Implications of memory CD8 T cell quality for protective immunity

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IMPLICATIONS OF MEMORY CD8 T CELL QUALITY FOR PROTECTIVE IMMUNITY

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CHAPTER 1

General Introduction


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GENERAL INTRODUCTION

In a highly sophisticated manner, the immune system combats malignant cells and invading pathogens. Within the immune system two branches can be distinguished: the innate and adaptive system. The innate immune system forms the first line of host defense, and occurs immediate after pathogen invasion. The innate immune response is indispensable for instigating the adaptive immune response, which in contrast to innate immunity confers lifelong protective immunity. Activation of the innate response occurs via recognition of conserved pathogen-specific structures by pathogen recognition receptors (PRRs) and is regarded as relatively non-specific. The adaptive response is highly specific and depends on the presence of antigen-specific receptors on lymphocytes derived from the thymus (i.e. T cells) and from the bone marrow (i.e. B cells). Antigen presenting cells (APC), and in particular dendritic cells, orchestrate the initiation of adaptive immune responses by presenting the antigens to the lymphocytes, thereby linking the two systems. Important to note is that after establishment of immunological memory, the memory T and B cells are more rapidly activated upon re-encounter with a pathogen. Upon activation of B cells by antigen binding to the B cell receptor, these cells differentiate not only into memory B cells but also develop into plasma cells that secrete antigen-specific antibodies, which can neutralize pathogens or mark infected cells for elimination. The latter is called humoral immunity. T cells on the other hand are mainly responsible for cell-mediated immunity.

T CELL ACTIVATION

For proper T cell activation three signals are required (1, 2). The first signal is established by interactions between the T cell receptor (TCR) and peptide-MHC complexes presented by antigen presenting cells (APC). $CD8^+$ T cells recognize peptides presented by MHC class I molecules. These peptides can either originate from intracellular or extracellular proteins. The latter is referred to as cross-presentation, and is a unique property of specialized dendritic cells (3). $CD4^+$ T cells recognize their cognate antigen in the context of MHC class II molecules, which solely present peptides from extracellular sources. Complementary signals enhancing the TCR signal are essential for full T cell activation and survival. These signals are provided by interactions between costimulatory receptors on T cells and their ligands on APCs (signal 2). Costimulatory receptors are divided into two families: the immunoglobulin (Ig) superfamily and the tumor necrosis factor receptor (TNFR) superfamily. Two well characterized members of the Ig superfamily are CD28 and ICOS, binding to CD80/86 (B7.1/2) and ICOSL respectively (4). Costimulatory members of the TNFR superfamily and their ligands include CD27 - CD70, 4-1BB - 4-1BBL, OX40 – OX40L, GITR – GITRL (5). In addition to supporting TCR signaling and costimulatory interactions, APCs produce IL-12 and type I interferons (IFNs), thereby

providing the third signal that is required for full CD8 T cell expansion (6). In the context of CD4 expansion signal 3 is well explored to lesser extent, however IL-1 has been reported to serve as signal 3 for $CD4^+$ T cells (7). The TNFR family member CD40 plays a special role in T cell stimulation, as the ligand (i.e., CD40L) is upregulated on activated $CD4^+$ T cells. $CD40L^+$ CD4 T cells are then able to stimulate $CD40^+$ APCs.

T CELL EXPANSION AND FUNCTION

In order to achieve eradication of pathogens or malignant cells a small number of antigen-specific T cells (in viral or bacterial infections usually 100-200 cells) has to expand massively to reach sufficient numbers (8, 9). Depending on the nature of the stimulus, after activation $CD4^+$ T cells can differentiate into a variety of subsets, each supporting a different kind of immune response. These subsets include T helper 1 (T_H1), T_H2, T_H17, T follicular helper cells (T_{FH}) and regulatory $CD4^+$ T cells ($T_{REGS)}$ (10). By licensing DCs through CD40-CD40L interactions, T_H1 cells provide a critical signal that is required for the induction of an adequate $CD8^+$ T cell response. T_{FH} cells are important for the humoral response by providing help to B cells enabling antibody isotype switching (11, 12). In contrast, T_{REGS} are recognized for their capacity to dampen the immune response (13).

$CD8^+$ T cells can eradicate infected or malignant target cells directly by releasing cytotoxic molecules as perforin and granzyme or by the expression of apoptosis-inducing ligands Trail and FasL. Other mechanisms whereby $CD8^+$ T cells exert their effector function are mediated via production of the cytokines interferon-gamma (IFN-γ) and TNF. Important functions of IFN-γ include direct anti-viral effects, but also anti-proliferative effects on malignant cells are observed as well as the induction of MHC class I upregulation. TNF can induce apoptosis of target cells by activating the caspase cascade (14, 15).

IL-2 is a cytokine that plays an important role during immune activation, differentiation and homeostasis of both $CD4^+$ and $CD8^+$ T cells. The development of T_{REGS} and differentiation of $CD4^+$ T cells depend strongly on IL-2 mediated signals (16, 17). In addition, IL-2 is required for the successful programming of naive $CD8^+$ T cells into effector and memory cells. Only memory $CD8^+$ T cells that have received IL-2 signaling during priming are able to undergo secondary expansion upon re-challenge (18, 19). IL-2 is also required for $CD8^+$ T cell maintenance and expansion in non-lymphoid tissue (20-22). Cells that are capable of producing IL-2 include $CD4^+$, $CD8^+$ T cells and dendritic cells . Importantly, $CD8^+$ T cells depend on autocrine IL-2 production for secondary expansion (23).

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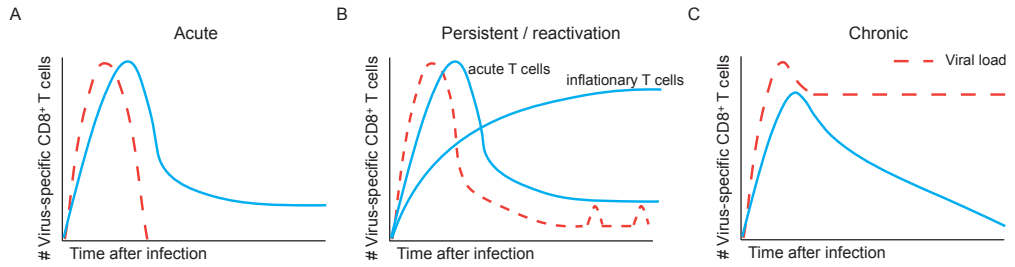
T CELL CONTRACTION AND MEMORY FORMATION

Upon pathogen clearance, the T cell response needs to be terminated to avoid immune-mediated pathology (24). Contraction of the T cell response is achieved by a variety of mechanisms, such as the elimination of APCs or upregulation of inhibitory co-receptors. Examples of inhibitory co-receptors are CTLA-4, which competes with CD28 for interaction with B7.1/2, and PD-1 that can interact with PD-L1 and PD-L2. Another mechanism involves the action of T_{REGS}, which can contribute to T cell contraction by the expression of inhibitory receptors, such as CTLA-4, the production of inhibitory cytokines, like IL-10 and TGF-β or by competing for IL-2 (25, 26). During the contraction phase, the majority of the antigen-specific T cells undergo apoptosis leaving a small but stable memory T cell population of predominantly central memory cells with the capacity to rapidly expand upon re-infection with the same pathogen (Figure 1A). These central memory T cells patrol the lymphoid organs for the presence of their cognate antigen, and for their maintenance they do not depend on antigen, but rely on homeostatic proliferation (27).

However, in some infections memory T cell formation is not primarily skewed to central memory T cells. Some viruses (e.g. Cytomegalovirus) are not completely eradicated by the immune system and establish a state of latency, which is occasionally interrupted by viral reactivation. In Cytomegalovirus (CMV) infection this viral reactivation results in the induction of a large subset of effector memory T cells that preferentially resides in tissues and depends on antigen for maintenance (Figure 1B). In addition, these CMV-specific effector memory T cells, also known as inflationary T cells increase in number over time and, although they show signs of repetitive antigen exposure, exhibit no features of exhaustion (28, 29). Finally, a third subset of memory T cells is formed, which in contrast to central and effector memory T cells do not circulate but remains present in tissue. These so-called tissue-resident memory T cells contribute to cell-mediated immunity as well.

Circumstances in which viral replication is high over prolonged periods of time, as observed in human immunodeficiency virus (HIV) and hepatitis C virus (HCV) infection often result in T cell exhaustion. In a hierarchical manner T cells then gradually lose their effector functions and at the final stage of exhaustion cells are physically deleted (Figure 1C) (30-32). Correspondingly, in cancer where antigen exposure is persistent T cell exhaustion is also observed. Loss of CD4 T cell help contributes to the severity of $CD8^+$ T cell exhaustion.



Different patterns of CD8+ T cell responses upon acute, latent and chronic infections. (A) Acute viral infections are rapidly cleared after which stable central-memory CD8+ T cell pools are formed. (B) Not all viruses are completely eradicated by acute T cells. Sporadic reactivation during persistent viral infection results in the accumulation of effector-memory CD8+ T cells. (C) High-grade chronic infections result in CD8+ T cell exhaustion and physical deletion of specific CD8+ T cells.

IMMUNOTHERAPY

Immunotherapy aims to induce or enhance the antigen-specific immune response and is considered to be potentially curative for various types of chronic viral diseases and cancers. Immunotherapeutic strategies include adoptive cell therapy (ACT), vaccination and administration of immune modulatory activators or inhibitors.

ACT, in which antigen-specific T cells are *ex vivo* isolated and expanded for transfer back into the patient, has proven to be successful in the treatment of several chronic viral infections and virus-associated malignancies (33, 34). Clinical benefit has also been observed for treatment of hematological malignancies and melanoma. However, in the majority of solid tumors clinical benefit is far limited due to failure of T cell persistence and function. Vaccination, on the other hand, is considered to be one of the greatest medical achievements that is available in public health, as preventive vaccination programs have drastically decreased morbidity and mortality of diseases such as smallpox, rubella and diphtheria (35). These vaccines are administered prophylactically and generally depend on the effective induction of humoral responses. However, eradication of numerous pathogens and cancers depend on T cells and although many vaccination strategies aiming to induce potent antigen-specific T cells are currently being investigated, thus far the clinical benefit of therapeutic vaccination as a treatment modality has been limited. Strategies include vaccination with DNA, synthetic long or short peptides and virus-based vaccines. Another approach is to enhance T cell expansion and function by triggering costimulatory pathways using agonistic antibodies such as anti- CD27, CD40, OX40, 4-1BB and GITR or by blocking inhibitory interactions between e.g. PD-1 / PDL-1/2 and CTLA-4 / B7.1/2 (36-39). Even as a monotherapy some of the above mentioned immune therapeutic strategies have proven to be able to achieve clinical benefit yet, combinations of one or more treatment modalities are probably required to obtain improved clinical success.

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SCOPE

The phenotype and functionality of T cells that are induced upon antigenic challenge (e.g. infection, malignancies or immunotherapy) are influenced by many factors, including the degree and duration of inflammation, antigenic load and the presence / absence of inhibitory or (co)stimulatory signals. Insight into how T cell phenotype and functionality as well as expansion capacity upon secondary challenge are regulated is particularly valuable for the development of immune therapeutic strategies. In this thesis we investigate factors that influence T cell function and phenotype and discuss how this knowledge can be used to improve current immune therapeutic interventions.

The first two chapters focus on IL-2 signaling in $CD8^+$ T cells. In **chapter 2** we demonstrate that the expansion potential of an antigen-specific $CD8^+$ T cell population is determined by the relative amount of IL-2 producers within that population. In addition we show by enhancing IL-2 production in $CD8^+$ T cells that the amount of IL-2 that is produced on a per cell basis is predictive for the degree to which a $CD8^+$ T cell can expand. In **chapter 3** we show that for instigation of autocrine IL-2 production by $CD8^+$ T cells costimulatory signals via B7.1/2 and CD70 are jointly required. **Chapter 4** describes how insight in the mechanisms that determine T cell function and phenotype can be applied to improve adoptive cell therapy (ACT). We address the role of IL-2 and other cytokine-mediated signals, and the potency of enhancing ACT efficacy via costimulatory signals and vaccination.

Upon infection, viruses such as CMV mount robust immune responses, allowing extensive studying of T cell responses and the mechanisms by which they are regulated. In **chapter 5** we show that in CMV infection the infectious dose determines the phenotype and function of the CMV-specific T cells and the degree to which they accumulate, stressing the importance of appropriate vaccine dosing in immune therapeutic strategies involving CMV as a vaccine vector and providing a plausible explanation for differences in T cell phenotype and frequency in CMV-seropositive individuals. Next we demonstrate that in addition to the antigen-specific T cell pool, the CMV dose also impacts the B cell response (**chapter 6**). In the following chapter (**chapter 7**) we investigate the impact of lifelong chronic CMV infection on the immune system. In particular, we address the influence of the viral dose, and show that high dose CMV inoculum impairs immunity to newly encountered viruses. Elaborating on this we discuss in **chapter 8** mechanisms whereby CMV compromises immune responses to new antigens. Subsequently we compared different routes of infection and demonstrate that the route does not differentially influence T cell phenotype and function (**chapter 9**). Finally, all studies comprising this thesis will be discussed in **chapter 10**.

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CHAPTER 2

The quantity of autocrine IL-2 governs the expansion potential of CD8$^+$ T cells

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ABSTRACT

Adequate responsiveness of $CD8^+$ T cell populations is of utmost importance for the efficacy of many vaccines and immunotherapeutic strategies against intracellular pathogens and cancer. Here, we show in a mouse model that the relative number of IL-2 producing cells within antigen-specific $CD8^+$ T cell populations predicts the population expansion capacity upon challenge, and further demonstrate that IL-2 producers constitute the best responding subset. Notably, we show that elevated production of IL-2 by $CD8^+$ T cells results in concomitant improved population expansion capacity and immunity. The amount of IL-2 produced on a per cell basis essentially connects directly to the superior $CD8^+$ T cell population expansion. Together, our findings identified that autocrine IL-2 production operates in a dose dependent fashion to facilitate the expansion potential of antigen-specific $CD8^+$ T cell populations, which may instigate ways to augment therapies depending on fit $CD8^+$ T cell.

INTRODUCTION

CD8+ T cells constitute an important branch of the adaptive immune system by providing vital contributions to eradicating acute infections, the control of persistent infections and cancer, and the maintenance of life-long protective immunity from secondary exposure to pathogens (1, 2). Consequently, successful immunotherapeutic strategies have been developed in which antigen-specific CD8+ T cells are infused to treat infections, hematological malignancies and solid tumors (3). Moreover, the induction of T cell immunity by vaccination or enhancement of the existing CD8+ T cell memory pool by immune checkpoint blockade is a powerful strategy to improve protection against infections and malignancies (1, 4, 5). However, despite the recent progress in developing successful adoptive immunotherapy and vaccination strategies, significant challenges remain as a consequence of incomplete knowledge of the signals required for CD8+ T cells to become and/or to remain potent mediators of immunity.

In order for individual CD8+ T cells to expand to a large pool of antigen-specific effector and memory cells, these cells have to undergo clonal proliferation and differentiation, and several distinct signals are needed for these processes. The primary signal is provided through interaction of the T cell receptor (TCR) with peptide-MHC complexes expressed on professional antigen-presenting cells, while costimulatory signals and certain pro-inflammatory cytokines provide additional signals for optimal expansion and differentiation (6, 7). One known important cytokine that is implicated in the programming of naive CD8+ T cells into effector and memory cytotoxic T cells is interleukin-2 (IL-2). IL-2 was first discovered over 30 years ago as a potent *in vitro* T cell growth factor present in the supernatants of activated human T cells (8). Thereafter, multiple studies have shown that in addition to a role pointing towards protective immunity, IL-2 also plays a crucial role in mediating tolerance by driving the development of CD4+ regulatory T cells (Tregs) (9, 10). Recent studies have shown the importance of IL-2 *in vivo* in shaping effector T cell responses (11, 12). Moreover, CD8+ T cells require IL-2 mediated signaling during the primary phase to be successfully programmed for the development into memory cells with the full capacity to expand upon secondary challenge (13, 14). Also, expansion and survival of CD8+ T cells in non-lymphoid tissue has been shown to be IL-2 dependent (15-17). The cellular source of IL-2 that is needed for the generation of CD8+ T cells that are able to re-expand was found to be CD8+ T cells themselves rather than IL-2 provision by CD4+ T cells or dendritic cells (18). We reasoned that the relative production of autocrine IL-2 by antigen-specific CD8+ T cells could be decisive for their expansion potential and here report on experiments confirming this hypothesis. Our finding that the amount of IL-2 produced on a per cell basis correspondingly connects to superior population expansion potential may have important implications for the development of immunotherapeutic strategies and vaccines that rely on CD8+ T cell mediated immunity.

MATERIALS AND METHODS

Mice

Wild-type (WT) C57BL/6 mice were purchased from Charles River. CD45.1 ($Ptprc^a$, Ly5.1) mice on a C57BL/6 background were obtained from The Jackson Laboratory and maintained in-house. The ovalbumin (OVA)-specific TCR transgenic mouse line OT-I was obtained from The Jackson Laboratory and backcrossed on a CD45.1+ background. All mice were maintained in individually ventilated cages (IVC) under specific pathogen free (SPF) conditions at the Central Animal Facility of Leiden University Medical Center (LUMC). Mice were 8-10 weeks old at the beginning of each experiment. All animal experiments were approved by the Animal Experiments Committee of the LUMC and performed according to the Dutch Experiments on Animals Act that serves the implementation of 'Guidelines on the protection of experimental animals' by the Council of Europe and the guide to animal experimentation set by the LUMC.

Viral infection and determination of viral load

Mouse cytomegalovirus (MCMV)-Smith was obtained from the American Type Culture Collection (ATCC VR-194; Manassas, VA) and salivary gland stocks were prepared from infected BALB/c mice. MCMV expressing OVA has been kindly provided by Dr. Geoffrey R. Shellam (School of Biomedical, Biomolecular and Chemical Sciences, Crawley, Western Australia, Australia). Female mice were infected i.p. with 5×10^4 PFU of salivary gland derived MCMV-Smith, 1×10^5 PFU MCMV-OVA, 2×10^6 or 4×10^6 PFU vaccinia virus (VV)-OVA. For determination of VV-OVA viral load, ovaries were homogenized and sonicated for 20 seconds. Serial dilutions were made and viral titers were determined by plaque assay on confluent Vero E6 cells. *Mycoplasma* tests routinely performed for all cell lines by PCR were negative.

Production of recombinant retrovirus

The LZRS-based recombinant retroviral constructs IRES-EGFP, IL-2-IRES-EGFP and OVA-IRES-EGFP were generated by standard methods and transfected into Phoenix ecotropic packaging cell lines using Fugene-6 reagent as described (19). Stable high-titer producer clones were selected using puromycin (1 μg/ml). Recombinant retrovirus-containing supernatant was harvested, filtered and stored at –80°C.

Retroviral transduction of T cells

Transduction of peripheral murine T cells was performed as described (20) with a few modifications. Single cell suspensions were prepared from spleens of CD45.1+ OT-I TCR transgenic mice by mincing the tissue through a 70 μm cell strainer after which cell suspensions were enriched for CD8+ T cells using CD8+ T cell isolation kits II from Miltenyi Biotec. The CD8+ OT-I T cells were pre-stimulated using αCD3/αCD28 Dynabeads (Thermo Fisher Scientific) in the presence of IL-7 and IL-15 (R&D Systems) for 22 h.

Next, 24-well non-tissue culture plates were coated with retronectin and blocked with 2% BSA. Retrovirus-containing supernatant was added and plates were centrifuged at 2.000×g at 32°C for 1 h. The pre-stimulated $CD8^+$ T cells in the presence of IL-7 and IL-15 were added and after centrifugation for 3 minutes at 100×g at 32° plates were incubated at 37°C for 48 h. For retroviral transduction of memory OT-I T cells, memory cells were first generated by infecting C57BL/6 mice with 1×10^5 PFU of MCMV-OVA that received 1×10^5 splenocytes of $CD45.1^+$ OT-I TCR transgenic mice. After 60 days the memory OT-I T cells were isolated from the spleen by magnetic separation based on CD45.1 expression and were transduced as described above.

Tumor challenge

The colon carcinoma cell line MC38 expressing OVA was generated by transduction of MC38 cells with LZRS-based OVA-IRES-EGFP retrovirus. Tumor cells were maintained in IMDM containing 9% heat-inactivated FCS, 2 mM glutamine, 2-mercaptoethanol, penicillin and streptomycin. WT mice were challenged by s.c. injection in the flank with 2×10^5 MC38-OVA tumor cells in 200 µl PBS. On day 15 after tumor challenge, when tumors were palpable, mice were split in three groups. One group was untreated while the other groups received either control (IRES-EGFP retrovirus) transduced OT-I T cells or IL-2 gene (IL-2-IRES-EGFP retrovirus) transduced OT-I T cells. After T cell transfer, mice were s.c. vaccinated with 50 µg OVA plus 5 µg CpG in the tail base. Tumor sizes were twice a week two-dimensionally measured with calipers to a maximum of 150 mm^2 after which the mice were sacrificed for ethical reasons.

Flow cytometry

Single cell suspensions were prepared from spleen by mincing the tissue through a 70 µm cell strainer (BD Bioscience). Erythrocytes were lysed in a hypotonic ammonium chloride buffer. Before lungs were removed, mice were perfused with PBS containing EDTA to remove all blood associated lymphocytes. Lymphocytes were isolated from the lungs by collagenase (crude, type IA) (Sigma Aldrich) and DNAse I (Sigma Aldrich) treatment for 0.5 h followed by percoll (GE Healthcare, Uppsala) gradient. Tetramer staining and intracellular cytokine staining were used to determine the magnitude and characteristics of the MCMV-specific T cell responses as described elsewhere (21). Gating strategies for MHC class I tetramer staining and intracellular cytokine staining are shown in Supplemental Fig. 1. Fluorochrome-conjugated antibodies specific for CD3 (500A2), CD8 (53-6.7), CD44 (IM7), CD45.1 (A20), CD45.2 (104), CD127 (A7R34), KLRG1 (2F1), Vα2 (B20.1), IFN-γ (XMG1.2), IL-2 (3ES6-5H4) and TNF (MP6-XT22) were purchased from BD Biosciences, BioLegend or eBioscience. Dead cells were excluded by positivity for 7AAD (Invitrogen). Cells were acquired using a BD LSR II flow cytometer, and data was analyzed using FlowJo software (TreeStar).

MHC class I tetramers and peptides

The following class I-restricted peptides were used: $M45_{985-993}$, $m139_{419-426}$, $M38_{316-323}$, $IE3_{416-423}$ as described elsewhere (22, 23) and $OVA_{257-264}$. MHC class I tetramer complexes for M45 (D^b-restricted), m139, M38 and IE3 (all K^b restricted) were produced as described elsewhere (24).

Adoptive transfers

To determine the secondary expansion capacity of MCMV-specific T cells after adoptive transfer, CD45.1+ mice were infected with 5×10^4 PFU MCMV-Smith. At day 60 post-infection, splenic M45, m139, M38 and IE3-specific CD8+ T cells were stained with MHC class I tetramers and purified by sorting using a BD FACSAria II flow cytometer. Next, 8×10^3 M45+, m139+, M38+ and IE3+ CD8+ T cells were transferred i.v. into the same naive CD45.2+ mice. The host mice were infected 3 h later with 1×10^5 PFU MCMV-Smith and at day 5 post-infection the absolute numbers of donor M45, m139, M38 and IE3-specific CD8+ T cells were determined in the spleen and lungs by tetramer staining.

For determining the expansion potential of cells selected for IFN-γ and IL-2 secretion, CD45.1+ mice were infected with 5×10^4 PFU MCMV-Smith and at day 60 post-infection CD8+ splenocytes were isolated using isolation kits and subsequently stimulated with class I-restricted peptides $M45_{985-993}$ or $M38_{316-323}$. IFN-γ and IL-2 secreting CD8+ T cells were enriched using cytokine capture assays according to the manufacturer's instructions (mouse IL-2 and IFN-γ Secretion Assays, Miltenyi Biotec). Next, 1×10^4 IFN-γ or IL-2 secreting M45- and M38-stimulated CD8+ T cells were transferred i.v. into naive CD45.2+ mice. The host mice were infected with 1×10^5 PFU MCMV-Smith and at day 5 post-infection the absolute numbers of donor M45-and M38-specific CD8+ T cells were determined in the spleen and lungs. The fold expansion of the IFN-γ or IL-2 secreting M45 and M38-specific CD8+ T cells was calculated by the number of splenic M45 and M38-specific CD8+ T cells present at day 5 after transfer divided by the exact number of IFN-γ or IL-2 producing M45 and M38-specific CD8+ T cells that were transferred. To determine the expansion capacity of IL-2 low and high producing memory CD8+ T cells, $M45_{985-993}$-specific T cells were sorted with flow cytometry after using a mouse IL-2 Secretion Assay.

For determining the expansion potential of cells selected for IFN-γ and IL-2 secretion using the same TCR Vα and Vβ, CD45.1+ OT-I T cells were transferred into CD45.2+ mice that were subsequently infected with 1×10^5 PFU MCMV-OVA. At day 30 post-infection, CD8+ splenocytes were isolated and stimulated with class I-restricted peptide $OVA_{257-264}$ and IFN-γ and IL-2 secreting CD8+ T cells were isolated using Mouse IL-2 and IFN-γ Secretion Assays. Next, 1×10^4 IFN-γ and IL-2 enriched CD45.1+ OT-I CD8+ T cells were transferred i.v. into naive CD45.2+ mice. The host mice were infected with 1×10^5 PFU MCMV-OVA and at day 5 post-infection the absolute numbers of CD45.1+ OT-I T cells were determined in the spleen and lungs.

For adoptive transfers of retrovirally transduced CD8+ OT-I T cells, cells were sorted

after transduction by flow cytometry based on GFP expression and indicated amount of cells were transferred into naive congenically different recipients or into tumor-bearing mice. Recipient naive mice were infected 3 h later with 1×10^5 PFU MCMV-OVA or challenged with irradiated (3000 Rad) OVA loaded splenocytes, and at day 7 post-infection the absolute numbers of OT-I $CD8^+$ T cells were determined in the spleen and lungs.

Statistical analysis

Statistical significance of 2 groups was assessed by unpaired two-tailed Student's *t*-tests and for comparing more than 2 groups one-way ANOVA with Tukey's post hoc test was used. GraphPad Prism software was used for all calculations and justifications. Sample size was chosen based on previous experiments and preliminary experiments. *P* values < 0.05 were considered significant.

RESULTS

The percentage of IL-2 producing $CD8^+$ T cells predicts the secondary expansion capacity of the total antigen-specific population

To examine the contribution of the IL-2 producing $CD8^+$ T cell subset to the expansion potential of antigen-specific T cell populations, we used the mouse cytomegalovirus (MCMV) infection model in which virus-specific memory $CD8^+$ T cell populations, as defined by the capacity to produce IFN-γ and bind MHC class I tetramers, have differential capacities to produce IL-2 (Supplemental Fig. 2 and Fig. 1A and 1B). Congenically (CD45.1) marked IL-2 producing MCMV-specific memory $CD8^+$ T-cell populations that contained high (M45-specific), moderate (m139 and M38-specific) and low (IE3-specific) percentages of IL-2 producers among the total antigen-specific population were adoptively transferred from latently infected mice into naive (CD45.2) recipients, which were subsequently challenged with MCMV (Fig. 1C). Evidently, the antigen-specific $CD8^+$ T cell population (i.e. M45) comprising the highest frequency of IL-2 producing cells, expanded most vigorously after re-challenge, followed by the moderate and low IL-2 producing antigen-specific $CD8^+$ T cell pools, demonstrating a correlation between the percentage of IL-2 producers and the secondary expansion capacity of the total antigen-specific T cell population (Fig. 1D**)**. The predictive value of the relative IL-2 producers among the total population for the secondary expansion capacity was observed in both lymphoid (spleen) (Fig. 1E**)** and non-lymphoid tissue (lung) (Fig. 1F), suggesting that the improved secondary expansion potential is not the result of altered migration characteristics. Thus, the percentage of IL-2 producing $CD8^+$ T cells within the total antigen-specific population is predictive for its expansion capacity as a whole.

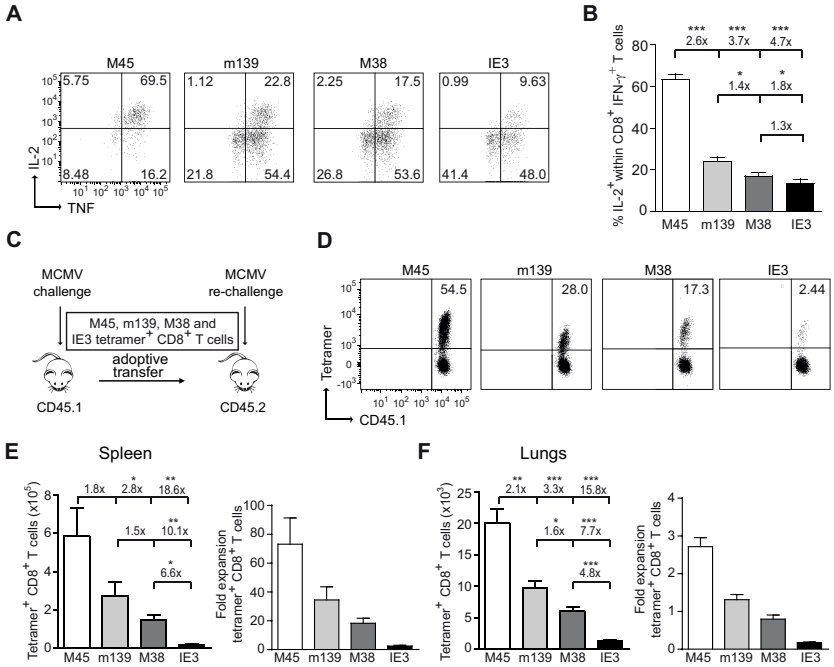

Figure 1. The frequency of IL-2 producing CD8+ T cells correlates with population expansion capacity. WT mice were infected with 5 × 10^4 PFU MCMV-Smith. (**A**) Intracellular TNF and IL-2 production within the splenic IFN-γ+CD8+ T cells after re-stimulation with M45, m139, M38 or IE3 class I peptides determined at day 60 post-infection. (**B**) Frequency of IL-2+ cells within splenic IFN-γ+CD8+ T cell populations at day 60 post-infection. (**C**) Schematic of the experimental setup: CD45.1+ mice were infected with 5 × 10^4 PFU MCMV-Smith, and after 60 days M45, m139, M38 and IE3 MHC class I tetramer+ CD8+ T cells were sorted and 8 × 10^3 cells were adoptively transferred into naive CD45.2+ recipients, which were subsequently infected with 5 × 10^4 PFU MCMV-Smith. (**D**) Staining of CD45.1+CD8+ T cells with M45, m139, M38 and IE3 MHC class I tetramers at day 5 after adoptive transfer. (**E**) Fold expansion and absolute numbers are shown for MCMV-specific CD8+ T cells in the spleen. (**F**) Fold expansion and absolute numbers are shown for MCMV-specific CD8+ T cells in the lungs. Data represents mean values + SEM (n=5 per experiment; **P* < 0.05, ***P* < 0.005, ****P* < 0.0005 (One-way ANOVA)), and are representative of three independent experiments.

Autocrine IL-2 producers contribute predominantly to CD8+ T cell population expansion

The predictive value of the IL-2 producing capacity for the secondary expansion capacity of antigen-specific T cell populations, poses the question whether this is an inherent property of IL-2 producers. To explore this question, we compared secondary proliferative responses between CD8+ T cell fractions with the same specificity that are enriched for either IFN-γ or IL-2 secreting cells. We isolated IFN-γ and IL-2 producing memory M45 and M38-specific CD45.1+ CD8+ T cells by cytokine capture assays, which allow enrichment of viable cytokine-secreting cells, and adoptively transferred these cells into naive CD45.2+ recipients that were subsequently challenged with MCMV (Fig. 2A and Supplemental Fig. 3A-C). The M45-specific CD8+ T cells enriched for IFN-γ show 2-3 fold increased expansion potential compared to M38-specific CD8+

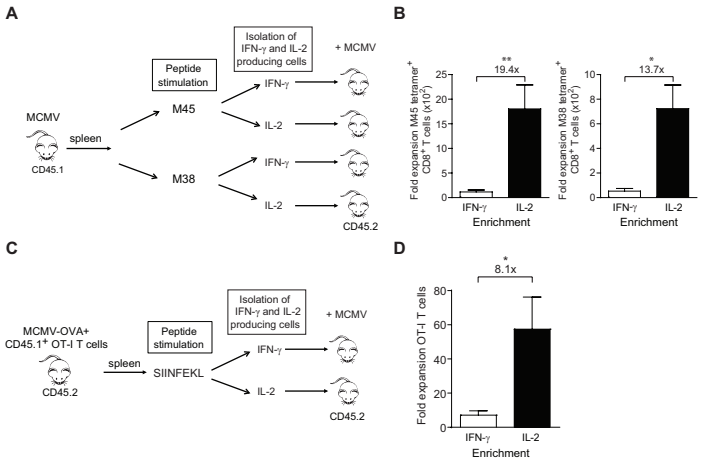

Figure 2. Autocrine IL-2 producers are crucial for CD8⁺ T cell population expansion. (**A**) Schematic of the experimental setup: CD45.1⁺ mice were infected with 5 × 10^4 PFU MCMV-Smith, and after 60 days splenic CD8⁺ T cells were isolated, re-stimulated with M45 or M38 class I peptide, and enriched for IFN-γ or IL-2 production by cytokine secretion capture assays. Isolated cells (1 × 10^4 of the enriched population) were transferred into naive CD45.2⁺ recipients that were subsequently infected with 5 × 10^4 PFU MCMV-Smith. (**B**) Fold expansion is shown for the IFN-γ or IL-2 producing M45 and M38-specific CD8⁺ T cells in the spleen at day 5 post-infection. (**C**) Schematic of the experimental setup: CD45.2⁺ WT mice received 2.5 × 10^4 CD45.1⁺ OT-I cells, and were subsequently infected with 1 × 10^5 MCMV-OVA. After 30 days splenic CD8⁺ T cells were isolated, re-stimulated with $OVA_{257-264}$ class I peptide and enriched for IFN-γ or IL-2 producing cells by cytokine secretion capture assays. Isolated cells were transferred into naive CD45.2⁺ recipients that were subsequently infected with 1 × 10^5 PFU MCMV-OVA. (**D**) Fold expansion of the IFN-γ or IL-2 producing CD45.1⁺ OT-I T cells in the spleen at day 5 post-infection. Data represents mean values + SEM (n=5 per group; $*P < 0.05$, $**P < 0.005$ (two-tailed unpaired *t*-test)), and are representative of 2-3 independent experiments.

T cells enriched for IFN-γ, which is consistent with the difference in expansion capacity observed between M45 and M38 MHC class I tetramer-enriched CD8⁺ T cells (Fig. 1D and 2B), and relates to the presence of a higher percentage of IL-2 producers among the IFN-γ producers in the M45-specific population as compared to the M38-specific population. Importantly, the IL-2 secreting enriched CD8⁺ T cells of both the M45- and M38-specific populations displayed superior expansion potential compared to the populations that were enriched for IFN-γ production (Fig. 2B). The IL-2 secreting M45-specific population is about 2 fold greater as compared to the M38-specific population, suggesting a potential difference in the differentiation status of these cells but this may also relate to differences in TCR affinity or in levels of cognate antigen. All, together these results indicate that the IL-2 producers within antigen-specific CD8⁺ T cell populations are predominantly contributing to the population expansion upon secondary challenge. Thus, the secondary expansion potential is not predicted by merely activated CD8⁺ T cells producing cytokines, but is confined to the IL-2 producers.

The IL-2 producing CD8⁺ T cells might have undergone a different clonal expansion resulting in altered selection of the TCRs leading to differences in expansion potential. To rule out possible confounding effects of TCR affinity and usage, we investigated whether the autocrine IL-2 producers also have a superior expansion capacity within the total

antigen-specific T cell population in settings in which all CD8+ T cells have the same TCRs. To asses this, we used TCR transgenic OT-I T cells, which have identical TCRs (Tcra-V2 and Tcrb-V5) recognizing the H-2K^b-restricted SIINFEKL epitope of ovalbumin (OVA) (25). Memory CD45.1+ OT-I T-cells were generated by infection with recombinant MCMV expressing OVA (MCMV-OVA), and these cells were subjected to cytokine capture assays (Fig. 2C). Following adoptive transfer of OT-I T cell populations enriched for either IFN-γ or IL-2 secreting cells into naive CD45.2+ mice that were challenged with MCMV-OVA, we observed that the IL-2-enriched OT-I T cells were markedly more potent in expanding upon antigen re-encounter compared to the IFN-γ-enriched counterpart (Fig. 2D). Thus, also in CD8+ T cell populations with identical TCRs (and thus TCR affinity), the IL-2 secreting cells are the main contributors to the secondary expansion potential.

Enhancement of autocrine IL-2 production directly improves the CD8+ T cell expansion capacity

We next hypothesized that enhancing the relative autocrine IL-2 production within an antigen-specific CD8+ T cell population could possibly improve the proliferative capacity. To assess this, we used a gain of function approach by generating retroviral constructs containing the IL-2 gene, EGFP sequences, and IRES sequences located between IL-2 and EGFP to allow translation of both genes. Subsequent, CD45.1+ OT-I T cells were transduced with control retrovirus (IRES-EGFP) or with the IL-2 gene containing retrovirus (IL-2-IRES-EGFP) (Fig. 3A). Transduced cells were sorted based on GFP expression (Supplemental Fig. 4A) and stimulation with PMA and ionomycin revealed that IL-2 production was indeed increased in IL-2-IRES-EGFP transduced cells compared to cells that were transduced with the control vector (Fig. 3B). After adoptive transfer into naive (CD45.2+) recipients that were challenged with MCMV-OVA, the transduced OT-I T cells showed no difference in phenotypical markers (i.e. CD127 and KLRG1) between control transduced and IL-2 gene transduced cells. In addition, no difference was observed in the IFN-γ producing capacity (Supplemental Fig. 4B and 4C). Importantly, we did observe that the proliferative capacity of OT-I T cells transduced with the IL-2 gene containing vector upon challenge with MCMV-OVA was considerably augmented compared to control vector transduced cells. The increased expansion capacity of the IL-2 gene transduced cells was found with both relatively low and high numbers of transferred cells, indicating that the IL-2 mediated effect operates without being influenced by clonal competition (Fig. 3C and 3D and Supplementary Fig. 4C and 4D). Moreover, the increased expansion population capacity due to the additional IL-2 production was observed in both lymphoid tissue (spleen) and non-lymphoid tissue (lungs) (Fig. 3C and 3D and Supplementary Fig. 4D and 4E).

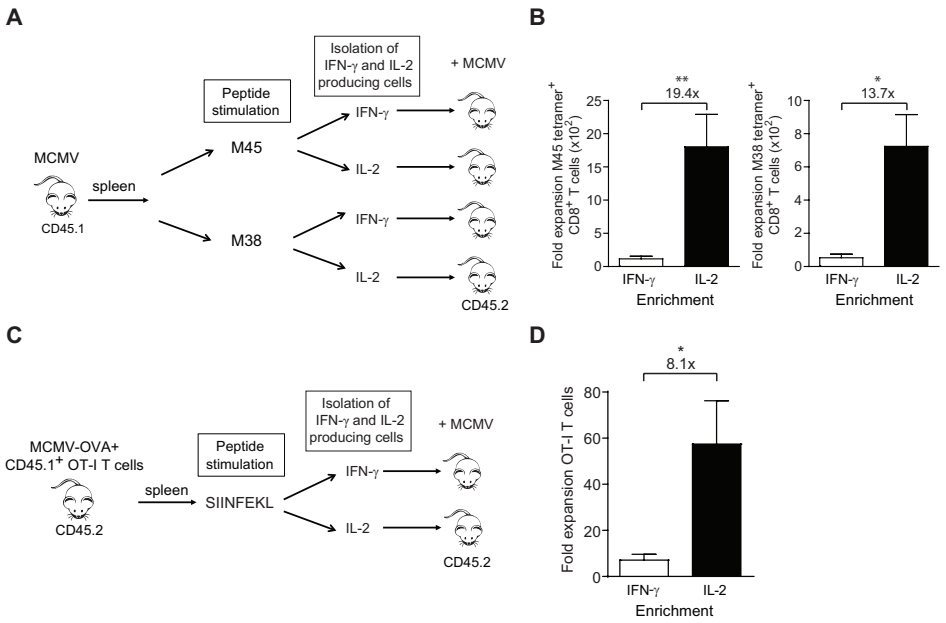

Figure 3. Improved autocrine IL-2 production increases CD8⁺ T cell expansion. (**A**) Schematic of the experimental setup: CD45.1⁺ OT-I T cells were transduced with IRES-EGFP or IL-2-IRES-EGFP retrovirus, sorted for GFP expression and 1×10^3 or 1×10^4 cells were transferred into naive CD45.2⁺ recipients, which were subsequently infected with 1×10^5 PFU MCMV-OVA. (**B**) IL-2 expression of transduced OT-I T cells as assessed by intracellular cytokine staining after stimulation with PMA and ionomycin. (**C**) Fold expansion of the transduced OT-I T cells in the spleen and in the lungs after transfer of 1×10^3 cells. (**D**) Fold expansion of the transduced OT-I T cells in the spleen and in the lungs after transfer of 1×10^4 cells. Data represents mean values + SEM (n=5 per group; ***$P < 0.0005$ (two-tailed unpaired *t*-test)), and are representative of 3 independent experiments.

To determine whether the autocrine IL-2-mediated effects are also obtained during non-inflammatory conditions, control and IL-2 transduced OT-I T cells were challenged with OVA-loaded splenocytes after transfer (Fig. 4A). We clearly observed that also during these conditions, elevating the autocrine IL-2 production results in a much better CD8⁺ T cell expansion (Fig. 4B). Thus, enhancing the IL-2 producing capacity of CD8⁺ T cells in both inflammatory and noninflammatory settings improves the expansion potential.

Enhanced autocrine IL-2 production by CD8+ T cells improves viral clearance and delays tumor progression

Next, we tracked the fate of the MCMV-OVA-challenged transduced OT-I cells in time. At day 60 post-challenge, the numbers of IL-2 gene transduced OT-I T cells compared to control transduced OT-I T cells were increased. Moreover, after re-challenge of these cells with vaccinia virus expressing OVA (VV-OVA), the memory OT-I T cells that had an increased capacity to produce IL-2 also had a superior proliferative capacity compared to control OT-I T cells (Fig. 5A and 5D). To examine the effector and memory phenotype of the transduced OT-I cells before and after re-challenge, we used the well-established

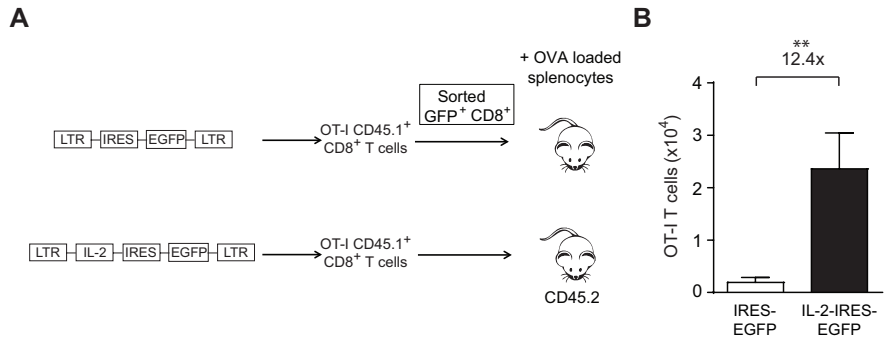

Figure 4. Enhanced autocrine IL-2 production increases CD8+ T cell expansion during noninflammatory conditions. (**A**) Schematic of the experimental setup: CD45.1+ OT-I T cells were transduced with IRES-EGFP or IL-2-IRES-EGFP retrovirus, sorted for GFP expression and 1×10^4 cells were transferred into naive CD45.2+ recipients, which were subsequently challenged with irradiated OVA-loaded splenocytes. (**B**) Absolute numbers of the transduced OT-I T cells in the spleen after transfer. Data represents mean values + SEM (n=4 per group; $^{**}P < 0.005$ (two-tailed unpaired *t*-test)). Experiment was performed twice, and similar results were obtained.

markers CD127 (IL-7Rα; marker for central-memory T cells) and KLRG1 (marker for effector-memory T cells (Fig. 5B). We found that the phenotype of the control transduced and the IL-2 gene transduced memory T cells was similar in CD127 and KLRG1 phenotype in a resting state, but after secondary challenge the expression of CD127 is higher on the IL-2 transduced T cells as compared to the control transduced T cells. This indicates that improved autocrine IL-2 can lead to increased CD127 expression after challenge, and is consistent with the fact that both IL-2 production and CD127 expression are markers for the central-memory T cell subset. The expression of the effector cytokines IFN-γ and TNF were comparable between control transduced and IL-2 gene transduced memory OT-I T cells, and this was observed before and after viral challenge (Fig. 5C).
Next, we assessed whether this IL-2 mediated superior secondary proliferation coincides with improved CD8+ T cell dependent immunity. Indeed, the viral titers of the VV-OVA challenged mice that received the IL-2 gene transduced OT-I cells were lower as compared to the control transduced memory OT-I T cells (data not shown). Moreover, the transfer of similar numbers of IL-2 gene transduced and control transduced memory OT-I CD8+ T cells into naive recipients that were subsequently challenged with VV-OVA also resulted in a decrease in viral load (~5 fold) in the group of mice that received the memory T cells with improved autocrine IL-2 production (Fig. 5E). Correspondingly, adoptive transfer of IL-2 gene transduced OT-I T cells in tumor bearing mice delayed tumor progression considerably as compared to control transduced OT-I T cells (Fig. 5F). Taken together, these data indicate that enhancing the autocrine IL-2 production extends the capacity to which CD8+ T cells can expand upon antigenic challenge leading to improved immunity.

