	0.07			This paper
34	$IE2_{463-480}$	DACNEGVKAAWSLKELHTHQLCPRSSDY	1/5	10	0.59			This paper
35	$IE2_{477-504}$	ELHTHQLCPRSSDYRNMIIHAATPVDLL	2/5	2	0.3	$IE2_{493-507}$	MIIHAATPVDLLGAL	(33), this paper
				3	0.26			
p8 36	$IE2_{481-518}$	RNMIIHAATPVDLLGALNLCLPLMQKFP	1/3	6	0.28	$IE2_{493-507}$	MIIHAATPVDLLGAL	(34), this paper
37	$IE2_{505-532}$	GALNLCLPLMQKFPKQVMVRIFSTNQGG	2/3	6	1.53			This paper
				8	3.23			
38	$IE2_{519-546}$	KQVMVRIFSTNQGGFMLPIYETAAKAYA	2/3	6	0.11	$IE2_{523-537}$	VRIFSTNQGGFMLPI	(30), this paper
				8	3.17			
39	$IE2_{533-560}$	FMLPIYETAAKAYAVGQFEQPTETPPED	2/3	6	0.15	$IE2_{550-558}$	FEQPTETPP	(31-33, 35), this paper
				8	1			
40	$IE2_{547-574}$	VGQFEQPTETPPEDLDTLSLAIEAAIQD	2/3	6	0.059	$IE2_{550-558}$	FEQPTETPP	(31-33, 35), this paper
				8	1.27	$IE2_{558-572}$	PEDLDTLSLAIEAAI	
41	$IE2_{561-580}$	LDTLSLAIEAAIQDLRNKSQ	2/3	6	0.12	$IE2_{558-572}$	PEDLDTLSLAIEAAI	(30), this paper
				8	1.63	$IE2_{563-577}$	TLSLAIEAAIQDLRN	

Table 3. Summary of all identified IE2-specific CD8$^+$ T-cell epitopes. Characteristics of the detected CD8$^+$ T-cell responses are shown. For all the immunogenic MHC class I epitope containing SLPs, submer peptides were synthesized and their binding status was validated in an ICS assay. In total 2 IE2 MHC class I T-cell epitopes were identified.

SLP #	Peptide	Sequence	Responder/ donors tested	Responding donors	ICS (%)	Identified epitope	Identified epitope sequence	Reference
p1 3	$IE2_{29-56}$	VTKATTFLQTMLRKEVNSQLSLGDPLFP	1/5	2	0.38	$IE2_{42-50}$	KEVNSQLSL	(30, 36-39), this paper
p6 27	$IE2_{365-392}$	**EVSRMFRNTNRSLEYKNLPFTIPSMHQV**	2/7	1	0.29	$IE2_{382-390}$	LPFTIPSMH	This paper
				10	0.17			
28	$IE2_{379-406}$	YKNLPFTIPSMHQVLDEAIKACKTMQVN	2/7	1	0.23			
				10	0.13			

co-secreting all three, IFN-γ^+/ TNFα^+/ IL-2$^+$, antiviral cytokines. In conclusion, 5 highly immunogenic IE2 SLPs, capable of inducing polyfunctional type 1 cytokine T-cell responses (Figures 6A-F) were identified.

DISCUSSION

Controlling viral infection at an early phase may prevent wide dissemination and full blown infection. Reinforcement of the T-cell response to highly immunogenic antigens expressed immediately after infection could be key in the development of protective vaccines. The IE2 protein, which is expressed early after HCMV infection and reactivation, is likely to be a particularly valuable target for vaccine strategies against HCMV. In this study, we assessed the immunogenicity of the HCMV IE2 protein to identify amino acid stretches that were frequently targeted by T cells of healthy HCMV-protected donors. This led to the identification of numerous new and previously found [28-37] MHC class I- and II-restricted epitopes, to which T-cell reactivity could be detected directly *ex vivo*. The majority of the identified epitopes are located across the $IE2_{351-434}$ residues, stressing that this is a very immunogenic area. Furthermore, we could single out five particularly interesting SLPs that were frequently recognized by donors with different HLA restriction elements. These SLPs contained epitopes recognized by CD4$^+$ T cells and/or CD8$^+$ T cells [28,34-37] and could form the core of a IE2-targeting T-cell based vaccine.

Unexpectedly, IE2 specific CD4$^+$ T cell responses were detected *ex vivo* and in the 10 day cultures from 3 out of 6 HCMV seronegative donors. This is not uncommon among individuals who are immune against viral infections. For instance, HIV-specific memory T cells accompanied with a complete lack of humoral immunity has also been observed in seronegative highly HIV-1-exposed individuals [38,39]. This finding supports the idea that CMV exposure does not inevitably lead to persistent infection and that heterogeneity in infection susceptibility could be due to natural protective immunity to CMV. One potential contributor to this difference may be the size of the initial infectious inoculum as it dramatically influences the antibody and T-cell response to the virus [40,41]. Phenotypic and functional characterization of cellular responses in HCMV resistant individuals or donors, who elicit merely virus-specific T-cell reactivity but lack humoral immune responses, might reveal distinct T-cell features and biomarkers of protection. However, the very low response rate in this study did not allow us to determine the T-cell activation status and to distinguish between effector or memory T-cell populations.

Analysis of the T-cell response against IE2 revealed a predominance of *ex vivo* detectable CD4$^+$ T-cell mediated reactivity (11 out of 15 seropositive donors) whereas CD8$^+$ T-cell reactivity was seen in 3 donors only. This is consistent with a recent study [28] and not a technical problem since pp65-specific reactivity was readily detected *ex vivo* in the CD8$^+$ T-cell population in parallel. A concomitant induction of CD4$^+$

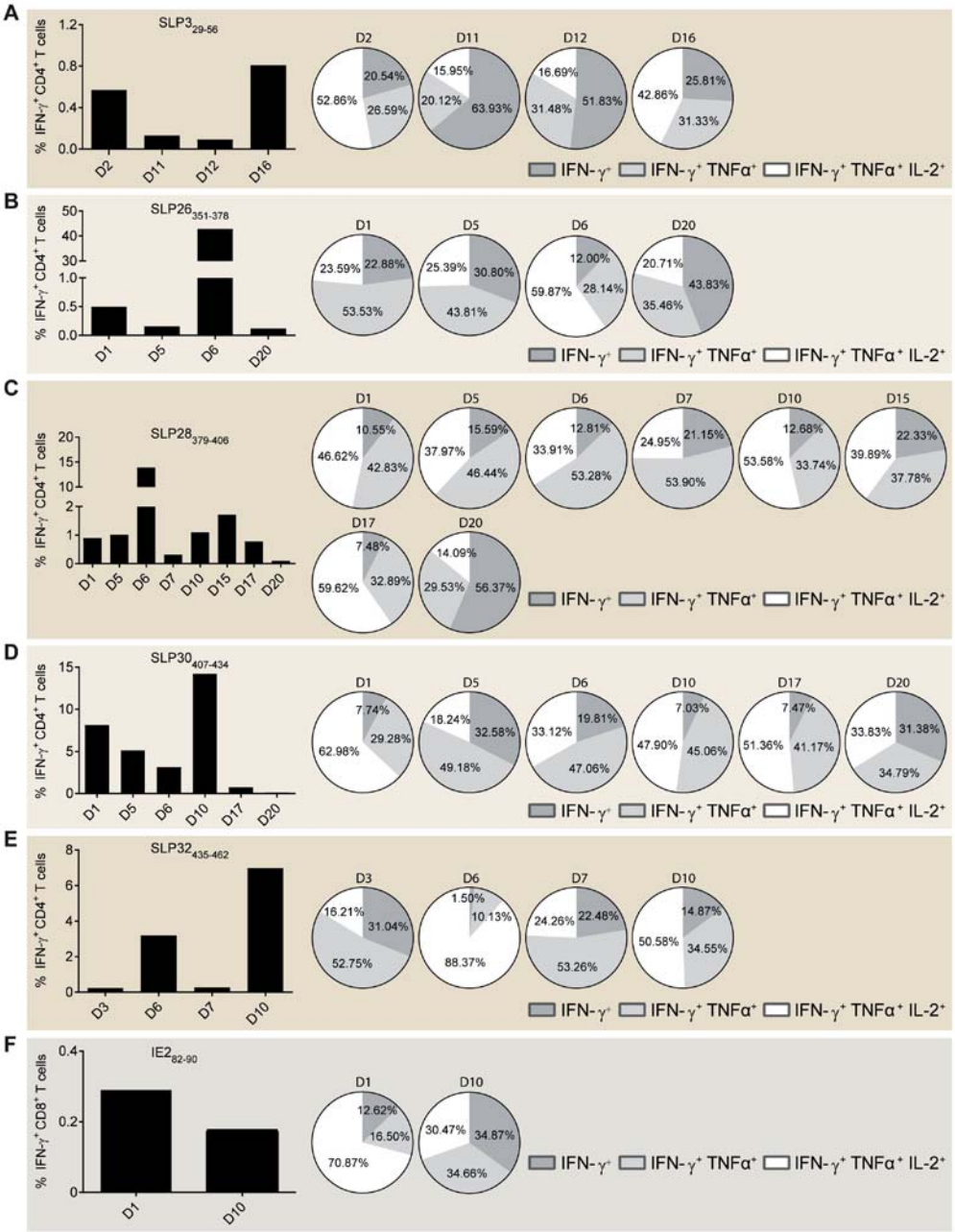

Figure. 6. Functional profiling of the most immunogenic MHC class I and II IE2 T-cell epitopes identified. (A-E) Percentages of IFN-γ$^+$ producing CD4$^+$ and **(F)** CD8$^+$ T cells elicited by responder donors following re-stimulation with relevant peptide. Cytokine production was measured in (*in vitro)* expanded donor T cells by polychromatic ICS assay. Pie charts show the percentages of the single (IFN-γ$^+$), double (IFN-γ$^+$/ TNFα$^+$) and triple (IFN-γ$^+$/ TNFα$^+$/ IL-2$^+$) cytokine producers within each antigen-specific CD4$^+$ or CD8$^+$ T-cell population and across all the responder donors.

and $CD8^+$ T-cells responses of a good magnitude, breadth and functional profile are crucial preconditions of vaccine efficacy [42-45]. Indeed, in the mouse model for CMV prophylactic vaccination with a mixture of SLPs comprising MHC class I- and II-restricted T-cell epitopes derived from different immunogenic proteins resulted in the best containment of virus dissemination after a challenge with lytic MCMV [12,14]. The detection of several immunodominant T-cell epitopes in the most immunogenic HCMV open reading frames (ORFs proteins) makes the translation of these findings in human clinical vaccine applications a valid and promising research target. Interestingly, while IE2 predominantly activates a strong *ex vivo* detectable $CD4^+$ T-cell response, the IE1 protein largely triggers an *ex vivo* measurable response to $CD8^+$ T cells [28]. These data would argue that an optimal SLP vaccine targeting the early phase of HCMV infection might require a mixture of IE1 and IE2 SLPs to achieve induction of a balanced $CD4^+$ and $CD8^+$ T-cell response. Of note, the SLP3 is shared between IE1 and IE2. Potentially the processing and presentation of this epitope is stronger in infected cells, however, whether such an epitope is more protective remains to be elucidated.

The development of efficient immunotherapeutic approaches against HCMV disease is an important research priority. The IE2 protein has never been explored in antibody or T-cell based vaccine platforms. We here showed that the spontaneous immune response to IE2 is characterized by highly polyfunctional $CD4^+$ T-cell reactivity that can be measured directly *ex vivo*. Moreover, the similar detection frequency of IE2- and pp65-specific T-cell reactivity validates IE2 as a strongly immunogenic HCMV target and identifies IE2 as a potential vaccine candidate. With that in mind, our study identified 5 highly immunogenic regions containing clusters of IE2 T-cell epitopes which could be exploited in vaccine development strategies but also in adoptive transfer therapies.

MATERIALS AND METHODS

Donors and sample

PBMCs from 15 HCMV-seropositive and 6 HCMV-seronegative subjects were isolated from buffy coats obtained after informed-consent (Sanquin, The Netherlands) by Ficoll (Ficoll-Amidotrizoaat, pharmacy LUMC) density gradient centrifugation. PBMC were cryopreserved in 80% Fetal Calf Serum (FCS; PAA laboratories) and 20% DMSO (Sigma) and stored in the vapour phase of the liquid nitrogen until further use. Handling and storage of donor PBMCs were performed according to the standard operating procedure (SOP) of the department of Medical Oncology at the Leiden University Medical Center [46].

Peptides and preparation of peptide pools

The IE2 amino acid sequence of the laboratory strain AD169 was used to synthesize 41 peptides of 28 amino acids in length and overlapping by 15 amino acids, together

covering the entire IE2 sequence, at the GMP-peptide facility of the LUMC. The purity (75-90%) of the synthesized peptides was determined by HPLC and the molecular weight by mass spectrometry. Additionally, a pool of peptides spanning the whole pp65 protein and a HLA A*0201-restricted $pp65_{495-503}$ (NLVPMVATV) peptide were included in the T-cell detection assays. The peptides were dissolved in DMSO at a stock concentration of 50 mg/ml and then further diluted to a concentration of 2 mg/ml and stored in -20°C. Subsequently, 8 peptide pools, each comprising either 5 or 6 SLPs, were made and stored under the same conditions as the individual peptides. All individual IE2 peptides, and IE2 peptide pools used in this study are listed in Supplementary Table 1.

Ex vivo detection of antigen-specific T cells

Direct *ex vivo* detection of HCMV-specific T cells was performed as described before [27]. Briefly, autologous plastic adherent monocytes were cultured in X-vivo 15 medium (Lonza) supplemented with granulocyte-macrophage colony-stimulating factor (800 IU/ml GM-CSF; Invitrogen) for 2-4 days at 37°C, 5% CO_2 in a humidified incubator. Then, cells were incubated for 5 h with each of the IE2 peptide pools, a pool of all IE2 peptides, a pool of all pp65 peptides or the pp65 short peptide (SP) in a final concentration of 50 µg/ml and for 24 h in the presence of poly (I:C) (25 µg/ml; Invivogen). A medium control sample served as negative control and *Staphylococcal* enterotoxin B (SEB; final concentration of 2 µg/ml; Sigma) was used as a positive control. Autologous donor PBMCs at a final concentration of 2-4 $\times$ 10^6 cells/well and Roferon (interferon-alpha 2a, 3 $\times$ 10^6 U/0.5 ml; Roche) were added to the cultured monocytes. An hour later, Brefeldin A (Sigma) was added and cells were incubated for 16-20 h (37°C, 5% CO_2) to allow the accumulation of intracellular cytokines. Subsequently, the cells were stained for live/dead marker (Yellow ARD, Life Technologies), CD3 (clone UCHT1), CD4 (clone SK3), CD8 (clone SK1), CD14 (M5E2), IFN-γ (B27), IL-2 (clone 5344.111), CD137 (clone 4B4-1), CD154 (TRAP1) all from BD and TNFα (clone MAb11), CD45RA (clone HI100) both from Biolegend according to SOP of the department of Medical Oncology. Samples were acquired at a LSRFortessa cytometer (BD Biosciences) and analysed using FlowJo-V10 software (Tree star). Flow cytometry gating strategies are shown in Supplementary Fig. 1. An immune response was considered positive when donor T cells produced both IFN-γ and TNFα and the response was ≥ 2 than the background (medium control).

In vitro expansion of low frequency antigen-specific T cells

To confirm the *ex vivo* detected T-cell responses against the IE2 SLP pools and to determine the reactivity to the individual SLPs within each pool, PBMC were subjected to a 10 day antigen-specific T-cell stimulation culture [47]. Briefly, donor PBMCs were thawed, resuspended in IMDM + 10% human AB serum (HAB, Life Technologies) and seeded in triplicate wells (3 ml/well) in a 6-wells plate (Costar). Tested peptide pools

were added to the PBMCs in a final concentration of 2.5 µg/ml and cells were cultured overnight at 37°C, 5% CO_2. The following day, 5 ng/ml IL-15 (Peprotech) and T cell growth factor (TCGF, Zepto Metrix) to a final concentration of 10% were added to the cultured T cells (referred to as bulk cultures) and cells were maintained for 10 days. In parallel on day 7, autologous donor PBMCs were thawed, seeded in 24-wells plates and monocytes were cultured for 2 days and loaded with indicated peptide pools and all individual SLPs that the pool contained in a concentration of 5 µg/ml. On day 10, bulk cultured T cells were harvested and $7 \times 10^5 - 1 \times 10^6$ cells/ml were added to the loaded monocytes. An hour later, Brefeldin A was added and cells were incubated for 16-20 h (37°C, 5% CO_2). The same positive and negative controls as in the *ex vivo* detection assay were also included. Subsequently, an intracellular cytokine staining (ICS) was performed and samples were measured and analysed as described above. An immune response was considered positive when donor T cells produced IFN-γ and the response was ≥ 2 than the background (medium control).