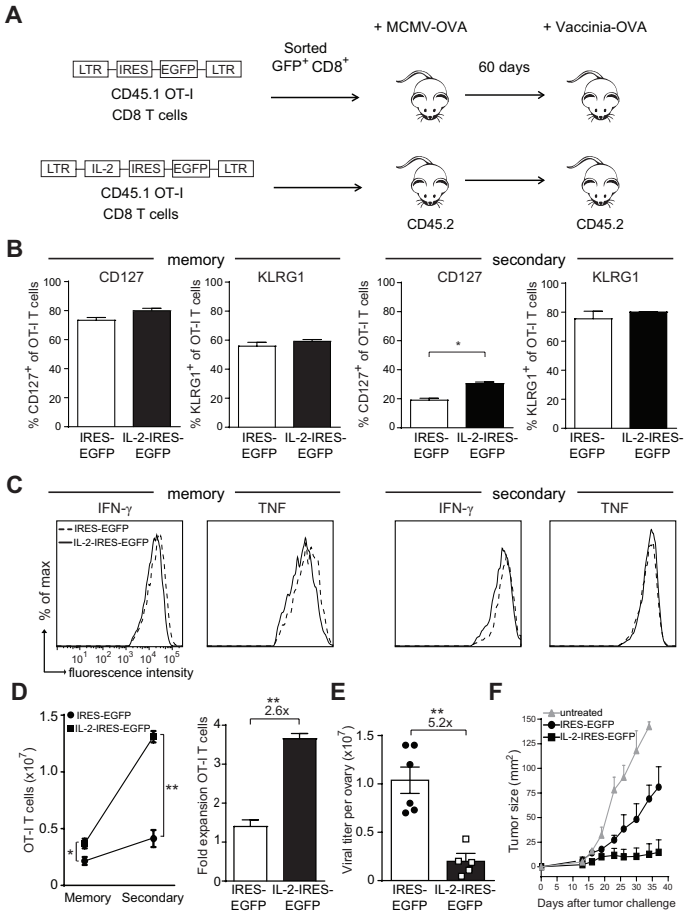

Figure 5. Enhanced autocrine IL-2 production by CD8⁺ T cells improves viral clearance and delays tumor progression. (**A**) Schematic of the experimental setup: naive CD45.1⁺ OT-I T cells were transduced with IRES-EGFP or IL-2-IRES-EGFP retrovirus, sorted for GFP expression and 1 × 10^4 GFP⁺ cells were transferred into naive CD45.2⁺ recipients, which were subsequently infected with 5 × 10^4 PFU MCMV-OVA. At day 60 post-infection, half of the mice were challenged with 2 × 10^6 PFU vaccinia virus expressing OVA (VV-OVA) to determine secondary responses. Both challenged (secondary) and unchallenged (memory) groups were analyzed 65 days after adoptive transfer of the OT-I T cells. (**B**) Frequency of CD127 and KLRG1 expressing transduced CD8⁺ OT-I T cells in the spleen. (**C**) Histograms show the IFN-γ and TNF expression of the transduced CD8⁺ OT-I T cells in the spleen. (**D**) Absolute numbers of control and IL-2 gene transduced CD8⁺ OT-I T cells in the spleen of challenged and unchallenged mice. Bar graph shows fold expansion of splenic CD8⁺ OT-I T cells after secondary challenge. Data represents mean values + SEM (n=5 per group; **P* < 0.05 and ***P* < 0.005 (two-tailed unpaired *t*-test)). Experiment was performed twice, and similar results were obtained. (**E**) Similar experimental setup as in (A) but at day 60 post transfer the transduced memory CD45.1⁺ OT-I T cells were sorted and 1 × 10^4 cells were transferred into naive CD45.2⁺ recipients that subsequently received a challenge with 2 × 10^6 PFU VV-OVA. After 5 days, VV-OVA titers were determined in the ovaries of the mice.
Each symbol represents an individual mouse. Data represents mean values + SEM (n=5-6 per group; **P < 0.005 (two-tailed unpaired t-test)). Experiment was performed twice, and similar results were obtained. (F) WT mice were injected s.c. in the flank with 2 × 105 MC38-OVA tumor cells. At day 15 post tumor challenge, when tumors were palpable, mice were left untreated or received 2 × 105 OT-I T cells that were transduced with either IRES-EGFP or IL-2-IRES-EGFP retrovirus. After OT-I transfer, mice were vaccinated with OVA plus CpG at the tail base. Shown is the tumor outgrowth measured two-dimensionally (mm2). Data represents mean values + SEM (n=4 per group) of 2 independent experiments.

The proliferative capacity of CD8+ T cells is autocrine IL-2 dose dependent

To demonstrate a potential difference in expansion capacity among IL-2 producing CD8+ T cells that relates to the amount of IL-2 produced on a per-cell basis, we examined the effect of differential autocrine IL-2 production on the proliferative potential. To address this point, we transduced CD45.1+ OT-I T cells with control retrovirus (IRES-EGFP) or retrovirus containing the IL-2 gene (IL-2-IRES-EGFP) and sorted GFP low, intermediate and high expressing cells (Fig. 6A). As we used a bicistronic construct with an IRES sequence between the IL-2 and the EGFP cDNAs, it is expected that the translation of both IL-2 and EGFP occurs and is related to each other, as has been shown before (19). Indeed, transduced CD8+ T cells sorted for high GFP expression contained the highest amount of IL-2 protein (Fig. 6B). After adoptive transfer and subsequent re-challenge, cells transduced with IL-2-IRES-EGFP and sorted for high GFP/IL-2 expression displayed significant increased expansion compared to cells sorted for GFP/IL-2 low expression. OT-I T cells with intermediate expression obtained a correspondingly in-between capacity to expand. OT-I T cells transduced with the control vector and sorted for high, intermediate and low expression of GFP did not show a differential ability to proliferate, showing that the dose dependent expansion potential relates to IL-2 production and is not due to confounding effects of increased transduction efficiency (Fig. 6C).

Next, we aimed to validate the dose dependent fashion of the autocrine IL-2 mediated CD8 T cell expansion in memory T cells from an endogenous repertoire. For this, we sorted of IL-2^{low} and IL-2^{high} expressing M45-specific memory CD8+ T cells from MCMV-infected mice, and transferred these cells into naive mice that were challenged with MCMV. As observed with transduced OT-I T cells (Fig. 6D), endogenous memory T cells with high expression of IL-2 expanded significantly better than IL-2 low expressing CD8+ T cells (Fig. 6E). Together, these data show that the extent to which a CD8+ T cell can expand depends on the amount of autocrine IL-2 that is produced.

DISCUSSION

Our study has identified that the percentage of IL-2 producers is predictive for the expansion capacity of the antigen-specific CD8+ T cell population as a whole, and that these IL-2 producers themselves possess superior expansion potential. Moreover, enhancing the autocrine IL-2 production of CD8+ T cells results in improved expansion of these cells and to heightened immunity. In addition, we show that the amount of IL-2 that is produced on a per-cell basis is predictive for the degree of the proliferative capacity. Thus, CD8+ T cell derived IL-2 could not only be used as an indicator for the expansion population potential but could also be exploited therapeutically as a direct mediator to increase the proliferative potential of antigen-specific CD8+ T cells.

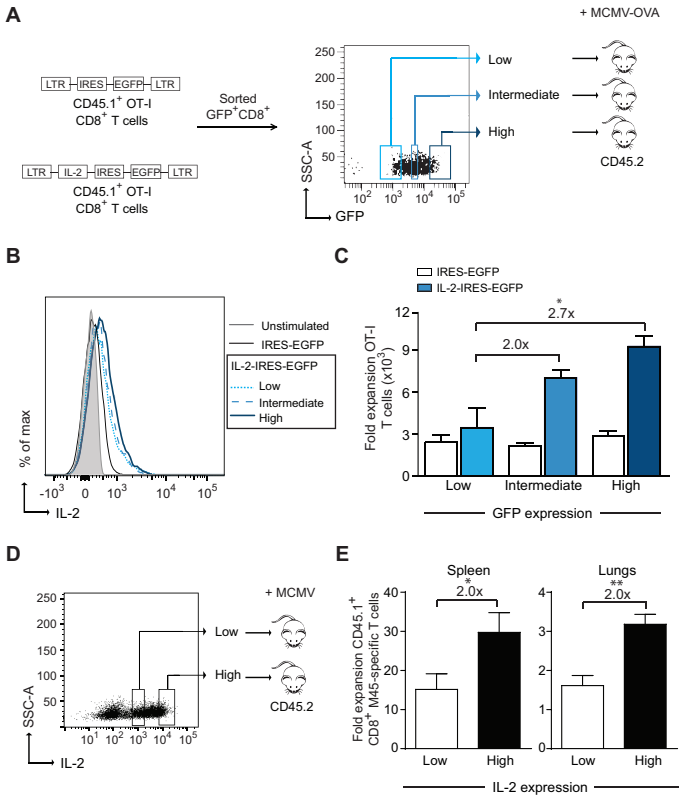

Figure 6. The expansion capacity of CD8⁺ T cells is autocrine IL-2 dose dependent. (**A**) Schematic of the experimental setup: naive CD45.1⁺ OT-I T cells were transduced with IRES-EGFP or IL-2-IRES-EGFP retrovirus, and sorted for low, intermediate and high GFP expression. 1×10^4 sorted cells were transferred into naive CD45.2⁺ recipients, which were subsequently infected with 1×10^5 PFU MCMV-OVA. (**B**) IL-2 expression of transduced low, intermediate and high GFP expressing OT-I T cells as assessed by intracellular cytokine staining after stimulation with PMA and ionomycin. The blue lines represent the IL-2-IRES-EGFP transduced OT-I T cells. The black line represents the high GFP expressing IRES-EGFP transduced OT-I T cells. (**C**) Fold expansion of IRES-EGFP and IL-2-IRES-EGFP transduced OT-I T cells, which were sorted for low, intermediate or high GFP expression, in the spleen. (**D**) CD45.2⁺ mice were infected with 5×10^4 PFU MCMV-Smith, and after 60 days splenic CD8⁺ T cells were isolated, re-stimulated with M45 class I peptide, and enriched for IL-2 producing cells by cytokine secretion capture assays. After isolation cells were sorted by flow cytometry for low or high IL-2 production and transferred into naive CD45.1⁺ recipients that were subsequently infected with 5×10^4 PFU MCMV-Smith. (**E**) Fold expansion of CD45.2⁺ CD8⁺ T cells, which were sorted for low or high IL-2 expression, in the spleen and lungs. $^{**}P < 0.005$ and $^{***}P < 0.0005$ (two-tailed unpaired *t*-test). Data represents mean values + SEM (n=5 per experiment) from two independent experiments.

Several studies have provided evidence that the quality of the T cell response against pathogens could be used as a correlate of protection, and the cytokine polyfunctionality of the CD8⁺ T cells is considered as an important parameter to determine this (26). Cytokine polyfunctionality of T cells is an important characteristic of the so-called central-memory T cells, and several studies indicated that the expansion potential of central-memory T cells is superior as compared to effector-memory T cells with respect to expansion (27-31). Autocrine IL-2 production is part of the polyfunctional cen-

tral-memory T cell profile and our study now provides direct evidence that autocrine IL-2 production is contributing to a greater expansion capacity of $CD8^+$ T cells. Also in case of anti-tumor immunity, it has been suggested that IL-2 production of tumor infiltrating $CD8^+$ lymphocytes could be considered as a pharmacodynamic biomarker for clinical responses (32, 33). However, IL-2-independent processes are likely important as well and may also be implicated in the self-renewal of central-memory $CD8^+$ T cells (34, 35).

In addition to demonstrating the importance of the autocrine IL-2-producing antigen-specific $CD8^+$ T cells for the total expansion population capacity, we show that the quantity of IL-2 produced on a per cell-basis is related to the expansion potential. This finding is consistent with the report by Cheng et al. wherein they suggest that the availability of IL-2R signals to $CD8^+$ T cells limits the size of the response (36). This also implies that autocrine secretion of IL-2 by itself, irrespective of the differentiation status of the $CD8^+$ T cell, is directly responsible for increasing the expansion potential. However, it may be that autocrine IL-2, besides providing direct signals for expansion via the activation of several signaling pathways (including JAK/STAT, PI3K/AKT, and MAPK pathways) (10), also impacts the differentiation status of T cells leading to other factors that could further improve T cell expansion and survival. Indeed, we found increased CD127 expression of the IL-2 gene transduced $CD8^+$ T cells after challenge, suggesting that CD127-mediated signals may be one of such factors.

Improving the autocrine IL-2 production is likely more advantageous in clinical settings as compared to exogenous administrated IL-2, which is not only available to $CD8^+$ T cells but also to other cell types expressing the IL-2 receptor, of which for example T_{regs} can exert negative effects on $CD8^+$ T cell responses (37-39). Although, it has previously been suggested that some of the IL-2 that is produced by $CD8^+$ T cells can stimulate Tregs (40), we did not observe alternations in the homeostasis of $CD4^+CD25^+Foxp3^+$ Tregs (data not shown). In addition, we did not observe significant effects of the IL-2 gene transduced $CD8^+$ T cells on the endogenous antigen-specific $CD8^+$ T cell responses (data not shown). One of the major challenges regarding vaccine development is the induction of T cells that have potent expansion capacity and also form long-lasting memory. IL-2 producing $CD8^+$ T cells meet these criteria and since it has been shown that these cells indeed confer improved protection, dissecting the precise requirements for induction of IL-2 producing antigen-specific $CD8^+$ T cells would be of great interest for the development of novel vaccine strategies (26). Such strategies might include blocking of inhibitory molecules like PD-1 and CTLA4 or providing agonistic antibodies to enhance costimulatory signaling. An alternative way of manipulating T cells to optimizing their fitness is gene transfer using retroviral vectors. In clinical trials retroviral vectors have been used already successfully to engineer T cells for example to express tumor- or virus-specific TCRs; chimeric antigen receptors (CARs); immunosuppressive cytokines or enzymatic genes

(41, 42). Although clinical responses are promising, some responses seem to be transient (43, 44). Combining *ex vivo* expansion of disease-specific T cells with transduction of the IL-2 gene and subsequent vaccination might be a promising approach to treat persistent viral infections and cancer.

To conclude, we propose that the increment of IL-2 producers and/or improvement of autocrine IL-2 production on a per cell basis should be considered in future efforts regarding the development of anti-tumor and anti-viral $CD8^+$ T cell dependent vaccines and immunotherapies.

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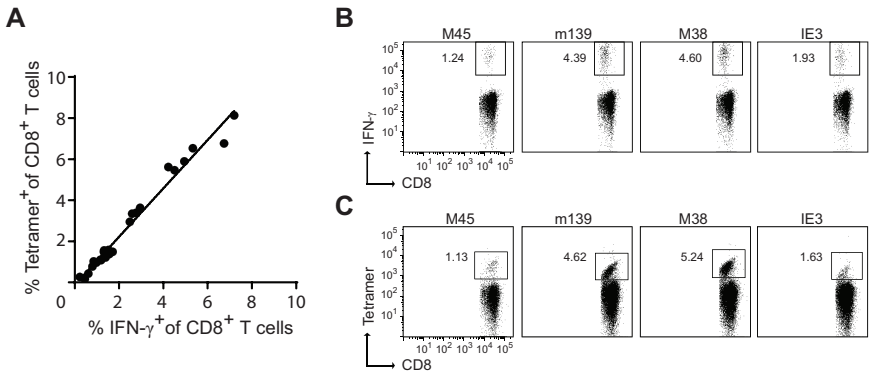
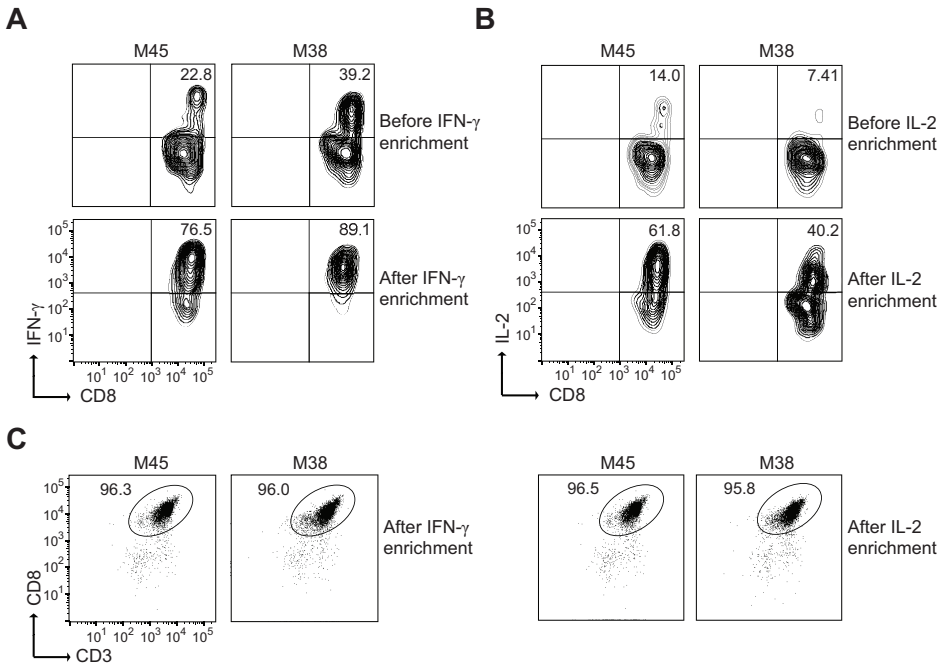
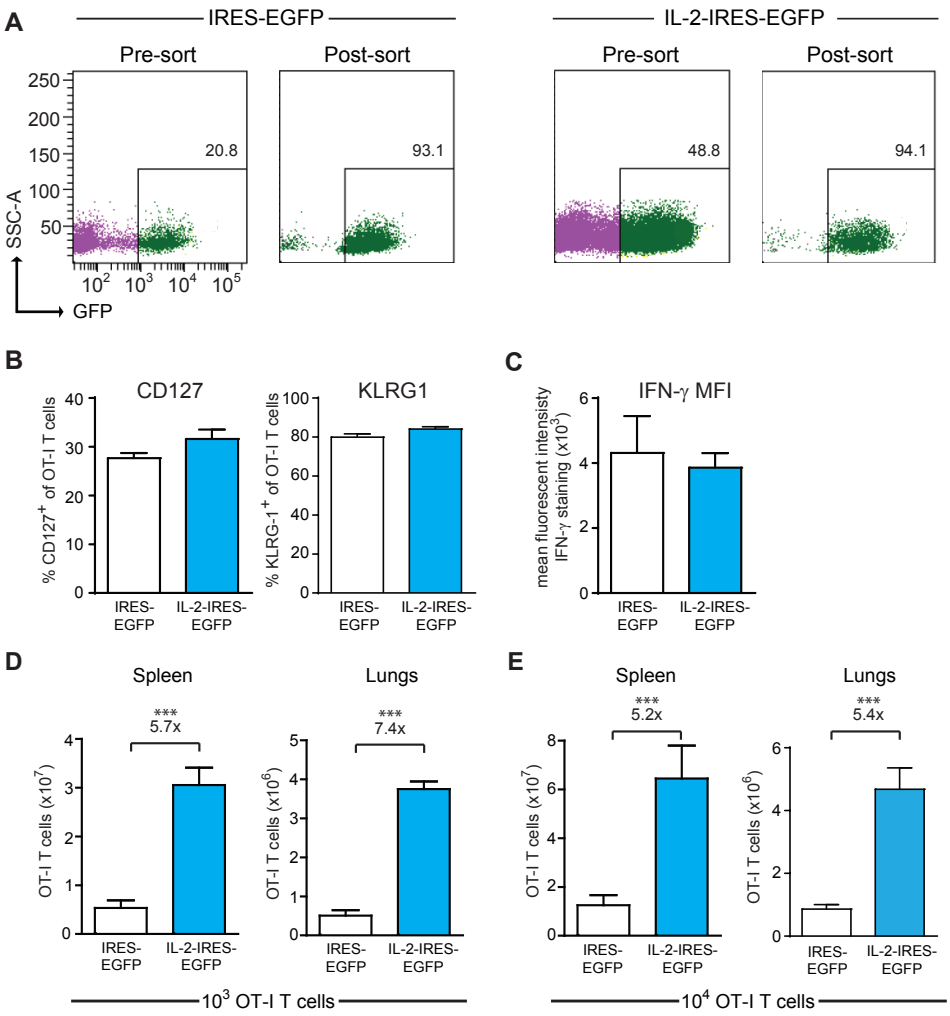
SUPPLEMENTARY INFORMATION



Supplementary Figure 1. MCMV-specific T cells as determined by MHC class I tetramer staining correlate with intracellular staining for IFN-γ. (A) WT mice were infected with 1 × 10^4 PFU MCMV-Smith and 60 days post-infection the frequency of MCMV-specific $CD8^+$ T cells was measured by IFN-γ production after restimulation with MCMV peptides and by class I tetramer staining. Graph shows % of MCMV-specific IFN-γ producing $CD8^+$ T cells plotted against the % of $tetramer^+$ $CD8^+$ T cells. (B) Gating of MCMV-specific $CD8^+$ T cells. Representative plots show M45, m139, M38, and IE3-specific T cells gated on basis of IFN-γ production. Data are representative of three independent experiments. (C) Gating of MCMV-specific $CD8^+$ T cells. Representative plots show M45, m139, M38, and IE3-specific T cells gated on basis of class I tetramer staining. Data are representative of three independent experiments.



Supplementary Figure 2. Flow cytometric analysis of IFN-γ and IL-2 enriched CD8+ T cell populations using cytokine capture assays. (A) Representative plots show the frequency of M45 and M38-specific CD8+ T cells producing IFN-γ. (B) Representative plots show the frequency of M45 and M38-specific CD8+ T cells producing IL-2 before and after enrichment. (C) Representative plots show the frequency of CD3+ CD8+ T cells after enrichment for CD8+ T cells and subsequent enrichment of IFN-γ and IL-2 by cytokine secretion assays. Data are representative of two independent experiments (n=5 per experiment).



Supplementary Figure 3. Flow cytometric analysis of IFN-γ and IL-2 enriched CD8⁺ T cell populations using cytokine capture assays. (A) Representative plots show the frequency of M45 and M38-specific CD8⁺ T cells producing IFN-γ. (B) Representative plots show the frequency of M45 and M38-specific CD8⁺ T cells producing IL-2 before and after enrichment. (C) Representative plots show the frequency of CD3⁺ CD8⁺ T cells after enrichment for CD8⁺ T cells and subsequent enrichment of IFN-γ and IL-2 by cytokine secretion assays. Data are representative of two independent experiments (n=5 per experiment).

CHAPTER 3

Programming of IL-2 production by CD8$^+$ T cells requires collective CD27 and CD28 signaling

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ABSTRACT

Defining the critical molecular interactions for induction and maintenance of effective antiviral T cell responses is important to improve vaccine strategies. By interrogating which costimulatory molecules were critical for polyfunctional cytokine production, we found that in particular IL-2 production by virus-specific $CD8^+$ T cells was collectively dependent on both the CD27-CD70 and CD28-CD80/86 costimulatory pathways. This costimulation-dependent effect on the quality of viral-specific $CD8^+$ T cells is programmed early after the onset of viral infection and remains established. The induction of the nuclear transcription factor c-Rel, a NF-kB family member known to be critical for IL-2 transcription, was found to differently regulated by CD70 and CD80/86-mdeiated costimulatory signals, and full induction was only achieved by collective signaling. These results reveal that IL-2 production by $CD8^+$ T cells is determined by distinct costimulatory pathways that act jointly. Thus, incorporation of targeting the critical costimulatory pathways could expand the current T-cell based immunotherapies.

INTRODUCTION

Upon infection, antigen-specific T cells are primed via binding of their unique T cell receptors (TCRs) to MHC-peptide complexes expressed by antigen presenting cells (APCs). Costimulatory receptor/ligand interactions augment TCR triggering and provide crucial signals to promote T cell proliferation, survival, cytokine production and effector functions (1). T cell costimulation mediated via CD28 upon binding its ligands CD80 (B7.1) and CD86 (B7.2), and costimulatory interactions between members of the TNF receptor (TNFR)/TNF ligand superfamily such as CD27/CD70, OX40/OX40L and 4-1BB/4-1BBL are particularly important in this respect. The quality of antigen-specific T cell populations is directly connected to their functional properties including the cytotoxic potential and ability to produce multiple effector cytokines upon antigenic stimulation. As polyfunctional T cells have been positively associated with virus control in chronic infections of EBV, CMV and HIV (2-4), an important aim of vaccination and immunotherapeutic strategies is to induce and/or preferentially use polyfunctional T cells. Important effector cytokines produced by polyfunctional $CD8^+$ T cells include interferon-γ (IFN-γ), tumor necrosis factor (TNF) and interleukin-2 (IL-2). IFN-γ production usually identifies all antigen-specific $CD8^+$ T cells (except in case of functional exhaustion), while TNF and IL-2 are produced by subsets of the antigen-specific $CD8^+$ T cell pool. Nevertheless, IL-2 production impacts all aspects of a T cell response (5) including effector and memory T cell formation (6). Furthermore, secondary $CD8^+$ T cell expansion is highly dependent on IL-2 signaling (7) in an autocrine manner (8). Recently, we showed that the relative amount of IL-2 on a per-cell basis is associated with a superior expansion potential of the total antigen-specific $CD8^+$ T cell population (9). Therefore manipulation of IL-2 production in antigen-specific $CD8^+$ T cells could be of great value to exploit in vaccination and in immunotherapeutic settings.

Factors that control IL-2 production include TCR triggering and costimulatory signals provided by the CD28/B7 pathway. The significance of CD28-mediated IL-2 production is exemplified in the reduction of IL-2 producing antigen-specific $CD8^+$ T cells upon abrogation of CD28/B7 costimulation during infection with vaccinia virus (VV), *Listeria monocytogenes* (LM), lymphocytic choriomeningitis virus (LCMV), murine gammaherpesvirus, and cytomegalovirus (CMV) (10-14). The mechanisms via which CD28 triggering stimulates IL-2 production are diverse, ranging from binding of transcription factors and epigenetic modifications along the IL-2 promoter region to IL-2 mRNA stabilization (15-18). IL-2 production in $CD8^+$ T cells is also affected by CD27 costimulation (19), and diminished IL-2 producing T cells are found upon abrogation of CD27/CD70 interactions (20). Thus both CD28/B7 and CD27/CD70 interactions are implicated in the induction of IL-2 production, yet whether these molecules act in a redundant or collective manner is unclear.

Here we show that CD70 and CD80/86–mediated signals function in a cooperative

manner via joint induction of the nuclear factor c-Rel to induce IL-2 production in virus-specific $CD8^+$ T cells. Furthermore, we found that IL-2 and costimulatory signals act synergistically to optimally enhance T cell expansion. These results have important implications for vaccination strategies, where incorporation of dual CD27 and CD28 triggering needs to be considered to enhance the fitness of vaccine induced $CD8^+$ T cells.

MATERIALS AND METHODS

Mice

C57Bl/6 mice were obtained from Charles River and used as wild type (WT) mice. *Cd70*$^{-/-}$ (37) and *Cd80/86*$^{-/-}$ on a C57BL/6 background were bred in house. *Cd70/80/86*$^{-/-}$ mice were generated by crossing *Cd70*$^{-/-}$ mice with *Cd80/86*$^{-/-}$ mice and are described (22) (Welten). CD11c Cre/iDTR mice Ly5.1 (CD45.1) and Ly5.1 TCR transgenic OT-I mice all on a C57BL/6 background were bred in house. All animals were maintained on specific pathogen free conditions at the animal facility in the Leiden University Medical Center. Mice were matched for age and gender and were between 8-12 weeks at the start of each experiment. All experiments were performed according to the Dutch guidelines of animal experimentation and were approved by the animal ethics committee.

Viral quantification and infections

MCMV-Smith was obtained from the American Type Culture Collection (VR-194; Manassas, VA). Stocks were generated from salivary glands of infected BALB/c mice as described elsewhere. Viral titers were determined by propagating serial dilutions of the virus stock on 3T3 cell monolayers. After 1 hour incubation and subsequent washing, cells were covered in carboxymethylcellulose (CMC) medium and incubated for 5-6 days at 37°C. Next cells were fixed in 25% formaldehyde and plaques were visualized using crystal violet solution. For an *in vivo* MCMV infection, mice were infected intra peritoneal (i.p.) with 1×10^4 PFU MCMV-Smith. MCMV-ΔgL is described elsewhere (24), and mice received a dose of 1×10^5 PFU i.p. for infection. Vaccinia Virus strain WR was purchased from the American Type Culture Collection (VR-194; Manassas, VA) and grown on HELA cells. Virus was quantified on VeroE6 cells as described (38) and mice were infected i.p. with 2×10^5 PFU. LCMV Armstrong was propagated on BHK cells, and the titers were determined by plaque assays on Vero cells. For *in vivo* experiments 2×10^5 PFU was administrated in mice via i.p. injection.

In vivo antibody treatment and DC depletion

For blockade of costimulatory interactions early in MCMV infection, mice received either 150 μg αCD70 (clone FR70) or a combination of 200 μg αCD80 (clone 16-10A1) and 200 μg αCD86 (clone GL1) on day -1, 0 and 3 post-infection. For blocking of OX40L

(clone RM134L, BioXCell) and 4-1BBL (clone TKS-1, BioXCell) 250 μg of blocking antibodies were administrated on day -1, 1, 3 and 5. For blocking of costimulatory molecules in the chronic phase of infection 200 μg αCD80 combined with 200 μg αCD86 or 150 μg of αCD70 was administrated starting on day 15 post-infection and administration was repeated every 3 days. OX40L and 4-1BBL were blocked starting on day 33 by administration of 250 μg αOX40L or α4-1BBL and this was repeated every 3 days. For CD4 depletion, mice received 200 μg of CD4 depleting antibodies (Clone GK1.5) on day -1, 0 and 3. All antibodies were administrated via an i.p. injection. For DC depletion CD11c Cre/iDTR mice received 25 ng/gram body weight DT (Sigma Aldrich) via i.p. injection on day -1, 1 and 4 post CMV infection.

Intracellular cytokine staining and flow cytometry

Single cell suspensions of spleens and cell surface staining of splenocytes were performed as described elsewhere (20). For cell surface staining of DCs, spleens were injected with 1 mg/ml collagenase and 0.02 mg/ml DNAse in IMDM without FCS, after which spleens were chopped in small pieces and incubated for 25 minutes at RT. Subsequently EDTA was added and cells were transferred through a 70 μm cell strainer to make a single cell suspension. Cells were pre-incubated with normal mouse serum and Fc-block (Clone 2.4G2) before fluorochrome conjugated or biotinylated antibodies were added. For intracellular cytokine staining 1.5×10^6 splenocytes were restimulated for 5 hours in the presence of 1 μg/ml brefeldin A (Sigma Aldrich) and 1 μg/ml MHC Class I restricted peptides at 37°C in a flat bottom plate. Cells were transferred to a U bottom plate, and subsequently, cell surface was stained, cells were washed and fixed with 0.5% PFA overnight at 4°C. The following day cells were washed with Perm/Wash buffer (ebioscience) and stained intracellularly with fluorochrome-conjugated antibodies in Perm/Wash buffer for at least 30 minutes at 4°C, after subsequent washing steps flow cytometric acquisition was performed on a BD LSR II flow cytometer (BD Biosciences). Cell sorting was performed on a BD FACSAria II (BD Biosciences). Data were analyzed using FlowJo software (TreeStar).

Imaging flow cytometry

Peptide (gp100) loaded bone marrow-derived DCs were incubated with Pmel-1 TCR transgenic $CD8^+$ T cells (recognizing gp100) in the presence of CpG, fixed and permeabilized. Next, cells were stained with antibodies against CD8, CD69 and antibodies against the transcription factors NFAT, c-Rel and c-Fos. Cells were acquired on the ImageStream X100 (Amnis-Merck Millipore). A minimum of 15,000 cells was acquired per sample at a flow rate ranging between 50 and 100 cells/s. Internalization and co-localization scores were calculated as previously described (39). Briefly, cells were acquired on the basis of their area. Analysis was performed with single cells after compensation (with a minimum of 5000 cells). Firstly, a mask was designed based on the surface of BECs

in the brightfield image. This mask was then eroded to exclude the cell membrane. Finally, the resulting mask was applied to the fluorescence channel. The internalization score was then calculated on this mask using the Internalization feature provided in the Ideas v6.0 software (Amnis-Merck Millipore). Internalization can be interpreted as a log-scaled ratio of the intensity of the intracellular space versus the intensity of the entire cell. Co-localization is calculated using the bright detail similarity R3 feature in the Ideas software. This feature corresponds to the logarithmic transformation of Pearson's correlation coefficient of the localized bright spots with a radius of 3 pixels or less within the whole cell area in the two input images.

Peptides and MHC tetramers

The following class I restricted peptides were used for MCMV; $M45_{985-993}$, $m139_{419-426}$, $M38_{316-323}$, $M57_{816-824,}$ for LCMV; GP_{33-41}, $NP_{396-404}$, $GP_{276-286}$, $NP_{238-248}$ $GP_{118-125}$, for VV; $B8R_{20-27}$, $A3L_{270-277}$, $A8R_{189-196}$, $B2R_{54-62.}$ The following MHC Class I tetramers were used M45 (D^b restricted), M57, m139 and M38 (all K^b restricted).

Adoptive transfer experiments

To determine the role of costimulatory molecules during T cell priming for the secondary expansion capacity, WT, *Cd70*$^{-/-}$, *Cd80/86*$^{-/-}$or *Cd70/80/86*$^{-/-}$ mice received 1 × 10^4 TCR transgenic CD45.1 OT-I cells and were subsequently infected with 2 × 10^5 PFU MCMV-OVA. On day 30 post-infection CD45.1 OT-I cells were sorted from each host and 1 × 10^4 OT-I memory cells derived from either WT, *Cd70*$^{-/-}$, *Cd80/86*$^{-/-}$ or *Cd70/80/86*$^{-/-}$ were adoptively transferred in CD45.2$^+$ WT hosts that were subsequently infected with 1 × 10^5 PFU MCMV-OVA and responses were analyzed 7 days post-infection.

To determine the role of costimulatory molecules for the secondary expansion, Ly5.1 congenic mice were infected with 1 × 10^4 MCMV-Smith and at day 77 post infection M45-specific CD8$^+$ T cells were sorted by flow cytometry. 1.25 × 10^3 M45- and 5 × 10^3 M38-specific CD8$^+$ T cells were transferred in WT, *Cd70*$^{-/-}$, *Cd80/86*$^{-/-}$ or *Cd70/80/86*$^{-/-}$ mice that were subsequently infected with 1 × 10^4 MCMV-Smith, at day 7 and day 28 post infection the magnitude of the transferred MCMV-specific CD8$^+$ T cells was determined.

The retroviral plasmids LZRS-IRES-EGFP (control) and LZRS-IL2-IRES-EGFP were constructed as described (9). For transduction of OT-I cells, CD8$^+$ T cells were MACS purified using a negative selection kit (Miltenyi Biotec). OT-I T cells were pre-stimulated for 22-24 hours with αCD3/αCD28 dynabeads (Life Technologies) at a ratio of 1:1 in the presence of IL-7 and IL-15 (R&D systems). The following day, transduction was performed as described (9). In short, 24-well non-tissue culture plates were coated with 50 μg/ml retronectin (Takara) for 3 hours and blocked with 2% BSA solution. After subsequent washing, viral supernatant was added and plates were centrifuged at 2000×g, 32°C, for 1 hour. Viral supernatant was removed and pre-stimulated OT-I cells were added in combination with IL-7 and IL-15. Plates were centrifuged for 3 minutes at 100×g at 32°

and incubated at 37°C for 2 days after which GFP$^+$ cells were FACS sorted. Either 1 × 10^4 GFP$^+$ CD45.1$^+$ or IL-2 GFP$^+$ CD45.1$^+$ cells were transferred in WT, *Cd70*$^{-/-}$, *Cd80/86*$^{-/-}$ or *Cd70/80/86*$^{-/-}$ hosts that were subsequently infected 2 × 10^5 MCMV-OVA. Seven days post infection the fold expansion of transferred OT-I was determined.

Bisulfite conversion

Splenic naive CD8$^+$ T cells (CD44$^-$, CD62L$^+$) or M45-specific CD8$^+$ T cells were sorted using a BD FACS Aria II flow cytometer (BD biosciences). DNA was isolated and bisulfite converted using the EZ DNA methylation-direct kit (zymo research) according to manufacturer's protocol. A nested PCR was performed, using the bisulfite converted DNA as template and IL-2 promoter specific primers as described elsewhere (16, 33). PCR product was purified using the Qia-quick gel extraction kit (Qiagen) and cloned into a pGEM easy T vector (Promega). At least 11 individual clones per condition were Sanger sequenced at the Leiden Genome Technology Centre (LGTC) and compared to the mouse IL-2 promoter using the online QUMA tool.

shRNA transduction of EL-4

The c-Rel pLMN (ZsGreen-neomicin) lentiviral shRNA plasmids were obtained from LIAI RNAi Center of the La Jolla Institute for Allergy and Immunology. For transduction, 24-well non-tissue culture plates were coated with 50 μg/ml retronectin (Takara) for 3 hours and blocked with 2% BSA solution. After subsequent washing, viral supernatant was added and plates were centrifuged at 2000×g, 32°C, for 1 hour. Viral supernatant was removed and EL-4 cells were added. Plates were centrifuged for 3 minutes at 100×g at 32° and incubated at 37°C. After 24h G418 was added and cells were cultured in the presence of G418 hereafter. The cells were screened using flowcytometry for the presence of ZsGreen$^+$ cells, and were FACS sorted.

In vitro CD8$^+$ T cell activation

For experiments using bone marrow-derived DCs (BMDCs), peptide loaded BMDCs from WT, *CD70*$^{-/-}$, *CD80/86*$^{-/-}$ and *CD70/CD80/86*$^{-/-}$ mice were incubated with OT-I or Pmel-1 TCR transgenic CD8$^+$ T cells in the presence of LPS.

In experiments using agonistic antibodies, CD8$^+$ T cells, cells were stimulated with indicated combinations of αCD3, αCD27 and αCD28 agonistic antibodies (Becton Dickinson). αCD3 (1μg/ml) and αCD27 (5μg/ml) were coated on 96-well plates for 2h at at 37°C after subsequent washing, CD8$^+$ T cells and αCD28 (2μg/ml) were added.

For experiments in which nuclear translocation of c-Rel was inhibited, cells were pre-incubated for 10 minutes at 37°C with indicated concentrations of pentoxifylline (Sigma-Aldrich). CD8$^+$ T cells were harvested and analyzed at indicated time points.

EL-4 cells that were transduced with c-Rel shRNA were stimulated with PMA / ionomycin.

IL-2 ELISA and c-Rel staining

For the IL-2 ELISA, 96-well plates (Nunc Maxisorp) were coated overnight at 4°C with IL-2 purified antibody (Biolegend) in bicarbonate buffer (pH 9.6). Plates were subsequently incubated for 1 h at 37°C with blocking buffer (PBS/5% milk powder). Supernatants were diluted 1:2 in PBS/1% milk powder and incubated in the blocked wells for 2 h at 37°C. IL-2 biotinylated antibody (Biolegend) was diluted in PBS/1% milk powder and incubated 1 h at RT. Next, streptavidin-HRP was diluted in PBS/1% milk powder and incubated 1 h at RT. To develop the plates 100 µL of TMB (3,3',5,5'-tetramethylbenzidine) (Sigma Aldrich) was added to each well and incubated for 15 min at room temperature, after which 100 µL stop solution (1M H_2SO_4) was added. Plates were measured within 5 min after adding stop solution at 450 nm using a Microplate reader (Model 680, Bio-Rad).

Intracellular c-Rel stainings were performed according to the manufacturer's protocol (eBioscience).

FlowFISH

For simultaneous flow cytometry analysis of cytokine protein and mRNA, Flow-FISH was used as previously described (40). Briefly, after T cell activation in the presence of 1 µg/ml monensin (eBioscience), T cells were labeled with anti-CD8a (53-6.7, BioLegend). Near-IR (Life Technologies) was added to exclude dead cells from analysis. Intracellular cytokine staining with anti-IL-2 (JES6-5H4, eBioscience) was performed with Cytofix/Cytoperm kit according to the manufacturer's protocol (BD Biosciences). For RNA labeling, cells were washed once with wash buffer (ddH_2O containing 12.5% formamide (Sigma Aldrich), 2X SSC and 4 units/ml murine RNAse inhibitor (New England Biolabs)), and then incubated with 15nM *Il2* mRNA FISH probes (Stellaris) in hybridization buffer (ddH_2O containing 10% formamide, 1X SSC, 0.1 g/ml dextran sulfate salts (Sigma Aldrich) with 40 units/ml RNAse inhibitor for 16 hours at 37°C (FISH probe sequences upon request). Cells were washed once with wash buffer, and acquired with FACS LSR Fortessa (BD Biosciences). Data were analyzed using FlowJo version 10.1 (TreeStar Inc.)

Statistical analysis

T cell responses and cytokine production were assumed to follow a normal distribution. For analyzing T cell responses between more than two groups, one-way ANOVA was used and Tukey's post-hoc test was performed to correct for multiple comparisons. P-Values <0.05 were considered as significant. Graphpad prism 6.0 was used to determine statistical significance.

RESULTS

Costimulatory signals mediated by CD70 and CD80/86 are collectively required for inducing IL-2 production by viral-specific CD8$^+$ T cells

The TNFR family member CD27 and the Ig family member CD28 are costimulatory receptors constitutively expressed on naïve T cells, providing these receptors to aid in the initiation of the T cell response. To study whether T cell costimulation can differentially affect the polyfunctional cytokine profile of antigen-specific CD8$^+$ T cells, we used mouse cytomegalovirus (MCMV) as an infection model. This DNA herpesvirus elicits large pools of central memory (CM)-like non-inflationary CD8$^+$ T cells (i.e., M45, M57) and effector memory (EM)-like inflationary (i.e., m139 and M38) CD8$^+$ T cells that produce distinct cytokine levels (14, 21). Wild-type (WT) mice and mice with abrogated costimulatory receptor/ligand interactions (i.e., *Cd70*$^{-/-}$ and *Cd80/86*$^{-/-}$ mice) were infected with MCMV, and the ability of the IFN-γ^+ CD8$^+$ T cell populations to co-produce the cytokines TNF and IL-2 was determined. In addition, MCMV-specific CD8$^+$ T cell responses were additionally evaluated by MHC class I tetramer binding. We found that the absolute numbers of tetramer-binding CD8$^+$ T cells were similar to the absolute numbers of IFN-γ^+ CD8$^+$ T cells, indicating no functional defects in IFN-γ production by virus-specific CD8$^+$ T cells due to the lack of costimulatory signals (Figure S1A). Consistent with previous reports, the MCMV-specific CD8$^+$ T cell responses were affected by the lack of CD70 and CD80/86-mediated cosignals (Figure S1B-D) (14, 20). Importantly, during acute infection the IL-2 production of the non-inflationary and inflationary MCMV-specific CD8$^+$ T cells was significantly decreased when either CD70 or CD80/86-mediated signalling was absent, while TNF production was only slightly affected (Figure 1A and 1B). Also, at later phases post-infection this particular effect on IL-2 production by CD70 and CD80/86-mediated costimulation was detected and observed in MCMV-specific CD8$^+$ T cell subsets (Figure 1C).

To examine whether IL-2 production by the MCMV-specific CD8$^+$ T cells is affected by CD70 and CD80/86 in a collective manner we analysed mice deficient in both CD70 and CD80/86 (*Cd70/80/86*$^{-/-}$) (22). The autocrine IL-2 production in the viral-specific CD8$^+$ T cells was more profoundly hampered in *Cd70/80/86*$^{-/-}$ mice as compared to *Cd70*$^{-/-}$ and *Cd80/86*$^{-/-}$ mice during acute and chronic infection, indicating collective mechanisms to induce IL-2 production (Figure 1A-C). MCMV-specific CD8$^+$ T cells in *Cd70/80/86*$^{-/-}$ mice maintained a strongly diminished IL-2 production throughout infection (Figure 1D). The decreased IL-2 production was not a result of antigen-mediated exhaustion, as occurs during e.g. chronic LCMV infection (23), since a) the total number of virus-specific CD8$^+$ T cells detected by MHC class I tetramers was comparable to the number of IFN-γ^+ CD8$^+$ T cells (Figure S1A), b) the TNF production was still abundantly detected (Figure 1B and C), and c) PD-1 expression was not found on the cell surface of MCMV-specific memory CD8$^+$ T cells (Figure S3A).