Statistical analysis

Statistical significance was calculated using the unpaired Student's t-test or ANOVA in GraphPad Prism software version 6 (GraphPad Software Inc., USA). Statistical significance levels were *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

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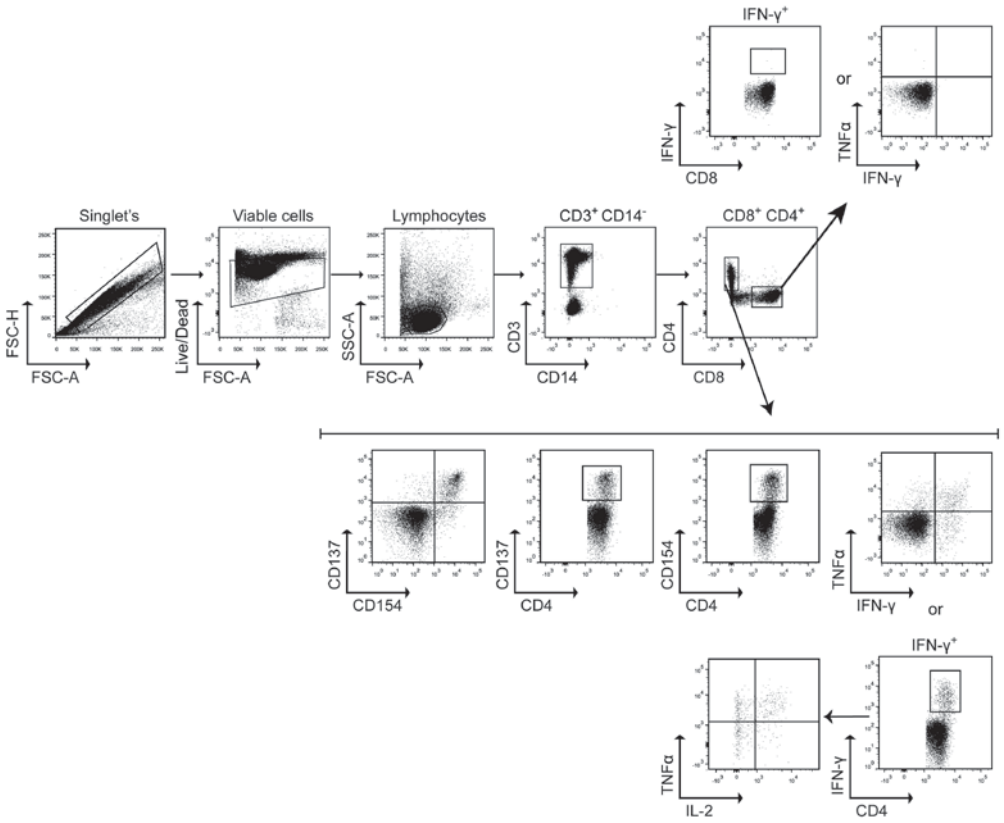
SUPPORTING INFORMATION

Supplementary Table 1. List of the IE2 SLP pools and individual SLPs used in this study.

	SLP #	Peptide residues (aa)	SLP Sequence
pool 1	1	$IE2_{1-14}$	MESSAKRKMDPDNPDEGPSSKVPRPETP
	2	$IE2_{15-42}$	DEGPSSKVPRPETPVTKATTFLQTMLRK
	3	$IE2_{29-56}$	VTKATTFLQTMLRKEVNSQLSLGDPLFP
	4	$IE2_{43-70}$	EVNSQLSLGDPLFPELAEESLKTFEQVT
	5	$IE2_{57-84}$	ELAEESLKTFEQVTEDCNENPEKDVLAE
pool 2	6	$IE2_{71-98}$	EDCNENPEKDVLAELGDILAQAVNHAGI
	7	$IE2_{85-112}$	LGDILAQAVNHAGIDSSSTGPTLTTHSC
	8	$IE2_{99-126}$	DSSSTGPTLTTHSCSVSSAPLNKPTPTS
	9	$IE2_{113-140}$	SVSSAPLNKPTPTSVAVTNTPLPGASAT
	10	$IE2_{127-154}$	VAVTNTPLPGASATPELSPRKKPRKTTR
pool 3	11	$IE2_{141-168}$	PELSPRKKPRKTTRPFKVIIKPPVPPAP
	12	$IE2_{154-182}$	PFKVIIKPPVPPAPIMLPLIKQEDIKPE
	13	$IE2_{169-196}$	IMLPLIKQEDIKPEPDFTIQYRNKIIDT
	14	$IE2_{183-210}$	PDFTIQYRNKIIDTAGCIVISDSEEEQG
	15	$IE2_{197-224}$	AGCIVISDSEEEQGEEVETRGATASSPS
pool 4	16	$IE2_{211-238}$	EEVETRGATASSPSTGSGTPRVTSPTHP
	17	$IE2_{225-252}$	TGSGTPRVTSPTHPLSQMNHPPLPDPLG
	18	$IE2_{239-266}$	LSQMNHPPLPDPLGRPDEDSSSSSSSSC
	19	$IE2_{253-280}$	RPDEDSSSSSSSSCSSASDSESESEEMK
	20	$IE2_{267-294}$	SSASDSESESEEMKCSSGGGASVTSSHH
pool 5	21	$IE2_{281-308}$	CSSGGGASVTSSHHGRGGFGGAASSSLL
	22	$IE2_{295-322}$	GRGGFGGAASSSLLSCGHQSSGGASTGP
	23	$IE2_{309-336}$	SCGHQSSGGASTGPRKKKSKRISELDNE
	24	$IE2_{323-350}$	RKKKSKRISELDNEKVRNIMKDKNTPFC
	25	$IE2_{337-364}$	KVRNIMKDKNTPFCTPNVQTRRGRVKID
pool 6	26	$IE2_{351-378}$	TPNVQTRRGRVKIDEVSRMFRNTNRSLE
	27	$IE2_{365-392}$	EVSRMFRNTNRSLEYKNLPFTIPSMHQV
	28	$IE2_{379-406}$	YKNLPFTIPSMHQVLDEAIKACKTMQVN
	29	$IE2_{393-420}$	LDEAIKACKTMQVNNKGIQIIYTRNHEV
	30	$IE2_{407-434}$	NKGIQIIYTRNHEVKSEVDAVRCRLGTM
pool 7	31	$IE2_{421-448}$	KSEVDAVRCRLGTMCNLALSTPFLMEHT
	32	$IE2_{435-462}$	CNLALSTPFLMEHTMPVTHPPEVAQRTA
	33	$IE2_{449-476}$	MPVTHPPEVAQRTADACNEGVKAAWSLK
	34	$IE2_{463-480}$	DACNEGVKAAWSLKELHTHQLCPRSSDY
	35	$IE2_{477-504}$	ELHTHQLCPRSSDYRNMIIHAATPVDLL
pool 8	36	$IE2_{481-518}$	RNMIIHAATPVDLLGALNLCLPLMQKFP
	37	$IE2_{505-532}$	GALNLCLPLMQKFPKQVMVRIFSTNQGG
	38	$IE2_{519-546}$	KQVMVRIFSTNQGGFMLPIYETAAKAYA
	39	$IE2_{533-560}$	FMLPIYETAAKAYAVGQFEQPTETPPED
	40	$IE2_{547-574}$	VGQFEQPTETPPEDLDTLSLAIEAAIQD
	41	$IE2_{561-580}$	LDTLSLAIEAAIQDLRNKSQ

Supplementary Table 2. Summary of IE1 and IE2 $CD4^+$ and $CD8^+$ T cell responses identified per donor. 21 donors were evaluated for $CD4^+$ and $CD8^+$ T-cell recognition of 28-mer IE2 peptides by ICS assay. Because the initial 85 amino acids of the IE1 and IE2 proteins are identical, the responses have been divided into three segments: shared IE1 and IE2 and unique IE2. The total number of the combined single and shared $CD4^+$ and $CD8^+$ T-cell responses measured is depicted. $CD4^+$ T-cell responses were confirmed in 11 donors, including 3 HCMV-seronegative (donors 16, 17 and 20). $CD8^+$ T-cell responses were detected in 3 HCMV-seropositive donors.

	IE1/IE2 shared segment*		Unique IE2 segment		IE2 total	
Donor #	**CD4**	**CD8**	**CD4**	**CD8**	**CD4**	**CD8**
1			5	2	5	2
2	1	1	1		2	1
3			2		2	
4						
5			5		5	
6			13		13	
7			2		2	
8			5		5	
9						
10			10	2	10	2
11	1		2		3	
12			1		1	
13						
14						
15			1		1	
16	5		1		6	
17			2		2	
18						
19						
20			4		4	
21						



Supplementary Figure 1. Gating strategy for intracellular cytokine staining to identify CD4$^+$ and CD8$^+$ IE2 T-cell responses. In sequential gating, cells were initially gated for singlet's (SSC-A vs. FSC-H) and then for viability (FSC-A vs. live/dead) and lymphocytes (FSC-A vs. SSC-A). Next lymphocytes were gated on CD3$^+$ CD14$^-$ to exclude monocytes and subsequently analysed for the expression of IFN-γ$^+$ producing CD4$^+$ and CD8$^+$ T cells. CD4$^+$ and CD8$^+$ T-cell subsets were further analysed for expression of TNFα and IL-2 cytokines and several activation markers (CD154, CD137).

Supplementary Figure 2. Chart of all the IE1 and IE2 HCMV T-cell identified epitopes.

Chapter 5

The level of pre-existing immunity determines the efficacy of MCMV-based vaccine vectors

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ABSTRACT

Cytomegalovirus (CMV) infection elicits long-lasting strong T cell responses. This property along with the ability to engineer the genome of this DNA virus, and the capacity of CMV to re-infect hosts despite pre-existing immunity has led to studies on the potential of CMV as vaccine vector. Pre-existing immunity, however, is known to reduce the infectious effect of CMV. Given the high variability in the magnitude of CMV-specific T cell responses in the human population, we here determined whether the level of pre-existing immunity influences the efficacy of CMV-based vaccine vectors in tumor mouse models. Immunization with CMV-based vaccines via the intraperitoneal or subcutaneous route eliciting strong vaccine-induced $CD8^+$ T cell responses that fully protected mice against lethal challenge with subcutaneous tumors expressing the model tumor antigen E7 of human papillomavirus type 16 (HPV16). Previous exposure to CMV via low dose or intranasal infection elicited weak CMV-specific immunity and did not result in an altered efficacy of the anti-tumor response following subsequent immunization with the CMV-based vaccine vector. In contrast, strong pre-existing immunity due to high dose CMV infection prevented vaccine-induced T cells to control tumor outgrowth. Together, our studies highlight the prospective of CMV-based vaccines, yet warrant that the level of pre-existing immunity should be considered when aiming for optimal efficacy of such vaccines.

INTRODUCTION

The role of the immune system in cancer eradication has been firmly established, and immunotherapy of cancer has set itself as a mainstream therapy among the conventional therapies comprising chemotherapy, radiotherapy and surgery. Immunotherapeutic approaches in which the inhibitory pathways are blocked to rejuvenate tumor-specific T cells (immune checkpoint blockade) and adoptive cell therapy (ACT) with tumor-specific T cells have shown efficacy in a significant number of patients [1,2]. Vaccination, is another promising form of immunotherapy that has been extensively explored, yet currently not many vaccines show clear clinical benefit. The latter has been contributed to the lack of inducing substantial long-lasting functional T cell responses able to overcome the immunosuppressive environment [3].

Cytomegalovirus (CMV) is unique among other viruses as this common betaherpesvirus is characterized by a strong induction of so-called memory inflation [4]. This process is characterized by virus-specific T cell responses and antibody levels that do not decline after primary infection but remain high or even increase over time [5,6]. Inflationary T cells are mostly effector-memory like, remain life-long polyfunctional, and are found in both lymphoid organs and tissues [7]. Despite the induction of host immune responses, CMVs are still able to re-infect [8]. Based on these properties, together with the ability to engineer the genome of CMV to attenuate the pathogenicity and/or to express foreign genes/epitopes that are of interest for vaccination, CMV-based vaccines have recently been explored. In mice and non-human primates the efficacy has been greatly valued against pathogens including SIV and Ebola [9,10]. Moreover, CMV-based vaccines containing tumor antigens have also shown anti-tumor efficacy in prophylactic and therapeutic settings [11-13].

Although CMV has the capacity to re-infect the host despite the presence of CMV-specific T and B cell responses that were elicited upon primary infection, experimental and epidemiological data indicated that pre-existing immunity is lowering the pathogenicity of the virus [14]. For example, mothers previously exposed to CMV have a lower prevalence of transmitting congenital CMV infection compared to unexposed mothers [15]. Given the promising results of CMV-based vaccine vectors to elicit responses with persistent immune activity against cancer, we studied here the efficacy of such vaccines on protection against tumor progression but also addressed the impact of pre-existing immunity. This is highly relevant in light with the large prevalence of this virus world-wide, and the fact that there is a great diversity in the level of pre-existing immunity based on the large difference in the magnitude of CMV-specific T cell responses in the population, ranging from nearly detectable to 40% of the memory compartment [16]. Here we found that CMV-based vaccines eliciting strong vaccine-induced $CD8^+$ T cell responses fully protected mice against lethal tumor challenge. Previous exposure to CMV eliciting weak immunity did not impact vaccine efficacy,

however, strong pre-existing immunity prevented vaccine-induced T cells to control tumor outgrowth. The results highlight the potential of CMV-based vaccines, yet warrant that the level of pre-existing immunity should be taken into account when an optimal vaccine-induced immune response is required.

RESULTS

Recombinant MCMV induces large inflationary T cell responses to inserted antigens

To assess the immune protection induced by CMV-based vaccine vectors against cancer, we first measured the kinetics of the vaccine-induced response after immunization with MCMV-based vectors containing tumor-specific antigens. Mice were infected i.p. with recombinant MCMV (MCMV-IE2-E7) expressing a single tumor-specific CD8 T cell epitope RAHYNIVTF ($E7_{49-57}$) derived from the E7 oncogene of HPV16. This epitope was fused to the carboxyl terminus of the MCMV IE2 gene because epitopes at this position are known to induce strong inflationary responses [17]. The non-inflationary response to the $M45_{985-993}$ epitope present in the recombinant MCMV and in MCMV-Smith showed the classical response of rapid expansion till day 8 followed by swift contraction and stable memory formation (Figure 1A and 1B). The E7-specific $CD8^+$ T cell response in the blood showed similar inflationary response kinetics as the response to the MCMV $M38_{316-323}$ epitope in the same vector or in wild-type MCMV (Figure 1A and 1B).

Characteristic of inflationary T cells is their effector-memory (EM) like ($CD44^+CD62L^{low}$, $CD127^{+/-}$, $KLRG1^+$ appearance [4]. In the acute phase of infection, the percentage of T cells with a naïve phenotype ($CD44^-$ $CD62L^+$ $KLRG1^-$) dropped to 50%, and remained stable during memory phase. In contrast, the percentage of EM-like cells within the total $CD8^+$ T cell population increased to 15-20%. Detailed analysis of the phenotype of antigen-specific $CD8^+$ T cell populations indicated that 40% of the M45-specific $CD8^+$ T cells showed an EM phenotype throughout the memory phase. M38-specific $CD8^+$ T cells displayed a gradual increment of the EM phenotype up to 70-80 %. The E7-specific $CD8^+$ T cells acquired a similar high level of EM-like cells, albeit already early after immunization, which remained high throughout the memory phase. Taken together, these data show that immunization with MCMV-IE2-E7 vector induces inflation of the E7-specific $CD8^+$ T cells with an EM-like phenotype.