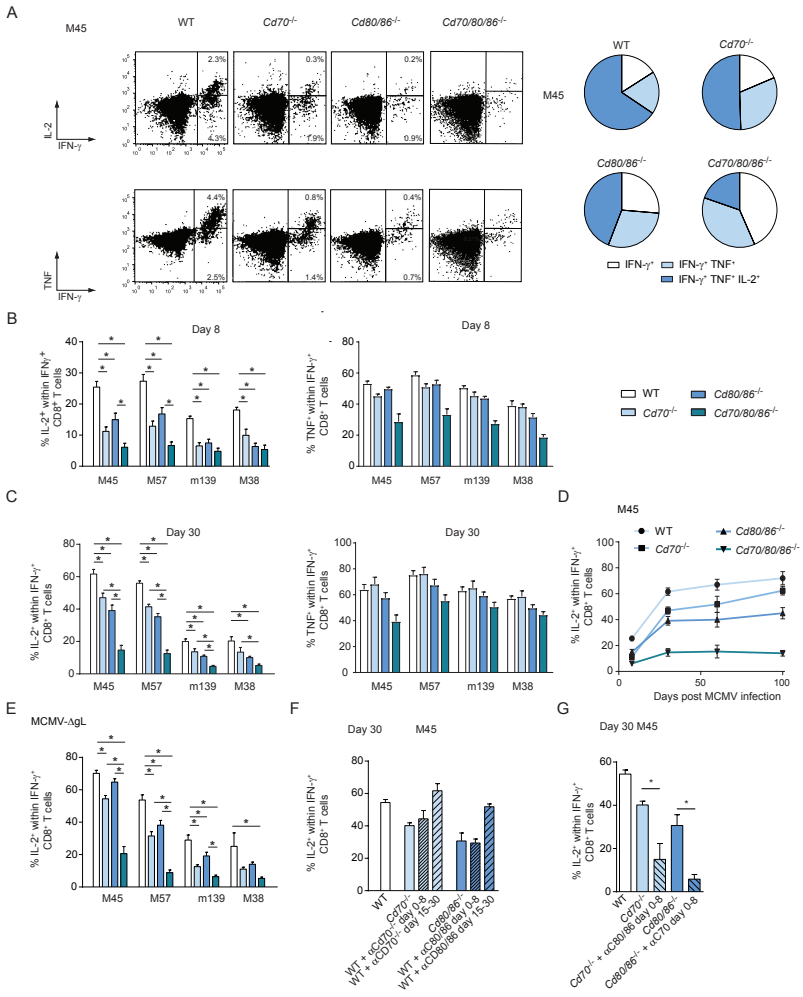

Figure 1. Costimulatory signals via CD70 and CD80/86 are jointly needed to induce IL-2 production in CD8⁺ T cells. Mice were infected with 1 × 10⁴ PFU MCMV-Smith and different costimulatory pathways were abrogated either by using gene targeted knockout mice or by administration of blocking antibodies. The cytokine production of the MCMV-specific CD8⁺ T cell pool was determined in the spleen by intracellular cytokine staining upon peptide restimulation. (A) Representative flow cytometric plots show intracellular IFN-γ, IL-2 and TNF production, gated on CD8⁺ T cells. Percentages indicate cytokine-producing cells within the CD8⁺ T cell population eight days post-infection. Pie charts show the poly-functional cytokine profile of IFN-γ+ CD8+ T cells. (B) Bar graphs indicate the percentage of cells producing IL-2 or TNF within the IFN-γ⁺ population eight days post-infection. Significant differences compared to WT mice are indicated. (C) The percentage of IL-2 and TNF producing CD8⁺ T cells within the IFN-γ⁺ population 30 days post-infection is shown. (D) The percentage of IL-2 producing CD8+ T cells within the IFN-γ+ population is indicated within time. (E) Mice were infected with 1× 10⁵ PFU MCMV-ΔgL and the percentage of IL-2⁺ T cells within the IFN-γ⁺ population and the total number of IL-2 producing T cells is shown. (F) CD70- or CD80/86-mediated interactions were abrogated at indicated times and responses were analyzed 30 days post-infection. The percentage of MCMV-specific CD8⁺ T cells producing IL-2 is shown. (G) CD70- or CD80/86-mediated interactions were blocked from day 0 to 8. IL-2 production was determined 30 days post-infection in IFN-γ CD8⁺ T cells.

To exclude possible confounding effects related to differences in viral replication, the T cell costimulation proficient and deficient mice were infected with a spread deficient

MCMV mutant lacking glycoprotein L (MCMV-ΔgL) (24). Essentially, the collective effects of CD70 and CD80/86-mediated costimulation on IL-2 production were nevertheless clearly evident, as the percentage of IL-2 producing $CD8^+$ T cells were severely hampered in the *Cd70/80/86*$^{-/-}$ mice (Figure 1E and S1G). Thus, CD70 and CD80/86-mediated costimulation have essential cooperative roles for inducing polyfunctional cytokine responses in $CD8^+$ T cells, and in particular these costimulatory molecules jointly regulate autocrine IL-2 production.

Next, we determined whether the autocrine IL-2 production is programmed early during infection by CD70 and B7-mediated costimulation or whether these costimulatory molecules are required throughout infection to sustain autocrine production in viral-specific T cells. WT mice received either CD70 or CD80/86 blocking antibodies at early and late times post-infection. When CD70 and CD80/86 interactions were abrogated from day 0 to 8, IL-2 production in M45-specific memory $CD8^+$ T cells was decreased, while blocking of CD70 and CD80/86-mediated costimulation from day 15 to 30 post-infection had no effect on IL-2 production (Figure 1F). Moreover, abrogation of CD70 interactions by administration of CD70 blocking antibodies in *Cd80/86*$^{-/-}$ mice from day 0 to 8, resulted in a further significant reduction of autocrine IL-2 in memory M45-specific $CD8^+$ T cells, and similar results were observed with CD80/86 antibody blockade in *Cd70*$^{-/-}$ mice (Figure 1G). This cooperative induction of IL-2 production by CD70 and CD80/86 mediated signals was also observed when M57, m139 and M38-specific $CD8^+$ T cell responses were analysed (data not shown). Abrogation of OX40L and 4-1BBL interactions at early and late times post-infection did not influence IL-2 production in MCMV-specific memory $CD8^+$ T cells (Figure S2A and B), indicating that these costimulatory interactions are dispensable for inducing IL-2 throughout the response. Together these results indicate that cooperative CD70 and CD80/86-mediated costimulation is required early during infection to induce autocrine IL-2 production in antigen-specific $CD8^+$ T cells.

Expression of CD70 and CD80/86 on APCs is required for autocrine IL-2 production by $CD8^+$ T cells

Next, we aimed to determine which cellular subset(s) is/are critical for the provision of the costimulatory signals inducing IL-2 production in $CD8^+$ T cells. Costimulatory ligands are expressed on diverse subsets of myeloid cells but can also be expressed by other cells including lymphocytes. Expression of CD70, CD80 and CD86 was considerably upregulated upon infection on class II^+ $CD11c^+$ and on $CD11b^+$ $CD11c^+$ cells (Figure 2A), but not on B and T cells (data not shown), indicating that $CD11c^+$ APCs might be implicated.

To determine whether costimulation deficient APCs indeed impact IL-2 production, we setup an *in vitro* system wherein peptide loaded BMDC's of WT or costimulatory deficient mice were used to stimulate TCR transgenic $CD8^+$ T cells. For both OT-I $CD8^+$ T cells (recognizing the $OVA_{247-256}$ epitope) and Pmel-1 $CD8^+$ T cells (recognizing the

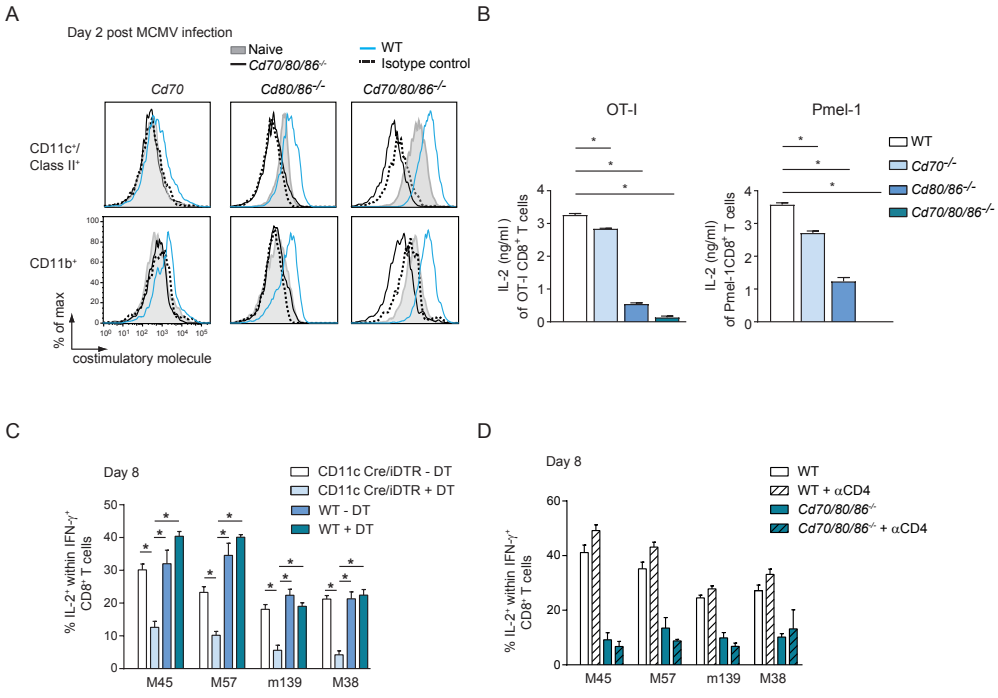

Figure 2. Costimulatory interactions mediated via CD70 and CD80/86 expressed on dendritic cells are crucial to induce IL-2 production. Mice were infected with 1 × 10^4 PFU MCMV-Smith. (A) Histograms show cell surface expression of CD70, B7.1 and B7.2 two days post MCMV-infection in the spleen. Cells are gated on $CD11c^+$/Class II^+ (top), $CD11b^+$/$F4/80^+$ cells (bottom, CD70 and B7.2), and $CD11b^+$ (bottom, B7.1). (B) Peptide loaded bone marrow-derived DCs from WT, $CD70^{-/-}$, $CD80/86^{-/-}$ and $CD70/CD80/86^{-/-}$ mice were incubated with OT-I or Pmel-1 TCR transgenic $CD8^+$ T cells. Bar graphs show concentrations of IL-2 in the culture supernatant as measured by ELISA. (C) DCs were depleted in CD11c Cre/iDTR mice by administration of DT and IL-2 production in MCMV-specific $CD8^+$ T cells was determined 8 days post-infection. The percentage of IL-2 producing $CD8^+$ T cells within the IFN-γ+ population is shown. (D) $CD4^+$ T cells were depleted by administration of a CD4 depleting antibody, and the cytokine production of MCMV-specific $CD8^+$ T cells was determined 8 days post-infection. For the whole figure, bar graphs are represented as mean + SEM, (N=3-8, *P<0.05).

$gp100_{25\text{-}33}$ epitope) we observed a significant decrease in the quantity of IL-2 that was produced when $CD8^+$ T cells were stimulated with costimulatory deficient BMDC's compared to WT (Figure 2B and S1H and I) while IFN-γ levels were to a lesser extend affected, implying a particular effect on IL-2 production

To specifically determine the role of $CD11c^+$APCs *in vivo* we used mice that express the diphtheria toxin receptor (DTR) under control of the CD11c promoter, which allows for selective depletion of $CD11c^+$ cells by administration of diphtheria toxin (DT) (25). Upon depletion a reduced population of MCMV-specific $CD8^+$ T cells was detected, thereby underscoring the importance of CD11c+ APCs for the development of viral-specific $CD8^+$ T cell responses (Figure S4A). Importantly, IL-2 production was hampered in the $CD8^+$ T cell populations that developed in the absence of $CD11c^+$ APCs, whereas TNF production was unaltered (Figure 2C and S4B). DT treatment in WT mice had no effect on the polyfunctional cytokine production excluding any possible effects of DT itself

(Figure 2C and S4A-B). To determine if help by $CD4^+$ T cells is additionally needed to induce IL-2 production in MCMV-specific $CD8^+$ T cells, $CD4^+$ T cells were depleted during MCMV infection in WT and *Cd70/80/86*$^{-/-}$ mice. However, no differences were found in IL-2 production between helped and helpless MCMV-specific $CD8^+$ T cells (Figure 2D). Thus, $CD11c^+$ APCs are foremost implicated in the provision of CD70 and CD80/86 costimulatory molecules that are required for the induction of autocrine IL-2 production by $CD8^+$ T cells, while $CD4^+$ T cell help is not required for this occurrence.

Impact of CD70 and CD80/86-mediated costimulation on IL-2 production of $CD8^+$ T cells is prevalent

To examine if CD70 and CD80/86-mediated costimulation influence autocrine IL-2 production of $CD8^+$ T cells in other viral infections, mice were infected with distinct viruses. Infection with vaccinia virus (VV; strain WR), a linear double-stranded DNA virus belonging to the poxvirus family, elicited somewhat reduced VV-specific $CD8^+$ T cell responses in *Cd70*$^{-/-}$ mice as compared to WT mice at day 30 post-infection, whereas responses in *Cd80/86*$^{-/-}$ and *Cd70/80/86*$^{-/-}$ mice were comparable (Figure S5A). Strikingly, also during VV infection, the percentage of IL-2 producing VV-specific $CD8^+$ T cells within the IFN-γ pool was lower in *Cd70*$^{-/-}$ and *Cd80/86*$^{-/-}$ mice and severely diminished in the absence of both CD70 and CD80/86-mediated costimulation (Figure 3A). Additionally, infection with the Armstrong strain of lymphocytic choriomeningitis virus (LCMV), a negative strand RNA virus that elicits an acute infection, resulted also in diminished IL-2 production in costimulation deprived $CD8^+$ T cells (Figure 3B) despite that the magnitude of the memory LCMV-specific $CD8^+$ T cell response was not abundantly dependent on costimulatory signals (Figure S5B). Notably, this specific effect on IL-2 production was clearly most pronounced when both CD70 and CD80/86 costimulatory pathways were abolished (Figure 3B). Thus, in different viral infections, CD70 and CD80/86-mediated costimulation are jointly critical for inducing IL-2 production in antigen-specific $CD8^+$ T cells.

CD70 and CD80/86-mediated costimulation act synergistically with IL-2 signals to enhance T cell expansion

Both T cell costimulation and IL-2 signals have been implicated to be important for secondary expansion (5, 26) (26, 27). To assess the inter-relationship between these two signals, we first tested the functional consequence of T cells primed in the absence of T cell costimulation for the recall response. To this end, WT, *Cd70*$^{-/-}$, *Cd80/86*$^{-/-}$ and *Cd70/80/86*$^{-/-}$ mice received 10^4 naive $CD45.1^+$ OT-I cells and were subsequently infected with MCMV expressing OVA (MCMV-OVA). OT-I T cells had a diminished IL-2 production when primed in the absence of costimulatory signals (Figure 4A, indicating that also in situations with a similar TCR, CD70 and CD80/86-mediated costimulation is critical for IL-2 production. In the memory phase of MCMV infection, OT-I T cells were sorted by flow

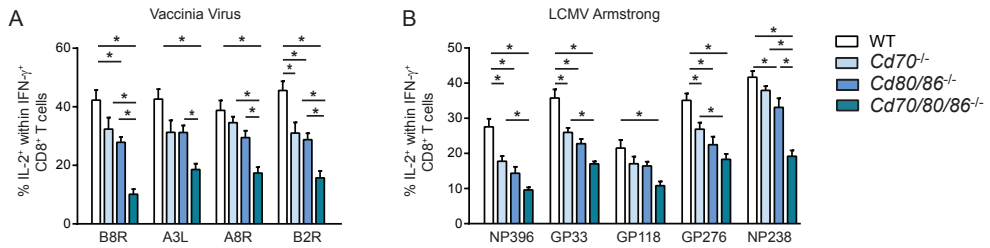

Figure 3. CD70- and CD80/86-mediated costimulation influence IL-2 production in different viral infection models. (A) Mice were infected with 2 × 10^5 PFU Vaccinia-WR and 28 days post-infection the cytokine production of the splenic VV-specific $CD8^+$ T cell response was determined after peptide restimulation with indicated MHC class I restricted peptides. Percentage of IL-2 producing T cells within the IFN-γ^+ $CD8^+$ T cells is shown (N=4-9). (B) Mice were infected with 2 × 10^5 PFU LCMV Armstrong and 28 days post-infection the cytokine profile of LCMV-specific $CD8^+$ T cell response was determined. The percentage of IL-2 producing T cells within the IFN-γ^+ $CD8^+$ T cell population is shown (N=8-14). For the whole figure, bar graphs are represented as mean + SEM, (*P<0.05).

cytometry and adoptively transferred into WT hosts that were challenged with MCMV-OVA. Consistent with the diminished IL-2 production, T cells primed in the absence of CD70 and CD80/86-mediated costimulation had an impaired secondary expansion capacity compared to T cells primed in WT mice (Figure 4B). Memory OT-I cells that developed in *Cd70/80/86*$^{-/-}$ host mice were severely hampered in their recall potential, indicating that dual costimulatory signals are required during primary infection to determine the secondary expansion potential of MCMV-specific $CD8^+$ T cells.

To determine if the diminished expansion of $CD8^+$ T cells in costimulation deficient mice is merely due to the hampered IL-2 production, IL-2 production was increased in OT-I T cells by retro-viral transduction. Transduced cells were sorted by flow cytometry and adoptively transferred into WT and costimulation deficient hosts that were subsequently infected with MCMV-OVA (Figure 4C). In WT mice, the fold expansion of transferred OT-I was rigorously enhanced by additional IL-2 production. Moreover, in *Cd70*$^{-/-}$ hosts OT-I T cells transduced with the IL-2 gene expanded better compared to OT-I cells transduced with the mock construct. This enhanced expansion of IL-2 gene transduced OT-I cells was also observed in *Cd80/86*$^{-/-}$ and *Cd70/80/86*$^{-/-}$ mice although the restoration of IL-2 production in the costimulation deficient host mice did not restore OT-I cell expansion to WT levels. Thus, both IL-2 and CD70/80/86-mediated signals are critical for the secondary expansion of MCMV-specific $CD8^+$ T cells, but the combination of these signals is even more vital and synergistically enhances $CD8^+$ T cell expansion.

CD70 and CD80/86-mediated costimulation does not directly regulate the methylation status of the *Il-2* promoter

The regulation of IL-2 expression is complex involving numerous epigenetic modifications and transcription factors. Specifically, the promoter-enhancer region upstream of the *Il-2* gene transcription start site has been shown to be implicated in driving the expression of IL-2 in a T cell-restricted and stimulation dependent manner (28).

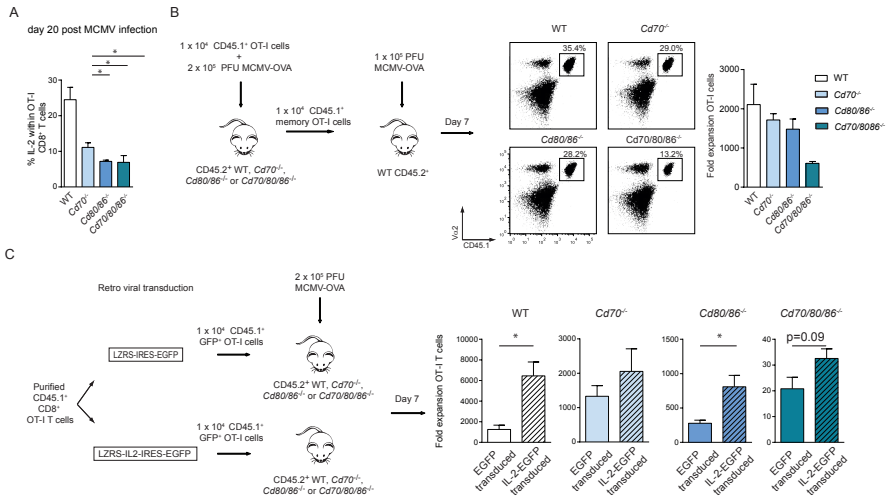

Figure 4. CD70- and CD80/86-mediated costimulation act synergistically with IL-2 to enhance CD8⁺ T cell expansion (A-B) Experimental setup: WT, CD70$^{-/-}$, CD80/86$^{-/-}$ and CD70/CD80/86$^{-/-}$ mice received 1 × 10^4 naive TCR transgenic OT-I cells and were subsequently infected with 2 × 10^5 PFU MCMV-OVA. (A) The percentage of IL-2$^+$ CD8$^+$ T cells within the IFN-γ$^+$ population is shown 20 days post-infection in the spleen (B) Memory OT-I cells were FACS sorted 26 days post-infection and were adoptively transferred into naive WT hosts that received 1 × 10^5 PFU MCMV-OVA. Seven days post-infection OT-I responses were analyzed in the spleen. Representative flow cytometric plots are gated on CD3$^+$/CD8$^+$ T cells. Fold expansion of splenic OT-I cells is shown 7 days post-infection. (N=4). (C) CD45.1$^+$ OT-I T cells were transduced with IRES-EGFP or IL-2-IRES-EGFP, sorted for GFP expression and 1 × 10^4 transduced GFP$^+$ OT-I cells were adoptively transferred into CD45.2$^+$ WT, CD70$^{-/-}$, CD80/86$^{-/-}$ and CD70/CD80/86$^{-/-}$ mice that were subsequently infected with 2 × 10^5 PFU MCMV-OVA. Seven days post-infection the fold expansion of the OT-I T cells was determined in the spleen. Bar graphs show fold expansion of OT-I T cells. The fold expansion was determined by dividing the absolute numbers of splenic OT-I by the numbers of transferred OT-I cells. Experiment was performed three times with similar results (N=4). For the whole figure, bar graphs are represented as mean + SEM, *P<0.05.

First, we interrogated whether T cell costimulatory signals could directly alter the methylation profile of the IL-2 promoter. As specific CpG sites in the promoter of the *Il-2* gene need to be demethylated (15) for the enhancement of transcription of IL-2 in T cells, we examined the DNA methylation status of these CpG sites within the *Il-2* promoter in naive and recently primed CD8$^+$ T cells of WT and costimulation deficient mice using bisulfite sequencing. In both WT and costimulation deficient mice, the CpG sites -379, -261 and -216 of the *Il-2* promoter in naive CD8$^+$ T cells were not heavily methylated while the CpG site -68 was >80% methylated. (Figure 5A and C). Upon infection, a profound loss of methylation of CpG site -68 to ~20% was observed in primed viral-specific T cells derived from either costimulation proficient or deficient mice. Loss of methylation was also evidenced for the CpG sites -261 and -216, but again no apparent differences were found between WT and costimulation deprived T cells (Figure 5B and D). These results indicate that the diminished IL-2 production in the antigen-specific CD8$^+$ T cells that develop in the absence of costimulation cannot be explained by differences in DNA methylation of the *Il-2* promoter.

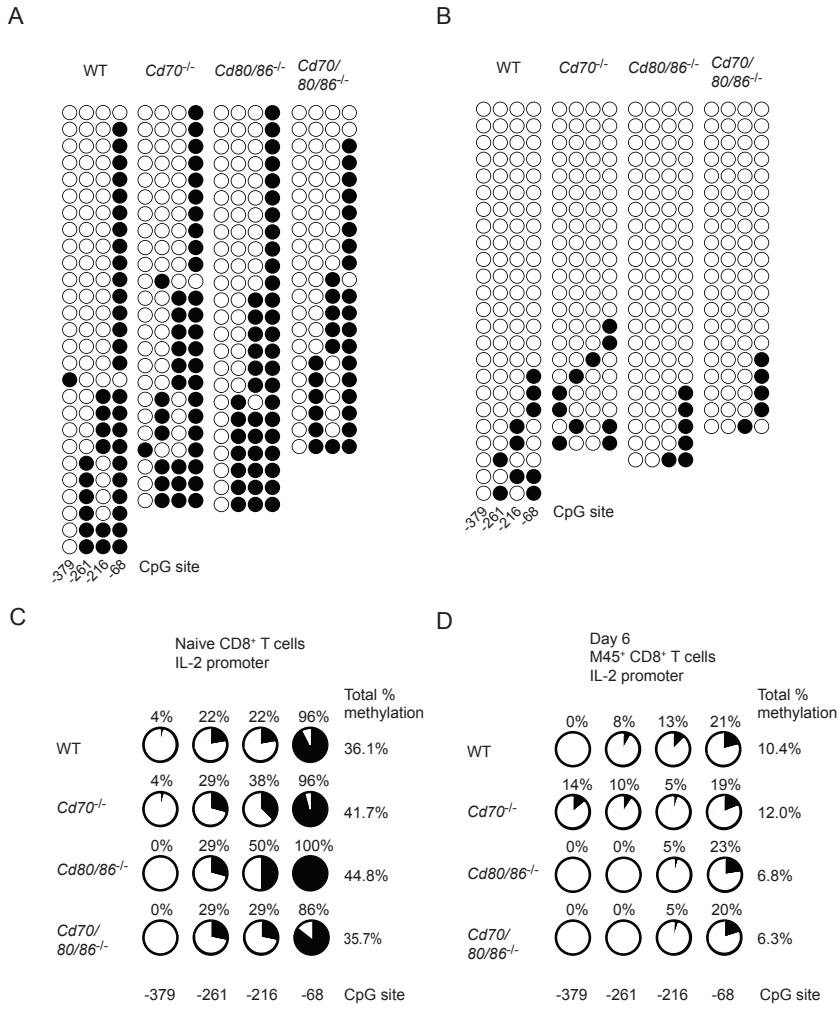

Figure 5. CD70 and CD80/86 mediated costimulation regulate IL-2 production on the transcriptional level. (A) The IL-2 promoter was amplified on bisulfite treated genomic DNA of naive CD8+ T cells (CD44-CD62L+). Each row represents one specific allele, and each circle represents 1 CpG site either unmethylated (◎) or methylated (●). (B) The IL-2 promoter 6 days post MCMV infection in M45-specific CD8+ T cells. (C) The IL-2 promoter was amplified on bisulfite treated genomic DNA of naive CD8+ T cells (CD44-CD62L+). Each row represents one specific allele, and each circle represents 1 CpG site. The average percentage of methylation at each CpG and the total percentage of methylation are shown. (D) The methylation status of the IL-2 promoter 6 days post MCMV infection in M45-specific CD8+ T cells is shown.

CD70 and CD80/86-mediated costimulation differentially regulate the nuclear localization of the *Il-2* gene controlling transcription factor c-Rel.

Within the promoter-enhancer region of the *Il-2* gene, binding sites for several inducible transcription factors (TFs) have been characterized including members of the NFAT, NF-κB and AP1 (c-Fos/c-Jun) families (29). To this end we studied the nuclear translocation of the prototypic TFs of these pathways by imaging flow cytometry. We incubated peptide-loaded bone marrow-derived DCs from WT, *CD70-/-*, *CD80/86-/-* and *CD70/CD80/86-*

$^{-/-}$ mice with Pmel-1 TCR transgenic CD8^{+} T cells and at indicated timepoints cells were stained and analyzed (Figure 6A). As shown in Figure 6B, the nuclear translocalization of NFAT and c-Fos was not influenced by costimulation via CD80/86 or CD70, while the translocalization of the NF-kB family member c-Rel was hampered by the lack of CD80/86 and of CD70. Moreover, DCs deficient in CD80/86 and in CD70 were the least efficient in inducing nuclear translocation of c-Rel in responding T cells, indicating a complementary role of CD28 and CD27 signaling in the activation of c-Rel

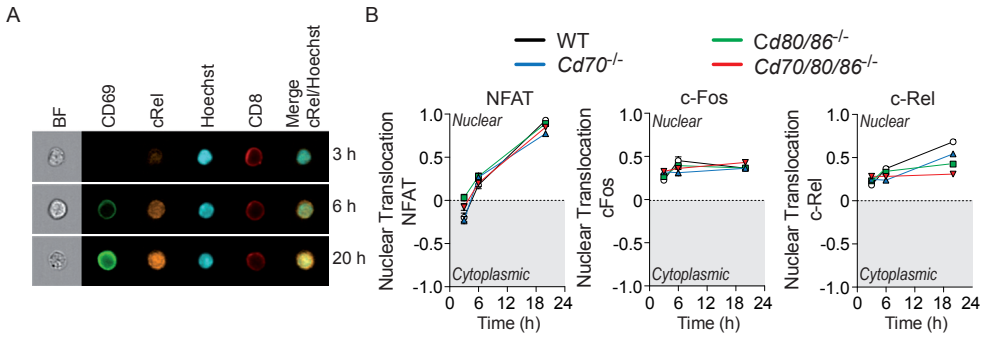

Figure 6. CD70 and CD80/86-mediated costimulation differentially regulates the nuclear localization transcription factor c-Rel. Peptide loaded bone marrow-derived DCs from WT, Cd70$^{-/-}$, Cd80/86$^{-/-}$ and Cd70/Cd80/86$^{-/-}$ mice were incubated with Pmel-1 TCR transgenic CD8^{+} T cells and at indicated time points cells were stained and analyzed by imaging flow cytometry (A). (B) Nuclear translocation of NFAT, c-Fos, and c-Rel in T cells, was followed in time after incubation with dendritic cells proficient or deficient in CD80/86 and/or CD70.

To validate whether CD70 and CD80/86-mediated costimulatory signals directly impact the expression and nuclear translocation of c-Rel, we used a gain-of-function approach in which we stimulated CD8^{+} T cells with agonistic antibodies to their respective co-stimulatory receptors. WT CD8^{+} T cells were stimulated with CD3 agonistic antibodies to mimic TCR signaling together with different combinations of CD27 and CD28 agonistic antibodies. After 5 h we determined the frequency of c-Rel expressing cells and observed that the frequency of c-Rel positive CD8^{+} T cells was highest when both CD27 and CD28-mediated signaling was provided. In accordance with this, we found that during the 5 h stimulation these dual stimulated T cells had produced more IL-2 as compared to T cells that were activated in the presence of CD27 or CD28 agonistic antibodies (Figure 7A).

The differential IL-2 production might be directly caused by differences in activation (and hence nuclear translocation) of c-Rel, however post-translational modifications of cytokine mRNA might be involved as well. Simultaneous flow cytometric analysis of IL-2 mRNA and IL-2 protein expression of T cells by FlowFISH revealed an increased presence of IL-2 mRNA in cells that had received both CD27 and CD28-mediated signaling, suggesting a stronger induction of IL-2 mRNA instead of a dominant role for

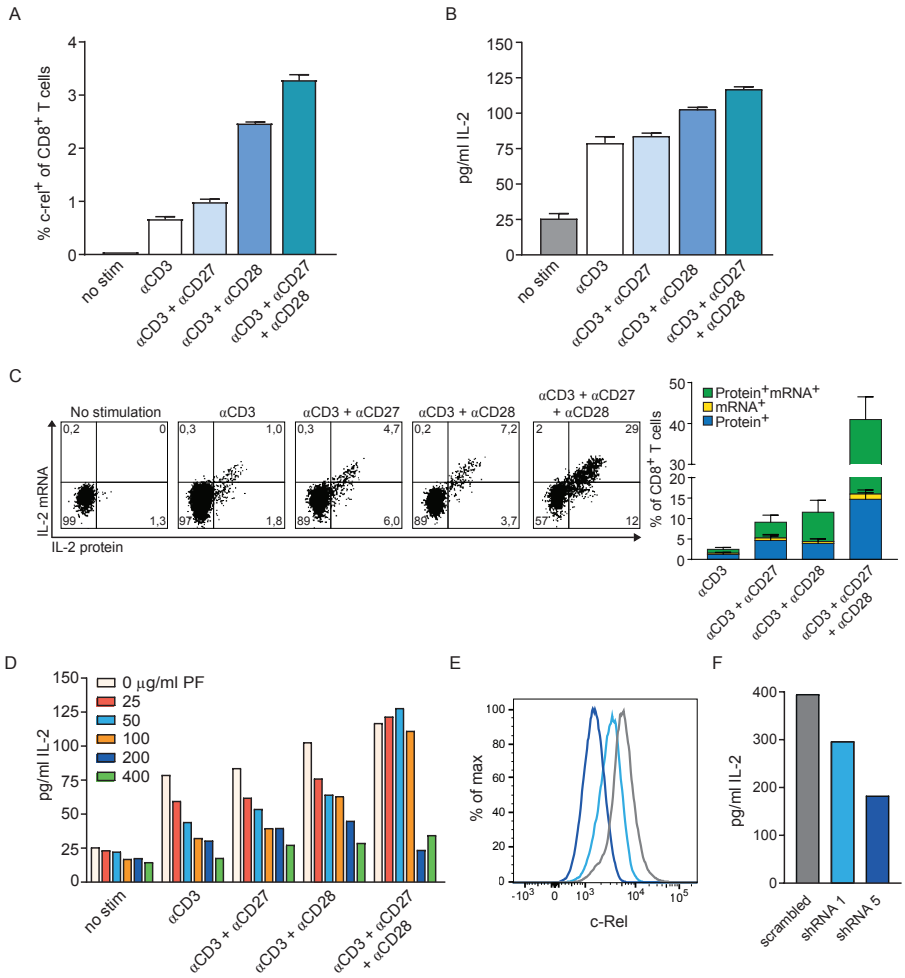

Figure 7. The transcription factor c-Rel differentially controls IL-2 production in CD8⁺ T cells. (A-B) WT CD8⁺ T cells were activated with indicated agonistic antibodies. After 5 h the frequency of c-Rel⁺ cells was determined (A) and supernatant was taken to measure IL-2 concentrations in the supernatants (B). (C). WT CD8⁺ T cells were activated with indicated agonistic antibodies for 5 h. IL-2 mRNA and protein production of activated CD8+ T cells were determined by FlowFISH analysis. Representative flow cytometry plots show IL-2 protein and mRNA staining. The bar graph shows frequencies of CD8⁺ T cells that produce IL-2 mRNA and / or protein upon activation. (D) WT CD8⁺ T cells were activated with indicated agonistic antibodies for 5 h in the presence of titrated concentrations of pentoxifylline (PF). Shown are the concentrations of IL-2 in the culture supernatant as determined by ELISA. (E-F) EL-4 cells transduced with c-Rel shRNA or scrambled shRNA were activated with PMA / ionomycin. (E) Intracellular c-Rel levels were determined by flowcytometry and (F) IL-2 secretion was determined by ELISA.

post-translational modifications of the IL-2 mRNA (Figure 7B). Next we aimed to directly link differential c-Rel activation to differential IL-2 production by CD8⁺ T cells. Therefore CD8⁺ T cells were activated in the presence of pentoxifylline (PF), a chemical compound known to specifically inhibit c-Rel induction but not other NF-kB family members (30) and measured IL-2 secretion. Importantly, we found that increasing concentrations of PF and hence decreasing activation of c-Rel resulted in diminished IL-2 secretion. Moreover, T cells that were activated in the presence of dual costimulation

were more resistant to PF-mediated inhibition of c-Rel compared to T cells that received CD27 or CD28 mediated-costimulation (Figure 7C). To exclude the possibility that our observations are due to confounding effects of PF, we used lentiviral transduction of c-Rel shRNA to generate T cell lines (EL-4) with differential c-Rel expression (7D). Importantly, also here we observed that differential c-Rel levels results in differential induction of IL-2 production.

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DISCUSSION

Polyfunctional T cells provide correlates of protection. Here we show that costimulatory interactions mediated via the costimulatory molecules CD70 and CD80/86 are both implicated for induction of IL-2 production in virus-specific $CD8^+$ T cells. Moreover, the collective signals mediated by CD70 and CD80/86 are in fact critical for IL-2 production. Furthermore, we show that costimulatory signals act synergistically with IL-2 to optimally enhance $CD8^+$ T cell expansion.

Recently, we showed that the relative amount of IL-2 produced within an antigen-specific population predicts the secondary expansion potential of the entire antigen-specific population (9). In the MCMV model, the percentage of IL-2 producers within the non-inflationary T cell population is higher compared to inflationary $CD8^+$ T cell populations. In agreement with this finding is that non-inflationary T cells are more dependent on costimulatory signals for their initial expansion (14, 20). It is likely that the dependence of the entire T cell population on costimulatory signals also dictates the relative amount of IL-2 that is produced within a given population. It is of interest to note that upon depletion of $CD11c^+$ cells, non-inflationary T cells are more hampered in their expansion compared to inflationary T cells, indicating that different antigen presenting cells might be providing the costimulatory signals and prime these T cells.

Signals mediated by IL-2 are critical for the development of effector and memory T cells, and also promote survival in non-lymphoid tissues. We observed that a combination of both IL-2 and costimulatory signals were important to optimally enhance $CD8^+$ T cell expansion as rescuing IL-2 expression was not sufficient to restore the expansion potential of CD8 T cells. Consistent with this observation is that CD28 signaling enhances T cell expansion in both an IL-2-dependent and independent manner (31). An IL-2 independent process for $CD8^+$ T cell expansion has also been found for the costimulatory CD70/CD27 pathway (32).

We found that costimulatory signals were needed during priming to program $CD8^+$ T cells for IL-2 production in the memory phase. This programming could occur epigenetically. Although CD28 signals have been implicated to induce DNA demethylation at the IL-2 promoter in $CD4^+$ T cells (16) we did not find differences in the methylation status of the IL-2 promoter between WT and costimulation deficient mice 6 days post-in-

fection in CD8+ T cells. Also histone acetylation most likely does not play a role, as the IL-2 promoter is hypoacetylated in naive CD8+ T cells, and remains unaltered in effector and memory CD8+ T cells (33). However, chromatin remodeling or other histone modifications might be affected by costimulatory signals.

NFAT, NF-κb and AP-1 (c-Fos/c-Jun) are key regulators for inducing IL-2 production (34). Although both CD27 and CD28 can signal via the NF-κB and the c-Jun pathways (35, 36), we observed that only the nuclear translocation of c-Rel was differentially regulated by these costimulatory molecules, thereby explaining the observed complementary role. Moreover, c-Rel controls IL-2 transcription based on the strength of its early activation.

To improve current vaccination settings and adoptive T cell transfers it could be of great value to provide dual triggering of CD27 and CD28, and subsequently induce optimal IL-2 production in antigen-specific CD8+ T cells. This can either be achieved *ex vivo* during the expansion of virus or tumor-specific T cells or *in vivo* by administration of agonistic antibodies to these receptors.

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SUPPLEMENTARY INFORMATION

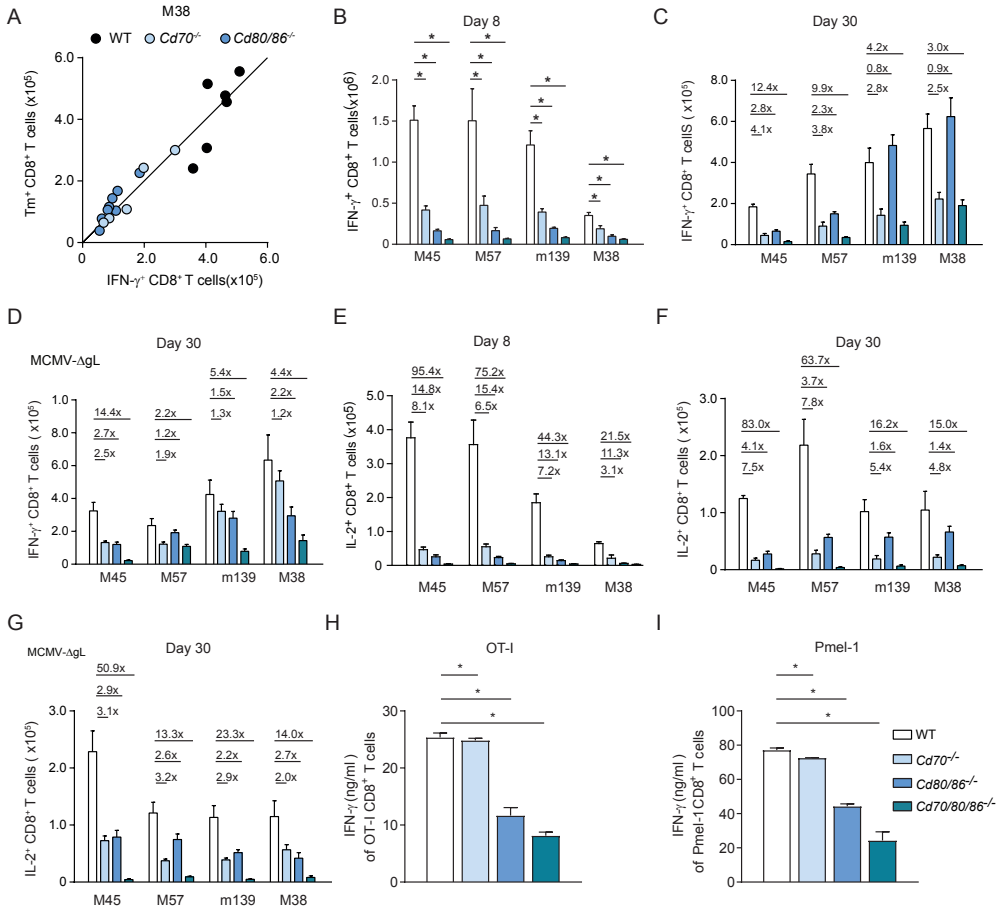

Figure S1. Impact of CD70 and CD80/86 on the magnitude of the MCMV-specific CD8⁺ T cell response. WT and costimulation deficient mice were infected with 1 × 10^4 PFU MCMV or 1 × 10^5 PFU MCMV- ΔgL. At day 8 and 30 days post-infection the splenic MCMV-specific CD8⁺ T cell responses were analyzed by intracellular cytokine staining upon MHC class I restricted peptide restimulation. (A) The magnitude of the splenic MCMV-specific CD8⁺ T cells was determined by MHC class I tetramer staining and intracellular cytokine staining 8 days after MCMV infection. The total number of tetramer⁺ CD8⁺ T cells is plotted against the number of IFN-γ⁺ CD8⁺ T cells. (B-D) The total number of IFN-γ⁺ CD8⁺ T cells is shown at day 8 (B) and day 30 (C) after MCMV infection. (D) The total number of IFN-γ⁺ CD8⁺ T cells 30 days after MCMV-ΔgL infection. (E-G) The total number of IL-2 producing CD8⁺ cells after infection is shown. Fold difference compared to WT mice is indicated. The total number of IL-2⁺ CD8⁺ T cells is shown at day 8 (E) and day 30 (F) after MCMV infection. (G) The total number of IL-2⁺ CD8⁺ T cells 30 days after MCMV-ΔgL infection. (H-I) Peptide loaded bone marrow-derived DCs from WT, CD70$^{-/-}$, CD80/86$^{-/-}$ and CD70/CD80/86$^{-/-}$ mice were incubated with OT-I or Pmel-1 TCR transgenic CD8⁺ T cells. Bar graphs show concentrations of IFN-γ in the culture supernatant as measured by ELISA.

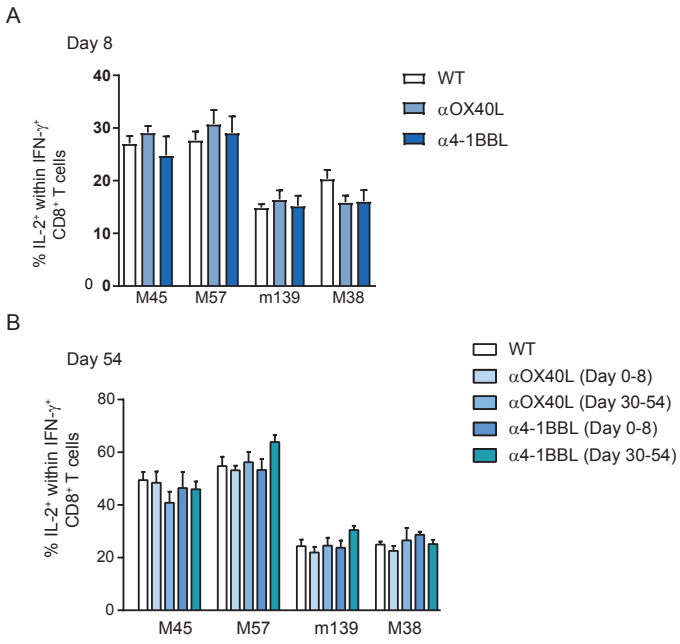

Figure S2. Requirements for OX40L and4-1BBL-mediated costimulation for the induction of IL-2 in MCMV-specific CD8⁺ T cells. Mice were infected with 1 × 10^4 PFU MCMV-Smith and different costimulatory pathways were abrogated by administration of OX40L and 4-1BBL blocking antibodies. The cytokine production of the MCMV-specific CD8⁺ T cell pool was determined in the spleen by intracellular cytokine staining upon peptide restimulation. (A) Bar graphs indicate the percentage of cells producing IL-2 within the IFN-γ⁺ population eight days post-infection. (B) OX40L or 4-1BBL interactions were abrogated at indicated times and 54 days post-infection, the percentage of MCMV-specific CD8⁺ T cells producing IL-2 was determined. For the whole figure, bar graphs are represented as mean + SEM (N=4-16).

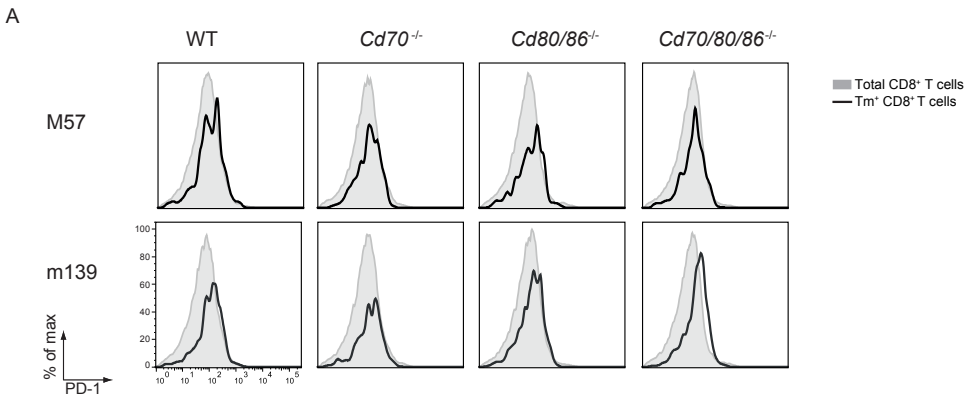

Figure S3. MCMV-specific CD8⁺ T cells in CD70/CD80/86$^{-/-}$ are not exhausted. Mice were infected with 1 × 10^4 PFU MCMV and 30 days post-infection MCMV-specific responses were analyzed by tetramer staining and intracellular cytokine staining. (A) Histograms show cell surface expression of PD-1 on the total CD8⁺ T cell population and on M57- and m139- specific CD8⁺ T cells (B) The total number of IFN-γ⁺ CD8⁺ was plotted against the total number of Tm⁺ CD8⁺ T cells for M57- and m139-specific responses.