The magnitude of inflationary T cell responses is influenced by the route of infection

To investigate the impact of the route of infection for the application of MCMV-based vaccine vectors, we compared the kinetics of the antigen-specific $CD8^+$ T cell responses upon i.p., i.n. and s.c. infection. Infection via i.p. and s.c. routes with MCMV-IE2-E7 elicited up to 3.5% non-inflationary M45-specific T cell responses within the total $CD8^+$ T cell pool at day 7 post infection, and during the memory phase of infection around 1% was stably lasting (Figure 2). However, i.n. infection induced only 0.1% M45-specific T cells,

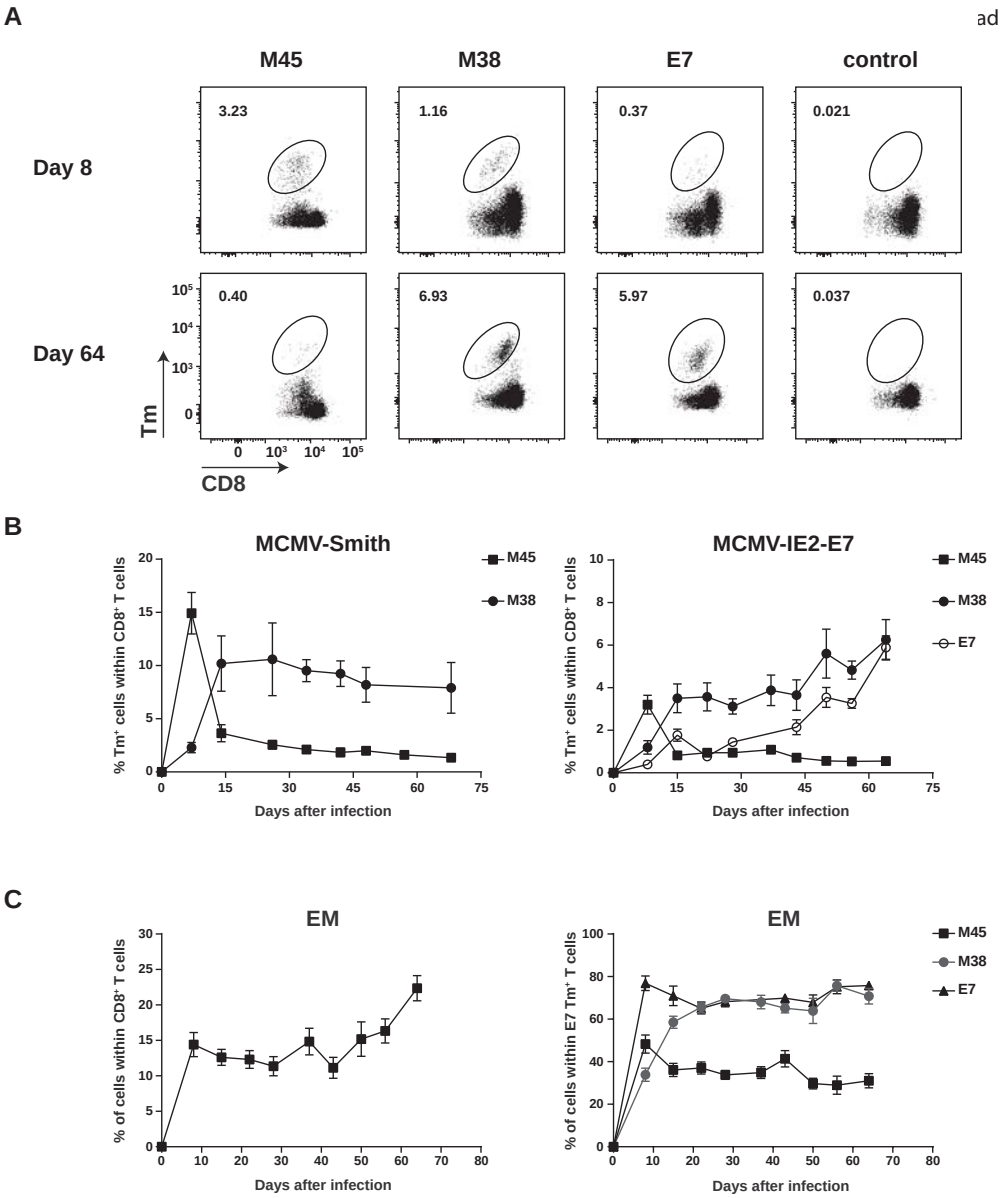

Figure 1. Recombinant MCMV vectors induce potent T cell responses resembling wild-type MCMV infection. Mice were infected with 1x10^5 PFU (i.p.) MCMV-Smith or MCMV-IE2-E7, and at the indicated times, the CD8 T cell responses were examined in blood. **(A)** Representative flow cytometry plots for MHC class I tetramer (Tm) staining to M45, M38 and E7$_{49–57}$ epitopes in blood of MCMV-IE2-E7 infected mice or naïve mice (control) during acute phase (day 8 post virus infection) and memory phase (day 64 post virus infection). Numbers represent the percentage of Tm$^+$ cells within the total CD8$^+$ T cell population. Flow cytometry plots show similar numbers of cells in each plot. **(B)** The frequency of MCMV- and tumor antigen-specific CD8$^+$ T cells in blood for each epitope, identified using MHC class I tetramers in mice infected with 1x10^5 PFU (i.p.) MCMV-Smith

or MCMV-IE2-E7. Data represents mean values ± SEM (n = 5-7 mice per group). **(C)** Percentages of EM cells ($CD44^+$ $CD62L^-$ $KLRG\text{-}1^+$ $CD127^-$) within the total $CD8^+$ T cells and within the $E7_{49–57}$ Tm^+ population. Data represents mean values + SEM (n = 7 mice per group), and are representative of two independent experiments.

and during the memory phase this response was close the detection limit. The M38-specific $CD8^+$ T cell response after both i.p. and s.c. infection was 1% and 3% of the total $CD8^+$ T cell population, respectively, at the acute phase of infection (Figure 2). During the persistent phase, i.p. infection resulted in a gradual increase of the M38-specific $CD8^+$ T cells (up to 6%), whereas the responses upon s.c. infection remained relatively robust, yet steadily declined. Similarly, E7-specific T cell responses raised gradually up to 6% upon i.p. infection while after s.c. infection responses remained high but did not inflate. Upon i.n. infection, the MCMV and E7-specific $CD8^+$ T cell responses were small (Figure 2). Taken together, these data show that i.p. and i.n. infection induces prominent T cell responses, albeit with different kinetics, while i.n. infection resulted in weak responses at best.

The strength of the vaccine-induced immune response determines the anti-tumor efficacy of MCMV-based vaccine vectors

Next, we investigated the influence of the route of infection and the resulting T cell response of the MCMV vectors on their vaccine efficacy in tumor challenge experiments. We prophylactically vaccinated mice with MCMV-IE2-E7 via the i.p., i.n. or s.c. routes and subsequently challenged them with TC-1 tumor cells expressing the HPV-16 E6 and E7 oncoproteins. While TC-1 tumors grew out progressively in all naïve mice, immunization with MCMV-IE2-E7 via the i.p. and s.c. route induced complete protection against TC-1

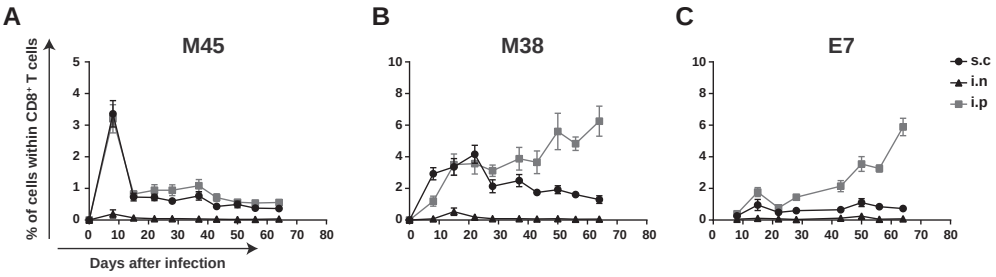

Figure 2. Impact of the route of administration of MCMV-IE2-E7 vaccination on the elicited CD8 T cell response. Mice were infected with $1x10^5$ PFU i.p., $1x10^5$ PFU i.n. and $5x10^5$ s.c. MCMV-IE2-E7 at day 0. The antigen-specific $CD8^+$ T cell responses were followed in blood for more than 60 days. Frequency of MCMV- and antigen-specific $CD8^+$ T cells to M45 **(A)**, M38 **(B)** and E7 (**C**) epitopes. Data represents mean values ± SEM (n = 7-8 mice per group). Data represents mean values + SEM (n = 7-8 per group), and are representative of two independent experiments.

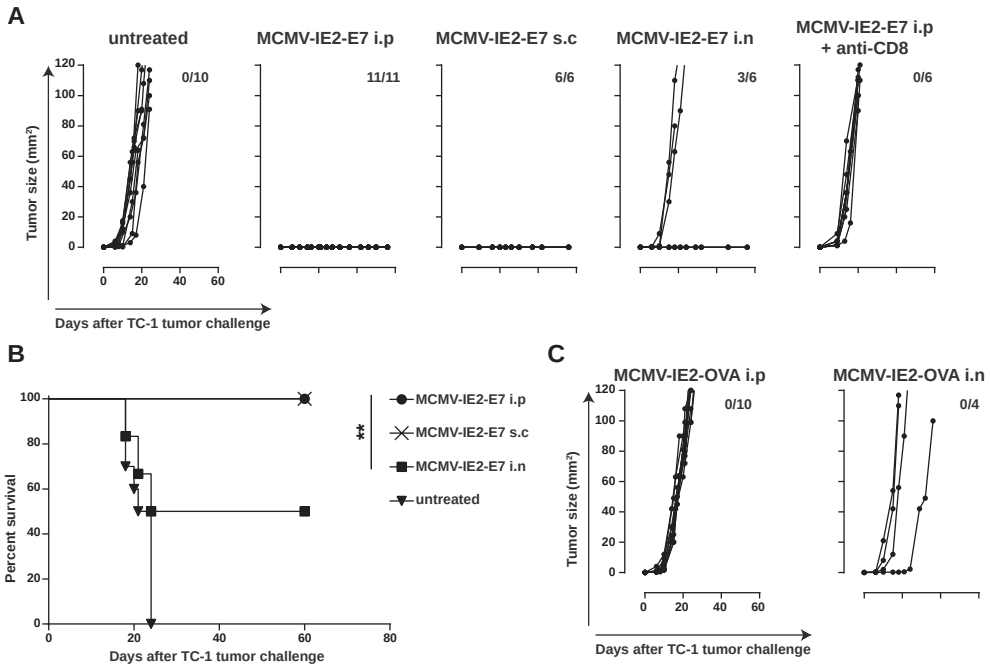

Figure 3. Different routes of infection with recombinant MCMV vaccine vectors induce various levels of protection against tumors. Mice were infected with $1x10^5$ PFU i.p., $1x10^5$ PFU i.n. and $5x10^5$ s.c MCMV-IE2-E7 at day -35 or kept uninfected (A-B). CD8 depleting antibody was given in the group of mice infected with MCMV-IE2-E7 via i.p. at the day -4. On day 0, all the mice were challenged with $1x10^5$ TC-1 tumor cells. The tumor outgrowth and survival of the mice was followed for 60 days. **A)** TC-1 tumor growth graphs of mice challenged with different routes of infection with MCMV-IE2-E7 compared to untreated mice. The number of tumor-free mice from the total mice is indicated above each graph. **B)** Survival graph of TC-1 tumor challenged mice shown in A. **C)** Tumor outgrowth kinetics of mice immunized with $5x10^5$ PFU MCMV-IE2-OVA via the i.p. or i.n. route. After 60 days, mice were challenged with TC-1 tumor cells. The number of tumor-free mice from the total mice is indicated above each tumor out growth graph.

tumor challenge (Figure 3A and 3B). However, intranasal immunization with MCMV-IE2-E7 only protected 50% of the mice (Figure 3A and 3B). Remarkably, all the mice which remained tumor free till day 60 were also protected against tumor rechallenge with unrelated C3 tumor cells expressing the E7 oncoprotein (data not shown), indicating the induction of immunological memory.

To assess if MCMV-based vaccine vector induced tumor protection is determined by $CD8^+$ T cells, we depleted these cells 4 days before TC-1 tumor challenge in mice infected earlier with MCMV-IE2-E7 via the i.p. route. Clearly, all the tumors grow out similar to untreated mice, suggesting the importance of vaccine-elicited tumor antigen-specific $CD8^+$ T cells (Figure 3A and 3B). To exclude possible bystander effects of virus infection on protection against tumor outgrowth, mice were infected with recombinant MCMV

not expressing the tumor antigen E7 (MCMV-IE2-OVA via the i.n. and i.p. route and challenged with TC-1 tumor cells. Infection with MCMV-IE2-OVA induced inflationary T cell responses against H-2kb-restricted $OVA_{257-264}$; SIINFEKL epitope and M38 and a non-inflationary response to the M45 epitope of the virus (Supplementary Figure 1). Importantly, TC-1 tumor cells grew out in all the mice immunized with MCMV-IE2-OVA via i.n. and i.p. route, suggesting that a vaccine-induced specific T cell response against antigens expressed by the tumor is required to induce protective immunity (Figure 3C). Thus, immunization with MCMV-IE2-E7 via the i.p. and s.c. routes of infection induces large tumor-specific T cell responses which are better capable of controlling tumor out growth.

To evaluate the impact of the magnitude of the elicited vaccine-specific $CD8^+$ T cell response by MCMV-based vaccine vectors in the same setting (i.e., route of infection), we immunized mice with different inoculum dosages (Figure 4A). Immunization of mice with 5×10^5 PFU MCMV-IE2-E7 via the s.c. route could protect all the mice from tumor outgrowth (Figure 4A, Group 2). However, immunization with 1000 fold less virus (500 PFU) could protect only 3 out of 12 mice (Figure 4A and 4B, Group 5). Similarly, i.n. immunization with 1×10^5 PFU MCMV-IE2-E7 resulted in 40% survival (Figure 4A, Group 3) whereas all the mice succumbed when a 200 fold lower vaccine dose (500 PFU) was provided (Figure 4A and 4B, Group 6). Also, i.p. infection with a lower dose led to loss of the vaccine-mediated protection (Figure 4A and 4B, Group 7).

To analyse the association between the strength of the vaccine-induced $CD8^+$ T cell response and tumor outgrowth, we determined the magnitude of the $CD8^+$ T cell response in blood. Infection with a high dose of MCMV-IE2-E7 via the i.p. and s.c. route induced stronger T cell responses against the E7 epitope compared to infection via i.n. route or lower dosages regardless of the infection route (Figure 4C). Taken together, these data suggest that the magnitude of the vaccine-induced $CD8^+$ T cell response is a key determinant for the efficacy of MCMV-based vaccine vectors against tumors.

The level of pre-existing immunity impacts the anti-tumor efficacy of MCMV-based vaccines

To investigate if pre-existing immunity can impact the induction of protective immunity by CMV-based vaccine vectors, we immunized mice with recombinant MCMV-IE2-OVA via the i.p. route. After 35 days, mice were inoculated with recombinant MCMV expressing the E7 tumor antigen via i.p, i.n and s.c. routes and subsequently challenged with TC-1 tumor cells (Figure 5A). Remarkably, tumor out growth was observed in all the mice which were previously infected with MCMV-IE2-OVA, despite the prophylactic vaccination with MCMV-IE2-E7 (Figure 5A and 5B), indicating that previous i.p. infection with MCMV hinders the anti-tumor immunity irrespective of the route of vaccine administration.

Next, we tested whether previous infections via routes that stimulate lower levels of MCMV reactivity can hamper the efficacy of the MCMV-based vaccine vectors.

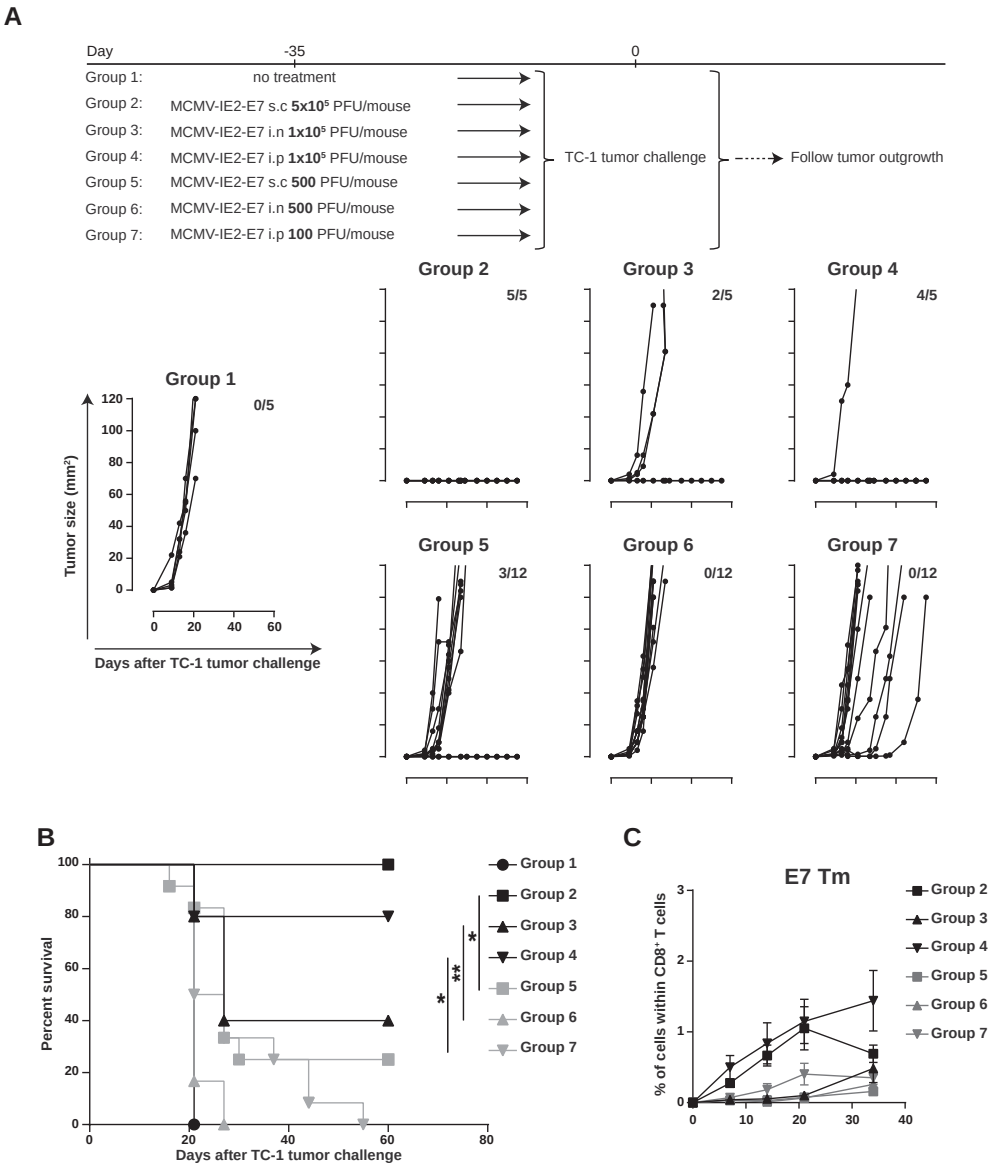

Figure 4. Efficacy of MCMV based vector vaccine depends on doses of immunization. A) Mice were infected with indicated doses and routes or kept uninfected as shown in the scheme of the experiment. After 35 days, all the mice were challenged with 1x10^5 TC-1 tumor cells. The tumor outgrowth and survival of the mice was followed for 60 days. TC-1 tumor growth graphs of mice challenged with different doses and routes of infection with MCMV-IE2-E7. The number of tumor-free mice from the total mice is indicated above each tumor out growth graph. **B)** Survival graph of TC-1 tumor challenged mice shown in A. **C)** Percentage of E7 Tm$^+$ CD8$^+$ T cells of the vaccinated mice shown in A and B.

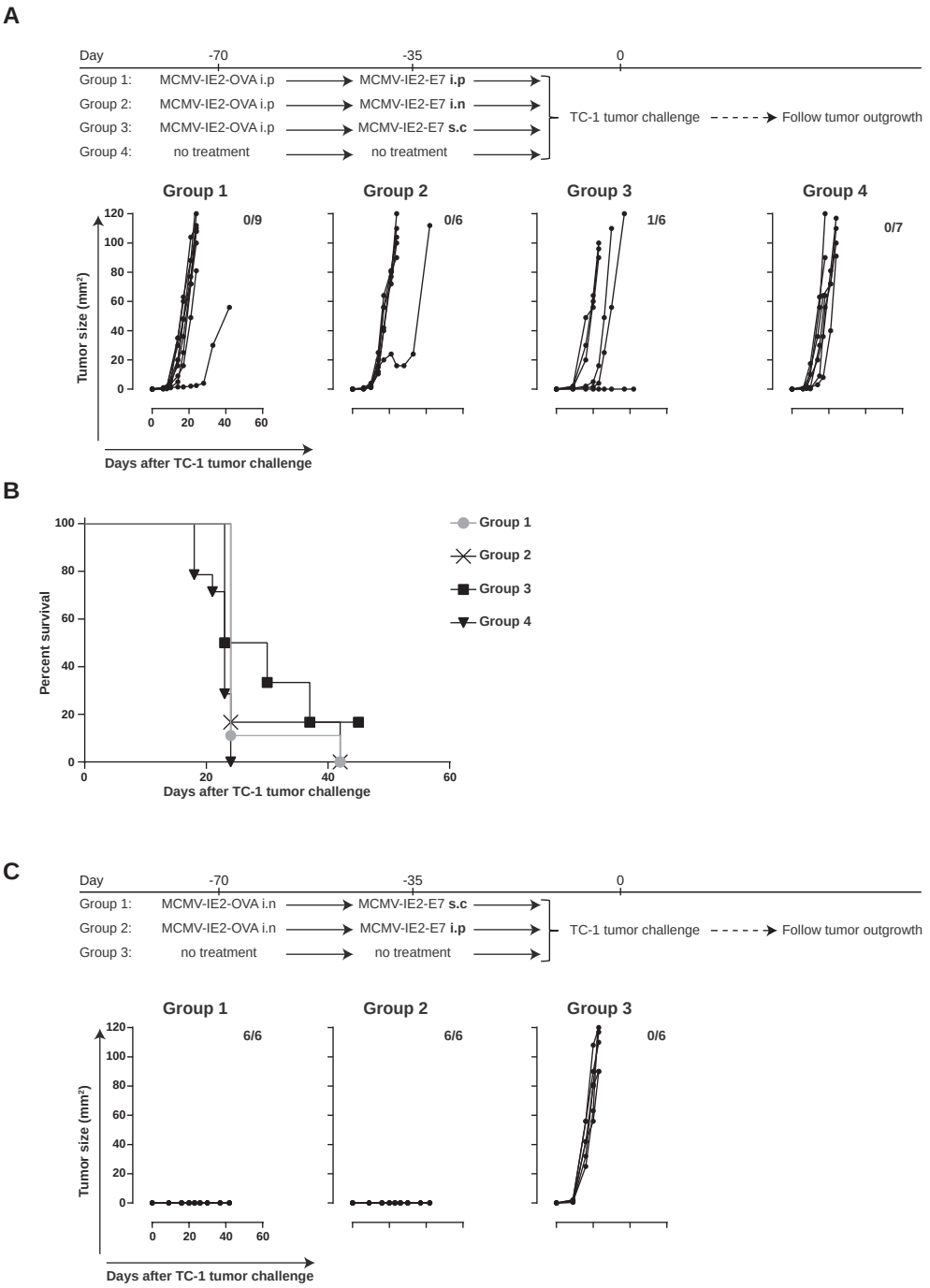

Figure 5. Previous MCMV infection can affect the efficacy of the anti-tumor efficacy of MCMV-based vaccine vectors. Mice were infected with 1x10^5 PFU i.p. or i.n. MCMV-IE2-OVA at day 0 or kept uninfected. After 35 days, the mice were challenged with 1x10^5 PFU MCMV-IE2-E7 via i.p. or i.n. route or with 5x10^5 MCMV-IE2-E7 via the s.c. route. All the mice were challenged with TC-1

tumor cells 35 days after the second infection. The tumor outgrowth and survival of the mice was followed for 60 days. **A)** and **C)** top: Scheme of the experiment. **A) and C)** below: TC-1 tumor outgrowth graphs of the mice. The number of tumor-free mice from the total mice is indicated above each tumor out growth graph. **B)** Survival graph of TC-1 tumor challenged mice shown in A.

Interestingly, mice infected with MCMV-IE2-OVA via the i.n. route and subsequently challenged with MCMV-IE2-E7 via the i.p. and s.c. route, did not develop tumor out growth but remained tumor-free (Figure 5C).

To address whether pre-existing MCMV immunity hindered the vaccine-induced $CD8^+$ T cell responses against MCMV and E7, we examined the antigen-specific T cell responses. Consistent with our earlier observation of the inflationary T cell response against E7, infection with MCMV-IE2-OVA via the i.p. route induced a strong and inflationary $CD8^+$ T cell response against $OVA_{257-264}$ epitope while via the i.n. route resulted in a low and non-inflationary response (Figure 6). The M38-specific $CD8^+$ T cell response was stronger following i.p. injection of MCMV-IE2-OVA than when given i.n. and after challenge with MCMV-IE2-E7 this response was less boosted than the M38-specific responses induced by i.n. injection followed by s.c. injection of the MCMV vectors. The E7-specific T cell response was higher after vaccination with MCMV-IE2-E7 via the i.p. and s.c. route in the mice previously infected with MCMV-IE2-OVA via the i.n. route than when MCMV-IE2-E7 was given s.c. following an initial infection with

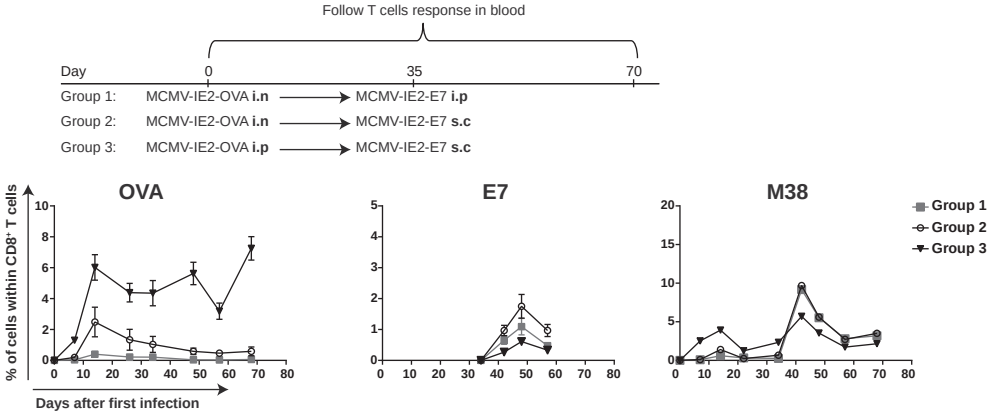

Figure 6. The strength of the T cell response induced by MCMV-based vaccine vectors depends on the levels of pre-existing immunity. Mice were infected with $1x10^5$ PFU i.p or i.n MCMV-IE2-OVA at day 0. After 35 days, the mice were challenged with $1x10^5$ PFU MCMV-IE2-E7 via i.p. or with $5x10^5$ MCMV-IE2-E7 via s.c. Blood was taken at different time points to follow the antigen-specific $CD8^+$ T cell response. Top: Scheme of the experiment. Below: Frequency of OVA, E7 and M38-specific CD8 T cells, identified using MHC class I tetramers. Data represents mean values ± SEM (n = 6 mice per group).

MCMV-IE2-OVA via the i.p. route. Together, these results suggest that the level of pre-existing immunity impacts the development of vaccine-induced $CD8^+$ T cell response induced by MCMV-based vector vaccine, and consequently the vaccine efficacy against tumor outgrowth.

DISCUSSION

Here we interrogated the impact of the pre-existing immunity on the efficacy of CMV-based vaccines against cancer. Such vaccines have the capacity to provide long-lasting robust tumor-specific T cell responses that protect against tumors. We found that the efficacy is however decreased in case of high-level pre-existing immunity, which warrants for the use of CMV-based vaccines without pre-screening for CMV immunity. Alternatively, recombinant CMV vectors that are still able to induce strong responses despite pre-existing immunity might be engineered in the future. Such vectors do exist for Rhesus macaques. RhCMV vectors containing SIV antigens provide strong immunity despite that the Rhesus macaques were all CMV positive. However, the used vaccine vector was unusual as it lead to the induction of unconventional $CD8^+$ T cells recognizing SIV epitopes presented by MHC class II and HLA-E molecules due to evasion of MHC class I and a limited tropism [18]. Testing HCMV-based vaccines with the same limited tropism leads however to conventional responses [19]. It remains to be determined whether efficacious HCMV vectors can be engineered for hosts with strong pre-existing immunity.

Inflating CMV-specific T cell populations are characterized by their EM-like phenotype and such responses seem to be qualitatively different with respect to their immediate effector functions and homing properties to tissues [20]. The latter is likely of importance to protect against viruses infecting mucosal areas, and may as well be crucial for clearance of tumors arising e.g. from epithelial or endothelial cells. Still, there may be settings in which vaccine-induced cells with an CM-like appearance, are beneficial. For example, the superior expansion capacity of CM-like cells upon challenge, may be crucial to fight-off systemic viral infections with highly replicating viruses [21].

In contrast to CMV-based vaccines, the majority of vaccines do not induce sustained high quantities of T cells with an EM phenotype. However, recently it became evident that memory inflation is not entirely restricted to CMV-induced responses. A relevant example of this are responses induced by adenoviral vectors. In mouse models, such vectors can lead to induction of inflationary responses resembling those induced by MCMV [22,23]. Interestingly, adenoviral vectors have been tested against human pathogens, including HCV, malaria and Ebola, and the vaccine-elicited responses are sustained over time and phenotypically resemble those induced by CMVs [24-26]. Whether CMV or adenovirus-based vaccines provide superior efficacy over each other in therapeutic settings remains to be determined.

Overall, our study shows the importance of determining the level of pre-existing immunity for CMV-based vaccines, and suggest to include stratification based on the magnitude of CMV-specific T cell responses in pre-vaccinated individuals. The route and dose of immunization may then even be adjusted to improve the vaccine efficacy. We conclude that further studies will be needed to clarify these questions with the anticipation to improve the efficacy of CMV-based vaccines.

MATERIALS AND METHODS

Mice

C57BL/6 mice were obtained from Charles River Laboratories (L'Arbresle, France). At the start of the experiments, mice were 6 to 8 weeks old. Mice were housed in individually ventilated cages (IVC) under specific pathogen-free conditions in the animal facility of the Leiden University Medical Centre (LUMC, The Netherlands). All animal experiments were approved by the Animal Experiments Committee of LUMC and were executed according to the animal experimentation guidelines of LUMC and were in compliance with the guidelines of Dutch and European committees.

Virus preparation, quantification, and infection

MCMV-Smith was obtained from the American Type Culture Collection (Manassas, VA, Unites States). Stocks were derived from salivary glands of infected BALB/c mice as described elsewhere [27]. Viral titers of virus stocks or infected tissues were determined by plaque assays as previously described [28]. MCMV-IE2-E7 (RAHYNIVTF) and MCMV-IE2-OVA (SIINFEKL) were generated as described [17].

Tumor challenge models and anti-tumor vaccination

The tumor cell line TC-1 (a kind gift from T.C. Wu, John Hopkins University, Baltimore, MD) was generated by retroviral transduction of C57BL/6 lung epithelial cells with the HPV16 E6/E7 and c-H-ras oncogenes [29] and cultured as previously described [30]. The tumor cell line C3 was developed by transfection of mouse embryonic cells with the HPV16 genome and an activated-ras oncogene and maintained as previously described [31]. The MC38-OVA tumor cell line is generated by a retroviral infection of the MC38 parental cell-line with PMIG/MSCV-IRES-GFP plasmid encoding cytoplasmic bound OVA [32]. Iscove's Modified Dulbecco's Media (IMDM) (Lonza, Basel, Switzerland) supplemented with 8% fetal calf serum (FCS) (Greiner), 2 mM L-glutamine (Life Technologies, Carlsbad, CA, Unites States), 50 IU/ml Penicillin (Life Technologies) and 50 µg/ml Streptomycin (Life Technologies) was used to culture tumor cell lines. Cells were cultured in a humidified incubator at 37°C and 5% CO_2. *Mycoplasma* tests that were frequently performed for all cell lines by PCR were negative.