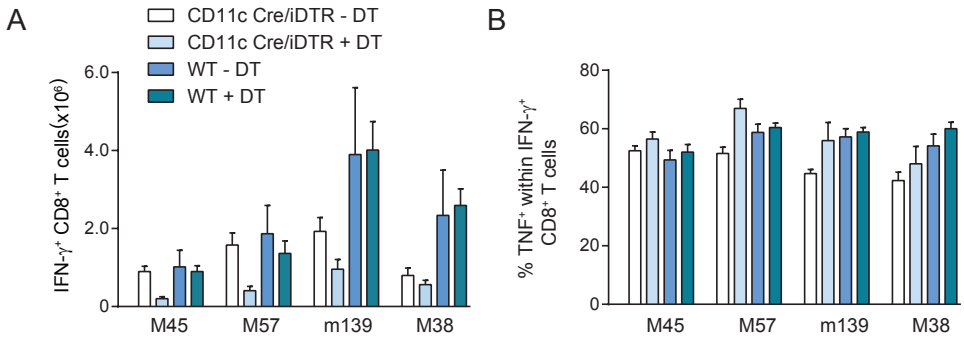

Figure S4: Major role for DCs in inducing IL-2 production in MCMV-specific CD8⁺ T cells. Mice were infected with 1 × 10^4 PFU MCMV. (A) DCs were depleted in CD11c Cre/iDTR mice by administration of DT. Bar graph shows the total number of MCMV-specific CD8⁺ T cells. Experiment was performed twice with similar results. (B) The percentage of TNF⁺ CD8⁺ T cells is shown within the IFN-γ⁺ CD8⁺ population. Bar graphs are represented as mean + SEM (N=3-5; *P<0.05).

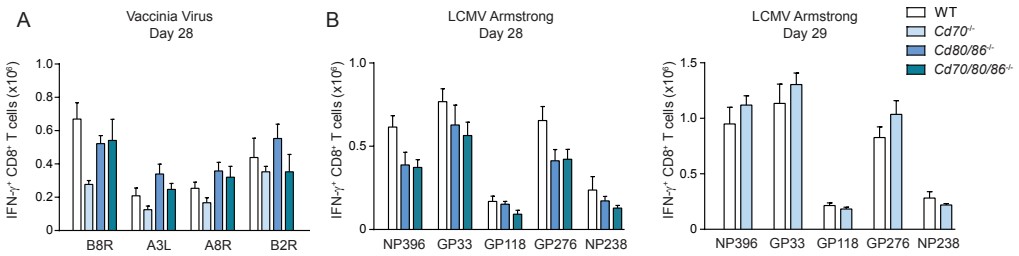

Figure S5: The requirements for CD70- and CD80/86-mediated costimulation on the magnitude of the antigen-specific memory CD8⁺ T cell pool is determined by the infectious pathogen. (A) Mice were infected with 2×10^5 PFU Vaccinia-WR and the magnitude of the splenic VV-specific CD8⁺ T cell response was determined 28 days post-infection by intracellular cytokine staining after peptide restimulation with indicated MHC class I restricted peptides. Absolute numbers of VV-specific IFN-γ⁺ CD8⁺ T cells are shown (N=4-9). (B) Mice were infected with 2×10^5 PFU LCMV Armstrong and 28 and 29 days post-infection the splenic LCMV-specific CD8⁺ T cell response was determined by intracellular cytokine staining. Total numbers of splenic LCMV-specific CD8⁺ T cells are shown (N=8-14).

CHAPTER 4

Improving adoptive T cell therapy: the particular role of T cell costimulation, cytokines and post-tranfer vaccination

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4

ABSTRACT

Adoptive cellular therapy (ACT) is a form of immunotherapy whereby antigen-specific T cells are isolated or engineered, expanded ex vivo and transferred back to patients. Clinical benefit after ACT has been obtained in treatment of infection, various hematological malignancies and some solid tumors, however, due to poor functionality and persistence of the transferred T cells, the efficacy of ACT in the treatment of most solid tumors is often marginal. Hence, much effort is undertaken to improve T cell function and persistence in ACT and significant progress is being made. Herein we will review strategies to improve ACT success rates in the treatment of cancer and infection. We will deliberate on the most favourable phenotype for the tumor-specific T cells that are infused into patients and on how to obtain T cells bearing this phenotype by applying novel ex vivo culture methods. Moreover, we will discuss T cell function and persistence after transfer into patients and how these factors can be manipulated by means of providing costimulatory signals, cytokines, blocking antibodies to inhibitory molecules and vaccination. Incorporation of these T cell stimulation strategies and combinations of the different treatment modalities are likely to improve clinical response rates further.

INTRODUCTION

During the recent years immunotherapy has emerged to be a powerful and potentially curative therapy for the treatment of various types of cancer and recurrent viral diseases. Adoptive cellular therapy (ACT) is a form of immunotherapy that involves the ex vivo isolation and expansion of antigen-specific T cells for adoptive transfer back to patients (1, 2). Although clinical benefit has been obtained in treatment of hematologic malignancies and melanoma, the efficacy of ACT in the treatment of most solid tumors is thus far limited since transferred T cells fail to function and persist in vivo. This is in sharp contrast to clinical results obtained with patients treated by ACT for virus-associated malignancies and recurrent viral infections. Here sustained presence of functional virus-specific T cells is observed, even up to 9 years post-infusion (3-5). This prolonged presence of transferred virus-specific T cells translates into a high clinical response rate that is being observed in patients that are treated with these cells. The superior efficacy obtained with ACT in the treatment of viral infections and virus-associated malignancies compared to the treatment of most solid cancers can be attributed to several factors, including tolerance to tumor-associated antigens (TAAs) and inhibition of tumor-specific T cells due to the suppressive tumor environment. Moreover, also the necessity for extensive culturing of tumor-specific T cells to obtain sufficient numbers for infusion into patients greatly influences the quality of the T cells and hence, persistence and anti-tumor efficacy in vivo. In addition to lessons that can be learned from studying T cells in a setting of viral infection, valuable lessons can also be learned by critical evaluation of results obtained with current protocols and importantly, by improving our understanding of the underlying mechanisms. In this review we will focus on current protocols of adoptive T cell therapy in cancer treatment, and discuss the various attempts to improve the clinical success rate of ACT by aiming to advance the quality of the infused T cells through delivery of costimulatory signals and cytokines, blocking of inhibitory signals and vaccination. As such, these developments are of interest for ACT improvement in cancer but also for other complicated T cell-dependent treatment modalities.

Approaches of ACT

One form of ACT involves expansion and infusion of natural T cells isolated from autologous tumors. Generation of tumor-infiltrating lymphocyte (TIL) cultures is performed by first culturing resected tumor fragments or tumor single-cell suspensions in medium containing IL-2 for 3-5 weeks followed by a rapid expansion protocol (REP) involving the activation of TILs using an anti-CD3 monoclonal antibody in the presence of irradiated peripheral blood mononuclear cells (PBMC) and IL-2 (2, 6, 7). Systemic administration of TILs to patients with advanced stage melanoma has mounted high and durable responses that resulted in objective clinical responses in >50% of the patients and

complete regression in up to 24% of the patients (6, 8-10). However, such results have only been described for ACT in melanoma patients and not for other solid tumors. This is probably due to the high mutational load in melanoma giving rise to neoepitopes, which can serve as neoantigens facilitating tumor recognition by T cells (11-14). The stability of these neoantigen expression, however, is altering upon ACT demonstrating a dynamic interaction of the transferred T cells with their targets and advocates for ACT procedures inducing a broad tumor-specific T cell response (15).
In most medical centers, lymphodepletion before transfer is a standard part of the treatment (16). However, ~50% of the patients experience side effects of this pre-treatment, which are mostly infection related, i.e. neutropenia and bacteremia (10). There is some evidence that alternative approaches can overcome the necessity to pre-condition the patient, e.g. selection of particular T cell clones, tailoring tumor-specific T cells to produce IL-12 or administration of low dose IFN-α (17-19). Another hurdle in ACT for solid tumors is the failure to successfully isolate TILs or expand TILs to sufficient numbers. In ACT for melanoma patients these procedures are usually very efficacious, showing a success rate of more than 50% (7, 8). However, TILs harboring sufficient anti-tumor activity can rarely be generated from tumors other than melanoma and moreover, for other types of cancer adequate amounts of surgical/bioptic material is often not available (20).

One strategy to circumvent these limitations is genetic engineering of autologous T cells by lentiviral or retroviral transduction to express TCRs that recognize TAAs. Although a promising clinical response rate of 30% was observed in a clinical trial for melanoma patients using a high-affinity HLA-A0201-restricted MART-1 TCR, in 29 out of 36 patients severe off-target toxicity was seen in the skin, ears and eyes as destruction of melanocytes also occurred at these sites (21). In a clinical trial where myeloma and melanoma patients were infused with autologous engineered T cells expressing an affinity-enhanced TCR against MAGE-A3, the first two patients died of cardiogenic shock. This severe cardiac toxicity was due to recognition of a MAGE-A3-unrelated protein expressed by normal cardiac tissue (22). This off-target activity is likely caused by the fact that an affinity-enhanced TCR was used instead of the low affinity parental TCR against MAGE-A3. Thus, a major drawback of this approach is the (sometimes unidentified) expression of target antigens on healthy tissue resulting in unwanted cross-reactivity. Nevertheless, certain antigens such as cancer-testis antigens (CTAs) do form an attractive target since they are expressed by a variety of tumor types, but usually not by adult tissue, with the exception of germline cells on which HLA class I and II is not expressed In clinical trials, targeting of the CTA NY-ESO-1 antigen 61% of synovial cell carcinoma patients and 55% of the melanoma patients demonstrated objective clinical responses without signs of off-target toxicity (23). Another report showed even an 80%

response rate in multiple myeloma (24). Although TCR transduction allows the generation of tumor-specific T cells without the necessity to isolate TILs, a major limitation of this approach is the HLA-restriction. For example, transduction of a TCR that recognizes its antigen in the context of HLA-A*0201 is only functional in patients with the same HLA type.

An alternative approach to obtain T cells with anti-tumor reactivity without the complication of HLA-restriction is genetic engineering of T cells to express chimeric antigen receptors (CARs). CARs are constructed by linking an antigen-binding domain, usually a single chain variable fragment (scFv), to an intracellular T cell signaling domain such as CD3-ζ (first generation CAR), and currently also including one or two costimulatory domains (second/third generation CAR). The specific binding of CAR T cells occurs thus in a non-MHC restricted fashion, yet antigen binding results in T cell activation. The most impressive clinical results so far have been obtained using CAR T cells targeting CD19 in patients suffering from B cell malignancies (25-30). Mixing defined populations of $CD4^+$ and $CD8^+$ CAR T cells recognizing both CD19 further improved this therapy (31, 32). However, since all CD19-expressing cells are targeted using this approach, also non-malignant B cells are depleted. The drawback that healthy cells expressing the antigen are also targeted by CAR T cells has also been reported for CARs directed to her2/neu and carboxy-anhydrase-IX (33, 34). In addition to this on-target off-tumor effect, acute anaphylaxis and tumor lysis syndrome (TLS) occurs frequently after CAR T cell therapy, but most often observed is cytokine release syndrome (CRS) (35-37). It has been suggested that the incidence and severity of the CAR T cell-mediated toxicity is related to tumor burden and T cell infusion dose. To minimize toxicity in patients with a high tumor burden treatment with a low T cell dose may be required (30, 32, 35, 37). Other strategies to overcome these adverse events include addition of a suicide gene (e.g. HSV-TK and *iCasp9*), whereby transferred cells can selectively be eliminated, and the generation of CAR T cells with dual specificity whereby each CAR targets a different tumor antigen and only engaging of both results in proper T cell activation and effector function (38-44).

Clinical trials using CAR T cells targeting other antigens than CD19 have thus far only shown limited anti-tumor efficacy. In a trial wherein neuroblastoma patients received CAR T cells recognizing the extracellular domain of L1-CAM, present on neuroblastoma cells, 2 out of 6 patients showed a clinical response (34). However in two other trials, using CAR T cells specific for carboxy-anhydrase-IX and alpha-folate receptor to treat renal cell carcinoma and ovarian cancer, respectively, no clinical responses were observed (45, 46). The limited success for these CAR T cells may in part be due to antigen-independent CAR signaling due to clustering of CAR scFvs resulting in their early exhaustion. This tonic CAR signaling is observed for several CARs, except the CD19 CAR (47).

Incorporation of the endodomain of 4-1BB (CD137), a costimulatory member of the TNF receptor (TNFR) superfamily, rather than a CD28 domain ameliorates this induction of exhaustion (47, 48). In addition, novel targets for CAR T cell therapy for solid tumors are on their way, which may have high clinical potential. For example, it was recently reported that CAR T cells can be engineered to target aberrantly glycosylated antigens on MUC1, which is expressed by multiple cancers, thereby providing a potential broad application (49).

Although CAR T cells and TCR transgenic T cells are favorable cancer treatment modalities, they usually target a single tumor antigen, which increases the chance of tumor escape (50, 51), and limits eradication of the often very heterogeneous tumors. The use of a combination of CARs with different antigen specificity or bispecific CARs could prevent antigen escape (52).

Quality of anti-tumor T cells

A major challenge in ACT is to obtain sufficient numbers of tumor-specific T cells for infusion into patients and importantly, since durable clinical responses profoundly depend on persistence of the infused T cells, cells that still have the capacity to persist long-term in vivo (53). Several reports suggest that the relative contribution to long-term persistence of T cells mainly comprises the least effector-differentiated memory T cells: central memory T cells (Tcm) and T memory stem cells (Tscm) (54). Tcm and Tscm circulate in the lymphoid organs and are endowed with an excellent expansion potential upon antigenic challenge as opposed to more differentiated memory T cells. Effector and effector memory T cells (Teff/Tem) home to tissues and respond to antigen with immediate effector function as compared to Tscm/Tcm, but have a reduced regenerative capacity (55). In addition, Tem in humans can be subdivided into cells that are either CD45RA- or cells that re-express CD45RA+. The re-expressing cells, termed Temra, are thought to be the most differentiated memory cells, as these cells have low proliferative capacity, strong cytotoxic potential and a higher susceptibility to apoptosis (56).

Tscm have the capacity to differentiate into Tcm and Tem and display a superior potential to self-renew as evidenced by a positive correlation of the amount of infused Tscm with early expansion after transfer and absolute numbers of long-term persisting cells (57-59). However, very low numbers of Tscm are found in the periphery and extensive expansion would be required, which likely results in loss of memory potential (60, 61). The limitation of low natural frequencies can be bypassed by targeting the Wnt/β-catenin pathway in naive cells which results in arrested effector T cell differentiation and promotion of memory-like CD8+ T cells with Tscm features. Although targeting the Wnt signaling pathway appears to be an effective method to promote stemness and inhibit differentiation this may restrict the proliferation and function, hence further research is required for its suitability to improve ACT (62). An alternative method to generate

sufficient Tscm is a procedure whereby human naive T cells are activated by CD3/CD28 engagement and culturing in the presence of IL-7, IL-15 and IL-21 (63, 64). Another approach currently being explored is based on inhibition of the Akt-signaling pathway during the ex vivo expansion of tumor-specific T cells resulting in the induction of early memory-like cells (65, 66). The advantage of this approach is that the ex vivo proliferation is not strongly inhibited and sufficient numbers of cells can be obtained for treatment. However, the role of Akt in T cell differentiation and metabolism needs to be further validated in order to determine if Akt inhibition could potentially be used in ACT protocols. Thus, although it is clear that Tscm have excellent stemness properties and much effort is being made to optimize isolation and expansion protocols, there are still some major hurdles and it is therefore not feasible yet to use these cells routinely for adoptive cell therapy.

A recent report demonstrates an alternative approach in which TCR transgenic CD8+ T cells were successfully reprogrammed into induced pluripotent stem (iPS) cells using a Sendai virus vector. After transfer into melanoma-bearing mice iPS-derived T cells mediated potent anti-tumor activity. These results show that it is feasible to reprogram TCR transgenic CD8+ T cells. Nevertheless, their anti-tumor activity and persistence were comparable with their non-reprogrammed counterparts (67). Importantly, the Busch laboratory convincingly showed in mice that also Tcm have stemness and long-term persistence potential after transfer. Actually, both naive T cells and Tcm cells were highly efficient in inducing epitope-specific T cell populations during single cell serial adoptive transfers (68). Also, infused Tcm clones in monkeys and humans have shown to have the capacity to mount long-term persistent clonotypes, and furthermore CD19 CAR T cells derived from Tcm have superior antitumor effects (31, 59, 69, 70).
In the current point of view both Tscm and Tcm, and if possible naive T cells, which may even be preferred, seem to be bona fide T cell subsets to use in ACT as these subsets have the potential to establish long-term persistence allowing for prolonged anti-tumor activity (71, 72). However, these less differentiated T cell subsets are not per definition superior in all tumor eradication settings. In cases of solid tumors where the level of tumor-antigen presentation by antigen presenting cells in lymphoid organs is low, these T cell subsets may not be activated sufficiently to exit the lymphoid organs and enter the circulation to exert their anti-tumor effects. One strategy to overcome this hurdle is increasing the level of antigen presentation in the lymphoid organs by vaccination resulting in appropriate T cell stimulation (as will be discussed later). Another approach is co-infusion of Teff and Tem cells. These cells have direct effector function and have (extralymphoid) tissue migrating properties leading to tumor destruction (55, 73-75). Consequently, this may also lead to sufficient activation of co-transferred Tcm/Tscm, which enables long-term anti-tumor immunity.

Ex vivo expansion protocols and costimulation

The expansion protocols that are currently used to expand TILs or generate engineered tumor-specific T cells often mount expanded T cell pools with a highly differentiated phenotype that have lost CD28 expression, decreased expression of the costimulatory TNFR family member CD27 and more susceptibility to activation-induced cell death (AICD) (76-78). Approaches to obtain sufficient numbers of TILs with a favourable phenotype or to reprogram TILs or TCR engineered T cells to the preferred phenotype during ex vivo expansion include manipulation of critical costimulatory and cytokine signaling pathways. Costimulatory signals can be provided via agonistic antibodies and artificial APCs (aAPCs), of which the latter can either be cell-based or non-cell-based (79). An advantage of non-cell-based aAPCs over cell-based aAPCs is that they can be engineered to be magnetic, which makes removal of the cells before infusion of the T cell product straightforward, also bio-degradable particles can be designed of which removal is no necessity. One of the costimulatory pathways known to be critical for priming T cells, the CD28 pathway, is currently implicated in ACT protocols for ex vivo expansion and transduction. Another candidate is 4-1BB, which is expressed on activated T cells and upon triggering enhances T cell responses by promoting proliferation, survival and effector function, and by regulating the suppressive potential of regulatory T cells (Tregs) (80). Comparison of aAPCs providing costimulation via CD28 or 4-1BB showed that signaling through 4-1BB preferably expands memory CD8+ T cells whereas CD28 costimulation favours expansion of naive cells. In addition, the CD8+ T cells that received 4-1BB signals displayed improved cytolytic function (81). Interestingly, enhanced 4-1BB costimulation through an agonistic antibody has been shown to rescue expression of CD27 and CD28 on post-REP CD8+ TILs, improved expansion of CD8+ T cells and increased responsiveness to antigenic re-stimulation and increased expression of the CD127 (IL-7Rα) (82, 83). Also, when combined with a potent TCR trigger, signaling through 4-1BB induces prominent upregulation of CD25 (IL-2Rα) and IL-2 (84). Thus, while generating tumor-specific T cell pools from naive cells, 4-1BB triggering could promote the generation of T cells capable of expanding upon secondary challenge. Another costimulatory molecule that could potentiate ex vivo culturing of tumor-specific T cells is CD27. The interaction of CD27 with its ligand CD70 has been shown to be important for IL-2-mediated T cell activation and in vitro activation of human T cells with anti-CD3 in the presence of an agonistic CD27 antibody showed comparable expansion potential as stimulation through 4-1BB (85). On the other hand, in vitro experiments have shown that in a co-culture of naive CD4+ T cells with CD70 expressing tumor cells, Tregs accumulate because of increased IL-2 production by non-Treg CD4+ cells (86). Other costimulatory members of the TNFR superfamily include OX40 (CD134), HVEM and GITR and agonistic antibodies targeting these molecules could also potentially be used to improve REP cultures. OX40 has been described to promote T cell expansion and

survival, the latter probably by regulating BCL-2 and BCL-xL expression (80, 87, 88). It has been shown that ligation of OX40 increases expression of IL-7Rα on antigen-specific CD8+ T cells, which leads to enhanced survival and accumulation upon IL-7 signaling, and combining OX40 and 4-1BB costimulation further enhanced this effect (89). Thus, to further improve the ex vivo culturing procedure, targeting of two or more costimulatory pathways simultaneously can be taken into consideration. Importantly, although the signal strength that is delivered to the T cells should be robust enough for proliferation, it should not result in an overall terminal differentiation of the T cells. An alternative approach is to make combinations of an agonistic antibody with cytokines that prevent overt differentiation, as will be discussed hereafter. To be able to select the most favourable agonist-cytokine combinations it would be highly recommendable to expand our knowledge regarding the effect on the expression of cytokines and cytokine receptors by targeting the costimulatory pathways simultaneously.

Ex vivo expansion and cytokines

An alternate strategy to boost cultured T cells and modulate the phenotype is via cytokine-mediated signals. The common-gamma chain (γ_c)-cytokine IL-2 is long been known to massively expand T cells, and high doses of IL-2 have been used to establish and expand ACT T cell cultures for more than 20 years (90). Enforced expression of IL-2 by the T cells themselves results also in prolonged survival in vitro and maintains the tumor specificity and function (91, 92). However, IL-2 can promote differentiation of T cells (93, 94), which may lead to an unfavourable phenotype for ACT usage. So strategies to optimize ex vivo T cell cultures for ACT involving the (co-)use of alternative cytokines is fully explored. Next to IL-2, other γ_c-cytokines such as IL-7, IL-15 and IL-21 have been described to play a role in memory T cell formation, proliferation and survival, yet result in a lower degree of T cell differentiation but are still able to enhance anti-tumor responses (95-99). Also IL-12 and IFN-α, non-γ_c-cytokines, hold promise to enhance the efficacy of ACT. IL-12 has been shown to play an essential role in T cell differentiation and memory formation and IFN-α is important in driving memory cell development (100-102). Use of these cytokines in ex vivo T cell cultures present a promising moiety to yield T cells with an improved capacity to respond (103, 104). T cells forced to secrete IL-12 benefitted also of this cytokine during culture (105).

In particular combinations of cytokines have shown encouraging results. In expansion protocols using naive T cells as a starting source, different combinations of IL-2, IL-7, IL-15 and IL-21 have proven to efficiently expand T cells and result in populations expressing early-differentiation markers such as CD27, CD28, CD62L and CD127 (64, 106-108). It has also been shown that CAR T cells can be efficiently expanded using a protocol devoid of IL-2, using CD3 and CD28 stimulation in the presence of IL-7 and IL-15 (109). Interestingly, also in TIL cultures and TCR/CAR engineered T cell cultures that are established in the presence of anti-CD3 and IL-2 and usually display a substan-

tial degree of differentiation, cytokine cocktails were able to establish T cell populations with a less differentiated phenotype (76, 110, 111). This suggests that cytokine cocktails can be used to reprogram late differentiated T cells. Besides delivery of cytokine-mediated signals via cytokine supplementation to the cultures, aAPCs can be designed to express cytokines or T cells can be triggered or engineered to produce abundant cytokines themselves (99, 112-114). The advantage of aAPCs is that they can be designed to simultaneously provide costimulation and cytokine-mediated signals (112, 115). Irrespective of the cytokine delivery manner, more research is required to pinpoint the amount, ideal timing and combination of cytokines for ex vivo cultures (108, 110). Moreover, requirements for establishing cultures from naive T cells or previously primed T cells are likely to be different. For example, addition of IL-21 causes naive T cells to significantly expand, while memory T cells fail to do so (98). Nevertheless, in both subsets IL-21 signaling increases CD28 expression.

In vivo costimulation

Another approach to improve ACT is enhancing the T cell expansion and function after transfer. The essentiality of costimulatory pathways has been demonstrated in experimental settings of adoptive transfers showing e.g. that CD27 and CD28-mediated costimulatory signals are important for expansion of both naive and memory $CD8^+$ T cells upon transfer (116, 117). To study the benefit of augmenting costimulatory pathways in patients after T cell infusion, preclinical studies and clinical trials have been performed exploring the use of agonistic antibodies against TNFR superfamily members 4-1BB, OX40, GITR, CD27, HVEM and CD40. The promise of these molecules in cancer immunotherapy has been reviewed recently (118-120).

Besides expansion an additional beneficial effect delivered by costimulation is induction of T cells with the capacity to produce IL-2 that acts in an autocrine manner *(84, 121). In contrast to exogenous IL-2, either provided in vitro or in vivo, autocrine IL-2 seems to be highly beneficial for both the (secondary) expansion potential and survival of CD8 T cells (113, 122).* However, in order to be able to optimize ACT protocols further and minimize the chance of severe adverse side effects as observed in the Phase I clinical trial with anti-CD28 and to a lesser extent in the Phase II study with the anti-4-1BB antibody Urelumab (NCT00612664), which was associated with a high incidence of severe hepatitis, a better understanding of the underlying mechanisms by which these antibodies exert their effects is crucial (123, 124). In pre-clinical models agonistic 4-1BB contributes to tumor regression by promoting survival and avoiding AICD of $CD8^+$ T cells and more importantly in the context of this review, in models using OVA-expressing tumors it has been demonstrated that a combination of agonistic 4-1BB antibody and transfer of OVA-specific $CD8^+$ T cells significantly improves tumor control (125, 126). Whether com-

bining ACT and 4-1BB agonists enhances anti-tumor activity in humans has not yet been assessed, but when used as a monotherapy 4-1BB antibodies seem to have some anti-tumor activity. Although two 4-1BB agonists have already been used in clinical trials only recently more insight into the mechanisms by which anti-tumor effect is exerted, is obtained and it has become clear that at least in pre-clinical models systemic 4-1BB activation induces a phenotype of $CD4^+$ and $CD8^+$ T cell characterized by high expression of the T-box transcription factor Eomes, $KLRG1^+$ and high cytotoxic capacity (125, 127-129). KLRG1 marks Tem and Teff cells and as already mentioned above, ACT of Tem and Teff cells combined with less-differentiated cells might be beneficial. Furthermore, agonistic 4-1BB antibody treatment correlated with decreased expression of the inhibitory receptors Programmed Death-1 (PD-1) and Lag3.

OX40 signaling can enhance T cell differentiation and survival via effects on IL-2 and IL-7-mediated signaling, and via increasing the anti-apoptotiac molecules Bcl-2 and Bcl-xL (130) Essentially, providing OX40 triggering augmented anti-tumor activity in a pre-clinical model of adoptive T cell transfer mediated by both $CD4^+$ and $CD8^+$ T cells (131). Conflicting results are reported on whether Treg responses are inhibited or promoted by OX40, which is most likely due to differences in dose and/or timing of OX40 ligation, and may depend on the model/setting (132). Although unraveling the precise mechanism of OX40 agonists remains a challenge, anti-OX40 has already been used in a Phase I clinical trial for patients with metastatic solid malignancies, albeit not in ACT settings (133). Results were promising, and indicated enhanced proliferation of $CD4^+$ and $CD8^+$ T cells that coincided with regression of at least one metastatic lesion in 12 out of 30 patients. Tregs in the tumor showed a higher expression of OX40 compared to peripheral blood Tregs.

Explored as well, albeit to a lesser extent are agonistic CD27 antibodies (134, 135). Promising results were reported in preclinical models, and are likely related to improved CD27-mediated T cell expansion, survival and function (77, 136-138). Conversely, it has also been reported that CD27 signaling can increase survival of Tregs in vivo and thereby promote tumor progression (86). The Teff:Treg ratio in the tumor has been suggested to determine whether CD27 agonist will promote or diminish tumor control (139).
An indirect way to improve the efficacy of transferred T cells is via administration of agonistic antibodies CD40 resulting in activation of APCs such as dendritic cells (DCs) (120). Consequent upregulation of costimulatory molecules on the APCs then provide the necessary stimulatory signals to activate tumor-specific T cells. In addition to DCs, CD40 antibodies also activate other myeloid cells (140) and the activity can also depend on complement-mediated cytotoxicity (CMC) or antibody-dependent cell-mediated cytotoxicity (ADCC), or even be immune effector independent when CD40 is expressed on tumor cells (141-146). Important to note is that CD40 triggering in malignant cells is

able to promote tumor cell proliferation leading to tumor progression (147, 148). Likely depending on one or more of the above described mechanisms, targeting of CD40 has already been proven a promising strategy in several preclinical models and clinical trials against solid cancer (120, 149-154), and also in preclinical models of ACT agonistic CD40 antibodies promote tumor-specific T cell expansion and enhanced anti-tumor activity in (155, 156). Thus, clinical effectiveness in ACT has potential given that CD40 antibody-associated toxicity is managed (152, 157). So far three agonistic CD40 antibodies, which differ in their agonistic activity, have been tested in clinical trials. The strongest agonistic antibody, CP-870.893, is a humanized antibody of an IgG2 isotype. Human IgG2 antibodies typically interact marginally with Fc receptors and are therefore not very effective mediators of CMC and ADCC (158). Nevertheless, this antibody is a potent activator of macrophages and DCs and can thereby mediate T cell-dependent anti-tumor immune responses, which suggests that it has the potential to enhance ACT. The two other antibodies that have been tested in the clinic (i.e., Dazetuzumab and ChiLob 7/4), displaying less agonistic activity compared to CP-870.89, are of an IgG1 isotype and hence, are more potent mediators of CMC and ADCC, making them less suitable for combinations with T cell transfer (120). Adverse effects that were observed after CD40 antibody treatment include CRS and liver damage (120). Targeting of CD40 is also possible by imposed expression of CD40L (CD154) on the transferred T cells. In an experimental model, CD19-specific CAR/CD40L T cells displayed increased cytotoxicity and enhanced tumor eradication (159).

Also currently under investigation in clinical trials is an agonistic antibody against GITR. In multiple animal models of cancer this antibody has proven to exert anti-tumor immune responses by providing costimulatory signals to T cells and skewing the balance between induced Treg and T_H9 cell differentiation in favor of T_H9 (160-162). In a preclinical adoptive transfer setting, agonistic GITR antibody has shown to increase the polyfunctionality of the transferred T cells and reduce the frequency of Tregs in the tumor, resulting in tumor regression (163). Repetitive doses of a GITR agonist is however potentially toxic (164).

Taken together, considerable progress has been made in dissecting the mechanisms by which agonistic antibodies to costimulatory molecules exert their anti-tumor effects but further unravelling is required to be able to implement this therapy into patients receiving ACT. Importantly, in case of treatment with such powerful agonists, also the mechanisms underlying the adverse immune-mediated side effects require attention. Undoubtedly, the effects of agonistic antibody administration are often multifaceted thereby making it challenging to predict treatment outcome. Attempts to minimize the chance of antibody-induced toxicity could include pretreatment with corticosteroids

and local administration of agonistic antibodies (151, 152, 154).
As mentioned before, second generation CAR T cells contain a costimulatory domain placed in series with CD3ζ and thereby costimulation is provided per definition upon target recognition. Since it is well appreciated that T cells require costimulation for proper activation, it is not surprising that incorporation of costimulatory domains advanced CAR T cell treatment. Alike for providing costimulation by agonistic antibodies, the choice of the costimulatory signaling domain influences CAR T cell functionality and persistence, i.e. by differential regulation of down-stream signaling expression. Most extensively explored are CAR T cells with incorporated CD28 and 4-1BB signaling domains and although treatment with both CAR T cells have resulted in clear clinical responses, comparisons showed prolonged persistence and ameliorated exhaustion of CAR T cells using the 4-1BB domain (47, 165, 166). Alternative signaling domains that have been integrated include domains of CD27, ICOS and OX40 (165, 167-169). In third generation CAR T cells, attempts to further enhance anti-tumor activity and long-term persistence rely on incorporation of two costimulatory domains. So far, combinations of CD28 with 4-1BB and CD28 with OX40 have shown to be promising, resulting in T cells having potent effector functions and improved capacity to persist long-term (168, 170). In a small pilot trial a CD20-specific CAR with CD28 and 4-1BB costimulatory domains has been tested in four relapsed indolent B cell and mantle cell lymphoma patients and the data suggest improved CAR T cell persistence (171).

In addition to improving T cell function by triggering costimulatory pathways, inhibitory pathways can be blocked and this strategy, also known as immune checkpoint blockade, has led to significant clinical advances in cancer immunotherapy (172-174). Several reports show that combinations of ACT and blockade of inhibitory molecules, such as CTL-associated antigen 4 (CTLA-4) and PD-1, have the potency to augment anti-tumor efficacy and increase T cell persistence (175-179). An alternative method in which PD-1-mediated inhibition was turned into CD28-mediated costimulation by generating PD-1-CD28 fusion receptors was also effective in ACT (180). Nevertheless, although targeting of either costimulatory or inhibitory pathways for the benefit of ACT may improve antitumor responses, for achieving greater clinical response rates, combinations of the two might be required and are currently under investigation. So far this approach has yielded encouraging results as evidenced by inhibition of tumor growth in preclinical settings including ACT cancer models (181-185). Decreased tumor progression coincided with an increase in effector T cells and a decrease in Tregs and myeloid suppressor cells at tumor sites. This shift from a more immunosuppressive to a more immunostimulatory tumor environment might explain the potent effects of these antibody combinations.

Cytokines in vivo

ACT using TILs generally includes in vivo administration of high dose IL-2 to improve proliferation and survival of the transferred TILs. Unfortunately, exogenous IL-2 treatment has two major drawbacks; it is often associated with severe toxicity and can promote Treg proliferation. It has been reported that the number of doses of IL-2 that are administered after adoptive TIL transfer is positively correlated with Treg reconstitution after lymphodepletion and furthermore, that the degree of Treg reconstitution is inversely correlated with the patient's response to treatment (186).

Attempts to circumvent IL-2-induced toxicity and Treg stimulation have been made. A straightforward measurement is by reduction of the IL-2 administration (187). Tailoring CD8+ T cells to augmented autocrine IL-2 production seems an alternative promising manner, which increases the availability of IL-2 to the right cell without promoting Treg proliferation. This can be achieved using retroviral or lentiviral transduction and this would especially be feasible in situations where transduction is already required. For instance, in case of generating TCR engineered T cells, but in fact also TILs can be transduced in the same manner. Recently, we have shown in a preclinical model that CD8+ T cells cultured in the presence of IL-7 and IL-15 that are forced to overexpress IL-2 display improved persistence and expansion potential after transfer and subsequent vaccination (113). Consequently, this heightened anti-viral and anti-tumor immunity in vivo compared to mock transduced cells. Notably, after in vivo secondary challenge the cells with elevated autocrine IL-2 efficiently re-expanded yet also expressed IL-7Rα, suggesting that although these cells underwent prolonged IL-2 signaling, they still seem to be of a less-differentiated phenotype, which may be related to the transduction procedure in the presence of IL-7 and IL-15. In addition, we did not observe any alterations in Treg homeostasis (113). ACT with human T cells overexpressing IL-2 has also been explored yielding promising results with respect to longevity but large clinical studies should be performed to determine if IL-2 over-expressing T cells result in clinical benefit (44, 92). Likely, ACT approaches with IL-2+ T cells are most successful when they are combined with vaccination given the prominent role of autocrine IL-2 production for secondary expansion of CD8+ T cells (113, 122). As discussed before, another strategy to circumvent exogenous IL-2 administration is to provide agonistic antibodies to costimulatory receptors that promote autocrine IL-2 production in T cells.

Other cytokines than IL-2 have also been explored to enhance ACT-mediated T cell responses. IL-7 and IL-15 are crucial cytokines for lymphoid homeostasis by playing an important role in orchestrating the survival of naive and memory T cells and memory cell differentiation (188, 189). Increased availability of IL-7 and IL-15 has been shown to be an important mechanism by which a lymphodepleting regimen improves the engraftment of the adoptively transferred T cells and hence the success of ACT (190-192).

Preclinical ACT models in which the effect of exogenous IL-7 and IL-15 on tumor outgrowth has been tested, demonstrated that both cytokines can improve tumor control including in vaccinated lymphodepleted or immunodeficient hosts (193-195). Recently a phase I clinical trial has been conducted to determine safety, adverse event profiles and the maximum tolerated dose of rhIL-15 in humans (196). Patients with metastatic melanoma and metastatic renal cancer were infused with different doses of IL-15 (0.3/1.0/3.0 μg/kg/day) for 12 consecutive days and this treatment regimen resulted in markedly altered homeostasis of mainly NK cells, γδ cells and to somewhat lesser extent of memory CD8+ T cells. No clinical responses according to the RECIST criteria (197), which includes the persistence of the cells after transfer, were observed and the maximum tolerated dose was determined to be the lowest used dose. Because of clinical toxicity caused by strong cytokine production, the authors stated that rhIL-15 is too difficult to administer intravenously and suggest developing alternative dosing strategies and new trials to assess this are being conducted (196). IL-7 administration is tolerated better in humans, but anti-tumor efficacy requires further evaluation (198, 199).
As for IL-2, systemic IL-15-mediated toxicity might be circumvented by tailoring tumor-specific T cells to express IL-15. In that way the IL-15 mediated effects are likely confined to the tumor environment, eluding systemic toxicity. Engineering T cells to produce IL-15 is another option. In experimental models it was shown that IL-15-expressing CD8+ T cells improve anti-tumor activity (95), and human IL-15 secreting cells perform also well in vivo (43, 200, 201).

The most recently discovered member of the γc cytokine family, IL-21, has also been explored as an anti-cancer treatment and IL-21 monotherapy of thymoma and melanoma in mice has shown to result in improved CD8+ T cell-mediated anti-tumor responses with augmented long-term survival (193, 202-204). IL-21 treatment prolonged persistence of endogenous and adoptively transferred tumor-specific transgenic CD8+ T cells, which was mainly attributed to IL-21-mediated promotion of survival (202). Additionally it has been shown that IL-21 is able to potentiate tumor-specific antibody responses, which resulted in complement-mediated tumor cell lysis (204). Combination of cytokines involving IL-21 demonstrated further enhancement of anti-tumor immunity compared to IL-21 as a monotherapy. In a study wherein mice were challenged with B16F10 melanoma, treatment by adoptive transfer of transgenic tumor-specific CD8+ T cells, combined administration of IL-21 and IL-2 and vaccination resulted in higher absolute number of circulating tumor-specific T cells and improved tumor-free survival compared to therapy with IL-2 or IL-21 alone (203). In addition, in a model using murine B16 melanoma cells that were transfected to secrete IL-21, it was shown that local presence of IL-21 can also promote anti-tumor immunity by preventing IL-2-mediated Treg induction (205). Experiments in the B16 model has shown that mixing IL-21 with IL-15 improves expansion of transferred tumor antigen-specific CD8+ T cells and enhances tumor control after

vaccination (106). Clinical trials using IL-21 as a single agent in melanoma and renal cell carcinoma show that this cytokine is well tolerated and favourable clinical responses have been observed as evidenced by patients in which disease stabilization (206, 207). To our knowledge the anti-tumor effect of IL-21 in human ACT has not been addressed yet. A study by Markley and Sadelain showed that forced expression of IL-7 and IL-21 by CD8+ T cells resulted in improved rejection of systemic lymphoma compared to T cells that overexpressed IL-2 or IL-15 (99). However, in these experiments vaccination was not provided post-transfer, which may have resulted in improved expansion of the transferred IL-2+ cells.

Anti-tumor efficacy of some cytokines that do not belong to the γc cytokine family has been explored as well. In preclinical models, administration of IL-12 caused tumor regression and promoted survival of tumor-bearing animals. This provided the rationale for applying IL-12 treatment in a clinical setting, however, translation into the clinic was hindered by severe toxicity (208, 209). To be able to explore IL-12 as a treatment modality multiple attempts have been made to design a safe IL-12-based treatment including different administration schedules and routes and intratumoral delivery (210). Several reports explored the therapeutic efficacy of tumor-specific T cells designed to express IL-12. In experimental settings T cells modified to produce IL-12 improved tumor eradication (19, 101, 211-213). Promising results were obtained using IL-12 gene transduced human TILs. Here different doses of IL-12 producing TILs were infused into metastatic melanoma patients and higher doses resulted in better clinical responses (214). Nevertheless, IL-12 transduced TILs did not persist for more than a month. In addition, higher cell doses led to severe adverse side effects attributable to the secreted IL-12 (214). The lack of persistence of the transferred T cells that is observed might be due to a negative feedback loop that involves IL-10 production to limit ongoing T cell activation (215). So far these reports suggest that ACT with IL-12 producing T cells has potential but improving the longevity of the cells and measures to prevent IL-12-mediated toxicity are vital for further application.

IFN-α is known for its direct anti-tumor effect and is currently a frequently used cytokine for the treatment of cancer. It has also been recognized that IFN-α promotes T cell activation, survival, expansion and memory formation through activation and differentiation of DCs (216). The mechanism by which IFN-α mediates memory formation is suggested to be mediated by enhancement of IL-15 presentation of DCs to T cells (217). Moreover, IFN-α can also affect T cell expansion directly. This so-called signal 3 further amplifies the signals T cells receive via the TCR (signal 1) and costimulatory receptors (signal 2) (218-220). However, due to severe adverse events caused by IFN-α administration treatment is discontinued in up to 50% of the cases. Frequently observed symptoms

of IFN-α-related toxicities include 'flulike' symptoms, fatique, anorexia and nausea (18, 221). To improve tolerability of IFN-α treatment, a polyethylene glycol (peg) moiety was added, resulting in a longer half-life. This allowed for less-frequent administration and hence, less toxicity. Accordingly discontinuation rates of IFN-α treatment were lower when a pegylated form was used (up to ~25%). To further enhance the anti-tumor effect of cytokines in ACT, blocking antibodies to inhibitory receptors can be co-administered, e.g. IL-21 and CTLA-4 (222).

Overall, we conclude that although combining ACT with cytokine treatment seems a promising approach, as hold true for treatments that trigger costimulatory pathways, great care must be taken in applying cytokine therapy. The immune effects are not solely confined to the infused T cells and affect many other cells, frequently leading to severe systemic toxicities.

Vaccination provided post transfer

Therapeutic cancer vaccines have the potential to mediate clinical benefit, even as a monotherapy, providing the rational to consider it as an approach to improve ACT (223). Preclinical tumor models provided the insight that vaccination can improve ACT and strategies that have been used to aid anti-tumor efficacy in ACT, include vaccination with viruses encoding tumor antigens, long peptides, peptide-pulsed DCs and DNA vaccination (224-229). Vaccination predominantly seems to improve anti-tumor responses by enhancing tumor infiltration, persistence and IFN-γ production of adoptively transferred T cells. Also in clinical trials the potential of vaccination to enhance ACT has been explored, but thus far clinical success is marginal (230-233). An encouraging approach by Rapoport and colleagues showed that in the setting of autologous HSC transplantation for multiple myeloma pre-transplant vaccination, adoptive transfer of in vivo vaccine-primed T cells and subsequent vaccinations led to significant improvement of immunity in patients that would otherwise suffer from severe immunodeficiency due to high-dose chemotherapy (233). Since then, similar strategies have been applied in anti-cancer treatment in patients; autologous vaccine-primed lymphocytes were expanded ex vivo and adoptively transferred accompanied by vaccinations. Using this strategy, promising results have been obtained with respect to enhancement of the tumor-specific T cell response, but clinical activity remains to be further validated (234-237). More recently two preclinical reports showed enhancement of CAR T cell-mediated anti-tumor responses by vaccination. Both studies were conducted using bispecific T cells targeting CMV and CD19 and vaccination consisted of CMV peptide pp65 presented by either T cells or CD40L and OX40L expressing K562 cells (238, 239). Compared to mice receiving vaccination with an irrelevant peptide, control of tumor cell growth was improved and this coincided with increased frequencies of CAR-CMV-CTLs, suggesting that CMVpp65 stimulation expanded the bispecific T cells efficiently.

Vaccination as a modality to enhance ACT has so far not been explored in great detail

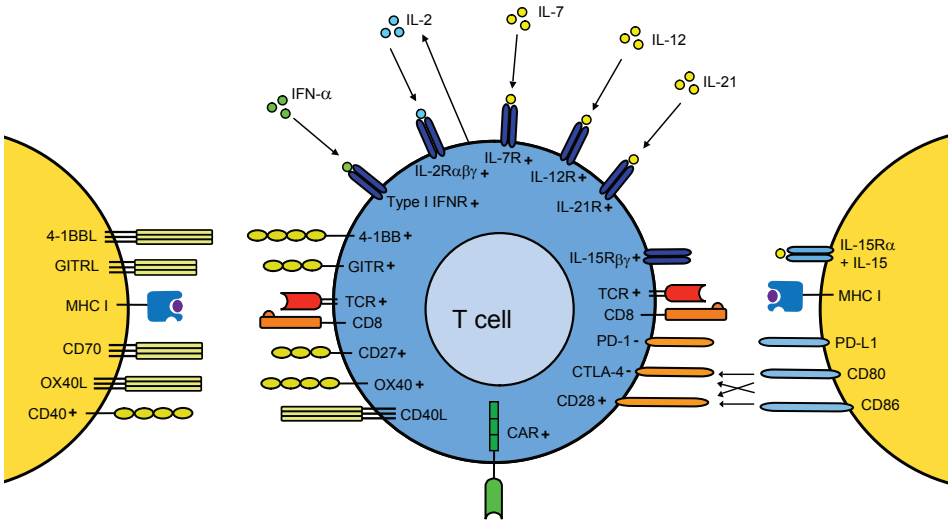

Figure 1. A T cell centric view of improving adoptive T cell therapy by provision of T cell stimulation signals. Upon transfer of ex vivo expanded disease-specific T cells into the host, these cells recognize their cognate antigen in the context of MHC molecules via their TCR. Amplification of costimulatory signals (e.g., via agonistic antibodies) can be used to additionally stimulate T cells. Antibodies against CD28, CD27, 4-1BB, OX40 and GITR have been evaluated in pre-clinical models and clinical trials for their capacity to enhance T cell function. Targeting of costimulatory receptors can also be used during the ex vivo expansion of the T cells. In case of CAR T cells, recognition occurs via the CAR, a chimeric TCR that already provides a certain degree of costimulation. Blockade of inhibitory molecules like CTLA-4 and PD-1 by antibodies after transfer, counteracts suppressed T cells thereby improving T cell activity. In addition, inflammatory cytokines are able to provide signals for enhancing expansion, differentiation, and migration. Cytokines such as IFN-α, IL-2, IL-7, IL-12, IL-15 and IL-21 have shown to have the capacity to enhance T cell efficacy either during ex vivo culturing or after adoptive transfer. Autocrine production of IL-2 is a vital property for secondary population expansion, and enhancing autocrine IL-2 is a promising way to improve T cell therapies. The + and – symbols indicate positive and negative signaling.

and clinical trials using this approach have so far not yielded outstanding results. One of the reasons for this might be that the responsiveness of the adoptively transferred T cells to the vaccine is poor. As aforementioned pointed out, vaccination may be best suitable for less differentiated T cells producing IL-2. Another possible explanation is that the immunosuppressive tumor environment is hampering T cell activation (240). Strategies to improve vaccination in the context of ACT include combination with peritumoral administration of TLR ligands and TLR-based adjuvants (226, 241).