Treatment schedule of each experiment is indicated in the respective figures and legends. Mice were infected with 1×10^5 PFU MCMV intraperitoneally (i.p.) or

intranasally (i.n.) or with 5×10^5 PFU MCMV subcutaneously (s.c.) unless otherwise mentioned. In tumor experiments, mice were inoculated subcutaneously in right flank with 1×10^5 TC-1 or 5×10^5 C3 tumor cells in 200 µl PBS containing 0.2% BSA on day 0. Tumor size was measured two times a week using a calliper. Mice were euthanized when tumor size reached >1000 mm^3 in volume or when mice lost over >20% of their total body weight (relative to initial body mass).

In vivo antibody usage

CD8 T cell depleting monoclonal antibodies (clone 2.43) were purchased from Bio X Cell (West Lebanon, NH, United States) and administered i.p. twice weekly (200 µg/mouse) for 2-3 weeks. CD8 T cell depletion was started 4 days before tumor challenge. Depletion was checked by staining for CD3 and CD8 followed by flow cytometric analysis.

Flow cytometric analysis of blood immune cells

For analysis of blood immune populations, blood cells were collected at different time points. Erythrocytes were lysed in a hypotonic ammonium chloride buffer. Cells were resuspended in staining buffer (PBS + 2% FCS + 0.05% sodium azide) and incubated with various fluorescently labelled antibodies against CD8 (clone 53-6.7), CD62L (clone MEL-14), CD44 (clone IM7), KLRG1 (clone 2F1), CD3 (clone 500A2), CD127 (clone A7R34). Antibodies were obtained from eBioscience (San Diego, CA, United States), BD Biosciences (San Jose, CA, United States) and Biolegend (San Diego, CA, United States). For dead cell exclusion, 7-Aminoactinomycin D (Invitrogen, Carlsbad, CA, United States) was used. To measure the MCMV-specific and tumor antigen-specific T cell response, the following class I-restricted tetramers were used: MHC class I Db restricted tetramers for the $OVA_{257-264}$ epitope (SIINFEKL) and HPV $E7_{49-57}$ (RAHYNIVTF), the MCMV epitope $M45_{985-993}$, and MHC class I Kb restricted tetramers for the MCMV epitopes $M38_{316-323}$, and were produced as described [33]. Samples were analysed with a BD LSRII or LSRFortessa flow cytometer, and results were analysed using FlowJo software (Tree Star, Ashland, OR, United States).

In vivo cytotoxicity assay

Splenocytes of naïve CD45.1 (Ly5.1) mice were isolated and loaded with MHC class I Db restricted-$E7_{49-57}$ or $OVA_{257-264}$ for 1 hour at 37°C at the final concentration of 1µg/ml. After extensive washing, cells which were pulsed with specific peptide or irrelevant peptide were labelled with high and low concentrations of CFSE (Invitrogen), respectively. Next, 5×10^6 peptide pulsed cells were pooled and injected i.v. via the retro-orbital route into the mice that have been vaccinated 70 days earlier with MCMV-based vaccines. After 24 hours, spleens of the recipient mice were isolated, stained for CD45.1, and subjected to flow cytometry. The cytotoxic capacity was calculated relative to naïve mice by using the following formula: {100 – ((percentage of pulsed peptide in infected

mice/percentage of unpulsed peptide in infected mice)/(percentage of pulsed peptide in naive mice/percentage of unpulsed peptide in naive mice)} × 100.

Statistical analysis

Statistical analyses were performed using GraphPad Prism (La Jolla, CA, Unites States). Survival data were analysed by Kaplan-Meier and the log-rank (Mantel-Cox) test. Statistical significance was determined by Mann Whitney. P-values of ≤ 0.05 were considered statistically significant.

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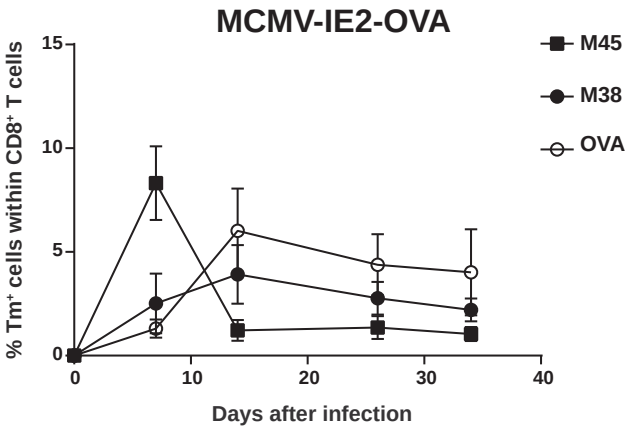
SUPPORTING INFORMATION



Supplementary Figure 1. Immunization with MCMV-IE2-OVA induces vaccine-specific CD8$^+$ T cell responses. Mice were infected with 1x10^5 PFU i.p. and i.n. MCMV-IE2-OVA. Frequency of antigen-specific CD8$^+$ T cells for the M45 and M38 epitopes of MCMV and of the inserted OVA antigen were identified using MHC class I tetramers. Data represents mean values ± SEM (n = 6 mice per group).

Chapter 6

Requirements for effective T cell-inducing vaccines against chronic viral infections

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ABSTRACT

For many years the focus of prophylactic vaccines was to elicit neutralizing antibodies but it has become increasingly evident that T cell-mediated immunity plays a central role in controlling persistent viral infections such as HIV, CMV, and HCV. Currently, a variety of promising prophylactic vaccines, capable of inducing substantial vaccine-specific T cell responses, are investigated in preclinical and clinical studies. There is compelling evidence that protection by T cells is related to the magnitude, breadth and quality of the T cell response as well as the type of the activated T cell subsets, and their characteristic homing properties, cytokine polyfunctionality, and metabolic fitness. In this review, we evaluate the main factors that determine the qualitative and quantitative properties of $CD4^+$ and $CD8^+$ T cell responses in the context of chronic viral disease and prophylactic vaccine development. Elucidating the mechanisms through which T cells mediate protection against chronic viral pathogens will facilitate the development of more potent, durable and safe prophylactic T-cell based vaccines.

INTRODUCTION

Our body is persistently exposed to a variety of pathogens present in the environment. The immune system is fortified with physical barriers and with diverse immune cell populations that play an integral role in protection from disease. Long-term immune protection is mediated by antigen-specific lymphocytes and antibodies that are formed upon pathogen entry. Memory B and T cells are numerically and functionally superior to their naïve-antigen precursors cells that are present prior to infection, and upon encounter with the same pathogen memory immune cells are able to induce a more rapid and powerful recall response (i.e., immunological memory) [1,2].

The majority of prophylactic vaccines against viral infections have focused on the induction of neutralizing antibodies. Indeed, potent antibody inducing vaccines against virally-induced diseases are available. Nevertheless, failures are demarcated in the case of providing long term efficacy and protection against certain complex chronic viruses. A series of studies in mice, non-human primates and humans provide evidence that effective prophylactic vaccines against chronic (low-level and high-level) replicating viruses (i.e. herpesviruses, HIV, HCV) should engage strong cellular T cell immunity [3,4,5]. The development of T cell-eliciting prophylactic vaccines has gained increasing attention despite that such vaccines are not always able to provide sterilizing immunity. The latter may relate to the fact that the immune mechanisms related to protection against chronic infections have not been clearly defined. There is still a lack of knowledge to be able to tailor vaccines to induce long-lasting $CD4^+$ and/or $CD8^+$ T cell responses of sufficient magnitude and phenotype that effectively contributes to pathogen clearance. Oftentimes, the memory cellular immune response provoked by vaccines is not sustained and frequently fades in time [6,7]. Elucidating the mechanisms through which antigen-specific T cell populations mediate long-term protection against viruses at body surfaces and (lymphoid) tissues remains an important goal, and will facilitate the development of more effective and safe prophylactic T cell-eliciting vaccines. Here we review determinants and mechanistic factors of T cell responses implicated in vaccine efficacy against chronic viral infections, and discuss how this knowledge can be utilised to maximize the possibility of creating effective vaccine platforms for persistent viral infections.

THE COMPLEXITY OF THE ANTIGEN-SPECIFIC T CELL RESPONSE DURING INFECTION

T cells acquire their activation signals as the interaction with the DCs becomes stable and reaches a duration of 12 h [8,9]. For proper activation of naïve $CD4^+$ and $CD8^+$ T cells, cognate antigenic signals through the TCR (signal 1), costimulatory signals (signal 2) and signals provided by inflammatory cytokines (signal 3) is required [10,11]. Expression

of particular chemokine receptors such as CCL19 and CCL21 by fibroblastic reticular cells (FRCs) enhance immune responses by stimulating the interactions between T cells and DCs during antigen presentation [12,13,14,15]. Additionally, the secretion of the CCL3 and CCL4 chemokines by activated DCs and $CD4^+$ T cells enhances $CD8^+$ T cell accumulation and help attract rare antigen-specific T cells [16,17]. The activation of T cells results in alteration of the expression of various molecules including integrins, selectins and chemokine receptors, resulting in modulating key intracellular signalling events that promote proliferation, differentiation and migration of T cells to inflamed tissues [18,19,20].

After resolution of the infection the majority (90-95%) of the effector T cells are eliminated by the immune system due to programmed cell death (PCD) and only a small diverse pool of memory cells remains [21,22]. Traditionally, memory T cells were classified into two major categories based on their proliferation capacity, phenotypic features and migration potential [23]. Specifically, effector-memory T (T_{EM}) cells are identified based on combined expression and/or lack of certain cell surface markers including $KLRG1^{hi}/CD44^{hi}/CD127^{lo}/CD62L^{lo}$. These cells have limited proliferation capacity upon TCR triggering, yet rapidly produce effector molecules and cytokines such as IFN-γ and TNF [24,25]. Central-memory T (T_{CM}) cells are distinguished by the expression of $KLRG1^{lo}/CD44^{hi}/CD127^{hi}/CD62L^{hi}$ surface markers, exhibit a superior proliferation capacity and produce cytokines that are directly associated with better secondary expansion such as interleukin (IL)-2. Secondary lymphoid organs are the main homing tissues of T_{CM} cells whereas T_{EM} cells are more dominantly present in tissues [26,27,28,29]. Both T_{CM} and T_{EM} cells can circulate, whereas a recently discovered new category of T cells present in tissues lacks migration capacities [30]. These cells, named tissue-resident memory T (T_{RM}) cells, permanently reside in peripheral tissues after an infection is cleared and are present in most organs and tissues. T_{RM} cells can be defined based on the expression of $CD62L^{lo}/CD44^{hi}/CD69^{hi}/CD103^{hi}$ surface markers, yet the composition of these markers depends on the tissue-specific cues [31,32,33]. Furthermore, a small subset of memory T cells exhibit advanced stem-cell like qualities and proliferation capacities compared to the conventional T cells [34]. These memory T cells, which were designated stem cell memory T cells (T_{SCM} cells), display a phenotype highly similar to naïve T cells (T_N cells), $KLRG1^{lo}/CD44^{lo}/CD127^{hi}/CD62L^{hi}/CD69^{lo}$, but they co-express stem cell antigen (Sca-1), the β chain of the IL-2 and IL-15 receptor (CD122, IL-2Rβ), and the chemokine receptor CXCR3 [34,35,36,37,38,39].

Notably, T cell immunobiology is fundamentally similar between human and mice, and the concepts of T_{CM}, T_{EM}, T_{RM}, and T_{SCM} cells are matching. Evidently, both live attenuated and synthetic or subunit vaccines are able to elicit T_{CM}, T_{EM}, and T_{RM} cells [30,33]. With respect to live attenuated vaccines, the vaccine-induced T cells subsets are in general similar to those subsets that develop upon infection [40]. However, the T cell subsets that develop upon immunization with synthetic or subunit vaccines is highly dependent on the route of administration and the adjuvant [41]. Whether sufficient

amounts of T_{SCM} can be generated with live attenuated or synthetic vaccines needs further exploration.

THE MAGNITUDE OF THE T CELL RESPONSE IS IMPORTANT FOR OPTIMAL PROTECTION

The magnitude of the viral-specific T cell responses is highly dictated by the infectious dose and route of infection [42]. Higher infectious dosages lead generally to higher peak values of effector T cells and correspondingly larger amounts of memory T cells in the circulation are found. However, if the immune system is overwhelmed and virus replication is uncontrolled this leads to immunopathology and subsequently this leads to exhaustion of T cells and poor memory formation [43].

Given the frequently observed correlation between the magnitude of T cell response and establishment of immunity during infections, in vaccination settings simply determining the magnitude of the vaccine-elicited T cell response may already serve as a predictor of efficacy. A number of studies have shown a direct association of the vaccine-elicited T cell response size and the ability for virus control [5,44,45,46]. Several parameters directly impact the magnitude of the vaccine-induced T cell response. Clearly, in case of live (attenuated) viruses the size of the initial dose of the inoculum correlates to the magnitude until a threshold is reached [47]. To reach the same level as compared to virulent virus, the inoculum sizes are, not surprisingly, higher for replication-deficient or single-cycle viral vectors. In case of synthetic vaccines, however, the saturation threshold may not be reached because of lack of sufficient inflammatory signals. However, recent discoveries in adjuvant development and synthetic (nano) particles provide promising results [48,49,50]. Besides the initial inoculum dosage, booster vaccine regimens impact evidently the magnitude of the T cell response, and are likely essential for the majority of vaccines including live vaccines [51].

MEMORY INFLATION AND THE MAGNITUDE OF RESPONSES TO RECOMBINANT VACCINES

An alternative mechanism which leads to an increased magnitude of memory T cells (especially $CD8^+$ T cells) is observed for certain specific responses following infection by CMVs - described as memory "inflation" [52,53]. Here, antigen-specific T cell responses to a subset of peptides show an unusual dynamic, whereby they expand gradually over time and are maintained at high frequencies as T_{EM} populations - as opposed to the classical expansion and contraction described above. These inflationary responses show maintained effector functions, tissue homing and can provide protection against challenge. Interestingly in the case of mouse cytomegalovirus (MCMV) there are two classes of response, a subset of inflationary responses and some which show classical

contraction and T_{CM} phenotype [52,54]. Memory inflation has also been observed for CMV-specific antibodies, which levels gradually incline over time [55]. Although the rules that determine which kind of memory have not been fully defined, it is clear that for inflation to occur viral antigen must persist long term and since CMV is reactivating from latency this condition is clearly fulfilled. Inflation appears to be restricted by antigen presentation, since peptides which are dependent on the interferon-inducible molecule LMP7 to form the immunoproteasome are not apparently presented long term, although such responses may be primed [56]. Modifying the context of the peptide can convert a classical response to an inflationary one [57].

Recombinant CMVs may provide important vectors for vaccines, although they are highly complex viruses, containing multiple immune evasion genes. In rhesus macaque experiments, the T cell responses induced against a recombinant CMV expressing SIV antigens include, in addition to $CD8^+$ T cell inflationary responses, responses mediated by class II-restricted $CD8^+$ T cells and also HLA-E restricted cells [58,59]. These unconventional responses likely arise because of the restrictions placed on normal antigen presentation by the attenuated CMV vectors used. More work is needed to identify which of these populations is critical for protection, and whether this additional protection – which can be very robust – is mediated via magnitude, function, breadth or targeting of particular peptides not normally presented.

Memory inflation is not restricted to CMV-induced responses. Similar phenomena have been seen with other viruses, including vaccine vectors. The most relevant of these are responses induced by adenoviral vectors. In mouse models, adenovirus-based vectors can lead to induction of inflationary responses which closely resemble those induced by CMV [60,61]. In this vaccine platform it is possible to generate inflationary responses against otherwise non-inflationary epitopes by removing the requirements for processing and presenting it in the form of a "minigene" [62]. Human adenoviral vectors are in use in a range of settings, including HCV, malaria and Ebola vaccines[4,63,64]. Although these do not show numerical inflation, the responses are sustained over time and phenotypically and functionally resemble those seen in mice and also those induced by CMVs [61].

THE BREADTH OF THE INDUCED IMMUNE RESPONSE IMPACTS ON PROTECTION

An increased breadth of the vaccine-induced T cell response has been found beneficial against many chronic viral pathogens [5,65,66,67,68]. Development of T cells with multiple antigen-specificities correlated with advanced capacity for virus control or even complete eradication during primary infection with HCV and superior protection upon reinfection [69]. Similarly, the breadth of Gag-specific responses was linked with low viremia as shown by analysing the $CD8^+$ T cell responses of untreated HIV-infected subjects [70].