Additional of great importance is the antigen that is used for vaccination. Often TAAs are used for vaccination. The advantage of this approach is that it is broadly applicable as it allows treatment of most patients with a certain tumor type. However, the specific T cell response toward the TAA can be blunted by central tolerance mechanisms. In contrast, T cells reacting to neoantigens expressed by tumors are not centrally tolerized (240). However, these antigens harbor unique mutations in a patient and thus targeting these antigens would require the production of personalized vaccines. This is a topic

of intense interest and future studies should resolve the feasibility of such approaches. Taken together, it is clear that strategies to improve ACT by vaccination need to be optimized and it seems that vaccination as a single modality to enhance this treatment is not sufficient.

CONCLUDING REMARKS

Currently multiple clinically approved immunostimulatory antibodies and cytokines are available that target a multitude of receptors expressed by T cells (Figure 1). It is expected that the agents targeting these receptors as well as the number of receptors that are targeted will increase in the coming years. The anti-tumor activity and persistence of infused T cells is highly dependent on the costimulatory pathways that are triggered after T cell transfer and on the expressed cytokine receptors. Unfortunately, the question as to which of the many T cell stimulating pathways need to be activated during ACT to attain T cells that exert a superior anti-tumor effect and are able to persist long-term has no unanimous answer. Ex vivo culture methods should be designed in such way that the expression of the appropriate receptors on particular T cell subsets is induced, and this holds true for (autocrine) cytokine production as well. To predict the expression pattern, a detailed understanding of the regulation of these receptors is essential. Once this question has been addressed, the next challenge would be to make sure that the transferred T cells remain functional longitudinally, which involves likely a certain degree of heterogeneity of T cell subsets expressing various costimulatory and cytokine receptors. Moreover, it is important to keep in mind that the effect of delivery of antibodies or cytokines to patients in order to improve survival, accumulation and anti-tumor efficacy of the transferred T cells, is not confined to the transferred T cells alone, but can affect also host cells bearing the appropriate receptor, potentially resulting in severe toxicity. Due to these toxicity issues the overall results of using cytokines and agonistic antibodies against immune costimulators may have been modest with respect to the anti-tumor activities in clinical trials. It is conceivable that also by enhancing the quality of engineered T cells (including CAR T cells), which generally already recognize their targets with good affinity, the provision of (additional) costimulation or cytokines might potentiate cross-reactivity or toxicity. An approach to circumvent treatment-related adverse effects includes local administration or specific targeting to the tumor site (152, 226, 242). Furthermore, combinations of treatment modalities are likely to reduce the dose that is required for clinical responses and this might avoid severe adverse effects. On the other hand, certain combinations, even at lower concentrations might result in unexpected toxicity. In addition to strategies to enhance ACT that have been discussed in this review, i.e. by providing costimulation, blocking inhibitory molecules, cytokines, and/or vaccination, the T cell quality can be further enhanced by changing the tumor

microenvironment to induce a more favorable milieu for T cells. Those strategies, which are beyond the scope of this review to be discussed in detail, include therapies counteracting myeloid-derived suppressor cells (MDSCs), neutralization of tumor acidity, chemotherapy and inhibition of IDO, and treatment with antibodies against immune suppressive cytokines (243-248). Finally, as discussed vaccination provided post transfer as an approach to enhance the efficiency of ACT is promising, yet such a combined treatment requires substantial effort to make it clinically successful.

Taken together, ACT holds its promise as an effective anti-cancer treatment but improvement is required. Concluding from the aforementioned discussion, the inclusion of T cell costimulation and cytokines should be an integral part for optimization of ACT protocols. In addition, combinations with other immune therapies such as vaccination are expected to further improve the clinical success rates of ACT.

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CHAPTER 5

Viral inoculum dose impacts memory T-cell inflation

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ABSTRACT

Memory T cell inflation develops during certain persistent viral infections and is characterized by the accumulation and maintenance of large numbers of effector-memory T cell populations, albeit with varying degrees in size and phenotype among infected hosts. The underlying mechanisms that control the generation of memory T cell inflation are not yet fully understood. Here, we dissected CMV-specific memory T cell formation and its connection to the initial infectious dose by varying the inoculum size. After low dose inoculum with mouse CMV, the accumulation of inflationary memory T cells was severely hampered and correlated with reduced reservoirs of latent virus in non-hematopoietic cells and diminished antigen-driven T cell proliferation. Moreover, lowering of the initial viral dose turned the characteristic effector memory-like inflationary T cells into more central memory-like cells as evidenced by the cell-surface phenotype of $CD27^{high}$, $CD62L^{+}$, $CD127^{+}$ and $KLRG1^{-}$, and by improved secondary expansion potential. These data show the impact of the viral inoculum on the degree of memory T cell inflation and provide a rationale for the observed variation of human CMV-specific T cell responses in terms of magnitude and phenotype.

INTRODUCTION

Memory T cell inflation arises during certain persistent viral infections and is characterized by the accumulation and maintenance of large numbers of effector-memory T cell populations (1). It is especially prominent during the course of cytomegalovirus (CMV) infection and observed in different species including humans, monkeys, and mice (2-5). Studies in experimental models of CMV infection imply that memory T cell inflation is driven by repetitive antigen exposure likely due to sporadic viral re-activation (6-10). The characteristic effector-like phenotype of the inflationary memory $CD8^+$ T cells (i.e. $CD27^{low}$, $CD28^-$, $CD62L^-$, $CD127^-$, $KLRG1^+$, IL-$2^{+/-}$) (8, 11, 12) further underscores the influence of antigen-driven differentiation but costimulatory signals provided by TNFR family members such as CD27, OX40 and 4-1BB are required for inflation as well (13-15). In addition, CD4 T cell help facilitates the inflationary $CD8^+$ T cell response (16, 17). In contrast to exhausted T cells, which develop during infection with high-level replicating viruses (18), inflationary T cells remain functional throughout the lifetime of the host (5, 19-21), which provides prospects for the use of CMV-based vaccine vectors (1, 22-24).

The percentages of human CMV-specific T cells occupying the memory T cell compartment vary greatly among seropositive individuals (25, 26). Moreover, the effector memory phenotype of CMV-specific T cells varies among individuals and seems to correlate with the memory $CD8^+$ T cell pool size (26-29). In this respect, it is of interest to note that the viral dose which humans become infected with likely varies within a large range as bodily fluids such as breast milk, saliva and urine, causing horizontal transmission of CMV, contain different quantities of CMV among individuals ranging from 10^1 to 10^5 copies/µl (30, 31). Thus, it appears that the degree of memory T cell inflation differs per CMV-infected host but whether this is linked to the initial viral inoculum dose is unknown.

In this study, we examined the influence of the viral inoculum size on the course of CMV infection and on memory T cell inflation in particular by using an experimental model of CMV, i.e. mouse CMV, which mimics human CMV infection. We found that low dose viral inoculum in contrast to high dose resulted in severely hampered memory T cell inflation, which is accompanied by a more central memory phenotype of the inflationary T cells and improved capacity to expand after re-challenge. These findings are of importance for vaccination strategies against CMV and for the development of CMV-based vaccine vectors.

MATERIALS AND METHODS

Mice

Wild-type (WT) C57BL/6 and BALB/c mice were purchased from Charles River. Thy1.1 (CD90.1) mice (obtained from The Jackson Laboratory) on a C57BL/6 background were

bred in-house. All mice were maintained under specific pathogen free (SPF) conditions at the Central Animal Facility of Leiden University Medical Center (LUMC) and were 8-10 weeks old at the beginning of each experiment. All animal experiments were approved by the Animal Experiments Committee of the LUMC and performed according to the Dutch Experiments on Animals Act that serves the implementation of 'Guidelines on the protection of experimental animals' by the Council of Europe and the guide to animal experimentation set by the LUMC.

MCMV infection and determination of viral load

MCMV-Smith was obtained from the American Type Culture Collection (ATCC VR-194; Manassas, VA) and salivary gland stocks were prepared from infected BALB/c mice. Mice matched for gender and age were infected i.p. with different dosages of salivary gland derived MCMV-Smith ranging from 10^1 to 10^4 PFU. For determination of viral load, genomic DNA was isolated from snap frozen tissues or from hematopoietic and non-hematopoietic cell splenic populations that were sorted based on the expression of CD45 using a DNAeasy blood and tissue kit (Qiagen, Venlo, Netherlands). MCMV glycoprotein B (gB) was then assayed by quantitative PCR using an IQ5 real time PCR detection system (Bio-Rad, Hercules, CA) and IQ SYBR Green MasterMix reagent (Bio-Rad). Aliquots (100 ng) of DNA were used as templates for each reaction. MCMV gB was used to determine MCMV viral load and MCMV copy numbers were calculated using a standard curve generated with the K181 MCMV plasmid. Data are expressed as MCMV copy number per 100 ng genomic DNA and normalized to β-actin. The limit of detection was 400 genome copies/100 ng DNA. The primer sequences used for detection of gB were 5'-GAAGATCCGCATGTCCTTCAG-3' and 5'-AATCCGTCCAACATCTTGTCG-3'. Primers used for detecting β-actin were 5'-GATGTCACGCACGATTTCC-3' and 5'-GGGCTATGCTCTCCCTCAC-3'. Real-time PCR data was analyzed using the Bio-Rad IQ5 software.

MCMV-specific antibody detection

Blood samples were collected retro-orbitally from infected mice at day 8, 60 and 120 post-infection. After brief centrifugation, sera were transferred to new tubes and stored at −20°C. MCMV-specific serum IgG levels were measured by ELISA. In short, 96 well plates (Nunc Maxisorp) were coated overnight at 4°C with NIH-3T3 derived MCMV-Smith in bicarbonate buffer (pH 9.6). Plates were subsequently incubated for 1 h at 37°C with blocking buffer (PBS/5% milk powder). Sera were diluted 1:500 in PBS/1% milk powder and incubated in the blocked wells for 1 h at 37°C. HRP-conjugated IgG_1, IgG_{2b}, IgG_{2c}, IgG_3 antibodies (Southern Biotech, Birmingham, USA) were diluted 1:4000 in PBS/1% milk powder and incubated 1 h at 37°C. To develop the plates 100 µl of TMB (3,3',5,5'-Tetramethylbenzidine) (Sigma Aldrich) was added to each well and incubated for 15 minutes at room temperature, after which 100 µl stop solution (1M H_2SO_4) was

added. Plates were measured within 5 minutes after adding stop solution at 450 nm using a Microplate reader (Model 680, Bio-Rad).

Flow cytometry

Single cell suspensions were prepared from spleen and lymph nodes by mincing the tissue through a 70 μm cell strainer (BD Bioscience). For lymphocyte isolation from the bone marrow, the tibias and femurs were removed and flushed followed by filtering through a 70 μm cell strainer. Erythrocytes were lysed in a hypotonic ammonium chloride buffer. Before lungs and liver were removed, mice were perfused with PBS containing EDTA to remove all blood associated lymphocytes. Lymphocytes were isolated from the lungs and liver by collagenase (crude, type IA) (Sigma Aldrich) and DNAse I (Sigma Aldrich) treatment for 0.5 h followed by percoll (GE Healthcare, Uppsala) gradient. Tetramer staining and intracellular cytokine staining were used to determine the magnitude and characteristics of the MCMV-specific T cell responses as described elsewhere (32). Fluorochrome-conjugated antibodies specific for CD3, CD8, CD25, CD44, CD45.1, CD45.2, CD90.1, CD90.2, CD127 (IL-7Rα), IFN-γ, IL-2, KLRG1, NK1.1, TNF, Ki67 and Bcl-2 were purchased from BD Biosciences, Biolegend or eBioscience. Dead cells were excluded by positivity for 7AAD (Invitrogen). Cells were acquired using a BD LSR II flow cytometer, and data was analyzed using FlowJo software (TreeStar).

Tetramers and peptides

The following class I-restricted peptides were used: $M45_{985\text{-}993}$, $m139_{419\text{-}426}$, $M38_{316\text{-}323}$, and $IE3_{416\text{-}423}$ (described in (12, 33)). MHC class I tetramer complexes for M45 (D^b restricted), m139, M38 and IE3 (all K^b restricted) were produced as described elsewhere (34).

Re-challenge and adoptive transfers

To determine the effect of the inoculum size on protective immunity, mice were infected with 10^1 or 10^4 PFU MCMV-Smith and 60 days post-infection these mice were re-infected with 10^5 PFU MCMV-Smith. One day before and 5 days after re-challenge, blood was taken and the magnitude of the response was determined by tetramer staining. To determine the secondary expansion capacity of MCMV-specific T cells after adoptive transfer, Thy1.1 mice were infected with 10^1 PFU or with 10^4 PFU MCMV-Smith. At day 60 post-infection, splenic M45-, M38- and IE3-specific $CD8^+$ T cells were stained with MHC class I tetramers and purified by sorting using a BD FACSAria II flow cytometer. Next, 1×10^3 $M45^+$, 3×10^3 $M38^+$ or 3×10^3 $IE3^+$ $CD8^+$ T cells were transferred i.v. into naive Thy1.2 mice. The host mice were infected 3 h later with 10^4 PFU MCMV-Smith and at day 5 post-infection the absolute numbers of donor M45-, M38- and IE3-specific $CD8^+$ T cells were determined in the spleen by tetramer staining.

To compare the secondary expansion capacity of central memory MCMV-specific $CD8^+$ T cells induced in low and high dose infected mice, Thy1.1 mice were infected with 10^1

PFU or with 10^4 PFU MCMV-Smith. At day 60 post-infection, splenic $CD127^+KLRG1^-$ M38-specific $CD8^+$ T cells were purified by sorting using a BD FACSAria II flow cytometer. Next, 3×10^3 of the sorted cells were transferred i.v. into naive Thy1.2 mice. The host mice were infected 3 h later with 10^4 PFU MCMV-Smith and at day 5 post-infection the absolute numbers of donor M38-specific $CD8^+$ T cells were determined in the spleen by MHC class I tetramer staining.

Statistical analysis

Statistical significance was assessed by the Mann-Whitney test using GraphPad Prism software. P values < 0.05 were considered significant.

RESULTS

The degree of memory T cell inflation is determined by the size of the viral inoculum

To determine the effect of the inoculum dose on the inflation of MCMV-specific memory T cell populations, C57BL/6 mice were infected with either 10^1 PFU (low dose) or 10^4 PFU (high dose) of MCMV Smith strain. Accordingly, both in the acute and chronic phase of infection, viral load in tissues (liver, lungs and salivary glands) determined by quantitative real-time PCR (qPCR) was lower in mice infected with 10^1 PFU MCMV as compared to infection with 10^4 PFU (Fig. 1A). Furthermore, the number of activated NK cells, which play an important role in limiting acute CMV replication, correlated to the viral dose (Fig. 1B). The MCMV-specific IgG2c antibody titers were reduced in low dose infected mice at day 8 post-infection. Inflation of IgG2c levels, however, was observed in both low and high dose infected mice (Fig. 1C). Similar results were obtained with other antibody IgG isotypes, i.e. IgG1, IgG2b, and IgG3 (data not shown).

In the acute phase of infection, the absolute splenic numbers of the non-inflationary M45-specific $CD8^+$ T cells were ~5-fold reduced in mice receiving low dose inoculum, and $CD8^+$ T cell numbers specific for the inflationary epitopes m139, M38 and IE3 were ~2 fold reduced (Fig. 1D). After the peak of the acute response (day 8), the M45-specific $CD8^+$ T cell response underwent similar contraction in the low and high dose infected mice. Remarkably, although MCMV infection in C57BL/6 mice is usually characterized by accumulation of m139-, M38- and IE3-specific $CD8^+$ T cells during the chronic phase, inflation of these populations is either absent (m139- and M38-specific) or rigorously impaired (IE3-specific) when the inoculum size is lowered to 10^1 PFU (Fig. 1E). Analogous to the spleen, the degree of memory T cell inflation in the lungs was impaired after low dose inoculum as compared to high dose (Fig. 1F). Thus low dose inoculum of MCMV still results in a persistent infection, but in contrast to high dose inoculum such infection is not accompanied by overt memory T cell inflation.

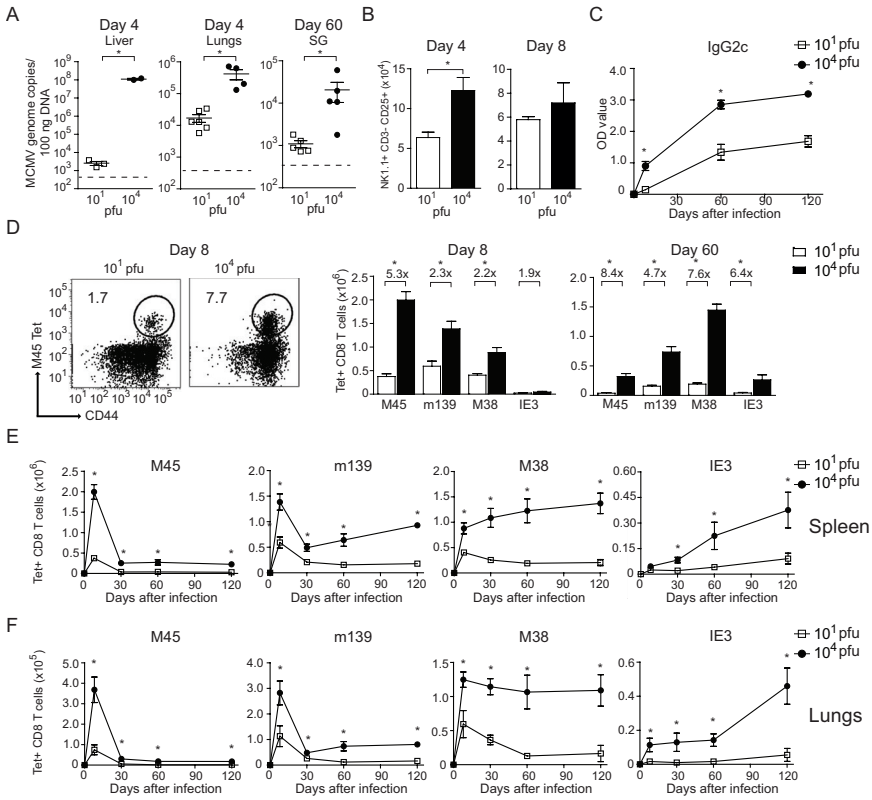

Figure 1. The degree of memory T cell inflation is determined by the size of the viral inoculum. WT mice were infected with 10^1 or 10^4 PFU MCMV-Smith. (A) Viral titers were determined in liver, lungs and salivary glands (SG) by quantitative real-time PCR at day 4 and 60 post-infection. Graphs depict MCMV genome copies per 100 ng tissue derived DNA. Each symbol represents an individual mouse. (B) Bar graphs depict the absolute number of splenic NK1.1$^+$/CD3$^-$ cells positively stained for CD25 at day 4 and 8 post-infection. (C) At day 8, 60 and 120 days post-infection serum samples were taken and MCMV specific IgG2c titers were determined by ELISA. Graph shows OD values measured at 450 nm. (D) At day 8 and 60 after infection, the magnitude of the CD8$^+$ T cell response to epitopes derived from the MCMV proteins M45, m139, M38 and IE3 was determined in the spleen using MHC class I tetramer staining. Representative flow cytometry plots show CD44 expression and binding to MHC class I M45 tetramers (Tet) on gated CD8$^+$ T cells at 8 day after infection. Bar graphs show the absolute number of splenic MCMV-specific CD8$^+$ T cells for each epitope at day 8 and 60 post-infection. (E and F) At day 8, 30, 60 and 120 after infection the magnitude of the CD8$^+$ T cell response to the indicated MCMV epitopes was examined in the spleen (E) and in the lungs (F). Graphs show the absolute number of MCMV-specific CD8$^+$ T cells for each epitope as determined by using MHC class I tetramers. Shown are mean values and SEM ($n = 4$). Statistical significance is determined by the Mann-Whitney test and indicated with an asterisk (* $p < 0.05$). Shown are representative data of three independent experiments.

Impaired memory T cell inflation during low dose infection relates to diminished latent virus in non-hematopoietic cells and to antigen-driven proliferation

Priming of CMV-specific T cells is thought to be dependent on cross-presenting hematopoietic antigen-presenting cells while memory T cell inflation, at least in the MCMV model, relies on infected non-hematopoietic cells (6, 10, 35). To evaluate whether the impaired memory T cell inflation after low dose inoculum relates to the amount and localization of (latent) MCMV, we aimed to quantify the presence of MCMV genomes by

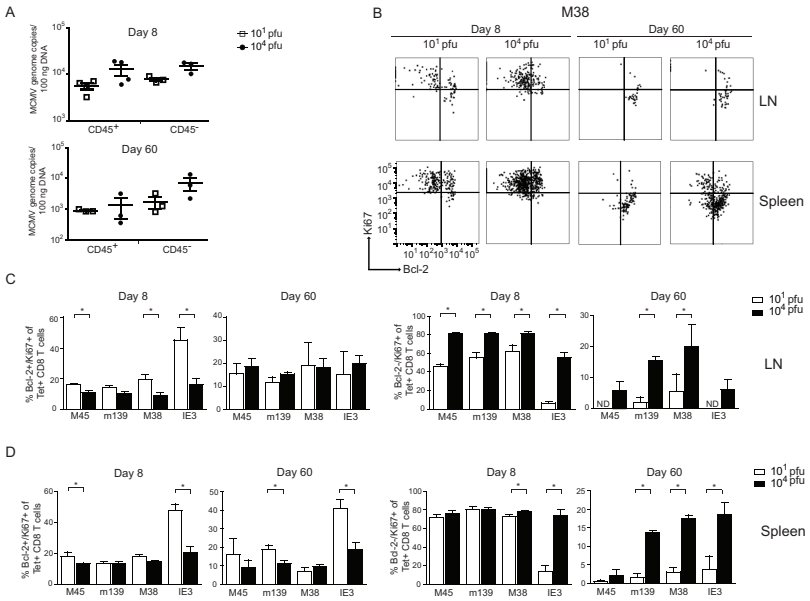

Figure 2. Reduced reservoirs of latent MCMV in non-hematopoietic cells and diminished antigen-driven T cell proliferation after low dose infection. WT mice were infected with 10^1 or 10^4 PFU MCMV-Smith. (A) Viral titers were determined in hematopoietic ($CD45^+$) and non-hematopoietic ($CD45^-$) spleen cells by quantitative real-time PCR at day 8 and 60 post-infection. Graphs depict MCMV genome copies per 100 ng DNA. Each symbol represents an individual mouse ($n = 3$). (B) Representative flow cytometry plots show expression of Bcl-2 and Ki67 on M38-tetramer$^+$ $CD8^+$ T cells derived from the spleen or LNs (axillary, brachial and inguinal) at day 8 and 60 after infection. (C) Bar graphs show the percentage of Bcl-2^+/Ki67^+ or Bcl-2^-/Ki67^+ expressing cells within the tetramer$^+$ $CD8^+$ T cell populations at day 8 and 60 post-infection in the LNs or (D) spleen. Shown are mean values and SEM ($n = 5$). Bcl-2^-/Ki67^+ M45- and IE3-specific T cells were not detectable (ND) in the lymph nodes at day 60 post-infection. Statistical significance is determined by the Mann-Whitney test and indicated with an asterisk (* $p < 0.05$). Shown are representative data of two independent experiments.

qPCR in hematopoietic ($CD45^+$) and non-hematopoietic ($CD45^-$) splenic cells during the acute and latent phase of infection. During the acute phase of infection, MCMV genomes were more abundantly present in both the hematopoietic and non-hematopoietic fraction of low and high dose inoculated mice while at day 60 post-infection, when memory T cell inflation occurs predominantly in high dose infected mice, MCMV localizes only in these mice at a higher level in the non-hematopoietic compartment (Fig. 2A). Thus besides affecting productive infection also the reservoir of latent MCMV genomes in non-hematopoietic cells is influenced by the initial viral dose. Consistent with previous findings (6, 10) this finding also suggests that latently infected non-hematopoietic cells are essential for driving memory T cell inflation.

To evaluate whether the reduced presence of MCMV genomes after low dose inoculum is related to impaired cycling activity of the inflationary memory T cells, we analyzed Ki-67 expression (associated with proliferation) together with the expression of the anti-apoptotic molecule Bcl-2 in order to discriminate between either antigen-driven proliferation (Bcl-2^-Ki-67^+) or cytokine-driven homeostatic proliferation (Bcl-2^+Ki-67^+). While

at day 8 post-infection, both in low and high dose infected mice the cycling activity of M45-, m139- and M38-specific CD8$^+$ T cells in spleen and lymph nodes was mainly antigen-driven, at late time-points post-infection the antigen-driven proliferation in these cells was still considerable in high dose infected mice but minute in low dose infected mice (Fig. 2B-D). Remarkably, IE3-specific CD8$^+$ T cells already show diminished antigen-driven proliferation at day 8 post-infection but show elevated cytokine-driven homeostatic proliferation at day 8 post-infection in spleen and lymph nodes and at day 60 in the spleen (Fig. 2B-D). Taken together, these results show that the impaired memory T cell inflation after low dose inoculum relates to reduced reservoirs of latent MCMV in non-hematopoietic cells and accordingly into diminished antigen-driven T cell proliferation.

Viral inoculum size influences central and effector memory CD8$^+$ T cell formation

Inflationary memory T cells are characterized by a predominant effector-like phenotype, whereas non-inflationary memory T cells have a principal central memory phenotype during the latent phase of infection (1). To evaluate whether the central/effector memory

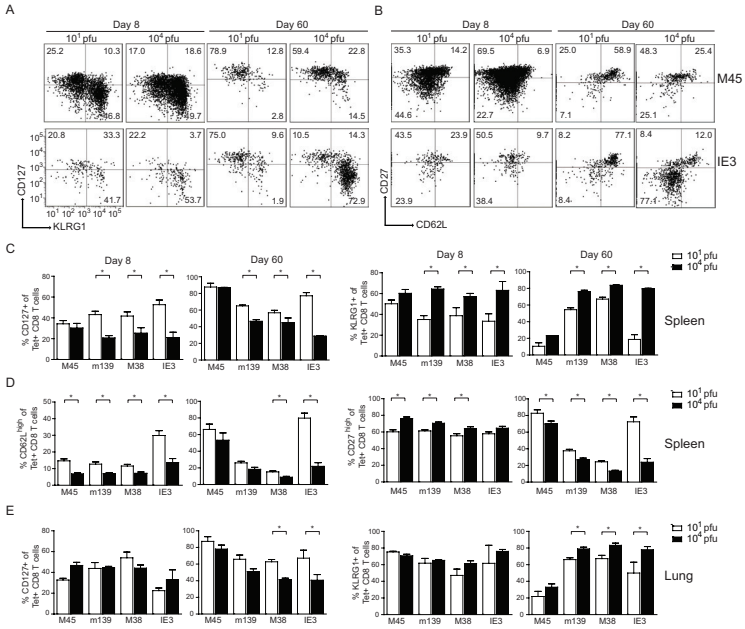

Figure 3. Viral inoculum size influences central and effector memory CD8$^+$ T cell formation. WT mice were infected with 10^1 or 10^4 PFU MCMV-Smith. (A) Representative flow cytometry plots show cell surface expression of CD127 and KLRG1 or (B) expression of CD27 and CD62L on splenic M45, m139, M38 and IE3 tetramer$^+$ CD8$^+$ T cells at day 8 and 60 after infection. (C and D) Bar graphs show the percentage of CD127, KLRG1, CD27 and CD62L expressing cells within the tetramer$^+$ CD8$^+$ T cell populations at day 8 and 60 post-infection in the spleen. (E) Bar graphs show the percentage of CD127 and KLRG1 expressing cells within the tetramer$^+$ CD8$^+$ T cell populations at day 8 and 60 post-infection in the lungs. Shown are mean values and SEM (n=4). Statistical significance is determined by the Mann-Whitney test and indicated with an asterisk (* $p < 0.05$). Shown are representative data of three independent experiments.

phenotype is related to the occurrence of inflation we examined the phenotype of MCMV-specific CD8$^+$ T cells after low and high dose viral inoculum. Regardless of the inoculum dose, the splenic M45-specific memory T cells cell surface markers associated with a central memory phenotype at day 60 post-infection (CD127$^+$KLRG1$^-$CD62L$^+$CD27^{high}) (Fig. 3A-D) while during acute infection (day 8), these cells display an effector-like phenotype (CD127$^-$KLRG1$^+$CD62L$^-$CD27^{low}). In contrast, the splenic m139- and M38-specific CD8$^+$ T cells display a reduced effector-like phenotype (CD127$^-$KLRG1$^+$CD62L$^-$CD27^{low}) in low dose infected animals during the acute and persistent phase of infection (Fig. 3A-D). Intriguingly, the CD8$^+$ T cells that react to the inflationary IE3 epitope have like the M45-specific CD8$^+$ T cells a predominant central memory phenotype (Fig. 3A-D). This shift in phenotype of the IE3-specific CD8$^+$ T cells in low dose infected mice is already clearly present in the acute phase of infection (day 8) and gradually becomes more pronounced. In the lungs, minimal differences in cell surface phenotype MCMV-specific CD8$^+$ T cells were observed between low and high dose infected during the acute phase of infection but at later time points the inflationary T cells were phenotypically less effector-like in low dose infected mice (Fig 3E).

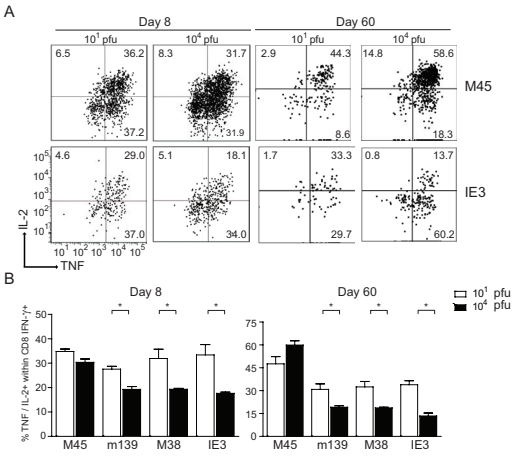

Figure 4. Inflationary MCMV-specific CD8$^+$ T cells in low dose infected hosts have increased IL-2 production. WT mice were infected with 10^1 or 10^4 PFU MCMV-Smith. (A) Representative flow cytometry plots show intracellular TNF and IL-2 production within the splenic IFN-γ^+ CD8$^+$ T cells after restimulation with M45 or IE3 class I peptides at day 8 and 60 post-infection. (B) Bar graphs show the percentages of TNF$^+$ IL-2$^+$ cells within splenic IFN-γ^+ CD8$^+$ T cell populations after restimulation with the indicated peptides at day 8 and 60 post-infection. Shown are mean values and SEM (n=4). Statistical significance is determined by the Mann-Whitney test and indicated with an asterisk (* $p < 0.05$). Shown are representative data of three independent experiments.

As observed with the analysis of the phenotypic cell-surface markers, the percentage of IL-2$^+$TNF$^+$ but not the IL-2$^-$TNF$^+$ expressing cells within the inflationary m139-, M38- and IE3-specific CD8$^+$ T cell pools was increased in low dose infected hosts, which also points to a more central memory phenotype (Fig. 4A and B). The IL-2 production by the non-inflationary M45-specific CD8$^+$ T cells was unaltered by varying the viral inoculum size. When intermediate inoculum sizes of 10^2 and 10^3 PFU were used to infect mice also intermediate phenotypes and degrees of memory inflation were observed (data not shown). Together, these results indicate that the phenotype of inflationary MCMV-specific CD8$^+$ T cells is influenced by the viral dose. In addition, the data instill that already early after infection phenotypic analysis of inflationary T cells can be used to predict the occurrence of inflation during the persistent phase of infection.

Organ distribution of IE3-specific CD8⁺ T cells correlates with the shift towards central memory phenotype in low dose infection

Central memory and effector memory T cells are characterized not only by their difference in effector function but also by their distinct homing capacity (36). While central memory T cells preferentially home to lymphoid organs, effector memory T cells have the capacity to migrate throughout the whole body. To address whether the skewing towards a more central memory-like phenotype in low dose infection resulted in alterations in tissue distribution of MCMV-specific CD8⁺ T cells, mice were infected with 10^1 or 10^4 PFU MCMV and absolute numbers of MCMV-specific CD8⁺ T cells were determined in lymphoid (inguinal lymph nodes (ILN), and bone marrow) and non-lymphoid tissues (lungs, and liver) at day 30 post-infection. In all these tissues the absolute numbers of MCMV-specific CD8⁺ T cells were as anticipated lesser in low dose infected mice (Fig. 5A and B). The organ distribution of M45-specific CD8⁺ T cells is not different between infection with 10^1 and 10^4 PFU MCMV, which is in accordance with the observation that M45-specific T cells display a central memory phenotype independently of the size of the viral inoculum. Despite the moderate differences in phenotype, the m139- and M38-specific CD8⁺ T cell populations exhibited minute differences in organ distribution when comparing the low and high inoculum sizes. During the course of low

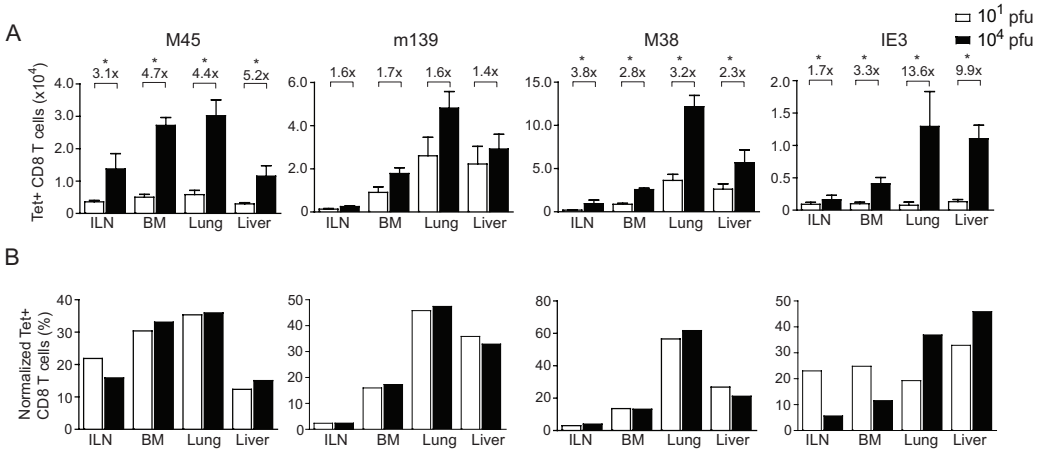

Figure 5. Organ distribution of IE3-specific CD8⁺ T cells correlates with a strong shift towards central memory phenotype in low dose infection. WT mice were infected with 10^1 or 10^4 PFU MCMV-Smith and at day 30 after infection the CD8⁺ T cell response to the indicated MCMV epitopes was examined in inguinal lymph nodes (ILN), bone marrow, lungs and liver. (A) Depicted are absolute numbers of M45-, m139-, M38- and IE3-specific CD8⁺ T cells as measured by MHC class I tetramer binding. (B) For each tetramer separately, the absolute numbers of tetramer⁺ CD8⁺ T cells per tissue were normalized to the total count of the tetramer⁺ CD8⁺ T cells in ILN, bone marrow, lungs and liver. Bar graphs show mean and SEM (n=4 per experiment). Statistical significance is determined by the Mann-Whitney test and indicated with an asterisk (* $p < 0.05$). Shown are pooled data of two independent experiments.

dose infection the IE3-specific CD8$^+$ T cells preferentially migrated to the ILN and bone marrow but homed lesser to the lungs and liver compared to high dose inoculum (Fig. 5A and B). Together, these data infer that the migratory properties of IE3-specific CD8$^+$ T cells are influenced by the initial viral dose, which is consistent with the strong shift of these cells towards a central memory phenotype.

Improved secondary expansion capacity of inflationary memory CD8$^+$ T cells elicited during low dose infection

Immunological memory is the ability of the immune system to respond faster and more effective upon secondary challenge with the same antigen and constitutes the basis for vaccination. To examine the effects of the inoculum size on protective immunity and secondary responses, mice were infected with 10^1 or 10^4 PFU MCMV and after 60 days the same mice were re-challenged using 10^5 PFU MCMV (Fig. 6A). The viral loads in the low and high dose infected mice were comparable after re-challenge and significantly lower as compared to mice that were not exposed to MCMV prior to the challenge, indicating that low dose inoculation protects as well as high dose (Fig. 6B). The secondary expansion potential of the inflationary m139-, M38- and IE3-specific but not of the non-inflationary M45-specific memory CD8$^+$ T cells was improved after low dose infection compared to high dose infection (Fig. 6C). However, certain conditions such as T cell competition and differences in innate and humoral immunity between low and high dose challenged mice could influence the T cell expansion rate. To create conditions in which the secondary expansion capacity of the MCMV specific CD8$^+$ T cells could be examined without such confounding factors, we adoptively transferred congenically marked (Thy1.1) non-inflationary (M45-specific) or inflationary (M38 and IE3-specific) memory CD8$^+$ T cells from low and high dose infected mice into naive (Thy1.2) recipients and subsequently challenged these recipients with MCMV (Fig. 6D). No difference was observed between the secondary expansion capacity of M45-specific CD8$^+$ T cells that originated from mice infected with either low or high dose PFU whereas M38 and IE3-specific T cells of low dose infected mice expanded better compared to M38 and IE3-specific CD8$^+$ T cells from high dose infected mice (Fig. 6E). Our observation that M45-specific T cells respond better than inflationary cells after adoptive transfer in naive mice, while in re-challenged mice that contain high numbers of inflationary T cells the expansion of M45-specific T cells is relatively reduced as compared to the inflationary T cells might be explained by competition among MCMV- specific T cell pools as has been described recently (37, 38).

As central memory T cells are known to have a better expansion potential as compared to effector memory T cells (39, 40), our findings are consistent with the raised central memory phenotype of the inflationary T cells in low dose infected mice. To determine whether there are differences among inflationary T cells with a central memory-like phenotype elicited either in a low or high dose infection, we sorted congenically marked

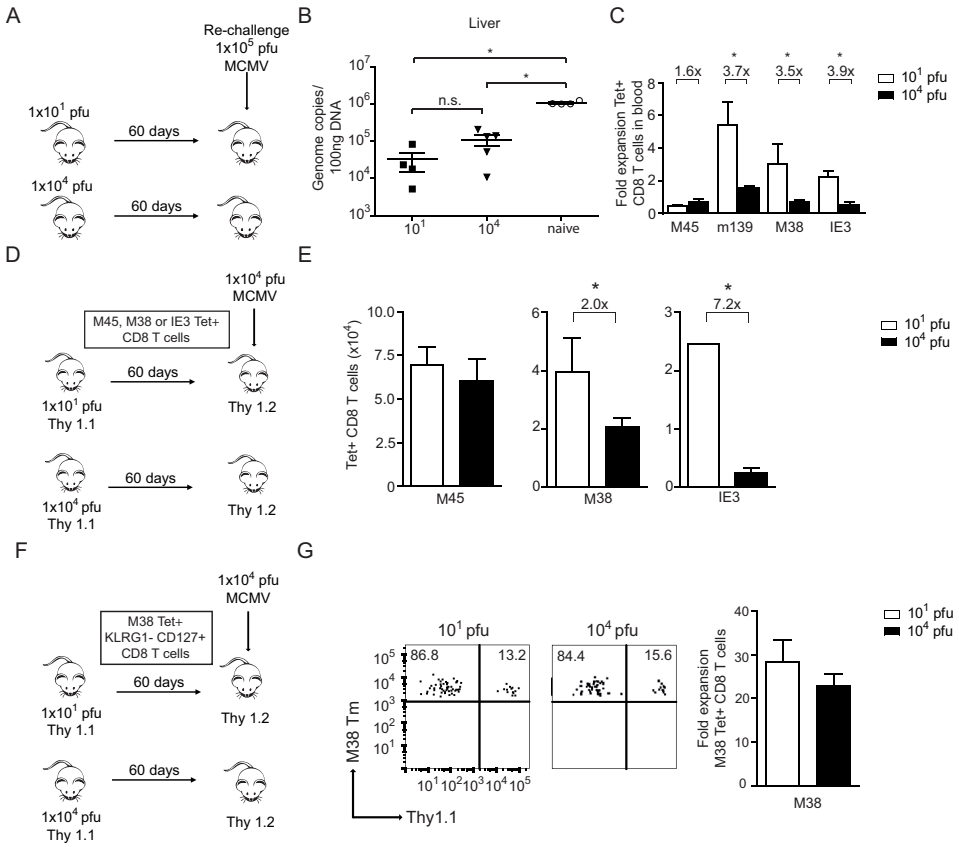

Figure 6. Improved secondary expansion capacity of MCMV-specific CD8⁺ T cells induced by low viral dose. (A) Schematic of the experimental setup. WT mice were infected with 10^1 or 10^4 PFU MCMV-Smith and 60 days after infection the same mice were re-challenged with 10^5 PFU MCMV-Smith. At the same time naive mice also received a challenge with 10^5 PFU MCMV-Smith. (B) Graphs indicate the number of MCMV copies per 100 ng liver derived DNA at day 5 post re-challenge. Each symbol represents an individual mouse. (C) One day before and 5 days after re-challenge the CD8⁺ T cell response to the indicated MCMV epitopes was determined by MHC class I tetramer staining in blood. Bar graph shows fold expansion of the MCMV-specific CD8⁺ T cell response after re-challenge. (D) Schematic of the experimental setup. Thy1.1 mice were infected with 10^1 or with 10^4 PFU MCMV-Smith. After 60 days M45, M38 and IE3 tetramer⁺ CD8⁺ T cells were sorted and transferred into naive Thy1.2 recipients, which were subsequently infected with 10^4 PFU MCMV-Smith. (E) Bar graphs show the absolute number of MCMV-specific CD8⁺ T cells in the spleen at day 5 post-infection. (F) Schematic of the experimental setup. Thy1.1 mice were infected with 10^1 or 10^4 PFU MCMV-Smith. After 60 days CD127⁺KLRG1⁻ M38-specific CD8⁺ T cells were sorted and transferred into naive Thy1.2 recipients, which were subsequently infected with 10^4 PFU MCMV-Smith. (G) Representative flow cytometry plots show cell surface expression of Thy1.1 (CD90.1) on splenic M38 tetramer⁺ CD8⁺ T cells at day 5 post-infection. Bar graph shows fold expansion of the MCMV-specific central memory CD8⁺ T cells in the spleen. Bar graph data show mean values and SEM (n=4). Statistical significance is determined by the Mann-Whitney test and indicated with an asterisk (* $p < 0.05$). Shown are representative data of two independent experiments.

(Thy1.1) central memory-like (CD127⁺KLRG1⁻) M38-specific T cells from low and high dose infected mice and adoptively transferred equal numbers of these cells in naive mice (Thy1.2 cells). After challenge, we observed a similar expansion capacity indicating that no intrinsic difference among central memory-like inflationary T cells occurs after a low or high dose inoculum. Thus, low dose viral inoculum elicits inflationary memory CD8⁺

T cells with an improved secondary expansion capacity on a population level consistent with a central memory phenotype.

DISCUSSION

In this study we show that the degree of memory T cell inflation is directly influenced by the size of the viral inoculum. This report also highlights the impact of the inoculum dose on the ratio of effector memory versus central memory phenotype within an antigen-specific T cell population; a low viral inoculum size skews the "inflationary" $CD8^+$ T cells, or to be more specific the $CD8^+$ T cells with the ability to undergo memory inflation, towards a central memory phenotype ($CD127^+$, $KLRG1^-$, $CD27^{high}$, $CD62L^+$, $IL\text{-}2^+$), whereas in high dose infection these T cells are more effector memory like ($CD127^-$, $KLRG1^+$, $CD27^{low}$, $CD62L^-$, $IL\text{-}2^{+/-}$). These data provide reasoning for the dissimilarity in terms of magnitude and phenotype of CMV-specific T cell responses as observed in human CMV-infected individuals (25-29).

Inflationary T cell responses are elicited by both immediate early (IE) (e.g. IE3 in C57BL/6 mice and IE1 in BALB/c mice) and early (E) antigens (e.g. m139 and M38 in C57BL/6 mice and m164 in BALB/c mice), albeit with different kinetics and magnitude (12, 41). We observed that the memory phenotype of the IE3-specific T cells was mostly affected by the viral inoculum size as compared to the m139- and M38-specific T cells. Nevertheless, strongly impaired but still detectable memory T cell inflation was detected against IE3 whereas no inflation was observed against m139 and M38 in low dose infected mice. These apparent differences between T cell responses against IE and E antigens point to different mechanisms how these antigens can provoke memory T cell inflation. Reddehase and colleagues have shown that transcriptional reactivation occurs for IE antigens in the lungs and liver (7, 42), and may drive memory inflation (42). Such transcriptional reactivation in non-lymphoid tissues is not found or at least to a lesser extend for E antigens, which could be related to a lower incidence or weaker promoter activity (43). Recent data by Oxenius and colleagues indicated that for memory inflation of M38-specific T cells, the lymphoid organs are instead important (10). Whether transcriptional reactivation is important to inflate memory T cells in the lymphoid organs is undetermined. Besides potential differences in antigenic triggering of IE and E antigen-specific T cells, these cells may also differentially depend on (inflammatory) cytokines and costimulation. If indeed different mechanisms exist for memory T cell inflation specific for the M38 (and m139) versus IE3 antigens, it may explain some of our findings. Such differences might surface more prominently when limited antigen or alternatively less inflammatory/costimulatory signals are available to sustain MCMV-specific T cell populations. It is thought that the generation and sustainment of high levels of effector-memory T cells induced by CMV-based vaccine vectors is important for protective immunity

against the targeted pathogens (22-24). Based on our results, this suggests that high dose inoculums are important for the success of such CMV-based vaccines. Conversely, in case of protection against CMV itself it seems that low dose infection is equally capable to induce protective immunity as compared to high dose infection. Nevertheless, neither low nor high dose inoculum is able to induce sterile immunity but whether sterile immunity against CMV is possible at all given the numerous immune evasion mechanisms of CMV (44, 45) remains an open question. Thus far it was found that the immune components that are involved in protective immunity against CMV re-infection/activation point to a role of antibodies and $CD8^+$ T cells (7, 46-51), but many particulars still need to be addressed. In this respect, it is also interesting to note that during primary acute infection NK cells and $CD4^+$ T cells but not antibodies and $CD8^+$ T cells are critically involved in controlling viral replication.

Related to the subject of protective immunity and vaccination is conceivably the importance of the balance between effector and central memory T cells. Depending on the features of the pathogen, either central memory or effector memory T cells constitute superior protection compared to the other (39, 40). The apparent influence of the viral inoculum size on the balance between central memory and effector memory $CD8^+$ T cells within the "inflationary" $CD8^+$ T cell pools upon MCMV infection might be exploited for future vaccine development.

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CHAPTER 6

Viral persistence induces antibody inflation without altering antibody avidity

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ABSTRACT

Antibodies are implicated in long-term immunity against numerous pathogens and because of this property antibody induction is the basis for many vaccines. Little is known on the influence of viral persistence on the evolving antibody response. Here we examined the characteristics of antibody responses to persistent infection employing the prototypic betaherpesvirus family member cytomegalovirus (CMV) in experimental mouse models. During the course of infection, mouse CMV (MCMV)-specific IgM and IgG responses are elicited but only IgG levels gradually inflate in the persistent phase of infection while IgM levels are stably maintained. Whereas CD27/CD70 interactions are dispensable, the CD28/B7 costimulatory pathway is critical for the class switching of MCMV-specific IgM to IgG B cell responses, which corresponds to the CD28/B7-dependent formation of CD4+ T follicular helper cells (TFH) and germinal center (GC) B cells. Furthermore, the initial viral inoculum dose dictates the height of the antibody levels during IgG antibody inflation, and relates to induction of long-lived plasma cells and memory B cells. Antibody avidity is nonetheless not altered after establishment of viral persistence and occurs independent of the inoculum dosages. However, repetitive challenge with intact viral particles, accompanied by increased GC reactivity, promotes the development of high avidity IgG responses with neutralizing capacity. These insights can be used for the rational design of CMV-based vaccines aimed at inducing antibody responses.

INTRODUCTION

The maintenance of long-lived humoral responses after infection and vaccination is attributed to both long-lived plasma cells that continuously produce antibodies and to memory B cells that are able to form antibody-secreting cells after re-exposure (1, 2). Antibodies can protect against numerous pathogens by direct neutralization and/or by supporting effector functions of immune cells (1, 3). Upon activation, B cells initially excrete antigen-specific IgM antibodies. This is followed by antibody isotype switching and affinity maturation when B cells receive the appropriate signals, including help signals by CD4+ T cells in germinal center (GC) reactions (4). During acute viral infections antibody levels increase followed by gradual decline once the antigen has disappeared. In case of appropriate induction of B cells leading to the generation of long-lived plasma cells, antibody levels eventually become stable and can mediate protection for many years.

Whereas memory B cells have self-renewal capacity in an antigen-dependent manner, long-lived plasma cells are thought to survive for decades (2). In case of antigen persistence, as is the case in chronic infections, one could argue that antigenic boosting effects humoral immunity. How this impacts the kinetics of antibody levels and antibody avidity maturation is, however, largely unknown. Recently, vaccines based on persistent viruses such as cytomegalovirus have shown their value by inducing either long-lasting effector-memory T cell responses (5-7) or protective antibodies (8, 9) but many particulars of such vaccines remain to be determined.

To gain more insight into the determinants of antibody responses that develop during persistent virus infection or after challenge with vaccines based on persistent viruses, we used mouse cytomegalovirus (MCMV), a prototypic member of the betaherpesvirus family. We found that similar to so-called inflationary MCMV-specific T cell responses, which gradually increase to high frequencies (10), MCMV-specific IgG antibody levels inflate in the persistent phase of infection. MCMV-specific IgM antibody levels, however, remain relative stable. Remarkably, this IgG antibody inflation is not accompanied by changes in antibody avidity after a single inoculum despite viral persistence. Instead, antibody avidity was amplified by repetitive challenge with virus, and correlated with elevated GC reactivity. Moreover, we show that operational GC reactions and T follicular helper cell (TFH) formation require the costimulatory CD28/B7 pathway while CD27/CD70 interactions are not critical.

MATERIALS AND METHODS

Mice and infection

C57BL/6 mice were purchased from Charles River. Cd70-/- (11), Cd80/86-/- (12), and Cd70/80/86-/- (13) mice all on a C57BL/6 background, were bred in-house. Mice between

8-12 weeks of age were infected intra-peritoneal (i.p.) with indicated doses of MCMV-Smith obtained from the American Type Culture Collection (Manassas, VA). Stocks were derived from salivary glands of infected BALB/c mice as described elsewhere (14). Viral load in mice was determined by quantitative PCR as described (15) and data is normalized to β-actin. All animals were maintained on specific pathogen free conditions at the animal facility in the Leiden University Medical Center (LUMC). All animal experiments were approved by the Animal Experiments Committee of LUMC (reference numbers: 10227, 12006, 13029) and performed according to the recommendations and guidelines set by LUMC and by the Dutch Experiments on Animals Act that serves the implementation of 'Guidelines on the protection of experimental animals' by the Council of Europe.

In vivo antibody usage

To deplete $CD4^+$ T cells, mice received 150 μg of CD4 depleting antibody (GK1.5) i.p. prior to infection. Depletion of $CD4^+$ T cells was maintained by administration of 100 μg GK1.5 antibody once a week. For blockade of costimulatory interactions during acute MCMV infection, mice received either 150 μg blocking CD70 antibody (clone FR70) or a combination of 200 μg blocking CD80 (B7.1) antibody (clone 16-10A1) and 200 μg blocking CD86 (B7.2) antibody (clone GL1) i.p. on day -1, 0 and 3 of MCMV infection.

Antibody detection by ELISA and antibody avidity assay

Blood of mice was collected retro-orbitally. Upon brief centrifugation, serum was collected and stored at -20°C until further use. MCMV-specific antibody levels were determined by ELISA as described (15) with minor alterations. In short, 96-well plates (Nunc Maxisorp) were coated overnight at 4°C with tissue culture-derived MCMV-Smith in bicarbonate buffer (pH 9.6). Plates were incubated for 1 hour at 37°C with blocking buffer (PBS containing 5% milk powder), followed by subsequent incubation with sera samples (diluted in PBS containing 1% milk powder) for 1 hour at 37°C. Plates were washed with PBS containing 0.05% Tween, after which HRP-conjugated IgM, IgG1 IgG2b, IgG2c, IgG3, IgA and IgE antibodies (diluted in PBS with 1% milk powder) were incubated for 1 hour at 37°C. To develop the plates, 50 μl of TMB (3,3',5,5'-tetramethylbenzidine) (Sigma Aldrich) was added to each well and incubated for 15 minutes at room temperature. To stop the reaction, 50 μl of stop solution (1M H2SO4) was added. Plates were measured within 5 minutes at 450 nm using a microplate reader (Model 680, Bio-Rad). To determine the avidity of the MCMV-specific antibodies, a serum dilution was used at which responses showed an OD Value of 1 at 450 nm. Plates were incubated with increasing concentrations of sodiumthiocyanate (NaSCN) for 15 minutes, followed by washing with PBS containing 0.05% Tween, and incubation with HRP-conjugated antibodies. The avidity of the MCMV-specific antibodies was determined by the ratio of the amount of antibodies bound after elution with different concentrations of NaSCN

relative to the amount of antigen bound in the absence of NaSCN (16).

Quantification of antibody secreting cells

Multiscreen-HA 96-well plates (Millipore) were coated overnight with MCMV in PBS at 4°C, subsequently washed with PBS and blocked for 1 hour with IMDM medium containing 8% FCS at 37°C. 2×10^5 splenocytes or 8×10^5 bone marrow cells of MCMV infected mice were added per well and incubated for 5 hours at 37°C. Plates were washed with PBS containing 0.05% Tween-20, followed by incubation overnight with HRP-conjugated IgG2c antibodies at 4°C. After subsequent washing, spots were visualized using TMB (Mabtech) and reaction was stopped with tap water. The total number of antibody secreting cells per organ was determined by dividing the absolute number of the organ by the amount of plated cells, and multiplying this number with the amount of spots that were counted per well. CMV-specific memory B cells were determined as described (17) with some modifications. In short, a concentration range from 1.8×10^5 to 2×10^4 splenocytes of MCMV infected mice were cultured in a flat bottom 96-well plate for 6 days in the presence of irradiated feeder splenocytes of naive mice (1200 rad), 0.4 μg/ml LPS and 1 μg/ml PHA. After 6 days of culture, cells were washed, transferred to ELISPOT plates and developed as described above.

Antibody neutralization assay

Different dilutions of serum of MCMV infected mice were incubated for 45 minutes with 50 PFU of MCMV-Smith at room temperature. The virus-serum inoculums were subsequently added to monolayers of M2-10B4 cells in 48-well plates. Cells were incubated for 1 hour at 37°C after which inoculums were removed and cells were covered in carboxymethyl cellulose-containing medium. After 5 days of incubation, cells were fixed with 25% formaldehyde and plaques were visualized using crystal violet solution.

Flow cytometry

Splenocytes were obtained by mincing the tissue through a 70 μm nylon cell strainer (BD). Erythrocytes were lysed in a hypotonic ammonium chloride buffer. The antigen-specific T cell response was determined by MHC class I tetramers and intracellular cytokine staining as described (18). T cell restimulation was performed with class I (i.e., $M45_{985–993}$, $m139_{419–426}$, $M38_{316–323}$ and $IE3_{416-423}$ (19)) and class II-restricted peptides ($M25_{409-423}$, $m139_{560-574}$ and $m142_{24-38}$ (20)). Fluorescently conjugated antibodies were purchased by Affymetrix, BD pharmingen or Biolegend. Flow cytometric acquisition was performed on a BD LSR II. Data were analyzed using FlowJo software (TreeStar).

Statistical significance

Mann-Whitney test was used to calculate the significance of viral titers. To evaluate significance between two groups the Student's t-test was used, and for more than two

groups one-way ANOVA was used. Tukey's post-hoc test was performed to correct for multiple comparisons. P-values <0.05 were considered as significant.

RESULTS

MCMV-specific IgG antibodies inflate during persistent infection without altered antibody avidity

We used MCMV as a model to determine antibody characteristics during a persistent virus infection and first examined if MCMV-specific antibodies provide protection upon re-infection. Following acute infection, IgG2b and IgG2c (the IgG2a equivalent in C57BL/6 mice) are the predominant isotypes that are produced (Fig 1A), which is not uncommon after viral infection (21, 22). Serum of naive mice and of latent MCMV-infected mice was transferred to naive mice (200 µl per recipient) that were subsequently challenged with MCMV. A ~10 fold diminished viral load was found in the liver and salivary glands of the mice that received serum containing MCMV-specific antibodies compared to the mice that received naive mouse serum (Fig 1A), indicating that MCMV-specific antibodies have protective capacity even in case of subordinate antibody titer (i.e., transfer of 200 µl of serum results in a much lower antibody titer in the recipient mouse than the donor). Consistent with the diminished viral load, also MCMV-specific

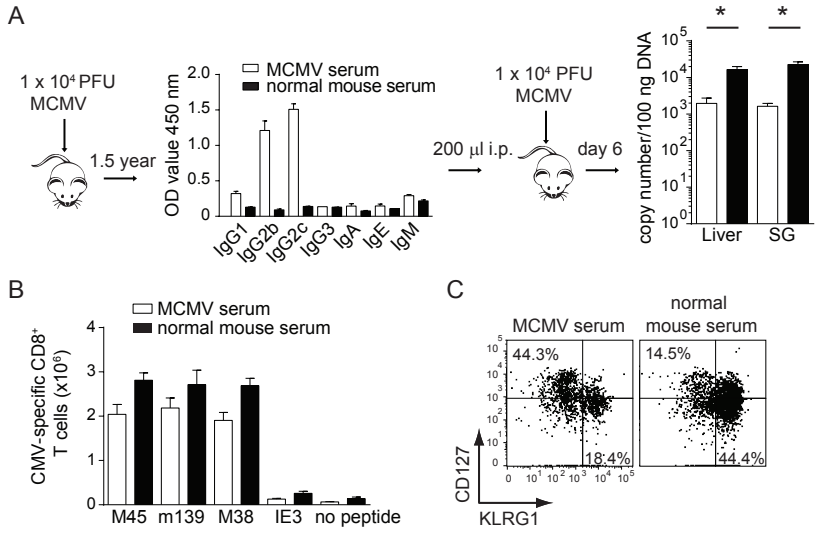

Fig 1. Protective properties of MCMV-specific antibodies. (A) C57BL/6 mice were infected with 1 × 10^4 PFU MCMV. After 1.5 years, immune serum was obtained and transferred to naive mice that were subsequently infected with 1 × 10^4 PFU MCMV. Serum of naive mice was used as control. Six days post serum transfer viral load was determined in the liver and the salivary glands by qPCR. (B) The magnitude of the MCMV-specific CD8+ T cell response was determined in the spleen by intracellular cytokine staining upon restimulation with the indicated MHC class I restricted peptides. (C) The cell surface expression of CD127 and KLRG1 on splenic M45 tetramer+ $CD8^+$ T cells (n=5 mice per group; *P<0.05).

T cell responses were reduced upon MCMV serum transfer and displayed a less activated phenotype as evidence by more CD127^{high} and KLRG1^{low} expression (15) (Fig 1B and 1C). Thus, although sterile immunity is not achieved under the conditions analysed, the antibodies that are generated during MCMV infection can reduce viral titers upon challenge.

Next, we determined the kinetics of MCMV-specific IgM antibodies and the predominant IgG2b and IgG2c isotypes. In the acute phase of infection, high levels of MCMV-specific IgM antibodies were detected in the serum. After acute infection, IgM levels declined but remained clearly detectable throughout the chronic phase of infection (Fig 2A), a phenomenon that is not observed after infection with viruses causing only acute infection (23). Another striking feature we observed was that MCMV-specific IgG2b and IgG2c antibody levels gradually accumulated in time (Fig 2A and 2B). This particular inflation of MCMV-specific IgG2b and IgG2c antibodies is reminiscent to certain inflationary MCMV-specific T cell responses that progressively accumulate in the chronic phase of infection (10).

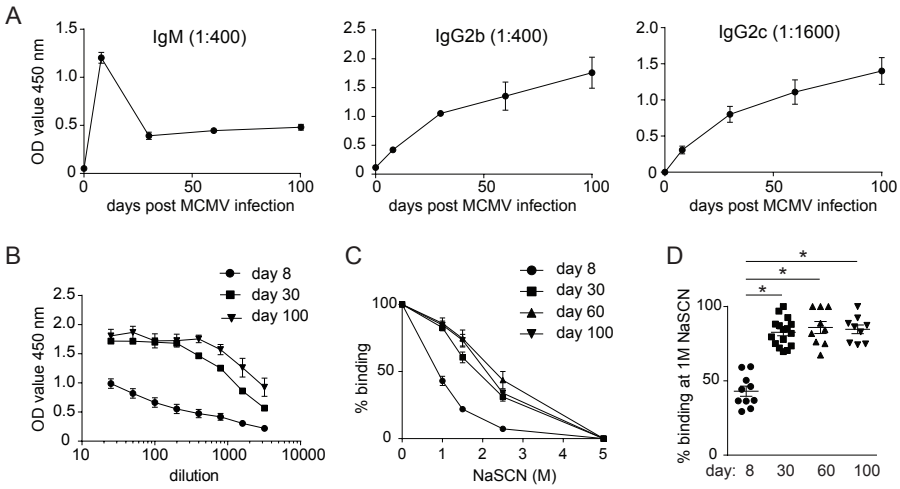

Fig 2. MCMV-specific IgG antibodies inflate during persistent infection while antibody avidity remains stable. (A) Mice were infected with 1 × 10^4 PFU MCMV. The MCMV-specific antibody levels are shown for the indicated serum dilution. (B) MCMV-specific IgG2c antibody titres in sera are depicted as mean ± SEM of day 8, 30 and 100 post-infection. (C) The elution profile of MCMV-specific IgG2c antibodies is shown as mean ± SEM. (D) The percentage of bound antibodies to MCMV in the presence of 1M NaSCN relative to the amount of antigen bound in the absence of NaSCN is shown. Data is pooled of 3 independent experiments (n=4 mice per group; *P<0.05).

To examine if the avidity of these inflating antibodies varies in time, an antibody avidity assay was performed with increasing concentrations of sodium thiocyanate (NaSCN) that accordingly disrupts the antigen-antibody bond. The avidity of MCMV-specific antibodies was relatively low at day 8 post-infection, as ~50% of the antibodies were eluted upon incubation with 1M of NaSCN (Fig 2C and 2D). However, 30 days post-infection

the avidity of MCMV-specific IgG2c antibodies was increased as only 20% of the total MCMV-specific IgG2c antibodies could be eluted with 1M of NaSCN. Throughout the ensuing persistent phase of MCMV infection, the avidity of the MCMV-specific antibodies did not further increase compared to the avidity detected at day 30 post-infection. Similar results were obtained with the IgG2b isotype (data not shown). Thus the levels of the MCMV-specific IgG2b and IgG2c antibodies inflate in chronic MCMV infection, but this is not accompanied by differences in antibody avidity during viral persistence.

MCMV-specific IgG antibody responses are dependent on CD28/B7-driven CD4+ T cell responses

To examine factors that influence antibody inflation and avidity during persistent viral infection, we first aimed to identify the signals that are critical for GC related processes such as isotype switching. In this respect, help signals provided by $CD4^+$ T cells are shown to be crucial for inducing class switching of antibodies. Upon depletion of $CD4^+$ T cells, the acute MCMV-specific IgM responses as well as the maintenance of the IgM levels were not much affected (Fig 3A). IgG2b and IgG2c responses were, however, severely hampered, also at late times post-infection. To identify the molecular interactions that provide the help signal we specifically focused on the role of costimulatory pathways involving the Ig superfamily member CD28 and its ligands B7.1 (CD80) and B7.2 (CD86) (hereafter referred as B7) and the TNFR family member CD27 and its ligand

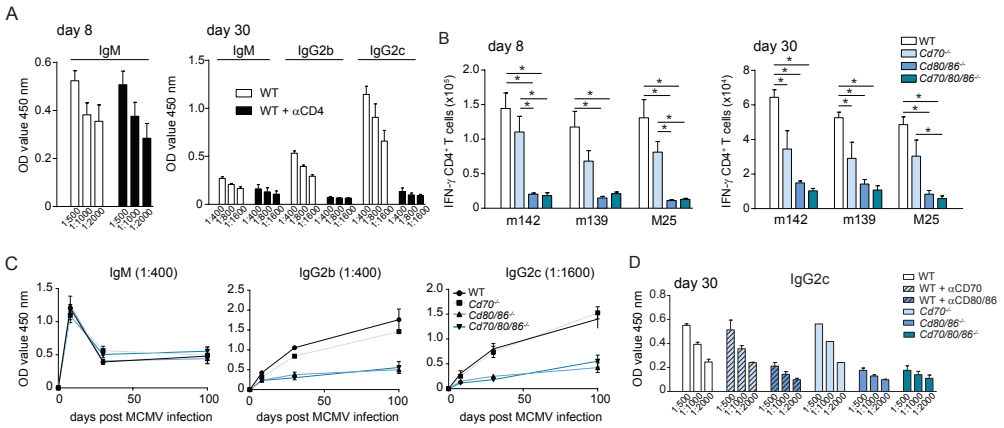

Fig 3. CD4 help and B7-mediated costimulation are critical for development of MCMV-specific IgG antibodies. (A) WT and $CD4^+$ T cell depleted mice were infected with 1×10^4 PFU MCMV and at day 8 and 30 post-infection the MCMV-specific antibody response was determined by ELISA. (B) WT and costimulation deficient (i.e. $Cd70^{-/-}$, $Cd80/86^{-/-}$, $Cd70/80/86^{-/-}$) mice were infected with 1×10^4 PFU MCMV and the MCMV-specific $CD4^+$ T cell response was determined in the spleen 8 and 30 days post-infection by intracellular cytokine staining upon restimulation with indicated MHC class II restricted peptides. (C) The MCMV-specific antibody response in WT and costimulation deficient mice in time is shown. (D) WT mice and costimulation deficient mice were infected with 5×10^4 PFU MCMV. CD70 and B7-mediated interactions were blocked in WT mice from day -1 to day 3 by administration of blocking antibodies. MCMV-specific IgG2c levels were determined in the serum 30 days post-infection. All bar graphs represent mean + SEM. Data is shown of one representative experiment of 3 independent experiments (n=4 mice per group; *P<0.05).

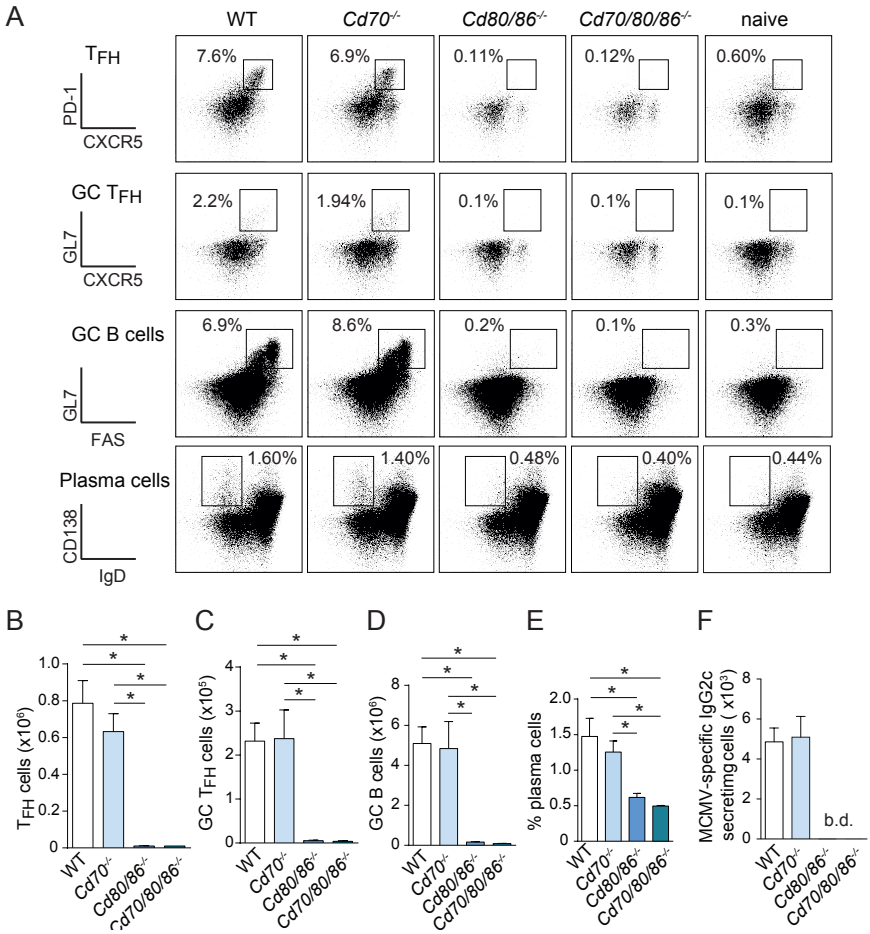

Fig 4. GC reactions in CMV infection are dependent on B7-mediated interactions. WT and costimulation deficient mice were infected with 1 × 10^4 PFU MCMV. (A) Representative flow cytometry plots of splenic cell populations at day 15 post MCMV infection. Depicted are TFH (PD-1$^+$CXCR5$^+$) and GC TFH (GL-7+CXCR5$^+$) gated on CD4$^+$CD62L$^-$ cells, and GC B cells (GL7$^+$FAS$^+$) and plasma cells (CD138$^+$IgD$^+$) gated on B220$^+$/CD19$^+$ cells. Numbers indicate percentages of positive cells within the gated population. (B-D) Total number of TFH, GC TFH, and GC B cells in the spleen at day 15 post-infection. (E) The percentage of plasma cells within the splenic B cell population at day 15 post-infection. (F) The number of MCMV-specific IgG2c secreting cells was determined in the spleen 15 days post infection using ELISPOT. All bar graphs represent mean + SEM. Data is shown of one representative experiment of 3 independent experiments (n=4 mice per group; *P<0.05).

CD70 because Cd80/86$^{-/-}$ and Cd70$^{-/-}$ mice have reduced MCMV-specific CD4$^+$ T cell responses (Fig 3B) (18, 24). No differences were found in the MCMV-specific IgM response during acute and persistent infection in all mice devoid of B7 and/or CD70-mediated costimulatory signals (Fig 3C), suggesting T cell costimulation is not required for the initiation and maintenance of viral-specific IgM antibody responses. In contrast, B7-mediated costimulation was crucial for the development of MCMV-specific IgG2b and IgG2c responses throughout the course of infection (Fig 3C). Despite the diminished MCMV-specific effector CD4$^+$ T cell response upon CD70 abrogation, comparable IgG2b

and IgG2c levels were found in WT and Cd70-/- mice. Mice deficient in both CD70 and the B7 molecules (Cd70/80/86$^{-/-}$) had a comparable defect in antibody inflation as Cd80/86$^{-/-}$ mice, indicating that the CD28/B7 pathway has a dominant effect on the development of antibody responses. This particular effect of CD28/B7 costimulation coincided with lower levels of MCMV-specific CD4+ T cells in Cd80/86$^{-/-}$ mice as compared to Cd70$^{-/-}$ mice (Fig 3B). Furthermore, MCMV-specific antibody isotype switching was not observed when mice were deprived of B7-mediated signals in the acute phase of infection, but did occur when CD70 signals were abrogated (Fig 3D). These data show that the B7-mediated signals are required at the beginning of infection.

Next, we aimed to explore the mechanisms underlying the dependence of IgG antibody responses on the CD28/B7 pathway. Most strikingly, and fully consistent with the phenotype was the virtual absence of the TFH subset (CXCR5^{+}PD1^{+}) and of the further differentiated GC-associated TFH cells (CXCR5^{+}GL7^{+}) in the Cd80/86$^{-/-}$ mice while Cd70$^{-/-}$ mice still had an induction of these cells (Fig 4A-C). Moreover, upon abrogation of B7 but not of CD70-mediated costimulation a huge reduction in GC B cells, identified by B220^{+}CD19^{+}CD95^{+}GL7^{+}, was observed at day 15 post-infection (Fig 4A and 4D). In line with this, diminished plasma cells characterized by B220^{+}CD19^{+}CD138^{+}IgD^{-} (Fig 4A and 4E), and no splenic MCMV-specific IgG2c-secreting cells were detected in Cd80/86$^{-/-}$ and Cd70/80/86$^{-/-}$ mice (Fig 4F). Together, these data indicate that the development of MCMV-specific IgG antibody responses is fully dependent on B7-mediated activation of CD4^{+} T cell helper subsets, and in particular of the TFH subset.

The initial viral inoculum dose affects antibody levels but not antibody inflation or avidity

Given that the initial viral inoculum dose influences memory T cell inflation (15), we determined if the viral dose impacts MCMV-specific antibody inflation as well. In mice infected with either a low (10^{1} PFU) or a high (10^{4} PFU) viral inoculum dose of MCMV, differences in antibody levels were determined. In low dose infected mice, IgM responses at day 8 were less pronounced compared to high dose infected mice but in the chronic phase of infection similar levels persevered (Fig 5A). MCMV-specific IgG2b and IgG2c levels were diminished upon a low dose infection at all times, but antibody inflation still occurred comparable to high dose infection (Fig 5A). The differences in antibody levels was reflected by distinct numbers of plasma cells (Fig 5B), the occurrence of IgG2c-secreting cells (Fig 5C) and increased numbers of splenic GC B cells (Fig 5D). Also in persistent CMV infection, more MCMV-specific IgG2c-secreting long-lived plasma cells were detected in the bone marrow upon a high dose infection (Fig 5E) but no IgG2c-secreting plasma cells were detected in the spleen in both low and high dose infected mice (data not shown). However, MCMV-specific IgG2c-secreting memory B cells persisted

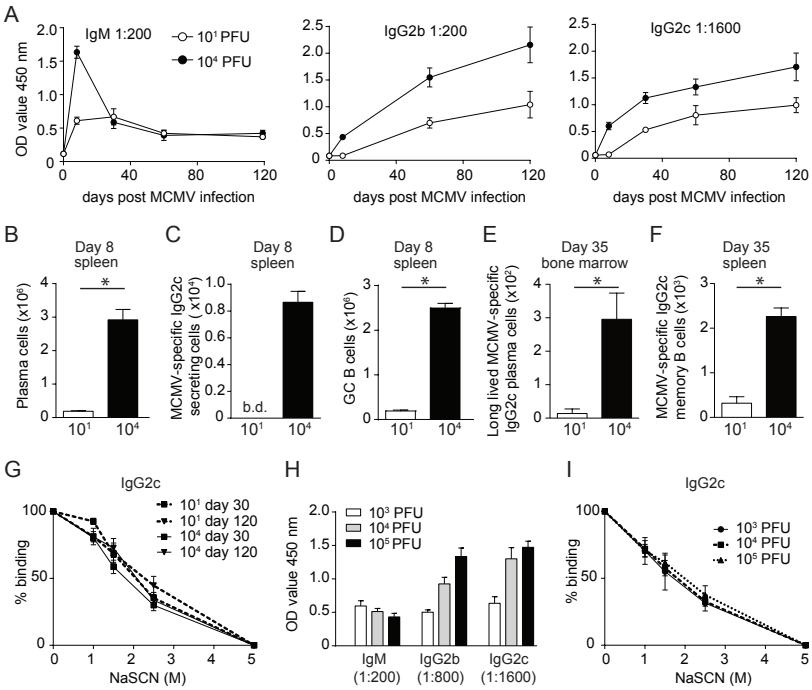

Fig 5. Impact of viral inoculum dose on antibody levels but not on antibody avidity. Mice were infected with either a low dose (10^1 PFU) or a high dose (10^4 PFU) of MCMV. (A) The levels of MCMV-specific antibodies within the serum. (B) The total number of plasma cells identified by $CD138^+/IgD^-$ in the spleen at day 8 post-infection. Cells are gated on $B220^+/CD19^+$ cells. (C) The amount of IgG2c-secreting cells in the spleen determined by ELISPOT at day 8 post-infection. (D) The number of GC B cells identified by $Fas^+/GL\text{-}7^+$ at day 8 post-infection. (E) The total number of IgG2c-secreting long-lived plasma cells determined in the bone marrow by ELISPOT at day 35 day post-infection. (F) The total number of MCMV-specific IgG2c memory B cells determined by ELISPOT at day 35 post-infection. (G) The elution profile of MCMV-specific IgG2c antibodies is shown as mean ± SEM. (H-I) Mice were infected either with 10^3 PFU, 10^4 PFU or 10^5 PFU MCMV and the MCMV-specific antibody levels were determined in the serum 1.5 years post-infection. (I) The elution profile of MCMV-specific IgG2c antibodies is shown as mean ± SEM. All bar graphs represent mean + SEM (n=4 mice per group; *P<0.05).

at higher levels in the spleen in high dose versus low dose infected mice (Fig 5F). Thus the initial viral inoculum dose impacts the number of long-lived plasma cells and memory B cells that are maintained during chronic infection, thereby impacting the amount of IgG antibodies that are present in the serum. IgG antibody inflation, however, occurs despite differences in the initial viral inoculum.

Although the viral dose had a major impact on the antibody levels, no differences were found in antibody avidity between low and high dose infected mice (Fig 5G). Moreover, when mice were infected with three different doses of MCMV, ranging from 10^3 to 10^5 PFU, the effect of the initial viral inoculum dose was again only reflected in the differences in the IgG2b and IgG2c antibody levels (Fig 5H), while no differences in the avidity of the MCMV-specific antibodies were detected (Fig 5I). Together, these data show that the initial viral inoculum dose impacts the antibody levels during antibody inflation but not antibody avidity.

Repetitive viral challenge stimulates the development of neutralizing CMV-specific antibodies and improves antibody avidity

To determine the development of MCMV binding antibodies that are able to neutralize the virus, in vitro neutralization assays were performed with serum of recently and latently infected mice. Neutralizing antibodies were below the detection limit in low and high dose infected mice during the first months post-infection (Fig 6A). Nevertheless, in high dose infected mice eventually neutralizing antibodies were detected. Next we examined if the level of neutralizing antibodies could be elevated by repetitive exposure to MCMV. We administrated a dose of 5 × 10^4 PFU MCMV every week in the same mice following the next year. The levels of the MCMV-specific antibodies were to some extent elevated in mice receiving MCMV repetitively (Fig 6B). Moreover, mice that were every week exposed to intact CMV particles, developed antibodies with a higher avidity (Fig 6C). Consistently, the levels of neutralizing antibodies was higher in the group of mice that received MCMV every week (Fig 6D). Notably, these effects coincided with higher amounts of GC B cells in the spleen (Fig 6E and 6F). These data show that repetitive challenge with intact virus as compared to a single inoculum promotes avidity maturation and development of neutralizing antibodies.

A

	10^1 PFU	10^4 PFU
day 8	<3	<3
day 30	<3	<3
day 60	<3	<3
day 120	<3	6

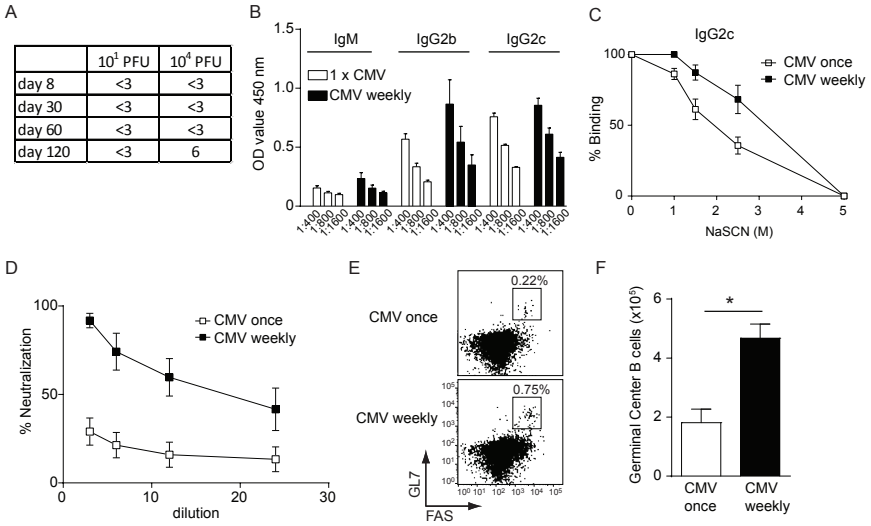

Fig 6. Repetitive antigen exposure increases the avidity and neutralization capacity of MCMV-specific antibodies. (A) Mice were infected with either a low dose (10^1 PFU) or a high dose (10^4 PFU) MCMV. At indicated times the neutralization capacity of the MCMV-specific antibodies was determined. Table indicates the neutralizing antibody titre expressed as the reciprocal of the serum dilution at which 50% of the total PFU was impaired. (B-F) Mice were infected with either a single dose of 5 × 10^4 PFU MCMV or received for one year a weekly dose of 5 × 10^4 PFU MCMV. (B) The levels of MCMV-specific antibodies in the serum at 1 year post-infection. (C) The elution profile of MCMV-specific IgG2c antibodies in the presence of NaSCN is shown as mean ± SEM. (D) The percentage of neutralization for each serum dilution is shown as mean ± SEM. (E) Representative plots show cell surface expression of GL7 and FAS gated on splenic $B220^+/CD19^+$ cells. Numbers indicate the percentage of GC B cells within the total B cell gate. (F) Total amount of GC B cells in the spleen. All bar graphs represent mean + SEM (n=7 mice per group; *P<0.05).

DISCUSSION

Memory T cell inflation is found in response to certain viral infections, most strikingly after persistent cytomegalovirus infection, and is characterized by the accumulation and maintenance of functional effector-memory $CD8^+$ T cells (10). Here we found that viral-specific IgG2b and IgG2c antibodies also inflate during persistent MCMV infection, and that the degree of antibody inflation relates to the dose of the initial viral inoculum. In other persistent infections such as chronic LCMV (25, 26) or HSV infection (27), virus-specific antibodies also accumulate in time, albeit in varying degrees. The serum levels of these virus-specific IgG antibodies however, eventually decline. Longitudinal follow-up of HCMV infected individuals indicates that HCMV-specific antibodies with different specificities expand over time (28, 29), suggesting similar mechanisms underlying antibody inflation. Gradual antibody accumulation is likely related to viral persistence, as it is not observed in various acute infections with influenza virus (30), VSV (31) and LCMV Armstrong (32).

Consistent with other studies in mice and humans (33-39), we found that immune sera of mice latently infected with CMV provide protection from a new CMV infection. It should be noted that we transferred 200 μl of serum from donor to recipient mice, which is actually an underestimation of the protective capacity. Whether these protective effects are mediated via neutralizing antibodies that limit cell-to-cell spread (39) and viral dissemination (35), or via non-neutralizing MCMV-binding antibodies is unclear. Neutralizing CMV-specific antibodies were detectable rather late in infection and the titers of these neutralizing antibodies were low, which is consistent with other low cytopathic viruses (40). Repetitive administration of viral particles, however, did improve the neutralization capacity of MCMV-specific antibodies.

CMV-specific memory B cells that are adoptively transferred have also protective capacity (36), indicating that antibodies and memory B cells together form the humoral immune response. We show that MCMV infection elicits both MCMV-specific long-lived plasma cells and memory B cells, and both are found in greater numbers after high dose inoculum compared to low dose. Consequently, IgG levels are as well higher after high dose infection, yet IgG inflation seems to occur alike after high and low dose infection. Furthermore, IgM levels are equally stably maintained after low and high dose infection. Long-term IgM maintenance is usually not observed after acute infection (23) but has been recently reported for responses to chronic bacteria and bacteria-associated polysaccharides (41-43). Whether the observed IgG inflation and IgM maintenance is directly connected to a gradual increment of the antibody-producing population driven by the persistence of antigen and/or to the induction of long-lived antigen-independent antibody producing cells remains to be explored.

Avidity maturation of CMV-specific antibodies occurred within a month after the initial viral inoculum and remained stable afterwards. This phenomenon is actually used as a diagnostic tool to identify a recent infection with human cytomegalovirus (HCMV) (44). Remarkably, we observed that avidity maturation during the persistent phase of infection can still increase but only by re-exposure to intact viral particles, which was accompanied by increased GC activity. In this respect, it is of interest to note that a study in humans with virosomal vaccines containing functional viral envelope glycoproteins demonstrated that the avidity of antibodies can be improved upon booster vaccination, suggesting that multiple encounters with intact rather than replicating virus is sufficient for avidity maturation (45).

If indeed avidity maturation during viral persistence is driven by newly induced GC reactions requiring intact viral particles one could argue that establishment of viral persistence after a single inoculum does not lead to sufficient viral particles to sustain GC reactions while re-exposure does. Such re-exposure however does not lead to increment of IgM levels, as occurs with primary infection, which may be related to pre-existing immunity preventing activation of IgM producing plasmablasts. In mice, replicating virus at late time points post-infection can however be observed in salivary glands but whether the viral production at this site contributes to the observed antibody inflation remains to be examined. Nevertheless, it has been observed that following intraglandular MCMV infection the salivary gland can operate as a mucosal inductive site for isotype switched IgG+ B cells (46). Moreover, studies by the Reddehase laboratory showed that in other tissues such as lungs, viral replication is abortive as only expression of (immediate) early genes are observed that nonetheless can lead to stimulating inflationary T cells (47).
MCMV-specific IgG2b and IgG2c levels were strongly decreased upon abrogation of B7-mediated costimulation but were intact in the absence of CD70-driven costimulation. Upon influenza virus infection similar viral-specific IgG levels are also found between WT and CD27-/- mice infection but a compensatory role for CD27 signalling is found in the absence of CD28 costimulation (30). We did not observe such a compensatory role but this may relate to a stronger dependence of the induced IgG responses on CD28/B7 costimulation in the MCMV model. In contrast to positive or neutral effects, in both acute and chronic LCMV infection, CD70 interactions have been described to have a negative effect on B cell responses (48, 49). As opposed to the low-level persistence of MCMV, LCMV persistence is accompanied with high-level replication and induction of profound expression of costimulatory molecules (13). Apparently, such high levels could lead to adverse effects of CD27/CD70 signalling on B cell responses and resembles findings in CD70 transgenic mice constitutively expressing CD70, in which deleterious effects on B cells occurs (50). In agreement with numerous other studies (51-53), a prominent role of CD28/B7 interactions for inducing proper B cell responses is found. We found that B7-mediated signals are clearly implicated in the induction of TFH but direct

effects on B cell responses might also be of importance. For example, via enhancing the survival of $CD28^+$ bone marrow-resident plasma cells (54).

CMV-based vectors have shown promising results in diverse infectious and cancer models, but the success of these vaccines is considered as T cell mediated (5, 55, 56). Recently, it has been shown that CMV vectors encoding additional antigens can also induce protective anti-melanoma or tetanus toxins antibodies (8, 9). Thus CMV-based vectors are also promising to apply in settings were antibodies can mediate protection. The results described here could help further support the use of CMV-based vaccines and may help to understand how our immune system coops with this persistent virus.

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CHAPTER 7

The contribution of cytomegalovirus infection to immune senescence is set by the infectious dose

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Submitted

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ABSTRACT

The relationship between human cytomegalovirus (HCMV) infections and accelerated immune senescence is controversial. Whereas some studies reported a CMV-associated impaired capacity to control heterologous infections at old age other studies could not confirm this. We hypothesized that these discrepancies might relate to the variability in the infectious dose of CMV occurring in real life. Here, we investigated the influence of persistent CMV infection on immune perturbations and specifically addressed the role of the infectious dose on the contribution of CMV to accelerated immune senescence. We show in experimental mouse models that the degree of mouse CMV (MCMV)-specific memory $CD8^+$ T cell accumulation and the phenotypic T cell profile are directly influenced by the infectious dose, and data on HCMV-specific T cells indicate a similar connection. Detailed cluster analysis of the memory $CD8^+$ T cell development showed that high dose infection causes a differentiation pathway that progresses faster throughout the life-span of the host, suggesting a virus-host balance that is influenced by aging and infectious dose. Importantly, short-term MCMV infection in adult mice is not disadvantageous for heterologous superinfection with lymphocytic choriomeningitis virus (LCMV). However, following long-term CMV infection the strength of the $CD8^+$ T cell immunity to LCMV superinfection was affected by the initial CMV infectious dose, wherein a high infectious dose was found to be a prerequisite for impaired heterologous immunity. Thus, stratification based on the size and differentiation of the CMV-specific memory T cell pools is of importance to the impact on immune senescence.

INTRODUCTION

Age-related decline in immunological competence, often referred to as immune senescence, is associated with an increased incidence of cancer, infections and a reduced efficacy of vaccines (1). Factors thought to contribute to this progressive decline of immune responses include genetic, molecular and cellular defects, but also environmental stressors such as chronic infections.

HCMV, a member of the betaherpesvirus family, persists in the majority of the adult population (2). Compared to other infections, extraordinary high memory T cell frequencies, on average ~10% of the total memory T cell compartment, are noticed in peripheral blood of healthy individuals (3). Especially at old age it has been observed that the HCMV-specific memory T cell pool can even occupy up to 50% of the total memory compartment (4). This phenomenon of high frequencies of circulating memory T cells that are maintained during the lifespan of the host or even can undergo gradual increment, aptly named memory inflation (5), is characteristic of CMV infection in general, as this is also found upon infection with the related mouse CMV (MCMV) and Rhesus Macaque CMV (RhCMV) (reviewed in (6, 7)).

Epidemiological studies have shown an association between HCMV positivity and manifestations of immune senescence by linking persistent HCMV infection to decreased overall survival in the elderly and to the immune risk profile (IRP), a cluster of parameters predictive for 2-year mortality in the elderly (8, 9). In addition, anti-HCMV IgG titers in aged individuals have been correlated with lower antibody responses to influenza (10-13), however other studies could not confirm this finding (14-17). Thus, whether HCMV infection is causally linked with accelerated immune senescence is a topic of debate (18, 19).