Successful induction of potent and broad T cell responses has been reported with DNA plasmid vaccines [71,72] and adenovirus serotype 26 vector-based vaccines [73]. The latter approach incorporated a combination of subdominant and dominant epitopes of rhesus macaques SIV, known as a HIV equivalent in monkeys, in a prime-boost vaccination schedules. In parallel with these findings, synthetic long peptide (SLP) T cell based vaccines, which induce memory $CD8^+$ T cells, exhibited increased protection against mouse cytomegalovirus (MCMV), when vaccination was performed with combinations of several distinct SLPs. The efficacy of the SLP vaccines to protect against MCMV was mainly driven by the breadth of the antigen-specific T cell response rather than the magnitude of the individual SLP vaccine-induced T cell responses [5]. These findings indicate that cytotoxic $CD8^+$ T cells with a broad repertoire of specificities are more capable for effective killing of virus infected cells than T cells of a single specificity. Possible explanations are that multiple encounters with T cells of diverse specificity results directly in enhanced killing of virus-infected cells or limits immune escape mechanisms. Moreover, an increase in epitope recognition may also contribute to protection against infection with heterologous viruses via cross-reactive responses [74]. Although the underlying mechanisms are still unclear the importance of the immune repertoire diversity should be taken into account while designing prophylactic T cell-based vaccines. As a consequence selection of the correct antigens that will steer the immune response at the correct direction is a very critical step of the vaccine development process. In this respect, it also of importance to mention that competition of antigens is apparent [5], warning that antigen selection is not simply the more the better. Overall, epitope-specific T cell repertoires elicited upon vaccination might serve as an evidence of vaccine efficacy, which will apply to many infections and not be limited to chronic viruses. Furthermore, not all antigen-specific T cell populations have the same efficacy. For example, T cell populations specific for CMV antigens that provoke inflationary responses show superior protective capacity [5]. Thus antigens provoking the most effective antigen-specific T cell populations should be selected to include in designing vaccine vectors or synthetic vaccines.

While both magnitude and breadth of the T cell response is of importance there is no direct association between protection and the frequency of the T cells in the circulation [75]. In mouse models it is becoming increasingly clear that, depending on the route of infection, T cells present in the mucosal or in the tissues (Tem and/or Trm) control the infection, and sufficient numbers are required [33]. Note, however, that besides the quantity and breadth also the quality of the T cell response is of crucial importance.

CYTOKINE POLYFUNCTIONALITY OF T CELLS IS AN IMPORTANT PARAMETER FOR VACCINE EFFICACY

Cytokine production is an important effector mechanism of T cell mediated immunity. Upon most viral and bacterial infections protective immunity consists of $CD4^+$ and $CD8^+$ T cells with a Th1 cytokine profile that is characterized by (co-)production of IFN-γ, TNF and IL-2 [76]. IFN-γ and TNF are pleiotropic cytokines with direct anti-viral properties [77,78,79]. Their receptors are broadly expressed, and signal via distinct pathways, which may explain why reciprocal production of IFN-γ and TNF leads to synergistic actions [80]. The predominant assessment method of vaccine-induced responses is the frequency of IFN-γ producing T cells. However, there are many examples showing that the magnitude of the IFN-γ secreting T cell response is not a sufficient immune correlate of protection. Single positive IFN-γ producing T cells can comprise a relatively large fraction of the total cytokine-producing $CD4^+$ and $CD8^+$ T cell population after immunization. Such T cells have a limited capacity to be sustained as memory T cells and are at the final stage of T cell differentiation [81]. Vaccines that elicit a high proportion of single, IFN-γ producing T cells would not be likely protective. On the other hand, studies characterizing vaccine elicited T cell responses against HIV, CMV and HBV revealed a strong correlation between the protection level of the vaccine regiments and their capacity to induce high frequencies of polyfunctional T cells (e.g. coproducing IFN-γ, TNF and IL-2 [4,82,83,84,85]. Similar results have been described in the course of infection with hepatitis C virus, CMV, influenza or M. tuberculosis [4,86,87,88,89]. Importantly, some of these studies showed that measuring the magnitude of IFN-γ producing $CD4^+$ and $CD8^+$ T cells alone was not sufficient to predict protection, and provided evidence that measuring the quality of the $CD4^+$ and $CD8^+$ T cell response, *vis-à-vis* polyfunctional T cells, is required.

IL-2 signals through a trimeric receptor comprised of CD25 (IL-2Rα), CD122 (IL-2Rβ) and the γ_c [90]. CD25 is not constitutively expressed but instead is transiently upregulated upon activation following exposure to certain inflammatory cytokines such as IL-12 [91]. Evidence examining he role of $CD4^+$ and $CD8^+$ T cells in HIV infected showed increased levels of T cells expressing IL-2 and IFN-γ in long-term non-progressors, or those on anti-retroviral treatment, but increased levels of T cells producing IFN-γ only in individuals with high viral loads (processors)[92]. Although IL-2 has no direct anti-viral function, it promotes proliferation and secondary expansion of antigen-specific T cells [93,94,95,96,97,98]. The ability of T cells to secrete IL-2 also relates to superior survival properties of these cells [81,99]. Additionally, IL-2 increases expression of the effector proteins perforin, granzyme B and IFN-γ that are all important for mediating cytolytic function [100,101]. IL-2 signals may also enhance NK cell activity that could contribute to the early control of infection following challenge [81,102,103,104,105].

In summary, the efficient vaccine protection medicated by $CD4^+$ and $CD8^+$ T cells is moderated by multiple mechanisms. First, $CD4^+$ and $CD8^+$ T cell have the highest

secretion rate of IFN-γ per-cell. Second, T cells that secrete both IFN-γ and TNF have enhanced effector activity compared with T cells that secrete IFN-γ alone. And third, autocrine IL-2 production promotes the secondary expansion of memory T cells, and is linked to other beneficial properties. Therefore, IL-2, TNF and IFN-γ comprise a simple set of cytokines that can be used to define the quality of the vaccine-elicited response against specific infections that require T cells for protection. As will be discussed hereafter, one way to improve the polyfunctionality of vaccine-induced T cells is by targeting of T cell costimulation It remains nevertheless necessary to better understand how the cytokine polyfunctionality is regulated during the programming of $CD4^+$ and $CD8^+$ T cell responses. Further dissecting these issues might provide fundamental insights into how T cell responses are controlled and may reveal potential strategies for superior vaccine-mounted T cell responses.

IMPROVING VACCINATION BY TARGETING T CELL METABOLISM?

The transition of naïve T cells to an active effector cell and to the formation of memory cells involves dynamic and coordinated metabolic modifications [106]. This reprogramming of the cellular metabolism is not a consequence of activation but is linked to the differentiation and activation processes, and reflect the fuel and substrates necessary to support the differentiation stages of a T cell [107,108]. Both naïve T cells and memory T cells rely primarily on oxidative phosphorylation (OXPHOS) and fatty acid oxidation (FAO) for fuel. This reflects the low level yet persistent need for energy as such cells are long-lived. Effector T cells on the other hand have extraordinarily high energetic and synthesis demands. These cells have enhanced glycolysis and employ the mitochondrial tricarboxylic acid (TCA) cycle to support their demand for *de novo* proteins, lipids and nucleic acids synthesis. It is becoming increasingly clear that metabolic reprogramming plays a critical role in T cell activation, differentiation and function. The distinct metabolic demands of different T cell subsets make them exquisitely sensitive to pharmacologic inhibitors of metabolism [109]. The different metabolic requirements of T cell subsets provide us with a promising therapeutic opportunity to selectively tailor (vaccine-induced) immune responses. Thus, targeting T cell metabolism affords the opportunity to additionally regulate vaccine-induced responses.

Upon T cell activation, there is an immediate uptake of amino acids such as glutamine and leucine that is critical for proper metabolic reprogramming. This is accompanied with the upregulation of amino acid transporters involved in glutamine (SLC1A5) and leucine (SLC7A5/SLC3A2 heterodimer) [110,111]. It is essential that these processes operate well to avoid suppression of the differentiation of T_H1 effector T cells while maintaining Treg differentiation. Whether this can be improved pharmacologically *in vivo* remains however to be further examined.

CD28-mediated costimulation leads to PI3K-dependent upregulation of surface GLUT1 to facilitate enhanced glucose influx [112]. This upregulation of GLUT1 is critical for T cell function, as genetic deletion of GLUT1 markedly inhibits effector T cells [113]. Concomitant with increased expression of glucose transporters is the upregulation of key glycolytic enzymes [114]. This metabolic reprogramming occurs simultaneously with T cell activation and is facilitated by mTOR [115]. mTOR activation promotes glycolysis, fatty acid synthesis and mitochondrial biogenesis. As such, targets upstream and downstream of the mTOR signaling pathway are potential therapeutic targets. Rapamycin, although known as an 'immunosuppressive' drug due to its ability to slow down T cell proliferation, promote robust responses to vaccination by enhancing $CD8^+$ T cell memory formation [116]. Correspondingly, deletion of the mTORC1 inhibitory protein TSC2 leads to enhanced mTORC1 activity and increased effector function [117]. Targeting of TSC2 or other molecules in the mTOR pathway might accordingly enhance immunity.

Targeting of glycolysis to inhibit immune responses in the setting of autoimmune disease and transplantation rejection is evolving, and this strategy is also used to enhance anti-tumor immunity by promoting long-lived memory cells ex vivo [118]. Whether this can be used in vaccination strategies remains to be examined. Although most studies have focused on the critical role of glycolysis in promoting effector T cell generation and function, it has become clear that mitochondrial-directed metabolism also plays an important role. Memory T cells rely for their energy upon OXPHOS and FAO. Because these metabolic pathways are dependent on mitochondria, the abundance and the organization of the mitochondria are instrumental for development of fit memory cells [119]. Alterations in the mitochondrial biogenesis can influence the differentiation of T cells, thereby providing opportunity to augment T-cell mediated immunity [120,121]. The transcription factor PGC1α promotes mitochondrial biogenesis and function [122]. Hence, pharmacologically or genetically enhancing PGC1α represents a potential strategy for improving vaccine-induced T cell responses. In *ex vivo* systems, it has already been shown that enforced overexpression of PGC1α, leads to improved metabolic fitness and effector cytokine function of $CD8^+$ T cells [123]. Again, whether *in vivo* targeting is possible remains to be examined, and in this respect a major challenge may be the specificity of metabolic inhibitors/enhancers as they may affect all cells of the body. However, inhibitors of glycolysis may preferentially affect effector T cells given their enhanced glycolytic need. The future will tell if indeed metabolic targeting is possible to enhance vaccines. Nevertheless, the metabolic profiles of (vaccine-induced) T cells are surely of interest and correlate to vaccine-mediated immunity [124].

COSTIMULATION EMPOWERS T CELL ELICITING VACCINES

Costimulatory signals transduced via the CD28 family members CD28 and ICOS, and via the tumor necrosis factor receptor (TNFR) family members CD27, 4-1BB, and OX40 play dominant roles in orchestrating the required signal 2 [125]. While CD28 and CD27 are constitutively expressed on naïve T cells ICOS, 4-1BB and OX40 are upregulated upon T cell activation [125,126]. The ligands for these costimulatory receptors are highly expressed on APCs upon activation, yet expression is also found on T cells, suggesting that these molecules may also mediate communication between T cells [127,128]. Synergy between these costimulatory molecules is expected [125,129], and is confirmed in experimental models [130].

There is extensive literature addressing the influence of the TNF/TNFR family interactions during a virus specific immune response. For instance, CD28 signals are required for sufficient T cell priming during the primary phase of an infection [131,132,133,134,135], while OX40 and 4-1BB gain importance during the late effector and memory stage of antigen-specific T cells either by providing pro-survival signals or by enhancing the quality of the memory T cells [136,137,138,139,140]. Thus, although an optimal immune response is the result of many receptor-ligand interactions, costimulatory signals dominate differentially during the diverse phases of the immune response (e.g. early *versus* late) to ensure optimal expansion and contraction of primary $CD8^+$ T cells and the generation of memory $CD8^+$ T cells.

Agonistic Abs against costimulatory receptors have shown efficacy in various preventive and therapeutic preclinical vaccination settings. Enforced engagement of costimulatory molecules results in improved T cell activation, expansion, survival and establishment of long-term memory [139,141,142,143,144,145,146], and has thus the potential to serve as effective immunomodulatory components of prophylactic vaccines against chronic viruses [143,147,148]. Indeed, this has already been observed for DNA and adenovirus based vector vaccines in which enforced expression of 4-1BBL, OX40L and CD70 leads to increased T cell expansion, enhanced CTL activity and antibody response [149,150]. Strikingly, agonistic antibodies to OX40 combined with synthetic peptide vaccines prompt robust effector and memory $CD4^+$ and $CD8^+$ antiviral T cell responses, improve T cell cytokine polyfunctionality and prophylactic vaccine efficacy against lytic MCMV infection [145]. Chronic viral infections are characterized by accumulation of functionally impaired antigen-specific $CD8^+$ T cells. Studies have shown that activation via 4-1BBL alone or in combination with CD80 can enhance the generation of primary $CD8^+$ T cell responses and induce expansion of the antigen-specific $CD8^+$ T cells from this pool of impaired T cells [136,151]. Similarly, 4-1BB stimulation has been shown to enhance the generation of primary $CD8^+$ T cell responses [139,152,153] and synergizes with attenuated vaccinia virus (VACV) vectors to augment $CD8^+$ T cell responses [139] .

Targeting of inhibitory molecules on T cells such as PD-1 and CTLA-4 have been shown to restore the effector function of activated T cells in settings of chronic viral infections and cancer [154,155,156,157]. Inhibitor blockade synergizes in combination with therapeutic vaccines [158]. Targeting of inhibitory pathways during primary immunization with prophylactic vaccines may advance the vaccine efficacy as well [159,160], but whether this results in significant improved vaccine efficacy remains to be established.

Although the use of antibodies targeting costimulatory/inhibitory molecules as immunostimulatory modalities in vaccines can facilitate antigen-specific T cell responses, the use of such Abs, however, is associated with toxicity as demonstrated in selected settings in rodents and in clinical settings [157,161,162,163]. Nevertheless, given the potential benefit to significantly increase the effectiveness of vaccines, both the efficacy and safety of targeting costimulation is currently extensively examined in various immunotherapeutic approaches against persistent viral infections. Examining the timing and/or the dosing is in this respect an important aspect to not only prevent unwanted side-effects but this may also lead to improved effectiveness. In addition, CD28 costimulation modulates T cell metabolism via activation of PI3K pathways, and this is essential to control effector cytokine production [112,164]. TNFR family members are also able to metabolically program T cells [165,166]. Collectively, targeting of T cell costimulation can impact the important quantitative (magnitude, breadth) and qualitative (cytokine polyfunctionality, metabolic fitness) determinants of vaccine-induced T cells, and provides thus major opportunities for further exploration in future vaccine designs.

CONCLUSIONS AND PERSPECTIVES FOR VACCINE DESIGN

The design of vaccines that imprint T cells with the ability to boost host defense against persistent viral pathogens has gained remarkable progress. An understanding of the appropriate initial programming signals is a key step, as is how the route of priming or boosting influences the development of effective memory T cells. A combination of several metrics such as the type of the vaccine elicited T cell response, breadth, polyfunctional quality and metabolic characteristics demonstrate a valid toolbox to define when a T-cell mediated response is protective. Unanswered questions about the anatomy, activation and differentiation of memory T cells in lymphoid compared to non-lymphoid organs need to be addressed. Costimulatory signaling pathways mediate basically all of the important T cell memory properties, and may serve as interesting targets for vaccine improvement. Experimental and clinical insight into their complex synergistic or antagonistic processes may identify requisite pathways and potentially other targets for immunotherapy. Identification of the best correlations of immunity with protection for persistent viral pathogens will enable the development of effective vaccination regimes.

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Chapter 7

General Discussion

GENERAL DISCUSSION

T cells are essential components of naturally acquired protective immune responses in many diseases. Although various ways of inducing potent T cell response by vaccination have been assessed, the majority result in low level, non-protective immune responses. Vaccine development has broadened and currently vaccine platforms range from replication-deficient (attenuated) or killed micro-organisms to viral vectored vaccines and more recently to protein subunit vaccines. Many highly effective vaccines designed to induce protective $CD4^+$ and/or $CD8^+$ T cells against chronic infections and cancer have been developed. Although their precise mechanisms of protection depend on the complexity and type of antigen, the quality of the vaccine-induced T cell response is gaining increasing attention.