To gain more insight into the possible connection between CMV infection and immune senescence, mouse studies have been performed previously in which it was demonstrated that long-term CMV infection impairs newly generated CD8+ T cell responses to heterologous infections (20-22). However, these studies have been conducted using relatively high doses of virus, and have not taken into account the variability in real life with respect to the infectious dose. For example, in the human population the percentages of CMV-specific T cells occupying the memory T cell compartment is highly variable (ranging from 0,01-50%) (3), which is likely related to the significant difference in the quantity of CMV found in bodily fluids such as breast milk, saliva and urine (101 - 105 copies/µl), causing horizontal transmission of CMV (23, 24). In this respect, we have previously shown that the degree of accumulation and phenotype of inflationary CMV-specific CD8+ T cells is corresponding to the size of the initial infectious dose (25). We anticipate that the viral inoculum size is highly variable between individuals and that this accounts for the large variance in the frequency, phenotype and accumulation of CMV-specific CD8+ memory T cells in infected humans, which may be an explanation

for the controversial results with respect to the possible contribution of CMV to immune senescence. Here we tested this hypothesis in a highly controlled prospective study using the mouse model of CMV infection, which mimics CMV infection in humans (26). We inoculated mice with different inoculum dosages of MCMV and investigated longitudinally the influence of life-long CMV infection on alterations within the peripheral T cell pool. To specifically assess the role of the CMV inoculum size on the development of heterologous anti-viral immunity, aged MCMV-infected mice received a challenge with lymphocytic choriomeningitis virus (LCMV). Our results show that the viral inoculum size determines the degree of CMV-induced immune alterations in life-long infection. Furthermore, we demonstrate that only infection caused by a high MCMV dose reduces newly generated CD8+ T cell responses to heterologous superinfection. To our knowledge, this is the first evidence that the inoculum size of CMV is a crucial determinant for the development of CMV-associated impaired immunity in ageing.

MATERIALS AND METHODS

Ethics Statement

The inclusion of patients has been conducted in accordance with the ethical principles set out in the Declaration of Helsinki. Both patient inclusion and blood sample collection were done with approval of the Amsterdam Medical Center Medical Ethical Committee. Written informed consent was obtained prior to data collection.

All animal experiments were approved by the Animal Experiments Committee of the Leiden University Medical Center (LUMC) and performed according to the Dutch Experiments on Animals Act that serves the implementation of 'Guidelines on the protection of experimental animals' by the Council of Europe and the guide to animal experimentation set by the LUMC.

Mice

Wild-type (WT) C57BL/6 mice were purchased from Charles River. All mice were maintained under specific pathogen free (SPF) conditions at the Central Animal Facility of Leiden University Medical Center (LUMC). Mice were housed under 12 h light/dark cycle, feed ad libitum and were 7-10 weeks old at the beginning of each experiment.

Viruses

MCMV-Smith was obtained from the American Type Culture Collection (ATCC VR-194; Manassas, VA) and salivary gland stocks were prepared from infected BALB/c mice. Mice matched for gender and age were infected i.p. with indicated dosages of salivary gland derived MCMV-Smith. For weekly infections with MCMV mice received 5 × 104 PFU MCMV weekly for 1 year. Vaccinia virus expressing IE1 of MCMV (VACV-

IE1) was produced as described elsewhere (27). LCMV Armstrong was propagated on BHK cells and titers of virus stocks and organ homogenates were determined by plaque assays on Vero cells as described. For LCMV Armstrong infection, mice were infected i.p. with 2 × 105 PFU. LCMV titers in the lungs and kidneys were determined by a virus focus forming assay on Vero 76 cells as described elsewhere (28).

Study subjects

For phenotypical analysis of HCMV-specific T cell responses, PBMCs from HCMV-seropositive healthy donors and from initially HCMV-seronegative recipients (HLA-A*0101$^+$, HLA-A*0201$^+$, HLA-B*0702$^+$, HLA-B*3501$^+$) receiving a HCMV-positive kidney transplant were isolated and labeled for flow cytometry analysis (29). Quantitative PCR for HCMV was performed in EDTA-treated whole-blood samples, as described elsewhere (30).

Flow cytometry

MHC class I tetramer staining combined with phenotyping, and intracellular cytokine staining were performed to determine the magnitude and characteristics of the mouse viral-specific T cell responses as described (31). Single cell suspensions were prepared from spleens obtained from uninfected and infected mice by mincing the tissue through a 70 μm cell strainer (BD Bioscience). Blood was collected from the tail vein. Erythrocytes were lysed in a hypotonic ammonium chloride buffer. Fluorochrome-conjugated antibodies specific for mouse CD3, CD4, CD8, CD27, CD44, CD62L, CD127 (IL-7Rα), IFN-γ, IL-2, KLRG1, and TNF were purchased from BD Biosciences, Biolegend or eBioscience. Analysis of human PBMCs was performed as described (29). Fluorochrome-conjugated antibodies specific for human CCR7, CD3, CD8, CD27, CD28, CD45RA, CD57, CD127 and KLRG1 were purchased from BD Biosciences, Biolegend or eBioscience. Cells were acquired using a BD LSR Fortessa flow cytometer, and data was analyzed using FlowJo software (TreeStar) and Cytosplore (32). Dead cells were excluded using live/dead markers. Gating strategies were performed as described (25, 29).

MHC class I tetramers and synthetic peptides

The following class I-restricted peptides were used: $M45_{985-993}$, $m139_{419-426}$, $M38_{316-323}$, $IE3_{416-423}$, $IE1_{168-176}$ (MCMV), $GP33_{33-41}$, $NP_{396-404}$, $GP_{276-286}$ (LCMV). A pool of the following class II-restricted MCMV peptides were used: $M09_{133-147}$, $M25_{409-423}$, $m139_{560-574}$ and $m142_{24-38}$ (33). The following class II-restricted LCMV peptide was used: GP_{61-80}. APC and PE-labeled MHC class I tetrameric complexes with the above described peptide epitopes were used. For analysis of HCMV-specific CD8$^+$ T cell responses MHC class I tetrameric complexes with the following peptides were used: $pp65_{363-373}$ (HLA-A*0101), $pp65_{495-503}$ (HLA-A*0201), $pp65_{417-426}$ (HLA-B*0702), $pp65_{123-131}$ (HLA-B*3501).

Multiplex

Blood was collected retro-orbitally and clotted for 30 min. After centrifugation serum was collected and stored at −80°C until further use. Cytokines were measured in serum using a mouse Bio-Plex Pro Mouse Cytokine 23-plex immunoassay (Bio-Rad, Herculus, CA, United States) according to manufacturer's protocol.

Serum antibody detection by ELISA

Total IgM and IgG concentrations were determined by ELISA in serum samples as described earlier (25). Briefly, Nunc-Immuno Maxisorp plates (Fisher Scientific) were coated overnight with virus in bicarbonate buffer, and after blocking (skim milk powder, Fluka BioChemika) sera from mice was added. Next, plates were incubated with various HRP-conjugated antibodies (SouthernBiotech) to detect IgM/IgG. Plates were developed with TMB substrate (Sigma Aldrich), and the color reaction was stopped by the addition of 1 M H_2SO_4. Optical density was read at 450 nm (OD_{450}) using a Microplate reader (Model 680, Bio-Rad).

Statistical analysis

To determine statistical significance between two groups an unpaired Student's *t*-test was performed. Significance between more than two groups was evaluated by one-way ANOVA and Dunnett's post-hoc test was performed to correct for multiple comparisons. Mann-Whitney U test was performed to determine statistical differences in the viral load in mice. To test the strength of the linear relationship between the frequency of effector memory $CD8^+$ T cells and the LCMV viral load, Pearson correlation was used. For correlations between the frequency of effector memory $CD8^+$ T cells and the magnitude of the response, Spearman correlation was used. GraphPad Prism 6.0 software (GraphPad Software, La Jolla, CA, United States) was used for statistical analyses.

RESULTS

Disparate effects of CMV infection and aging on naive and memory $CD8^+$ and $CD4^+$ T cell subsets

To investigate whether dissimilar infectious dosages of MCMV differentially affect the immune system in life-long infection, we inoculated C57BL/6 mice at 7 weeks of age with different dosages of MCMV-Smith (0, 10^3, 10^4 and 10^5 PFU) and monitored the $CD4^+$ and $CD8^+$ T cell frequencies over 300 days post-infection (dpi). In naive (uninfected) mice, the frequency of total peripheral $CD8^+$ T cells was unchanged in time. However, early after MCMV infection (acute phase), $CD8^+$ T cell frequencies were elevated corresponding to the inoculum size (Figure 1A). A strict impact of the inoculum size was also observed at later time-points: mice that were infected with the highest MCMV PFU

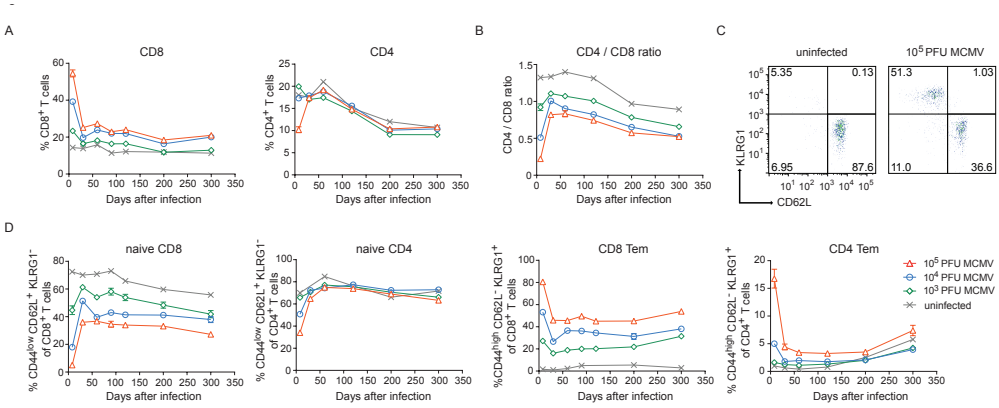

Figure 1. Disparate effects of CMV infection and aging on naive and memory CD8⁺ and CD4⁺ T cell subsets. Wild-type (WT) mice were infected with 10^3, 10^4 or 10^5 PFU MCMV-Smith (A) The average frequencies of CD8⁺ and CD4⁺ T cells in blood determined at the indicated time points post-infection. (B) The CD4⁺/CD8⁺ T cell ratio in blood determined at the indicated time points. (C) Representative flow cytometry plot showing the KLRG1 *versus* CD62L expression on CD8⁺ T cells of naive and 10^5 PFU MCMV infected mice. (D) Kinetic analysis of the average frequencies of naive ($CD44^{low}CD62L^{+}KLRG1^{-}$) or EM ($CD44^{high}CD62L^{-}KLRG1^{+}$) CD8⁺ and CD4⁺ T cells in blood. Error bars indicate SEM (n=16 mice per group).

quantities showed the highest frequency of CD8⁺ T cells. Only mice infected with the lowest inoculum dose (10^3 PFU) did not differ substantially during the persistent phase from naive age-matched controls (Figure 1A). The frequency of total peripheral CD4⁺ T cells in naive and infected mice showed an age-related decrease, yet effects of the dosage of MCMV infection were not apparent for this T cell subset (Figure 1A).

It has been suggested that HCMV-seropositivity is linked to the IRP and one of the parameters defining the IRP is an inverted CD4/CD8 T cell ratio (34). Moreover, it has been shown that latent MCMV infection decreases this ratio (21). By calculating, the CD4/CD8 T cell ratio we also observed a decreased CD4/CD8 T cell ratio during the persistent phase and markedly noticed an obvious MCMV-dose impact for this phenomenon (Figure 1B). A decreased CD4/CD8 T cell ratio was also most apparent in high dose infected aged mice based on the absolute counts of CD8⁺ and CD4⁺ T cells present in the spleen, and this was mainly caused by an increment of the absolute CD8⁺ T cell count (Figure S1A in supplementary material).

Next we assessed the influence of the different MCMV inoculum sizes on the naive and memory T cell subset composition during the course of infection. MCMV infection resulted in strongly reduced frequencies of naive circulating CD8⁺ T cells ($CD44^{low}CD62L^{+}KLRG1^{-}$), which inversely correlated with the inoculum dose (Figures 1C and D). Aging reduced the naive CD8⁺ T cell frequencies in both uninfected and infected mice. The differences between the groups of differently MCMV dosage inoculated mice remained over time. The naive CD4⁺ T cell pool was not overtly influenced by the inoculum size or ageing (Figure 1D).

Accumulation of effector-memory (EM; $CD44^{high}CD62L^{-}KLRG1^{+}$) CD8⁺ T cells was not observed in uninfected animals. However, this was clearly present after MCMV infec-

tion. Strikingly, increasing inoculum sizes correspondingly heightened the frequency of EM CD8$^+$ T cells. These differences in EM CD8$^+$ T cell frequency between the groups remained stable during persistent infection and somewhat inclined after 200 days post-infection (Figure 1D). The EM CD4$^+$ T cell frequencies were increased after the highest dose inoculum and remained higher compared to lower dose infected mice and uninfected mice (Figure 1D). Aging increased the EM CD4$^+$ T cell frequency in both infected and uninfected mice after 200 days post-infection. Thus, the size of the MCMV inoculum predominantly affects the formation of EM-like CD8$^+$ T cells throughout the course of infection while aging seems to predominantly impact the percentages of EM-like CD4$^+$ T cells.

The magnitude and phenotype of inflationary MCMV-specific CD8$^+$ T cells is strongly influenced by the inoculum dose

Next we determined if dissimilarities in inoculum dosage sizes would differentially affect MCMV-specific CD8$^+$ T cell populations during aging. In the acute phase of infection we observed that the magnitude of both non-inflationary (M45-specific) and inflationary (m139-, M38-, and IE3-specific) MCMV-specific CD8$^+$ T cell responses were correspondingly ~2 fold higher in the group infected with a 10-fold higher dose. During the chronic phase of infection the frequency of the M45-specific CD8$^+$ T cells is comparable between the different inoculum sizes. In contrast, the magnitude of the CD8$^+$ T cell response against the inflationary epitopes in m139, M38 and IE3 increased in a strict dose dependent manner (Figure 2A).

Induction of MCMV-associated immune senescence may be potentiated by the accumulation of virus-specific EM-like CD8$^+$ T cells. Here we assessed the phenotype of

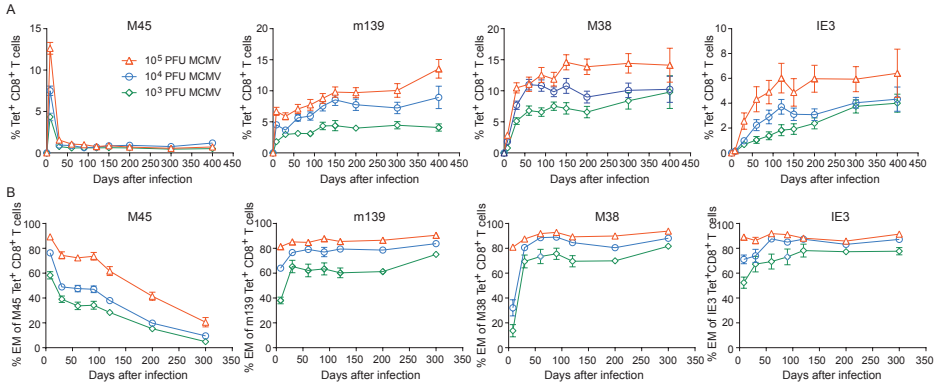

Figure 2. The magnitude and phenotype of inflationary MCMV-specific CD8$^+$ T cells is strongly influenced by the inoculum dose. WT mice were infected with 10^3, 10^4 or 10^5 PFU MCMV-Smith. (A) The average frequencies of MCMV-specific CD8$^+$ T cells (i.e., specific to epitopes derived from the MCMV proteins M45, m139, M38 and IE3) in blood were determined by MHC class I tetramer staining at the indicated time points post-infection. Error bars indicate SEM (n=16 mice per group). (B) Kinetic analysis of the average frequencies of EM (CD44^{high}CD62L$^-$KLRG1$^+$) type CD8$^+$ T cells within the total MCMV-specific CD8$^+$ T cell population in blood. Error bars indicate SEM (n=16 mice per group). Spearman's correlation was used to determine the strength of the correlations; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. (n=16 mice per group).

the antigen-specific CD8$^+$ T cells that were induced after inoculation with the different MCMV doses. For all MCMV-specific CD8$^+$ T cell responses measured, we observed that the size of the viral inoculum determines the frequency of effector CD8$^+$ T cells during the acute phase (Figure 2B). While the effector phenotypic profile of the non-inflationary M45-specific T cells is gradually turned into a predominant central-memory (CM; CD44^{high}CD62L$^+$ KLRG1$^-$) phenotype, the CD8$^+$ T cells that are specific for the inflationary epitopes exhibit an increased effector-type appearance even after the acute phase of infection (Figure 2B and Figure S1B in supplementary material). The percentage of inflationary MCMV-specific CM CD8$^+$ T cells is low throughout infection (Figure S1C in supplementary material). Thus, the initial infectious dose has a dominant influence and shapes both the magnitude and phenotype of inflationary MCMV-specific CD8$^+$ T cells.

Analogous correlations between the magnitude and phenotype of inflationary mouse and human CMV-specific CD8$^+$ T cells

Subsequently, we evaluated whether correlations exist between the magnitude and phenotype of the CMV-specific CD8$^+$ T cells. Strikingly, only in case of responses against inflationary epitopes, a direct correlation between the magnitude of the MCMV-specific CD8$^+$ T cell response and the frequency of the EM-like cells within the MCMV-specific CD8$^+$ T cell population is observed (Figure 3). In mice infected with the lowest dose (i.e., 1×10^3 PFU), the magnitude of the CD8$^+$ T cell response against the inflationary epitopes and the frequency of effector cells is correspondingly the lowest, whereas these are highest in infection with the highest dose (i.e., 1×10^5 PFU) (Figure 3A). Also, when we inoculated mice weekly with the same dose of MCMV for a period of 1 year, we found these correlations between the magnitude and phenotype of the inflationary CD8$^+$ T cells, and these correlations were similar to those found in mice infected with MCMV once (Figure 3B). Notably, responses against IE1 in the context of MCMV infection results in a similar correlation as observed for IE3 (data not shown) while IE1 expressed by recombinant vaccinia virus (VACV-IE1), eliciting acute but not persistent infection, did not elicit EM phenotype *versus* magnitude correlations (Figure 3C), indicating that viral antigen reactivation is crucial for such correlations.

Since humans are likely infected with varying doses of HCMV, we aimed to recapitulate whether the above described correlations also exist in HCMV-positive individuals. Therefore, we performed analysis of the human pp65-specific CD8$^+$ T cell response in a large cohort of healthy HCMV-seropositive individuals. Essentially, reminiscent to the correlations of the inflationary responses observed in the mouse (Figure 3A), a relationship was observed between the magnitude of the HCMV-specific CD8$^+$ T cell response and the frequency of specific CD8$^+$ T cells harboring an EM phenotype (Figure 3D). Remarkably, the development of the EM phenotype of CD8$^+$ T cells against HCMV pp65

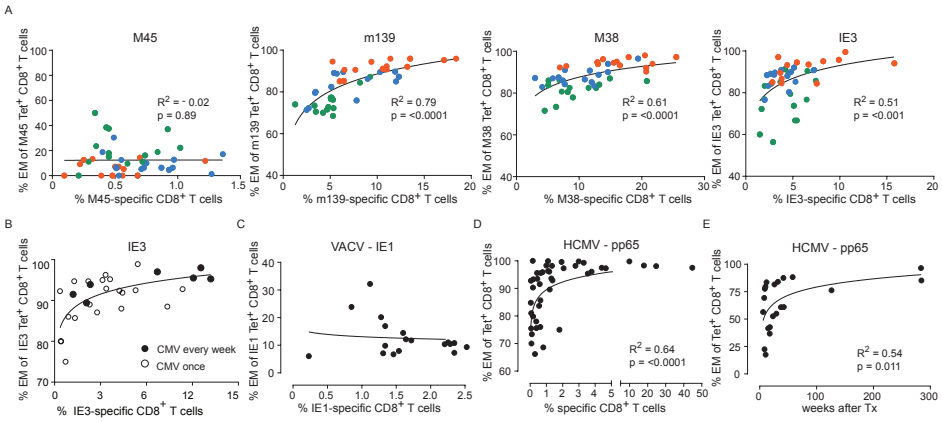

Figure 3. Correlations between the magnitude and phenotype of inflationary CMV-specific T cells (A). WT mice were infected with 10^3, 10^4 or 10^5 PFU MCMV-Smith. The percentage of MCMV-specific EM ($CD44^{high}CD62L^{-}KLRG1^{+}$) $CD8^{+}$ T cells *versus* the percentage of MCMV-specific $CD8^{+}$ T cells in blood at day 300 post-infection. (B) WT mice were infected with 5×10^4 PFU MCMV-Smith once (n=20) or weekly (n=7) for one year. At 1 year post-infection the average frequencies and phenotype of IE3-specific $CD8^{+}$ T cells in blood was determined by MHC class I tetramer staining. The graph shows the percentage of IE3-specific EM ($CD44^{high}CD62L^{-}KLRG1^{+}$) $CD8^{+}$ T cells *versus* the percentage of IE3-specific $CD8^{+}$ T cells in blood. (C) WT mice were infected with recombinant vaccinia virus (VACV-IE1) At day 200 post-infection the average frequencies and phenotype of IE1-specific $CD8^{+}$ T cells in blood was determined by MHC class I tetramer staining. The graph shows the percentage of IE1-specific EM ($CD44^{high}CD62L^{-}KLRG1^{+}$) $CD8^{+}$ T cells *versus* the percentage of IE1-specific $CD8^{+}$ T cells in blood (n=18). (D) Analysis of HCMV-specific $CD8^{+}$ T cell responses. The percentage of antigen-specific EM $CD8^{+}$ T cells *versus* the percentage of antigen-specific $CD8^{+}$ T cells in blood from healthy donors for HCMV pp65 (n=46) (E) The percentage of antigen-specific EM $CD8^{+}$ T in blood from initially HCMV-seronegative recipients receiving a HCMV-positive kidney transplant against the weeks after transplantation Spearman's correlation was used to determine the strength of the correlations; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

follows analogous kinetics upon primary infection of HCMV seronegative patients receiving a HCMV-positive kidney transplant as compared to the EM phenotype of the inflationary MCMV epitopes (Figures 2B and 3E). Together, these data indicate that $CD8^{+}$ T cell responses against viral epitopes in human and mouse CMV follow a similar developmental pathway in which the EM phenotype of the responding $CD8^{+}$ T cells correlate with the magnitude of the viral-specific $CD8^{+}$ T cell response.

Progressive effector-memory $CD8^{+}$ T cell differentiation is accelerated by high dose CMV infection

To gain a more detailed understanding of the phenotypic differences of the MCMV-specific $CD8^{+}$ T cells that develop during the low, intermediate and high dose infection, we used a novel analysis platform, termed Cytosplore (32), for immune cell phenotyping that incorporates approximated t-distributed stochastic neighborhood embedding (A-tSNE) for dimensionality reduction and –the mean- shift clustering algorithm for subset definition, based on the dimensionality reduced data (35). The A-tSNE algorithm lays out cells in a two-dimensional scatter plot, based on similarity of defined markers. Similar cells will be placed closed together in the plot, while slight variations in the

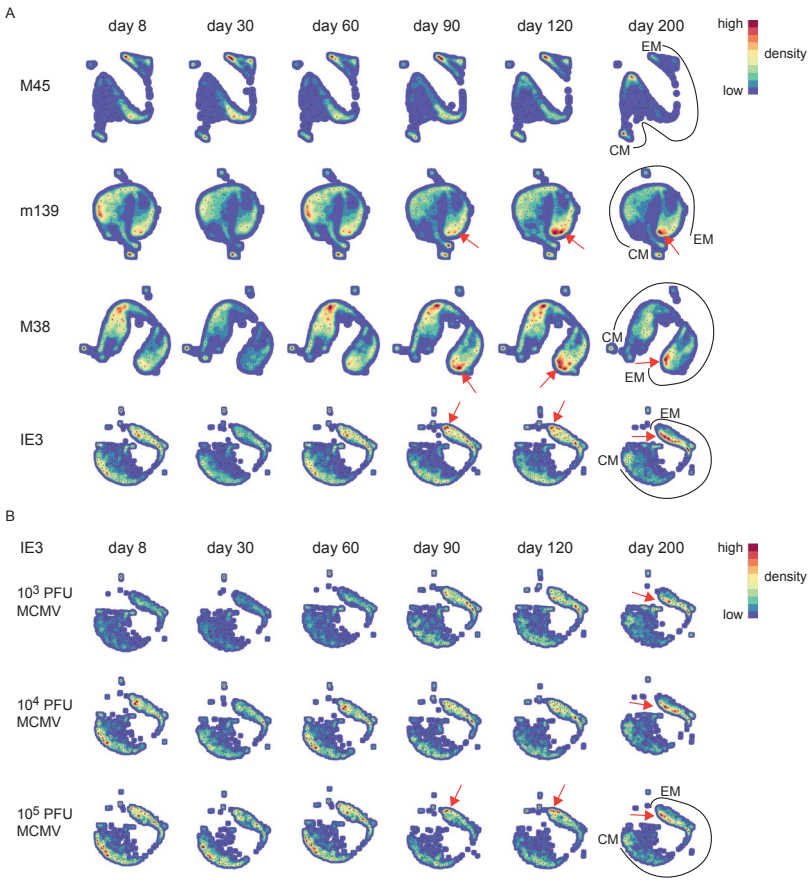

Figure 4. Progressive effector-memory CD8⁺ T cell differentiation is accelerated by high dose CMV infection. WT mice were infected with 10^3, 10^4 or 10^5 PFU MCMV-Smith. MCMV-specific CD8⁺ T cells in blood were stained with MHC class I tetramers combined with cell surface markers (CD62L, KLRG1, CD27 and CD44) at the indicated time points post-infection. (A) Cytosplore analysis of the MCMV-specific CD8⁺ T cells in time. A-tSNE plots depict the pooled data of MCMV-specific CD8⁺ T cell responses of the 10^3, 10^4 and 10^5 PFU MCMV-infected mice for each time point after infection. (B) A-tSNE plots depict the IE3-specific CD8⁺ T cell response of the 10^3, 10^4 and 10^5 PFU MCMV-infected mice for each time point after infection. (n=16 mice per MCMV dose).

level of marker expression will result in gradual positional transitions. The results of the A-tSNE algorithm are then visualized as density plots. This provides an unprecedented insight into the development of the cellular differentiation, which is not feasible by conventional flow cytometry data analysis. We performed Cytosplore analysis on the MCMV-specific CD8⁺ T cell populations we tracked in time by MHC class I tetramer staining combined with phenotypical markers comprising CD62L, KLRG1, CD27 and CD44, which allow discrimination between CM and EM-type T cells (Figure S2A in supplementary material). Such analysis should display the potential differentiation trajectory path of EM and CM-type T cells. In order to provide an initial global picture of the impact of CMV infection on the EM/CM T cell differentiation, we analyzed each

MCMV-specific CD8$^+$ T cell population in time using the combined data from the different infection dosages. We observed that the development of the EM phenotype of the inflationary T cells is progressive in time, as evidenced by an ongoing shift toward a higher advanced EM-phenotype (indicated with red arrows) (Figure 4A). Even between day 120 and day 200, the EM differentiation continues. To assess the role of dosing in this phenomenon, we performed the analysis with the separate dosages. Compared to low dose infection, higher dose infections accelerated the EM CD8$^+$ T cell differentiation throughout the infection (Figure 4B; shown for IE3,). We also observed that the impact of the dosage effect on the EM CD8$^+$ T cell differentiation is irrespective of the infection duration (Figure S2B in supplementary material). Together these data suggest that high dose infection causes an EM CD8$^+$ T cell differentiation that not only develops faster but also continues to segregate throughout the life-span of the host, suggesting a virus-host equilibrium that is influenced by aging and the infectious dose.

The dichotomy in cytokine polyfunctionality of inflationary *versus* non-inflationary CMV-specific T cells increases with the infectious dose.

In elderly individuals a higher frequency of cytokine-deficient CD8$^+$ T cells recognizing the HCMV epitope pp65 is associated with all-cause mortality in these individuals, suggesting that the presence of these cells could predict age-related dysfunction and increased risk of death (36). To gain insight into the cytokine profile of the MCMV-specific T cells in differentially inoculated aged mice we assessed the polyfunctional cytokine profiles 400 days after MCMV infection. The majority of the M45-specific CD8$^+$ T cell pool in the spleen comprises the capacity to co-produce IFN-γ, TNF and IL-2 (Figure 5A). The frequency of these triple cytokine producers is elevated in mice infected with higher inoculums of MCMV. In contrast, CD8$^+$ T cell populations specific for the inflationary epitopes (m139, M38 and IE3), consist most frequently of double-producing cells (IFN-γ and TNF). These frequencies increase with higher MCMV inoculum dosages, while this coincides with decreasing frequencies of triple cytokine producing cells (Figure 5A). Correspondingly, high dose infection results in elevated absolute numbers of double IFN-γ/TNF producers, especially among the inflationary CD8$^+$ T cells (Figure 5B). In the MCMV-specific CD4$^+$ T cell pool a dose dependent increase of double cytokine producers is also observed, although mainly at the expense of the single IFN-γ producing cells (Figure S3A in supplementary material). Thus, whereas non-inflationary CD8$^+$ T cells have an increment of the most polyfunctional T cells with higher inoculums, inflationary CD8$^+$ T cells tend to lose this property.

Only high dose CMV infection impairs the development of heterologous T cell responses

To assess whether the initial infectious dosages that determine the degree of the CMV-

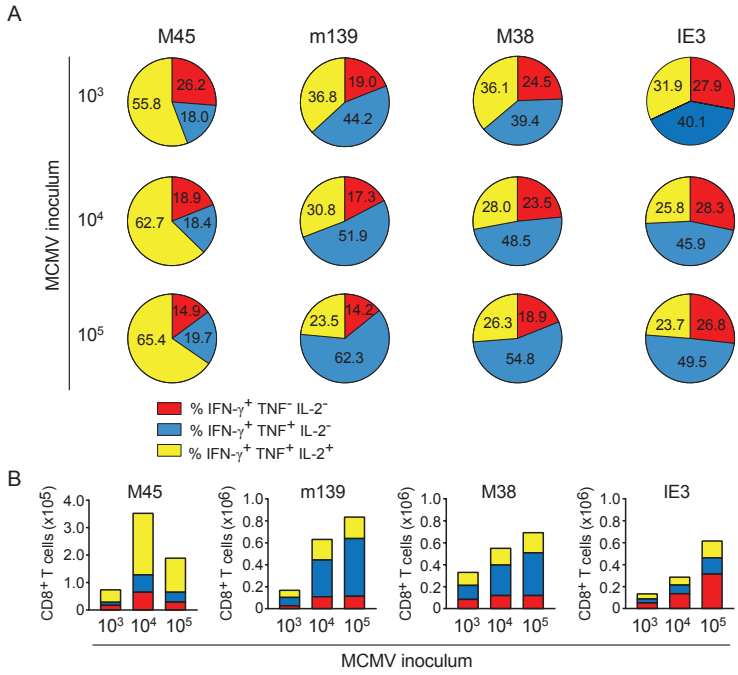

Figure 5. The dichotomy in cytokine polyfunctionality of inflationary *versus* non-inflationary CMV-specific T cells increases with the infectious dose. Following MCMV infection with different dosages (10^3, 10^4 or 10^5 PFU MCMV-Smith) the cytokine polyfunctionality of splenic CD8$^+$ T cells was determined after peptide restimulation at day 400 post-infection. (A) 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. (B) Absolute counts 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 (n = 8 per group).

induced perturbations of peripheral naive and memory T cell compartments is connected to differential impairment of the development of heterologous anti-viral immunity we challenged 400 day old naive and MCMV-infected mice (1×10^3, 1×10^4 or 1×10^5 PFU MCMV) with 2×10^5 PFU LCMV-Armstrong. LCMV infection significantly increased the absolute splenic CD8$^+$ T cell numbers of the naïve mice and of the mice infected with 1×10^3 or 1×10^4 PFU MCMV compared to age-matched mice that did not receive a LCMV challenge (Figure 6A). Strikingly, in mice that were long-term infected with the highest dose of MCMV (1×10^5 PFU), the LCMV-induced CD8$^+$ T cell expansion was impaired and numbers were not significantly increased (Figure 6A). Determination of the effector CD8$^+$ T cell quantities revealed a similar trend; albeit LCMV challenge caused a significant increase in all groups, the expansion was least pronounced when mice were previously infected with the highest dose of MCMV (Figure 6B), suggesting a reduction in LCMV-specific CD8$^+$ T cells when mice experienced high dose MCMV inoculums previously. To test this assumption we determined absolute numbers of LCMV-specific ($GP33_{33\text{-}41}$, $NP_{396\text{-}404}$, and $GP_{276\text{-}286}$) CD8$^+$ T cells by MHC class I tetramer binding (Figure

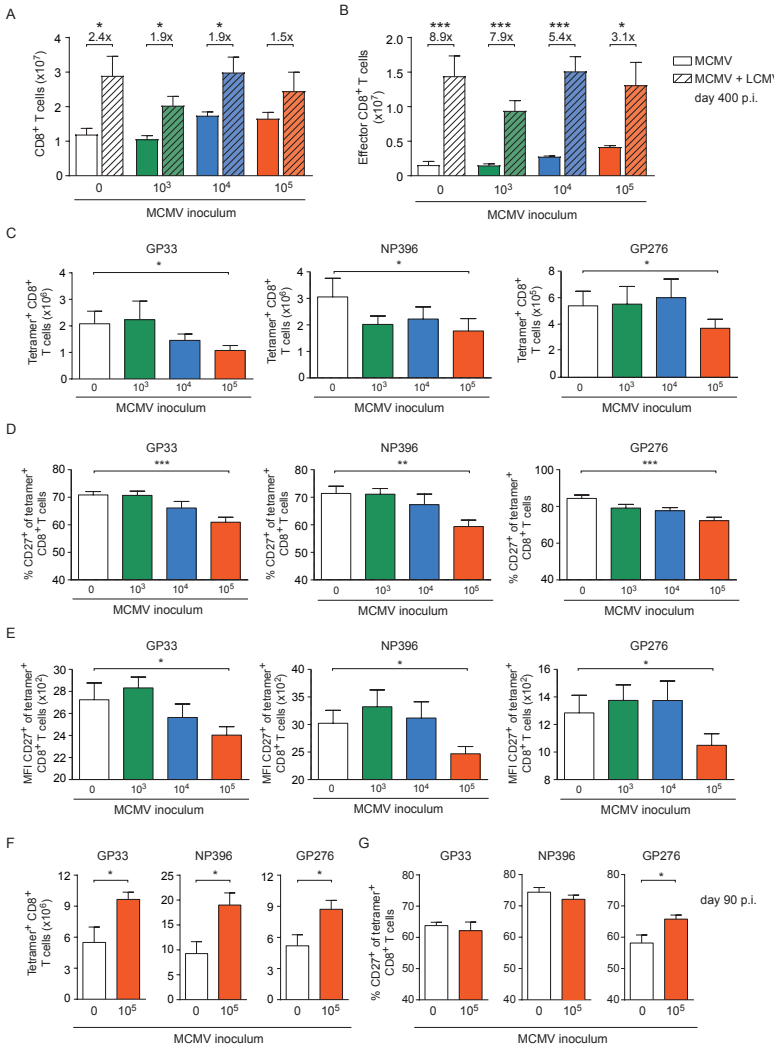

Figure 6. High dose CMV infection impairs the development of heterologous viral-specific T cell responses. (A-E) WT mice were kept uninfected or infected with 10^3, 10^4 or 10^5 PFU MCMV-Smith (n=16 mice per group), and at day 400 post-infection 8 mice per group were challenged with 2 × 10^5 PFU LCMV-Armstrong. At day 8 post LCMV infection, mice were analyzed and compared with uninfected littermate controls. (A) Numbers of total splenic $CD8^+$ T cells. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ by one-way ANOVA and Dunnett's post-hoc test was performed to correct for multiple comparisons. (B). Numbers of total effector ($CD44^{high}CD62L^-$ $KLRG1^+$) $CD8^+$ T cells. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ by one-way ANOVA and Dunnett's post-hoc test was performed to correct for multiple comparisons. (C) Numbers of splenic LCMV-specific $CD8^+$ T cells determined by MHC class I tetramer staining. *, $P < 0.05$ by one-way ANOVA and Dunnett's post-hoc test was performed to correct for multiple comparisons. (D) Percentage of LCMV-specific $CD8^+$ T cells expressing CD27. *, $P < 0.05$ by one-way ANOVA and Dunnett's post-hoc test was performed to correct for multiple comparisons. (E) Mean fluorescence intensity (MFI) of CD27 expression on LCMV-specific $CD8^+$ T cells. *, $P < 0.05$ by one-way ANOVA and Dunnett's post-hoc test was performed to correct for multiple comparisons. (F-G) WT mice were kept uninfected or infected with or 10^5 PFU MCMV-Smith (n=8 mice per group), and at day 90 post-infection challenged with 2 × 10^5 PFU LCMV-Armstrong. At day 8 post LCMV infection, mice were analyzed and compared with uninfected littermate controls. (F) Numbers of splenic LCMV-specific $CD8^+$ T cells determined by MHC class I tetramer staining.). *, $P < 0.05$ by unpaired Student's *t*-test. (G) Percentage of LCMV-specific $CD8^+$ T cells expressing CD27. Results are represented as means and error bars indicate SEM (n=8 mice per group). *, $P < 0.05$ by unpaired Student's *t*-test.

6C), and found indeed that the response against these immunodominant LCMV epitopes was mostly reduced in mice that were inoculated with the highest dose of MCMV (1×10^5 PFU). Analysis of the LCMV-specific $CD4^+$ T cell response (GP_{61-80}) as measured by IFN-γ production revealed no MCMV-induced perturbations (Figures S3B and C in supplementary material). Together, these data suggest that high dose MCMV infection affects the capacity of immunodominant $CD8^+$ T cells to expand upon heterologous infections.

To assess whether the increasing doses of MCMV could differentially affect the activation status of the LCMV-primed $CD8^+$ T cells, the cell surface expression of CD27, CD127, CD62L and KLRG1 was evaluated. Strikingly, we found that the frequency of the immunodominant LCMV-specific $CD8^+$ T cells expressing CD27, and correspondingly the expression of CD27 on a per cell basis, was mostly reduced in mice persistently infected with the highest MCMV dose (Figures 6D and E), while expression of CD127, CD62L and KLRG1 was not influenced by MCMV infection (Figure S4A-C in supplementary material). Next we investigated if the impairment of heterologous immune responses due to high dose CMV infection is related to long-term infection of aged mice. Strikingly, the LCMV-specific $CD8^+$ T cell response in young mice (5 months old) that were previously uninfected or infected with a high dose MCMV (1×10^5 PFU MCMV-Smith) revealed no reduction or even an increase in the amount or activation status of LCMV-specific $CD8^+$ T cells (Figures 6F-G and Figures S4D-G in supplementary material).

Next, we aimed to determine if the MCMV dose-associated alterations could also impact the cytokine polyfunctionality of the LCMV-specific $CD8^+$ T cell responses in the aged MCMV-infected mice after heterologous infection with LCMV. Following *ex vivo* stimulation with class I-restricted peptides GP_{33-41}, $NP_{396-404}$, and $GP_{276-286}$, the frequencies of the single (IFN-γ), double (IFN-γ and TNF) and triple (IFN-γ, TNF and IL-2) producing LCMV-specific $CD8^+$ T cells were determined. Although an obvious altered cytokine profile was not detected (Figure 7A), the responding LCMV-specific T cells exhibited a lower IL-2 expression on a per cell basis in only those mice that were previously inoculated with the highest dose of MCMV (Figure 7B). Analysis of the $CD4^+$ T cell response against LCMV epitope GP_{61-80} showed that LCMV-specific $CD4^+$ T cell responses are not affected by lifelong MCMV infection (Figures S3B, C in supplementary material).

Taken together, these results establish that persistent MCMV infection induced by a high inoculum dose is able to decrease the development of heterologous adaptive immune responses upon encountering a new pathogen.

The inflammatory milieu and antibody levels upon heterologous infection are influenced by the inoculum dose of MCMV.

Chronic low grade inflammation accompanied with persistent CMV infection might affect antigen-presenting cells in such a way that the priming of T cells against newly encountered antigens is hampered. To test whether the size of the inoculum dose might

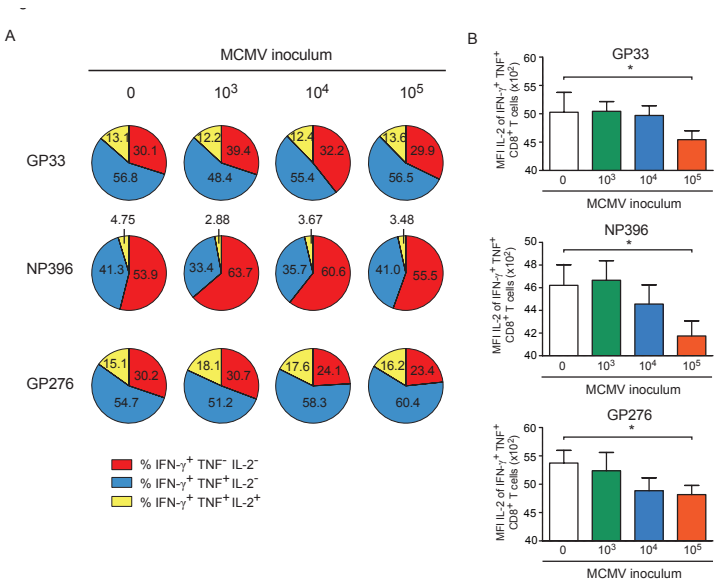

Figure 7. High dose CMV infection impairs the autocrine IL-2 production of heterologous viral-specific T cells. WT mice were kept uninfected or infected with 103, 104 or 105 PFU MCMV-Smith, and at day 400 post-infection 8 mice per group were challenged with 2 × 105 PFU LCMV-Armstrong. At day 8 post LCMV infection, mice were analyzed and compared with uninfected littermate controls. (A) The cytokine polyfunctionality of splenic CD8+ T cells was determined after peptide restimulation. 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. (B) MFI of IL-2 expression of LCMV-specific CD8+ T cells. All data represents mean values + SEM (n = 8 per group). *, P < 0.05 by one-way ANOVA and Dunnett's post-hoc test was performed to correct for multiple comparisons.

influence low grade inflammation in steady state and upon heterologous infection, we determined cytokine and chemokine serum levels. Analysis of the tested cytokine and chemokine levels revealed no substantial changes between mice that were infected with different doses of MCMV at day 400 post MCMV infection (Figure S5 in supplementary material). Upon LCMV infection, we observed for a number of cytokines/chemokines a major increase, albeit mostly this was not influenced by the MCMV inoculum dose. However, a MCMV dose dependent decrease in IFN-γ serum levels is apparent (Figure 8A). For the TNF serum levels the same trend was observed (Figure 8B). Analysis of the IL-2 serum concentration revealed that IL-2 levels were lower in the high MCMV dose inoculating mice (Figure 8C), which corresponds to the lower amounts of IL-2 produced by the inflationary T cells. In contrast to the other cytokines present in serum, upon LCMV infection the IL-2 levels decreased in the mice without prior MCMV infection and in the mice inoculated with 1×10^3 and 1×10^4 PFU. Whereas in mice that experienced lifelong infection with the highest MCMV dose, the IL-2 concentration increased, suggesting a lower rate of IL-2 consumption by the T cells (Figure 8C). Thus, the MCMV inoculum dose differentially shapes the inflammatory milieu with respect to effector T cell-associated cytokines (IFN-γ, TNF, IL-2) upon encountering a new pathogen.

Given the correlation between MCMV-specific antibody titers and the infectious dose

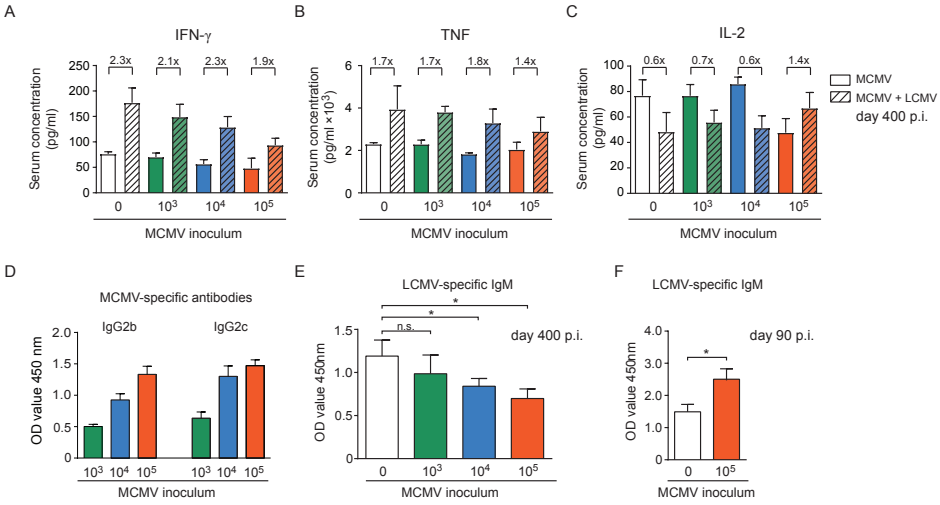

Figure 8. The inflammatory milieu and antibody levels upon heterologous infection are shaped by the inoculum dose of MCMV. (A-D) WT mice were kept uninfected or infected with 10^3, 10^4 or 10^5 PFU MCMV-Smith (n=16 mice per group), and at day 400 post-infection 8 mice per group were challenged with 2×10^5 PFU LCMV-Armstrong. At day 8 post LCMV infection, mice were analyzed and compared with uninfected littermate controls. Cytokines and chemokines in the serum were determined by mouse cytokine bio-plex immunoassays (A-C), and MCMV (D) or LCMV-specific (E-F) antibody levels were determined by ELISA . (A-C) Shown are the serum concentrations of IFN-γ (A), TNF (B), and IL-2 (D). MCMV-specific IgG antibody levels in LCMV unchallenged mice (E) Levels of LCMV-specific IgM antibodies in LCMV-challenged mice. *, $P < 0.05$ by one-way ANOVA and Dunnett's post-hoc test was performed to correct for multiple comparisons. (F) WT mice were kept uninfected or infected with 10^5 PFU MCMV-Smith, and at day 90 post-infection mice were challenged with 2×10^5 PFU LCMV-Armstrong. Blood was taken 8 days after LCMV infection and levels of LCMV-specific IgM antibodies within the serum were determined. *, $P < 0.05$ by unpaired Student's *t*-test. All data represents mean values + SEM (n = 8 per group).