In this doctoral thesis, the SLP T cell-based vaccination strategy alone or in combination with agonistic antibodies triggering the costimulatory TNFR superfamily member OX40 were tested for their capacity to induce potent $CD4^+$ and $CD8^+$ T cell responses and to confer protection against lytic MCMV infection. Vaccination with MHC class I- and II-restricted MCMV epitope containing SLPs successfully eliminated virus spread in naïve mice but failed to boost host's antiviral immune responses and treat established MCMV infection. Direct correlates of protection were thoroughly investigated. Another vaccination model tested was the MCMV-based vector vaccine against virus-induced cancer (HPV). Injection of the MCMV vectored vaccine expressing an immunodominant MHC class-I restricted HPV E7 epitope led to long term protection against tumor outgrowth in naïve hosts but exhibited limited efficacy in hosts with a strong pre-existing immunity to MCMV. To optimise both vaccination strategies, we explored essential components required to shape protective immune responses and ways to subvert existing immune responses and improve immune recognition.

COMBINATION IMMUNOTHERAPY AGAINST MCMV

Several approaches can be used to improve immunity to MCMV-associated disease. Concomitant stimulation of $CD8^+$ and $CD4^+$ T cell responses via MHC class I and II epitope-containing SLPs, respectively, and augmentation of costimulatory signals mediated by the TNFR family member OX40 are very promising tools, which will be extensively discussed below.

Maximum virus control through activation of $CD4^+$ and $CD8^+$ T cells

The last decades, numerous immunotherapeutic platforms mainly focused at the induction of neutralizing antibodies have been tested against CMV. The majority of these vaccines exhibited low or short-term efficacy [1]. Whether sterile immunity against CMV is an achievable goal remains questionable mainly due to the numerous immune evasion mechanisms exploited by the virus. T cell-based vaccines designed to

induce $CD4^+$ or/and $CD8^+$ T cell responses is a rapidly growing field. Many studies have demonstrated the importance of T cell immunity against CMV. There is solid evidence that $CD4^+$ T cells are crucial for controlling CMV replication during the acute phase of the infection while $CD8^+$ T cells play an essential role during latency and virus reactivation [2-4]. SLP T cell based immunotherapy is a safe and well-explored vaccination platform, which results in the activation of $CD4^+$ and $CD8^+$ T cells and has shown remarkable potency to treat HPV associated disease in both preclinical and clinical studies [5,6].

In **chapter 2,** MHC class I epitope containing SLPs along with TLR9 agonists were tested in a prime-boost vaccination setting in various mouse strains for their ability to contain high load systemic MCMV infection. Vaccination led to the induction of vigorous and poly-functional (IFN-γ^+/ TNF^+/ IL-2^+) cytotoxic $CD8^+$ T cell subsets mediating potent and long-term protective immunity against MCMV infection. Vaccine-induced $CD8^+$ T cells slowly converted to a unique memory T cell subject sharing features from both effector and central memory T cells while their functionality was significantly improved throughout time. Notably, the size of each distinct SLP vaccine-induced $CD8^+$ T cell response was found unrelated to the functional avidity and proportional to the naïve T cell precursor frequency. This finding suggests that T cell precursor frequencies may be considered as a powerful model to predict the subsequent size of the T cell response induced upon peptide vaccination. In shared antigen or neo-antigen cancer vaccine trials *in vitro* stimulation with the targeted antigen is used to provide insight into the patient's immune system ability to respond to potential vaccination [7,8]. However, determination of the targeted antigen/peptide precursor frequency will possibly ease classic *in vitro* antigen selection processes and may form an important parameter to be considered in immunotherapeutic strategies that their efficacy is based on the selection of antigens.

Despite the importance of $CD4^+$ T cells in the immune system, the role of CMV-specific $CD4^+$ T cell responses in CMV infection has not been completely understood. $CD4^+$ T cell ''help" is likely to be crucial for increasing the effectiveness of candidate vaccines for CMV. Therefore, in **chapter 3** the efficacy of MCMV-specific $CD4^+$ T cells to control MCMV was studied using a vaccine that comprises MHC class II epitopes. In addition, the capacity of the $CD4^+$ T cell responses to enhance the efficacy of a MCMV-specific $CD8^+$ T cell response was studied by simultaneous injection of MHC class I and II epitope SLPs. $CD4^+$ T cells induced after vaccination with various MHC class II SLPs and OX40 ligation elicited broad antiviral Th1 cytokine responses and showed direct antiviral function against MCMV infection. Interestingly, vaccine induced $CD4^+$ T cell responses conferred moderate protection against lytic MCMV infection in both lymphoid and non-lymphoid organ tissues. These findings advance the findings of other reports showing direct antiviral effector function of CMV specific $CD4^+$ T cells in both mice and human [2,9-12].

Moreover, inclusion of $CD4^+$ T cell ''help" during vaccination with a combination of MHC class I SLP vaccines significantly enhanced $CD8^+$ T cell expansion and remarkably

improved the overall prophylactic vaccine efficacy. Notably, $CD4^+$ T cell signals increased priming of vaccine-evoked $CD8^+$ T cells suggesting a direct synergy between these T cell subsets. The concept that $CD4^+$ T cell ''help" to license DC for proper $CD8^+$ CTL priming must be antigen specific has long been concluded [13-16]. Thus, MHC class II epitopes are likely to add value if included in the design of epitope-based vaccines against CMV. Consequently, in **chapter 5** a plethora of highly immunogenic IE2 MHC class II restricted T cell epitopes where identified following traditional *in silico* screening methods. Whereas the T cell response to the IE1 HCMV protein is dominated by MHC class I T cell responses, limited $CD8^+$ T cell reactivity was measured against the IE2 protein. Since T cell responses to IE antigens predominate the lytic phase of the CMV infection, a vaccine formulation that will comprise IE1-specific MHC class I epitopes and IE2-specific MHC class II epitopes targeting immune dominant $CD8^+$ and $CD4^+$ T cell viral regions respectively is possible to act synergistically and inhibit the establishment of CMV latency.

The need for enforced OX40 co-stimulation

Therapeutic targeting of immune check point inhibitors (PD-1/ PD-L1 and CTLA-4) or costimulatory receptors (41BB/ 41BBL and OX40/ OX40L) has been beneficial against many chronic viral infections and cancer [17-24]. Costimulatory molecules provide critical interactions for inducing and maintaining adaptive immune response against CMV. Antibodies can specifically bind to costimulatory receptors and boost or inhibit an immune response.

In **chapter 3** agonistic OX40 antibodies in combination with various MHC class I and MHC class II epitope containing SLPs of MCMV-encoded antigens were tested in a prime-boost vaccination schedule. OX40 ligation synergized with the SLP-based vaccines and empowered overall vaccine efficacy but did not exhibit any therapeutic or prophylactic reaction when provided as monotherapy. Enforced OX40 activation strongly increased the number of-vaccine-induced $CD4^+$ T cell and $CD8^+$ T cell responses, especially when provided during booster vaccination. The effect of OX40 triggering was more pronounced on $CD4^+$ T cells presumably due to the higher expression of the OX40 molecule on this T cell subset [25,26]. OX40 mediated signals were not short lived or limited on effector T cells but influenced also memory T cell formation. Specifically, memory vaccine-derived $CD8^+$ T cells treated with OX40 agonists during booster SLP vaccination exhibited improved functionality, survival and secondary clonal expansion compared to the untreated subjects.

Interestingly, the timing and the number of doses OX40 agonistic antibody was administered regulated both the size and the quality of the subsequent vaccine-mediated T cell response. Specially, when agonistic OX40 antibody was provided during both prime and booster SLP vaccination all the positive effects of the OX40 triggering on T cell induction were diminished or utterly lost. A possible explanation

for this outcome is that short time intervals between the two vaccinations did not allow adequate memory T cell development and upon secondary OX40 stimulation apoptotic cell death events were dramatically accelerated. T cell susceptibility to activation-induced cell death (AICD) can occur in a cell-autonomous manner and is regulated by previous T cell activation history and the stage of the T cell maturity [27]. Repeated TCR activation and co-stimulation may promote the Fas/CD95 pro-apoptotic pathway whereas correct timing of co-stimulation can promote the Bcl-2 anti-apoptotic pathway [28]. Consistent with this, upregulation of the Bcl-2 molecule, known as a target of OX40, was measured when agonistic anti-OX40 antibody was provided only during booster vaccination and correlated with prolong T cell survival. In contrast, repeated enforced OX40 stimulation during both prime and booster SLP vaccination downregulated Ki67 and Bcl-2 expression leading to decreased T cell proliferation and survival. Additionally, it has been previously reported that Bcl-2 upregulation accompanied with increased expression of IL-2 inhibits AICD of previously activated T cells [28,29]. OX40 stimulation during booster SLP activation dramatically escalated autocrine IL-2 cytokine production levels and AICD events were delayed. Whether further treatment with IL-2 at the time of stimulation with OX40 agonists could overcome T cell susceptibility to AICD remains to be explored.

Another interesting observation was that IL-2 secretion was tightly regulated by OX40 costimulatory signals. Enforced OX40 stimulation mainly during booster SLP vaccination promoted ''Th1'' cytokine production by both $CD4^+$ and $CD8^+$ vaccine-specific T cell subsets. Strikingly, IL-2^+ $CD4^+$ and/or $CD8^+$ cytokine T cell responses were significantly bolstered during the effector phase of the vaccine response by OX40 stimulation and maintained at high levels throughout time. As a future development, it would be particularly intriguing to test whether IL-2 induction, currently provided in many combination immunotherapeutic settings through laborious adoptive transfers or high toxicity direct infusions could be complemented or even replaced by providing OX40 co-stimulation using agonistic antibodies.

Due to its capacity to regulate both $CD4^+$ and $CD8^+$ T cells, OX40 is considered a promising candidate in immunotherapy of persistent viral infections and cancer. However, there is preclinical evidence that anti-OX40 antibodies could induce off-target toxicity causing deleterious immunosuppressive side effects by promoting the accumulation of MDSCs and the production of Th2 cytokines leading to autoimmunity or inflammatory diseases [30-32]. In **chapter 3**, NK cell augmentation and Th2 cytokine activity associated with pro-inflammatory and autoimmune conditions were not triggered by OX40 stimulation. Presumably, combination immunotherapy with peptide-vaccines and low dose schedules of OX40 stimulation did not allow development of off target toxicity events. Moreover, agonistic OX40 antibody treatment has exhibited mild toxicity and no expansion of Tregs, when applied as monotherapy for the treatment of solid tumours [24], compared to the FDA approved CTLA-4 (ipilimumab) or PD-1/ PD-L1 mAb blockade therapies [33,34].

Despite all the positive effects of OX40 agonists it is unlikely that anti-OX40 as single agent will be sufficient to cure patients with different tumour types or viral infections. However, there is great promise that combination immunotherapy incorporating OX40 stimulation and vaccination may be able to increase efficacy. Finally, it would be particularly informative to investigate whether enforced OX40 triggering could complement the therapeutic activity of other costimulatory antibody (anti-4-1BB), checkpoint inhibitor (PD-1/ PD-L1/ CDLA-4) and conventional treatments (i.e. radiotherapy, chemotherapy, cytokine infusions). Encouraging results come from recent studies where anti-OX40 antibody treatment uniquely synergized with PD1-blockade to promote tumour regression in experimental models [34,35].

DIVERSITY IN SPECIFICITY

A valuable observation in **chapter 2** was that the prophylactic efficacy of the distinct MHC class I MCMV epitope containing SLP vaccines was remarkably potentiated when all individual SLPs were combined and administered as a mixture. The efficacy of the MHC class I SLP vaccines to protect against lytic MCMV infection was primarily driven by the breadth of the $CD8^+$ T cell responses rather than the magnitude of the response to all individual SLPs. Although the magnitude of the individual antigen-specific $CD8^+$ T cell responses was significantly reduced when provided within a mixture and the overall T cell response size was approximately similar to individual SLP vaccines, the ability to contain virus spread was drastically enhanced.

An interesting observation of the present work was the superior capacity of the M38 and m139 MCMV MHC class I epitope containing SLPs to reduce viral titers upon lytic MCMV challenge. M38 and m139 antigens elicit strong inflationary $CD8^+$ T cell responses during MCMV infection and presumably play an important role in regulating virus replication and persistence [36,37]. Conceivably, induction of strong $CD8^+$ T cell vaccine responses to the M38 and m139 viral antigens in combination with high expression of these antigens on MCMV-infected cells may explain the advanced efficacy of the M38 and m139 specific SLP vaccines in controlling virus spread.

Associations of potent T cell responses with increased breadth leading to decreased viremia or complete viral eradication has been observed in other chronic viral infections [38-41]. A possible explanation for this finding is that development of a robust cytotoxic T cell response with a diverse repertoire of specificities, targeting viral antigens which are expressed at different stages in the viral life cycle increases the likelihood of immune recognition of viral infected cells and the probability of virus dissemination control. Especially, there is evidence that potential encounter of viral infected cells with vaccine-induced T cells of different specificities boost cytokine-mediated direct cell killing and promote CTL cooperation events [42].

Although the exact mechanisms through which immune T cell repertoire diversity influences anti-viral immunity are not yet defined, it is important to consider induction

of a T cell response with increased breadth when designing prophylactic T cell-based vaccines against MCMV infection. Based on the results obtained from the chapter 2 the breadth of the vaccine response may be an important goal in vaccine formulations and a determinant of vaccine efficacy.

SHAKING DOWN THE RIGID WALLS OF PRE-EXISTING IMMUNITY

In many infection models, immunological memory to a viral vector is considered a hindrance for subsequent induction of cell-mediated and humoral immune responses. Various components of the immune system, including neutralizing antibodies, vector-specific T cells and type I IFN-activated NK cells contribute to seriously compromised immune responses against the delivered heterologous antigen [43]. Additionally, pre-existing immunity to a viral vector impacts viral vector's expression level, virus trafficking and alters homing patterns of vaccine induced T cells. Similarly, pre-existing immunity may modulate the magnitude, breadth and immune system's antibody and T cell response to the inserted antigen [44]. CMV-based vectors are endowed with the capacity to stimulate robust CTL and humoral immune responses and have shown unprecedented efficacy against persistent viral infections and cancer [45-47]. Due to the high prevalence rate of CMV in the population, pre-existing immune responses in the host may exist. However, compared to other viruses and traditional viral vector systems (i.e. adenoviruses, lentiviruses), CMV has the ability to re-infect or even superinfect the same host and initiate a second cycle of immune responses.

Thus, in **chapter 5**, the capacity of MCMV-based vector vaccines to induce antitumor responses against HPV-induced cancer was explored. Whether the presence of pre-existing immune responses to MCMV is detrimental or can augment response to the vectored antigen was also investigated. MCMV vectors encoded a dominant MHC class I HPV16 E7 epitope in either inflationary of non-inflationary MCMV epitope regions were tested for both prophylactic and therapeutic efficacy against HPV^+ tumors in mice. Notably, systemic or subcutaneous MCMV-vectored vaccine administration stimulated vigorous HPV-specific T cell responses recapitulating MCMV response pattern and provided complete and long term protection against tumor challenge in naïve mice.

The therapeutic testing of the MCMV vector vaccine prolonged survival of challenged mice but exhibited moderate immunogenicity when compared to mice with no immunological memory to the vector, eventually leading to minor therapeutic effect. Interestingly, the efficacy of the vaccine was associated with the level of pre-existing humoral immunity to MCMV. Specifically, the initial viral infection dose determined the magnitude of the subsequent antibody and T cell vaccine- specific response. Mice initially infected through the systemic or subcutaneous routes developed strong anti-viral immunity, which severely diminished viral vector's impeding vaccine efficacy. In addition, there is supporting evidence that the therapeutic efficacy of the CMV vectors expressing tumour or viral antigens is significantly attenuated when the host harbours

latent CMV [47]. We and others have previously reported that the initial viral inoculum dose impacts virus immune response [48,49]. Hence, we tested whether different levels of pre-existing immunity to MCMV can influence the subsequent MCMV-vector vaccine efficacy. Strikingly, when initial infection administered orally, weak T cell and antibody responses were elicited, which were significantly boosted following systemic or subcutaneous MCMV- vector vaccine administration. Consistently, the vaccine efficacy was escalated leading to almost complete eradication of tumor-bearing mice. In contrast, strong pre-existing immunity to MCMV was not surpassed by any of the conventional vaccine injection routes tested.

It is not surprising that MCMV – when applied as a vaccine vector containing tumor antigens – is not capable to overcome its own immunogenicity. Even therapeutic targeting of persistent MCMV infection using the strong SLP T cell-based vaccine concept in **appendix of chapter 3** was not capable to boost the considerable high levels of pre-existing virus-specific T cells present 8 weeks after viral infection. Whether the efficacy of the peptide vaccines in therapeutic settings would have been improved in case of low dose pre-exposure to the virus remains to be tested.

Our results suggest that T cell-based vaccine vectors must be designed to generate sufficient quantities of antigen and induce broad cytotoxic $CD4^+$ and $CD8^+$ T cell responses. Key outstanding research challenges in the use of CMV vectors are dealing with the diversity in the level of pre-existing immunity of pre-exposed individuals and in discovering ways to minimize this response to ensure that all CMV vectored vaccines will reach the threshold levels of protective immunity needed for efficacy. Several strategies, such as augmentation of viral vector dose, immunization route and timing are important factors to circumvent pre-existing immunity to CMV and need to be considered. Moreover, strategies to lower pre-existing immunity through dynamic targeting of critical genes/proteins for virus replication and latency are likely to be particularly informative. Importantly, deletion of virally encoded inhibitors of MHC class I antigen presentation may be essential for blocking establishment of persistent secondary infection or superinfection in CMV rhesus macaques [50]. Finally, the design of distinct CMV serotypes to overcome immunological memory might be a feasible future direction. Analysis of the components involved at the regulation of pre-existing MCMV immunity will help further improve the development of therapeutic CMV vaccines and vector delivery systems for animal and human use. [51]

CONCLUDING REMARKS

T cell mediated protection is multifaceted and driven by several factors. Despite differences among viral infectious diseases, common determinants of the vaccine efficacy is the magnitude, breadth, availability of co-stimulation, tissue location and functionality of $CD8^+$ T cells **(chapter 2, 3, 4 and 6)**. The role of $CD4^+$ T cell ''help" in providing direct effector function and regulating the magnitude of the $CD8^+$ T cell

response is crucial (**chapter 3**). Therapeutic interventions with MCMV vectors appear to be promising for chronic viral infections (**chapter 5**). A fundamental research question which the findings of this thesis put forward is whether other factors such as innate immunity and B cells are required for sufficient vaccine function and how these factors can be co-manipulated to optimize vaccination.

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Chapter 8

Appendix

ENGLISH SUMMARY

The development of novel vaccine strategies for CMV is major research priority. Vaccines based on SLPs may form an efficient vaccination strategy against CMV infection. However, a detailed and systematic examination of the potentially promising prospects of SLP vaccines is lacking. This PhD thesis presents a series of experimental studies undertaking elaborate evaluations of the efficacy of SLP vaccines against MCMV infection. SLP vaccines alone or in combination with immunomodulatory agents of the TNFR superfamily were investigated for prophylactic and therapeutic efficacy in various mouse models of CMV infection. The pivotal knowledge obtained from the MCMV studies led to the investigation of highly immunogenic IE2 HCMV antigenic targets and formed the basis for the design of a HCMV vaccine in this doctoral thesis.

The first experimental study **(chapter 2)** tested the preventive efficacy of SLP vaccines comprising immunodominant MHC class I MCMV restricted epitopes that exclusively elicit cytotoxic MHC class I T cell responses against lytic MCMV infection in C57BL/6 and BALB/c mouse strains. Prime-booster vaccination induced strong and polyfunctional $CD8^+$ T cell responses. Interestingly, the degree of the $CD8^+$ T cell response was determined by the naive $CD8^+$ T cell precursor frequency of each epitope rather than the T cell functional avidity. Vaccination with a combination of SLPs produced the best reduction in viral titers, suggesting that the breadth of the vaccine-induced $CD8^+$ T cell response is critical for preventive vaccine strategies against MCMV.

The second study **(chapter 3)** examined whether the efficacy of the MHC class I SLP based vaccine is boosted with $CD4^+$ T cell "help" and/or OX40 co-stimulation. The prophylactic effects of SLPs containing MHC class II epitopes from 5 immunodominant MCMV antigens were examined alone or in combination with the MHC class I SLPs in C57BL/6 mice. Enforced OX40 signalling during booster SLP vaccination improved vaccine-specific $CD4^+$ T cell and $CD8^+$ T cell reactivity. The combined vaccination of SLPs containing various MHC class I and II SLPs plus OX40 agonistic antibodies produced the best protection and virus dissemination effects. Drawing on these promising findings, the therapeutic efficacy of the combinatorial MHC class I and II SLP vaccines was also tested in the C57BL/6 mouse strain **(appendix section of chapter 3)**. The strong T cell response to MCMV was not boosted following SLP vaccination and no therapeutic effect was observed. These results do not support the therapeutic prospects of the combinatorial MHC class I and II SLP vaccines against MCMV infection.

Chapter 4 presents an endeavour to translate this research in humans. A study to discover novel HCMV T cell epitopes, which would enlarge the choice of antigens for potential HCMV T cell- based SLP vaccines, was undertaken. IE2 HCMV T cell responses were tested in the peripheral blood of 15 HCMV- seropositive and 6 HCMV- seronegative healthy donors using IE2 SLP pools and cytokine flow cytometry. Whereas the IE1 protein is frequently recognized by $CD8^+$ T cells, a limited number of HLA-class I restricted IE2

T cell epitopes were found. Several new HLA class II-restricted IE2 T cell epitopes were detected in addition to previously detected epitopes. Five highly immunogenic IE2 SLPs recognized by polyfunctional Th1 cytokine (IFN-γ^+/ TNF^+/ IL-2^+) producing cells were identified during the functional characterization of the IE2-specific T cell responses. Testing of these 5 highly immunogenic IE2 SLPs as a potential T cell based vaccine for CMV is highlighted as a fruitful future research direction.

Since CMV has a unique ability to re-infect the same host and initiate a second cycle of immune responses, a study **(chapter 5)** was performed in which the protective and therapeutic efficacy of MCMV-based viral vector vaccines against HPV^+ tumours were evaluated in mice with and without pre-existing immunity. MCMV-based vaccine vectors expressing an immunodominant MHC class I-restricted HPV E7 epitope in either inflationary or non-inflationary T cell epitope regions were generated. The diversity of the inflationary versus non-inflationary HPV specific T cell responses was thoroughly examined over time and the vaccine effectiveness was compared. All MCMV vector-based vaccines exhibited long term prophylactic vaccine efficacy. Interestingly, therapeutic evaluation of the MCMV vectored vaccines showed that the level of pre-existing immunity determines the efficacy of MCMV-based vaccine vectors.

The empirical findings presented in the previous 4 chapters are complemented by a narrative review of the literature in **chapter 6,** which critically evaluates the research evidence on the potency of prophylactic T cell eliciting vaccines for chronic viral infections. It outlines the main factors which determine the quality of the $CD4^+$ and $CD8^+$ T cell responses. In parallel, the complexities in understanding the mechanisms underpinning potent T cell responses are discussed as T cell mediated protection is driven by several factors.

The final **chapter 7** provides an insightful discussion of the key knowledge gaps filled in this thesis, the core strengths and limitations of the utilised methodologies and findings, and the most fruitful research ideas to be prioritised by future studies in this area. Importantly also, a balanced debate is presented about the likely direct and indirect clinical implications of this thesis in the design of efficacious prophylactic T cell based vaccines.

NEDERLANDSE SAMENVATTING

De ontwikkeling van nieuwe vaccinatiestrategieën tegen humaan cytomegalovirus (CMV) is een belangrijke onderzoeksprioriteit. Een mogelijke efficiënte vaccinatiestrategie tegen CMV infectie vormen vaccins gebaseerd op synthetische lange peptiden (SLP). Echter, tot op heden ontbreekt een gedetailleerd en systematisch onderzoek naar het gebruik van SLP vaccins als potentieel veelbelovend therapie tegen CMV. In dit proefschrift worden een reeks experimentele studies beschreven waarin de werkzaamheid van SLP vaccins tegen muizen CMV (MCMV)-infecties zijn geëvalueerd. SLP vaccins, alleen of in combinatie met stimulatie van het TNFR superfamilielid OX40, werden getest op profylactische en therapeutische werkzaamheid in diverse muismodellen van MCMV infectie. De door de MCMV studies verkregen kennis leidde tot een studie om immunogene aminozuur sequenties binnen het CMV IE2 eiwit te identificeren als eerste opstap naar een mogelijk SLP vaccin voor CMV.

In het eerste onderzoek **(hoofdstuk 2)** werd de preventieve werkzaamheid van SLP vaccins, bestaande uit een aantal MHC klasse I-bindende MCMV peptiden, tegen een lytische MCMV infectie getest. Hieruit bleek dat vaccinatie met een combinatie van SLP's de sterkste werking had. Dit suggereert dat de breedte van de vaccin geïnduceerde $CD8^+$ T-cel reactie belangrijk is voor preventieve vaccinatie tegen MCMV. Daarnaast bleek dat de sterkte van de vaccin-geïnduceerde T-cel reactie afhankelijk was van het aantal circulerende naïeve $CD8^+$ voorloper T-cellen.

In het tweede onderzoek **(hoofdstuk 3)** werd getracht de werkzaamheid van het eerder genoemde SLP-vaccin te verhogen door het vaccin uit te breiden met SLP's, bestaande uit MHC klasse II-bindende MCMV peptiden, waardoor ook $CD4^+$ T-helpercellen worden geactiveerd. Daarnaast werd er ook een antistof tegen OX40 geïnjecteerd waardoor de T-cellen extra gestimuleerd werden. Uit deze studie kwam naar voren dat OX40 stimulatie tijdens booster SLP vaccinatie de vaccin-specifieke $CD4^+$ T-cel en $CD8^+$ T-cel reactiviteit sterk verbeterd. Tevens bleek dat de beste bescherming tegen MCMV infectie werd verkregen wanneer de muizen werden gevaccineerd met een combinatie van MHC klasse I en MHC klasse II-bindende SLP's en OX40 stimulatie tijdens de booster vaccinatie.

Op basis van deze veelbelovende bevindingen werd ook de therapeutische werkzaamheid van deze vaccinatiestrategie getest **(bijlage hoofdstuk 3)**. De al aanwezige T-cel response tegen MCMV werd niet verbeterd door het vaccin en er werd ook geen therapeutisch effect waargenomen. Dus een eventuele therapeutische toepassing van dit vaccin bij een CMV infectie wordt niet door de resultaten ondersteund.

In **hoofdstuk 4** werd een eerste stap gedaan om dit onderzoek te vertalen naar de mens door binnen het IE2 eiwit van humaan CMV te zoeken naar aminozuur sequenties die door de T-cellen van verschillende CMV-geïnfecteerde personen herkend

werden. Het IE2 eiwit werd vooral door $CD4^+$ T-helper cellen herkend en in mindere mate door $CD8^+$ T-cellen. Uiteindelijk werden er vijf immunogene SLP's geïdentificeerd die herkend werden door zogenoemde polyfunctionele type 1 T-helpercellen. Deze SLP's zouden de basis kunnen vormen voor een potentieel vaccin tegen CMV.

Aangezien CMV een uniek vermogen heeft om dezelfde gastheer opnieuw te infecteren en een tweede cyclus van afweerreacties te initiëren, werd een studie uitgevoerd waarin de beschermende en therapeutische werkzaamheid van MCMV-gebaseerde virale vector vaccins tegen humaan papillomavirus (HPV) veroorzaakte tumoren werd geëvalueerd **(hoofdstuk 5)**. Alle MCMV-vector gebaseerde vaccins vertoonden langdurige profylactische werkzaamheid. De therapeutische werkzaamheid van een MCMV-gebaseerd virale vector vaccin was afhankelijk van het niveau van de al bestaande immuniteit tegen MCMV. Naarmate de bestaande immuniteit sterker was nam de therapeutische werking af.

In **hoofdstuk 6** worden de empirische bevindingen uit de voorgaande 4 hoofdstukken aangevuld met een uitgebreide bespreking van de literatuur op het gebied van profylactische T-cel activerende vaccins. Het schetst de belangrijkste factoren die van invloed zijn op de kwaliteit van de $CD4^+$ en $CD8^+$ T-cel reacties. Tegelijkertijd wordt de complexiteit van de verschillende mechanismen die een rol spelen bij het ontstaan van krachtige T-cel reacties besproken.

Hoofdstuk 7 geeft een inzicht in hoe de studies in dit proefschrift bijdraagt aan de huidige kennis binnen dit onderzoeksgebied en beschrijft de sterktes en zwaktes van de gebruikte methodes en bevindingen. Tevens wordt er een evenwichtige beschouwing gegeven over de mogelijke directe en indirecte klinische implicaties bij het ontwerpen van effectieve profylactische T-cel gebaseerde vaccins en prioriteert het ideeën voor toekomstige studies.

ABBREVIATIONS

Ad	Adenovirus
AICD	Activation-Induced Cell Death
APCs	Antigen Presenting Cells
CTL	Cytotoxic T lymphocyte
CCMV	Chimpanzee CMV
CMV	Cytomegalovirus
DC	Dendritic Cell
GPCMV	Guinea Pig CMV
HCMV	Human CMV
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
HPV	Human Papilloma Virus
IE1	Immediate Early 1 protein
IE2	Immediate-Early 2 protein
IFN-γ	Interferon-γ
IL-2	Interleukin-2
IL-4	Interleukin-4
IL-12	Interleukin-12
MCMV	Mouse CMV
MHC	Major Histocompatibility Complex
MVA	Modified Vaccinia Ankara
NK cells	Natural killer cells
PAMPs	Pathogen-Associated Molecular Patterns
PBMCs	Peripheral Blood Mononuclear Cells
PCD	Programmed Cell Death
PFU	Plaque-Forming Units
pp65	protein 65
PRRs	Pattern Recognition Receptors
RCMV	Rat CMV
RhCMV	Rhesus CMV
SIV	Simian Immunodeficiency Virus
SLP	Synthetic Long Peptide
T_{CM}	Central Memory
T_{EM}	Effector Memory
T_{RM}	Tissue-Resident Memory
TNF	Tumor Necrosis Factor

CURRICULUM VITAE

Eleni Panagioti was born in 11 September 1988 in Athens, Greece. In 2011 she obtained first class bachelor degree in Biomedical Sciences from the University of Athens and then moved to England to pursue a Master's in Immunology and Immunogenetics at the University of Manchester. During her Master's she undertook an internship at the Immunology group of the Cancer research UK, Manchester Institute under the supervision of Prof. Peter Stern and Dr. David Gilham to characterize the functional properties of novel h5T4 monoclonal antibodies in malignant melanoma. In June 2013, she started a PhD on a prestigious Marie Curie Early Stage Research Fellowship, funded by the European Commission at the Department of Medical Oncology, at the Leiden University Medical Center under the supervision of Prof. Sjoerd van der Burg and Dr. Ramon Arens. During her fellowship, she received an excellent training in Vaccinology from world's leading universities and pharmaceutical companies.

LIST OF PUBLICATIONS

Beyranvand Nejad, E., **Panagioti, E.,** Redeker, A., Cicin-Sain L., van der Burg, S.H., and Arens, R. The level of pre-existing immunity determines the efficacy of MCMV-based vaccine vectors. *(in preparation*)

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Vanheusden, M., Broux, B., Welten, S. P., Peeters, L. M., **Panagioti, E.,** Van Wijmeersch, B., Somers, V., Stinissen, P., Arens, R., and Hellings, N. 2017. Cytomegalovirus infection exacerbates autoimmune mediated neuroinflammation. *Scientific Reports* 7:663.

Panagioti, E., Boon, L., Arens, R., and van der Burg, S. H. 2017. Enforced OX40 Stimulation Empowers Booster Vaccines to Induce Effective $CD4^+$ and $CD8^+$ T Cell Responses against Mouse Cytomegalovirus Infection. *Frontiers in Immunology* 8:144.

Panagioti, E., Redeker, A., van Duikeren, S., Franken, K. L., Drijfhout, J. W., van der Burg, S. H., and Arens, R. 2016. The Breadth of Synthetic Long Peptide Vaccine-Induced $CD8^+$ T Cell Responses Determines the Efficacy against Mouse Cytomegalovirus Infection. *PLoS Pathogens* 12: e1005895.

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