(Figure 8D), we aimed to address whether the MCMV inoculum dosage particularly affects heterologous T cell responses or whether also heterologous B cell responses are influenced. In the MCMV-infected mice 8 days after LCMV infection the LCMV-specific IgM antibody induction was hampered and only in those mice that were previously inoculated with high dose MCMV (Figure 8E). In young mice with and without a latent MCMV infection that were challenged with LCMV a reverse trend was observed. In these mice the LCMV-specific IgM production was increased in mice with a latent MCMV infection (Figure 8F). Thus, only in aged mice the MCMV inoculum size inversely correlates with LCMV-specific IgM induction after heterologous infection.

High dose CMV infection hampers control of heterologous infections and correlates to the phenotypical alterations of virus-specific CD8⁺ T cells.

To determine if the aforementioned MCMV-induced immune alterations resulted in hampered LCMV control, we determined the LCMV viral load in the lungs and kidneys of the uninfected and MCMV infected mice. We observed that compared to MCMV-naive mice the viral load was significantly increased in mice that were inoculated with 1 ×

10^5 PFU MCMV. Importantly, in mice that were inoculated with lower doses of MCMV, control of LCMV replication was not significantly affected (Figure 9A). Importantly in young mice inoculated with a high dose, LCMV infection was even slightly better controlled (Figure 9B).
The frequency of EM T cells in peripheral blood correlates with the inoculum dose while an inverse correlation is seen for CM $CD8^+$ T cells. To determine whether the frequency of EM $CD8^+$ T cells before challenge with LCMV also correlates with the ability to control heterologous infection, we plotted the EM frequencies of the total $CD8^+$ T cell population against the LCMV viral load in the lungs. Clearly, a strong connection between the EM phenotype and LCMV titers in the lungs is observed (Figure 9C). A reverse correlation was found when the frequency of CM T cells was plotted (Figure 9D). These data indicate that the frequency of EM $CD8^+$ T cells (or inversely the CM $CD8^+$ T cells) can be used as predictors of a possible defective capacity of the host to respond to newly encountered pathogens.

DISCUSSION

The possible contribution of CMV to immune senescence has received considerable attention but remained controversial (18, 19). To extend our knowledge with respect to the role of CMV in immune senescence we performed a highly controlled prospective study revealing that the size of the initial viral inoculum dictates the degree of CMV-induced immune alterations in long-lasting infection. Our data revealed a clear correlation between the magnitude of the $CD8^+$ T cell response and the frequency of

CMV-specific $CD8^+$ T cells exhibiting an EM phenotype, which was apparent in CMV-infected mice and humans. Importantly, we show that solely a high CMV infectious dose results in immune perturbations that are substantial enough to impair heterologous anti-viral immunity, thereby providing a better understanding of CMV in immune senescence and in addition settles previous controversies in this respect.

In agreement with our results, several studies have shown that MCMV infection with a high infectious dose causes immune perturbations that impair $CD8^+$ T cell immunity later in life (20-22). Previously we have shown that the influence of CMV on eliciting inflationary EM-like $CD8^+$ T cells populations is depending on the inoculum dose (25). In the current study we have appreciated this differential impact of dissimilar CMV dosages and show that CMV infection does not by definition impairs immunity, but specifically a high infectious dose is a prerequisite for CMV-associated immune senescence.

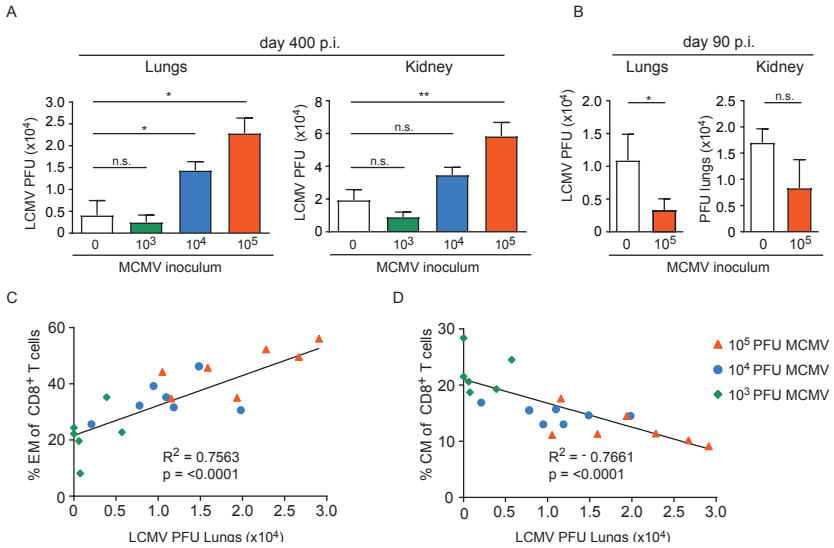

Figure 9. High dose CMV infection hampers control of heterologous infections and correlates to the phenotypical alterations of virus-specific CD8⁺ T cells. (A) WT mice were kept uninfected or infected with 10^3, 10^4 or 10^5 PFU MCMV-Smith (n=16 mice per group), and at day 400 post-infection 8 mice per group were challenged with 2 × 10^5 PFU LCMV-Armstrong. At day 8 post LCMV infection, mice were analyzed for the LCMV viral load in lungs and kidneys. All data represents mean values + SEM (n = 8 per group). *, $P < 0.05$; **, $P < 0.01$ by one-way ANOVA and Dunnett's post-hoc test was performed to correct for multiple comparisons. (B) WT mice were kept uninfected or infected with or 10^5 PFU MCMV-Smith (n=8 mice per group), and at day 90 post-infection challenged with 2 × 10^5 PFU LCMV-Armstrong. At day 8 post LCMV infection, mice were analyzed for the LCMV viral load. All data represents mean values + SEM (n = 8 per group). n.s., $P > 0.05$ by unpaired Student's *t*-test (C-D) Correlation between lung LCMV titers and the frequency of peripheral EM (C) and CM (D) CD8⁺ T cells 400 days post infection of the same mice. Pearson's correlations was used to determine the strength of the correlations ****, $P < 0.0001$.

Conceivably, in immune compromised hosts where viral control is hampered, already a low viral dose might result in CMV-related immune senescence. Although we have shown that weekly reinfection with the same dose does not increase EM T cell accumulation, it should be noted that in addition to the initial infectious dose, the CMV dosage during reinfection and the level of pre-existing CMV-specific neutralizing antibodies could impact the degree of CD8⁺ T cell accumulation. We assume that in humans, where reinfection is known to occur (37), the viral dose differs in subsequent reinfection. We consider it likely that CMV reinfection with increasing infectious doses does intensify the degree of EM T cell accumulation and hence, thereby possibly immune senescence.

In human individuals, correlations between HCMV positivity and impaired immunity are controversial since this relationship is observed in some but not all studies (10-16, 38-42). Our results can at least partly explain this controversy, because it is highly conceivable that in humans CMV infection occurs with infectious dosages that are highly variable. In addition, besides the infectious dose also host intrinsic factors like genetic predisposition can impact the CMV grade, and hence, contribute possibly to immune senescence.

The accumulation of CMV-specific EM CD8$^+$ T cells, which correlate with impaired heterologous immunity, might by itself be (partly) causative for the negative impact of long-standing latent CMV on heterologous immunity. In case of high dose infection the frequency of CMV-specific CD8$^+$ T cells might reach a certain threshold resulting in diminished priming of naive T cells against newly encountered pathogens. Indeed, detailed cluster analysis of the EM development showed that high dose infection causes a differentiation pathway that progresses faster throughout the life-span of the host, suggesting a virus-host equilibrium that is not fixed and apparently is influenced by aging and the infectious dose. A higher infectious dose is known to result in a higher level of reactivation from latency (43) and this is in line with the observation that a higher CMV dose results in larger reservoirs of latent CMV (25). As a result stimulation of CMV-specific T cells is increased, thereby accelerating differentiation. Moreover, we observed that the LCMV-specific CD8$^+$ T cells in mice infected with a high CMV dose are not properly primed as evidenced by decreased upregulation of CD27 and lower IL-2 production. It is considered that in aged individuals priming occurs less as compared to young (44). The additional amounts of (activated) T cells might further diminish this by competing for T cell growth factors or even by competition at the APC level. Nonetheless, we consider it likely that the accumulation of CMV-specific T cells is not solely responsible for impairing immune responses against newly encountered pathogens. For example, our analysis also revealed a weakened LCMV-specific B cell response in those mice that were infected with the highest dose of MCMV. In mouse and human CMV infection accumulation of CMV-specific antibodies has been reported (25, 45, 46). This may indicate that also accumulation of CMV-specific antibody-producing B cells occurs. Possibly these CMV-specific plasma cells compete with B cells that are specific for the newly encountered pathogen.

In contrast to what we observed in aged mice, in young mice CMV infection seems to improve immunity against other pathogens, which is in agreement with other reports showing beneficial effects of herpesvirus infection on protection from other infections (16, 47). However, the positive effects of herpesvirus infections in young mice is transient (16, 47, 48), which may be due to a temporary effect on both the innate and adaptive immune system. In addition, Furman et al showed that CMV-seropositive young adults displayed an increase in CD8$^+$ T cell sensitivity and an improvement in the antibody response upon influenza vaccination, and it was shown that IFN-γ was required for the CMV-associated improved cross-protection (16). Upon heterologous challenge with LCMV we observed that serum levels of IFN-γ were significantly reduced in mice that were infected with the highest dose of CMV compared to mice that were CMV negative. This suggests that a "fit" immune system in which IFN-γ production is properly induced is crucial for the CMV-associated enhancement of heterologous immune responses. We consider it likely that ageing by itself abolishes the positive effect of CMV

by decreasing the IFN-γ producing capacity and that a combination of old age and extensive CMV-induced immune aberrations shift the balance towards a negative effect of CMV on heterologous immunity.

In summary, our results demonstrate that the infectious dose of CMV is a key determinant for the immunological outcome and consequently, also projects the possible impact for heterologous immunity later in life. Importantly, here we show for the first time that only infection with a high CMV infectious dose impairs the immune response against newly encountered pathogens in aged hosts. Future prospective longitudinal studies are required that incorporate stratification based on the strength of the CMV-specific immune response.

ACKNOWLEDGEMENTS

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SUPPLEMENTARY FIGURES

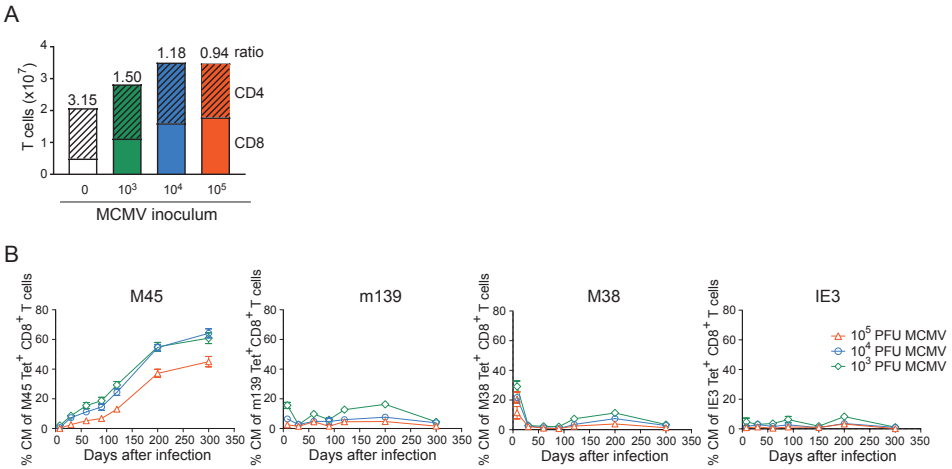

Figure S1. Disparate effects of CMV infection on CD8⁺ and CD4⁺ T cell subsets. WT mice were infected with 10^3, 10^4 or 10^5 PFU MCMV-Smith. (A) Absolute counts of the total CD8⁺ and CD4⁺ T cell populations in spleen at day 400 post-infection. The CD4/CD8 T cell ratio is specified. (B) Wild-type (WT) mice were infected with 10^3, 10^4 or 10^5 PFU MCMV-Smith (n=16 mice per group). Graphs depict the average frequencies of central-memory ($CD44^{high}CD62L^{+}KLRG1^{-}$) type CD8⁺ T cells within the MCMV-specific CD8⁺ T cell populations in blood. All data represents mean values (n = 16 per group).

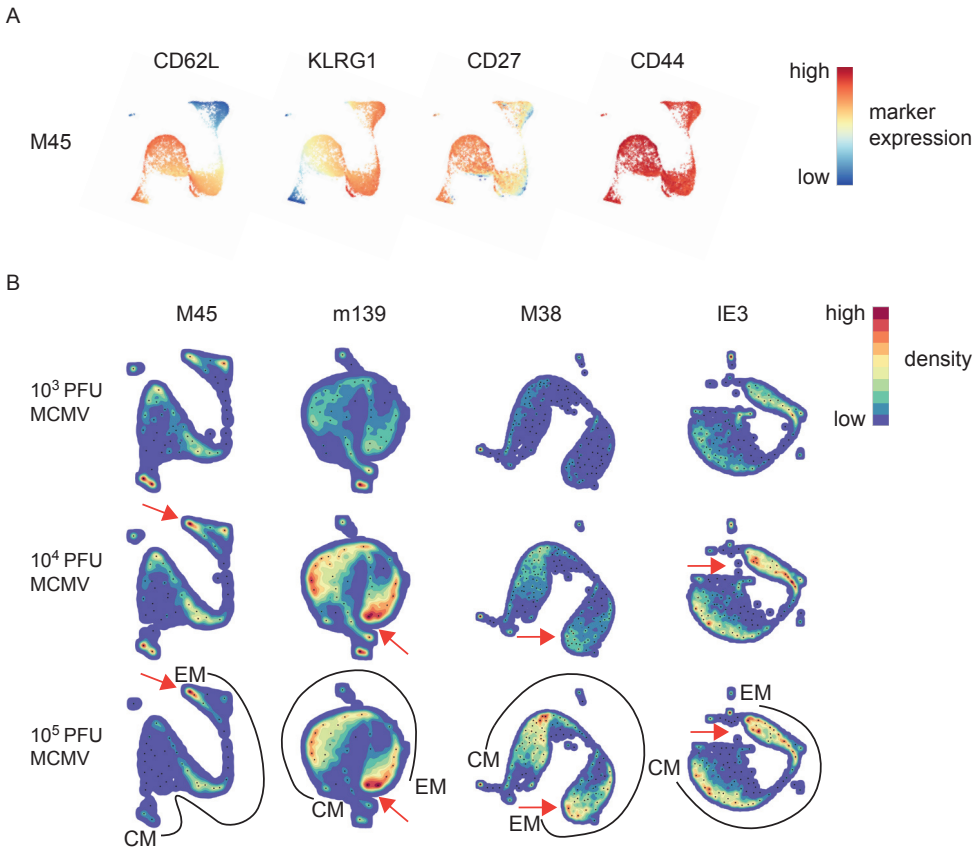

Figure S2. Increased effector-memory CD8+ T cell differentiation in high dose CMV infection. WT mice were infected with 103, 104 or 105 PFU MCMV-Smith. MCMV-specific CD8+ T cells (i.e., specific to epitopes derived from the MCMV proteins M45, m139, M38 and IE3) in blood were stained with MHC class I tetramers combined with cell surface markers (CD62L, KLRG1, CD27 and CD44). (A) A-tSNE plots visualize the intensity of single marker expression as a scatterplot. (B) Cytosplore analysis of the MCMV-specific CD8+ T cells in time. A-tSNE plots depict the pooled data of MCMV-specific CD8+ T cell responses of each time point after infection of the 103, 104 and 105 PFU MCMV-infected mice.

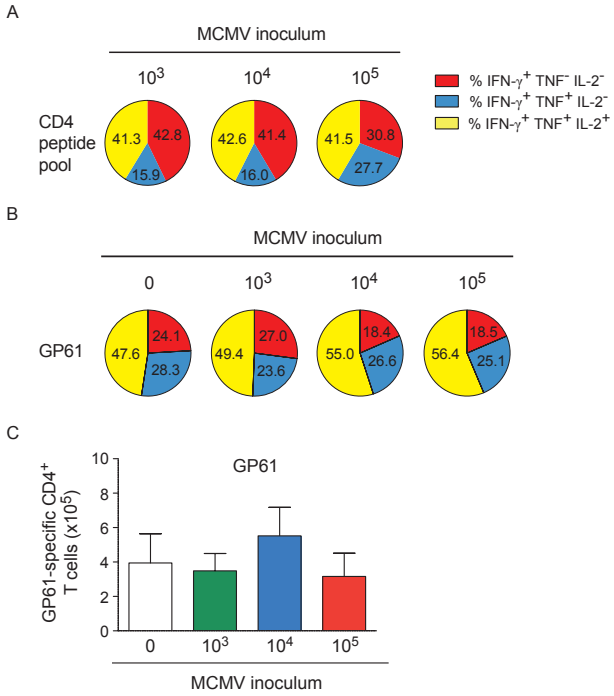

Figure S3. MCMV and LCMV-specific CD4⁺ T cell responses. WT mice were kept uninfected or infected with 10^3, 10^4 or 10^5 PFU MCMV-Smith (n=16 mice per group), and at day 400 post-infection 8 mice per group were challenged with 2×10^5 PFU LCMV-Armstrong. (A) The cytokine polyfunctionality of MCMV-specific splenic CD4⁺ T cells was determined after peptide restimulation at day 400 post-infection (of the LCMV-unchallenged group). (B) The cytokine polyfunctionality of LCMV-specific splenic CD4⁺ T cells was determined after peptide restimulation at day 400 post-infection (of the LCMV-challenged group). Pie charts depict the percentages of the single (IFN-γ), double (IFN-γ/TNF) and triple (IFN-γ/TNF/IL-2) cytokine producers upon peptide stimulation with (A) a pool of class II-restricted MCMV peptides or (B) GP61 peptide. (C) Absolute numbers of GP61-specific CD4⁺ T cells as determined by IFN-γ production. Data represents mean values (n = 8 per group).

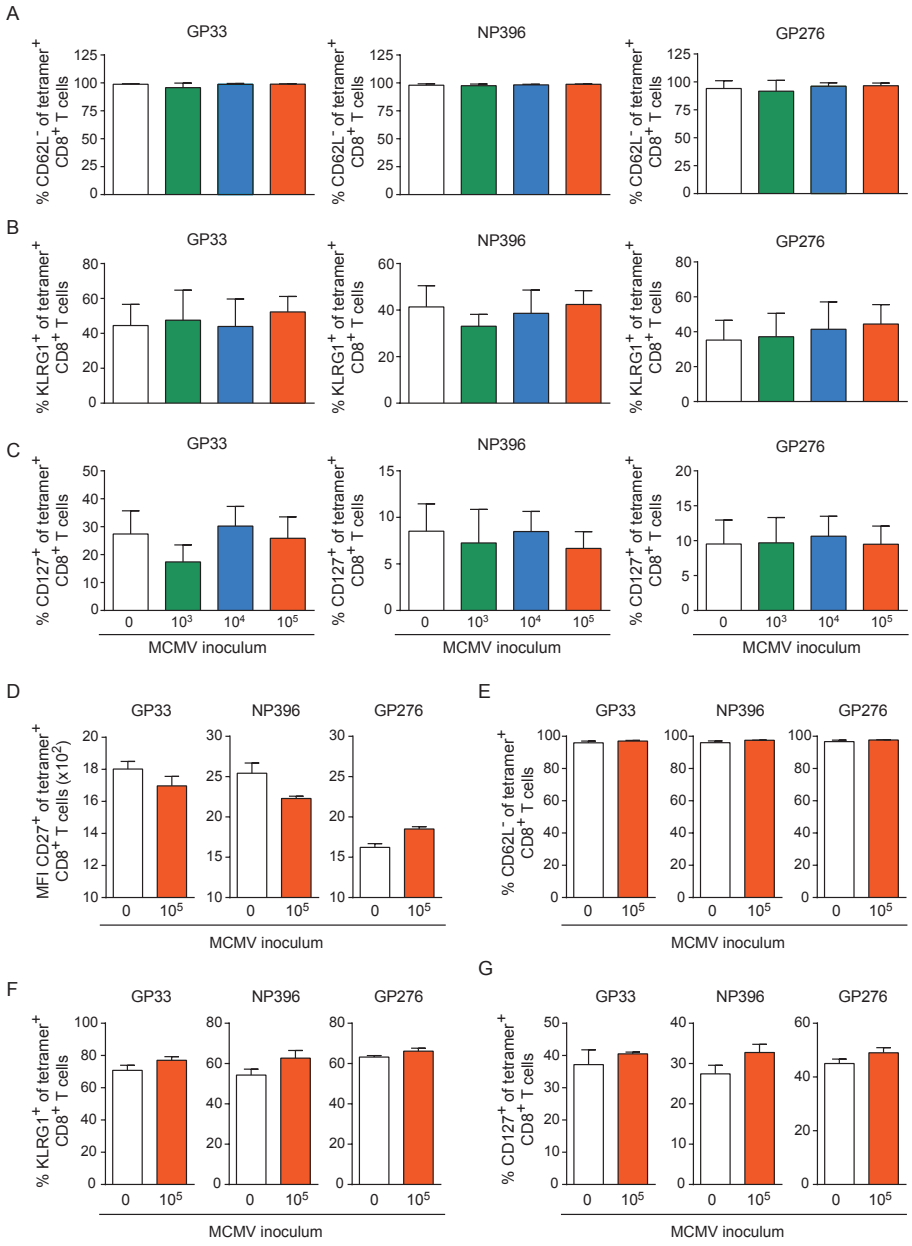

Figure S4. Expression of activation markers on LCMV-specific CD8⁺ T cells. (A-C) WT mice were kept uninfected or infected with 10^3, 10^4 or 10^5 PFU MCMV-Smith (n=16 mice per group), and at day 400 post-infection 8 mice per group were challenged with 2×10^5 PFU LCMV-Armstrong. At day 8 post LCMV infection, mice were analyzed and compared with uninfected littermate controls. (A) Percentage of LCMV-specific CD8⁺ T cells that are $CD62^{low}$. (B-C) Percentage of LCMV-specific CD8⁺ T cells expressing (B) KLRG1 or (C) CD127. (D-G) WT mice were kept uninfected or infected with or 10^5 PFU MCMV-Smith (n=8 mice per group), and at day 90 post-infection challenged with 2×10^5 PFU LCMV-Armstrong. At day 8 post LCMV infection, mice were analyzed and compared with uninfected littermate controls. (D) Mean fluorescence intensity (MFI) of CD27 expression on LCMV-specific CD8⁺ T cells. (E) Percentage of LCMV-specific CD8⁺ T cells that are $CD62L^{low}$. (F-G) Percentage of LCMV-specific CD8⁺ T cells expressing (F) KLRG1 or (G) CD127.

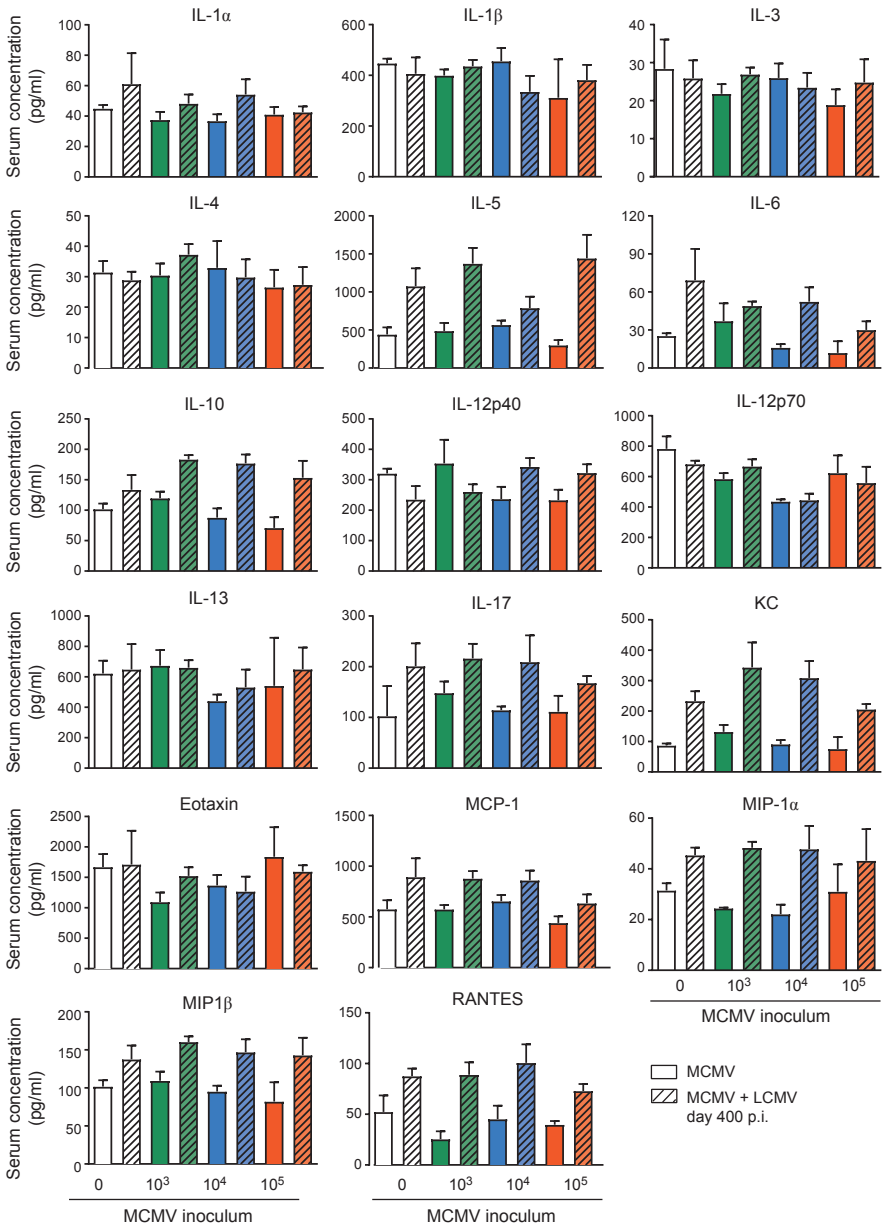

Figure S5. Cytokine and chemokine serum concentration.
WT mice were kept uninfected or infected with 10^3, 10^4 or 10^5 PFU MCMV-Smith (n=16 mice per group), and at day 400 post-infection 8 mice per group were challenged with 2×10^5 PFU LCMV-Armstrong. At day 8 post LCMV infection, mice were analyzed and compared with uninfected littermate controls. Blood serum was taken and cytokine and chemokine concentration were determined by mouse cytokine bio-plex immunoassays. Shown are the serum concentrations of IL-1α, IL-1β, IL-3, IL-4, IL-5, IL-6, IL-10, IL12p40, IL12p70, IL-13, IL-17, KC, Eotaxin, MCP-1, MIP-1α, MIP-1β and RANTES.

CHAPTER 8

CMV immune evasion and manipulation of the immune system with ageing

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ABSTRACT

HCMV encodes numerous proteins and microRNAs that function to evade the immune response and allow the virus to replicate and disseminate in the face of a competent innate and acquired immune system. The establishment of a latent infection by CMV, which if completely quiescent at the level of viral gene expression would represent an ultimate in immune evasion strategies, is not sufficient for lifelong persistence and dissemination of the virus. CMV needs to reactivate and replicate in a lytic cycle of infection in order to disseminate further, which occurs in the face of a fully primed secondary immune response. Without reactivation, latency itself would be redundant for the virus. It is also becoming clear that latency is not a totally quiescent state, but is characterised by limited viral gene expression. Therefore the virus also needs immune evasion strategies during latency. An effective immune response to CMV is required or viral replication will cause morbidity and ultimately mortality in the host. There is clearly a complex balance between virus immune evasion and host immune recognition over a lifetime. This poses the important question of whether long-term evasion or manipulation of the immune response driven by CMV is detrimental to health. In this meeting report, three groups used the murine model of CMV (MCMV) to examine if the contribution of the virus to immune senescence is set by the (i) initial viral inoculum; (ii) inflation of T cell responses; (iii) or the balance between functionally distinct effector $CD4^+$ T cells. The work of other groups studying the CMV response in humans is discussed. Their work asks whether the ability to make immune responses to new antigens is compromised by (i) age and HCMV carriage (ii) long term exposure to HCMV giving rise to an overall immunosuppressive environment and increased levels of latent virus or (iii) adapted virus mutants (used as potential vaccines) that have the capacity to elicit conventional and unconventional T cell responses.

INTRODUCTION

CMV Immune Evasion during lytic infection

It is clear that primary HCMV infection elicits a series of robust cell mediated immune responses initially by innate NK cells, followed by adaptive $CD4^+$ and $CD8^+$ T cells and B cell high avidity neutralizing antibodies [reviewed in (1)]. These responses are essential in controlling viral replication and dissemination as shown by primary infection in either the immune-naive or immunosuppressed. Here uncontrolled virus replication leads to end organ disease and morbidity and if left uncontrolled, mortality (2-4). Primary HCMV infection has a profound effect on the human immune system, leaving a permanent signature in the form of phenotypically distinct T and NK cell subsets at high frequencies (discussed in the accompanying article by Souquette et al.). However, despite this robust host immune response, HCMV is never cleared after primary infection, but persists for the lifetime of the host. Crucial to this life-long persistence is the ability of the virus to establish a latent infection, in which infected cells carry viral genome but with limited viral gene expression and the absence of production of new infectious virions (5). Importantly, the virus in these latently infected cells has the capacity to sporadically reactivate, leading to further rounds of antigenic stimulation and secondary immune responses with the associated release of inflammatory mediators. These rounds of virus reactivation and immune system stimulation can potentially drive further immune cell differentiation and increase the frequency of CMV specific T cells. The latter phenomenon has been termed "memory inflation" in the MCMV model and is characteristic of CMV infection (6).

Paradoxically, HCMV is recognized as a paradigm for a human pathogen encoding numerous viral immune evasion proteins and microRNAs (miRNA), which are able to orchestrate a sophisticated array of immune evasion mechanisms. The mechanisms that modulate the infected cellular environment to limit immune recognition are most extensively expressed during lytic infection, but it is starting to become clear that viral gene activity during latency also acts to prevent immune clearance.

During lytic infection, specific genes encoded by HCMV can directly modulate innate/intrinsic immune responses such as the interferon responses (7) as well as both intrinsic and extrinsic apoptosis pathways (8). The virus encodes proteins that act as receptors for host inflammatory cytokines, thereby reducing localized cytokine effectiveness by acting as cytokine sinks (9). HCMV encodes a number of viral homologues of cytokines like UL146 (IL-8 like) and UL111a (vIL-10), an immunosuppressive IL-10 homologue (10, 11). IL-10 is a powerful inhibitor of Th1 cytokines (such as IFN-γ and IL-2) and also inhibits inflammatory cytokine production from monocytes and macrophages which results in a decrease in surface MHC Class II expression and a reduction of antigen presentation to CD4+ T cells (12).

HCMV interference with normal MHC Class I expression to modulate $CD8^+$ T cell recognition (see below), would lead to reduced inhibitory signalling and NK cell recognition of infected cells if additional viral mechanisms were not utilized. It is of little surprise then that a substantial number of HCMV proteins target multiple different pathways in order to modulate NK cell recognition. These include preventing HCMV infected cells from expressing ligands at their cell surface that engage activating NK cell receptors, such as MIC A/B and the ULBP family which bind activating NKG2D; notably both MCMV (13) and RhCMV (14) also target NKG2D ligands. The virus also promotes the expression of ligands that are able to engage inhibitory receptors. For example, CMV encodes a viral MHC Class I like molecule, UL18, which binds the LIR-1 inhibitory NK cell receptor (only expressed by a subset of NK cells) and via a peptide processed from the viral UL40 protein is able to promote cell surface expression of HLA-E which binds the inhibitory heterodimer CD94/NKG2A. HCMV also targets PVR and Nectin 2, important ligands of activating receptors DNAM-1 and Tactile using UL141. UL141 of HCMV is further able to directly bind and inhibit expression of the TRAIL death receptors (15, 16), a second avenue to dampen NK-mediated killing and a mechanism also employed by MCMV via m166 (17), also reviewed in (13, 18).

A number of HCMV encoded genes expressed during lytic infection can interfere with both MHC Class I and II restricted antigen processing and presentation. Proteins encoded within the US2-11 gene cluster target MHC Class I and II molecules for retention within the endoplasmic reticulum (ER), re-direct MHC for degradation and inhibit normal loading of peptides onto MHC Class I. It is again noteworthy that MCMV and RhCMV have either viral homologues or other viral proteins which target normal MHC Class I processing and antigen presentation (19, 20). In addition, two structural tegument proteins introduced into cells immediately upon infection, UL82 (pp71) and UL83 (pp65) interfere with ER egress of MHC I to the Golgi apparatus and with viral peptide generation, respectively [reviewed by (21)]. As discussed earlier, while HCMV downregulates classic HLA A and B MHC I alleles it has a mechanism that preserves HLA-E expression, it is noteworthy that in RhCMV vaccine vectors, peptide presentation via non classical HLA-E molecules is the basis for protective T cell responses and work discussed by Ann Hill later investigates to what extent this is reflected in HCMV.

Immune evasion during latency

Virus-encoded micro RNAs (miRNA) which are expressed during the lytic viral cycle potentially provide an ideal mechanism to mediate immune evasion in latently infected cells. Recent evidence shows that many, if not all, of the HCMV encoded miRNAs are expressed during latency (22); miRNAs have a major advantage in that they are not antigenic as far as T cell recognition is concerned. At least five miRNAs have been shown to target components of the immune system during lytic infection. miR-UL112.1 targets

MICB, a ligand for NK cell activating receptor NKG2D (23); miR-US4.1 downregulates ERAP-1, an amino-peptidase which trims peptides for presentation by MHC Class I, leading to decreased HCMV specific $CD8^+$ CTL recognition of HCMV infected cells (24); miR-UL148D targets the chemokine CCL5 (RANTES), which is a T cell chemoattractant (25); miR-US25-2-3p targets inhibitors of TIMP3, leading to increased MICA shedding (also decreasing NK cell activation via NKG2D) and miRUl112-3p targets the Toll receptor TLR2 [reviewed in (26)].

Three HCMV proteins, US28, UL111A, and UL144 that are expressed in latently infected cells (27) have known immune evasion functions during lytic infection. US28 is a G-protein coupled receptor that can bind many chemokines including, CCL5, MCP-1, MCP-3, MIP1-α, MIP1-β and fractalkine. Binding of these chemokines to US28 results in their internalization and it has been suggested that US28 functions as a "chemokine sink", reducing the local concentration of these inflammatory and chemotactic cytokines during lytic infection (28). A splice product of UL111A is expressed during latency and encodes a viral IL-10 homologue (LAvIL-10) which acts to downregulate MHC Class II expression on experimental latently infected myeloid cells modulating $CD4^+$ T cell recognition (10, 29, 30). UL144 has sequence similarity with members of the tumour necrosis factor receptor superfamily (TNFRSF) (31-33) as well as the herpes simplex virus entry mediator (HVEM). UL144 has two putative immune evasion functions; the ecto-domain has been shown to interact with B and T lymphocyte attenuator (BTLA) inhibiting T cell proliferation *in vitro* (34) and the intracellular domain signals via NFκB, TRAF6 and TRIM23 to induce production of the chemokine CCL22 which acts as a Th2 type chemoattractant, possibly subverting the Th1 immune response (35-37).

Analysis of the secretome of cellular proteins produced by experimental latently infected $CD34^+$ progenitor cells has identified a number of proteins, including CCL8, cIL-10 and TGF-β, which are involved in immune response regulation and chemotaxis (38). CCL8 recruits $CD4^+$ T cells to latently infected cells, where the $CD4^+$ T cell encounters the substantial levels of the immune suppressive cytokines cIL-10 and TGF-β produced by the infected cell. IL-10 and TGF-β inhibit anti-viral IFN-γ and TNF-α cytokine secretion and cytotoxic effector functions of HCMV-specific Th1 CD4+ T cells. In addition, uninfected bystander $CD34^+$ cells were induced to secrete TGF-β and cIL-10 by the latent CMV infected cell secretome. This evidence strongly suggests that the microenvironment around latently infected CD34+ cells is immunosuppressive to T cell function.

Viral proteins expressed during latency have important roles in maintaining latency and preventing T cell recognition. As such are they themselves targets for T cells? LUNA, UL138, US28 and LAvIL-10 have been shown to be recognized by T cells, however, the predominant response was mediated by $CD4^+$ T cells, many of which were not classic

IFNγ Th1 cells but secrete the immunomodulatory cytokines cIL-10 and TGF-β (39). In MCMV infection, IL-10 producing $CD4^+$ T cells have been isolated from salivary glands and in IL-10 KO mice or following IL-10R blockage the latent MCMV load is reduced. This is consistent with the view that cytomegalovirus uses regulatory T cells to prevent latently infected cells from being recognised by the immune system (40, 41). Chris Benedict describes later in this review the current evidence for the immunomodulatory role of the IL-10 axis and its impact on $CD4^+$ T cell responses in MCMV infections.

Evidence for CMV manipulation of the immune system with ageing

As humans age there are alterations to the immune response that can be potentially detrimental to health. For example, there is evidence from many studies that people aged over 65 years are more susceptible to infectious diseases [reviewed in (42, 43)]. However in the healthy elderly overt disease caused by CMV is rarely seen (44), despite the dual manipulation of the immune response by the virus and the effects of natural ageing. There is some limited evidence in humans that modulation of the immune response by HCMV in the aged is occurring, potentially resulting in less effective control of viral replication following reactivation of virus. This hypothesis was suggested by the following observations; (i) CMV DNA was detectable in the urine of old but not young donors (44), (ii) an increase in viral DNA has been detected in the blood of older donors in a Japanese cohort (45) and (iii) a UK study detected a significant increase in latent CMV genomes (in peripheral blood $CD14^+$ monocytes) in people aged over 70 years (46). To counteract this evidence there have been studies of aged CMV positive people, which have been unable to detect viral DNA in the blood (44, 47). Wills and Jackson present in this review some experimental evidence, which suggests there may indeed be a slight loss of control of viral replication in the aged compared to younger individuals.

There is however evidence that CMV infection might be altering the balance of immune responses in ageing, from the strong association of CMV sero-positivity with increased mortality from cardiovascular disease (48-52). It has been proposed that the association of CMV infection with cardiovascular disease may be a direct result of pathology caused by the large expansion of $CD4^+$ CD28 negative T cell populations (53) commonly seen in CMV infected individuals (54).

As previously discussed HCMV infection modulates the hosts IL-10 pathway, both directly through expression of viral encoded IL-10 homologs (55) and indirectly by altering the secretome of the infected cell, in addition to generating cellular IL-10 secreting CMV specific $CD4^+$ T cells. The production of cellular IL-10 in response to HCMV antigenic stimulation by $CD4^+$ T cells has been observed in multiple studies (39, 56-58). Production of IL-10 is a characteristic feature of regulatory T cells (Tregs) (59). CMV specific Tregs in humans have been identified in a number of studies using various methods of Treg phenotyping (39, 56, 58, 60-62). The Schwele et al study demonstrated that the gen-

eration of CMV specific inducible Tregs (iTregs) is most likely to be related to frequent episodes of antigenic reactivation (58), a further method that the virus can use to modulate T cell populations. Suppressive CMV-specific iTregs have also been identified in the expanded $CD4^+$ $CD28^-CD27^-$ population (60) and their frequency increased with age and was associated with vascular pathology along with expanded CMV specific $CD8^+$ effector T cell populations (61). In the murine model it has been demonstrated that IL-10 secretion and Tregs reduce clearance of the virus and increase persistence (56, 63). This phenomenon has been demonstrated with CD4+ T cells resident in the salivary glands (41) and the liver (64).

While clinical studies in humans have allowed for important insights into pathologies associated with HCMV persistence, by their very nature they cannot define if CMV is the cause of (i) pathology (ii) a consequence of underlying predispositions for associated pathologies or (iii) merely an accidental and adventitious correlation. Defining cause-effect relationships in HCMV immunology requires an experimental method and such studies are hindered by the strict species specificity of this virus. Therefore, studies in animal experimental models have relied on CMV orthologue viruses in their natural host species. Whilst data from animal models are not necessarily a direct representation of natural events, this caveat holds for any dataset generated in a model, whether animal, experimental or mathematical.

Using the mouse model, it has been shown that CMV infection results in memory inflation of T cell populations specific to particular MCMV protein epitopes (65) and is another example of the impact CMV infection can have on host immune responses over a lifetime. Despite the dominance of a restricted clonal MCMV specific T cell populations as a proportion of total T cell compartment (65) it has been clearly shown that the expanded memory T cells are still functional and dynamic (66). These cells can be replenished to similar expanded levels following depletion (67) and the inflation of this population can occur, although less pronounced, in the absence of reactivating or replicating virus (68). The lessons that can be learnt from the manipulation of the immune response by MCMV infection in the mouse and its impact on aged T cell responses are discussed later in this review by Ramon Arens & Anke Redeker and Luka Cicin-Sain.

Previously it has been suggested that the expansion of CMV specific $CD8^+$ and $CD4^+$ T cells that has been reported in some human ageing studies is also evidence of "memory inflation" within HCMV infection (studies reviewed in (54). However unlike experiments in the murine model most of the evidence for "memory inflation" in humans comes from cross-sectional age studies (69), rather than longitudinal studies tracking the size of CMV specific $CD8^+$ and $CD4^+$ T cells within particular individuals over many years. It has been reported in individuals that as much as 10% of the total $CD8^+$ T cell

compartment (70) and 5% of the total CD4+ T cell compartment (71) are composed of CMV specific responses. The expansion in size of the CMV specific T cell response in humans is at the expense of T cell responses to other antigens. This hypothesis has been used previously to explain the observations from a number of studies where there is an increase in all-cause mortality and susceptibility to new infections in elderly CMV sero-positive donors (72-76). However there have been a number of studies that dispute this hypothesis. It has been shown that poor responses to vaccines in older people is irrespective of CMV sero-positivity (77) and the ability of older people to respond to novel infections is not impaired by an existing CMV infection (78, 79). Debbie Van Baarle and Sara van den Berg discuss the impact of CMV infection and consequential immune modulation on the ability to respond to neo-antigens in old age further later in this review.

CURRENT RESEARCH PERSPECTIVES

The impact of aging on IL-10 secreting HCMV latent antigen specific T cells and latent viral load (Sarah Jackson and Mark Wills)

Human cytomegalovirus (HCMV) infection and the periodic re-activation of latent virus is generally well controlled by T cell responses in healthy people (1, 80). Within older donors although overt disease caused by HCMV is rarely seen (44), there is some evidence discussed in the introduction, that suggests there may be immunomodulation of the immune response in the elderly, potentially resulting in low level viral replication and dissemination without causing overt disease. Latent CMV infection is characterised by limited viral transcription, with only a handful of viral genes, (e.g. UL138, LUNA, US28, UL111A and UL144), being transcribed (27). Previously, we have reported that there are IFNγ CD4+ T cell responses to UL138 and LUNA proteins that are cytotoxic and there are also cellular IL-10 responses by CD4+ T cells in response to these latent associated proteins (39). Therefore, IL-10 is a candidate to mediate immunomodulation of the CMV specific immune response during ageing (81). To address the question of whether long term carriage of HCMV changes the proportions of IL-10 and IFNγ secreting HCMV specific T cells populations, Jackson and Wills recruited a large cohort of CMV sero-positive donors aged 20 – 80 years in association with the Cambridge Bioresource. We correlated the CD8+ and CD4+ T cell responses to 11 HCMV proteins (5 latent ORFs and pp65, pp71, gB, IE1, IE2 and US3) with age, HCMV IgG levels, latent HCMV load in CD14+ monocytes, lytic HCMV DNA copies in whole blood and absolute T cell population counts from whole blood.

The recruited donor cohort had a significant decline in total and naïve CD8+ and CD4+ T cells numbers, which has been reported previously in different ageing studies (sum-

marised in (54)). The impact of CMV infection resulted in increased numbers of differentiated $CD8^+$ and $CD4^+$ T cell populations, which has often been described (54), however this phenomenon was irrespective of donor age in this cohort. Both $CD4^+$ and $CD8^+$ T cells responded to stimulation with all 11 HCMV proteins, there was no accumulation of specific IFN-γ T cell responses with increasing age; the $CD4^+$ T cell IL-10 response was less frequently observed, however overall the breadth and magnitude of the IL-10 response to HCMV proteins remains stable regardless of donor age. IL-10 secreted $CD4^+$ T cell responses were predominantly to Latency-associated proteins (LUNA, UL138, US28 and vIL-10); although the lytic-expressed proteins pp71 and US3 also triggered a number of donors to produce a $CD4^+$ T cell specific IL-10 response. Within this cohort CMV IgG levels remained stable and there was no inversion in the CD4:CD8 ratio with increasing donor age. Measurement of HCMV viral copy numbers in $CD14^+$ monocytes, a known cellular site of latent CMV carriage (82), did not reveal any increase in viral levels in older donors, in contrast to a previous UK study (46). Importantly however, we did see a significant correlation between increased latent viral copies and the breadth and magnitude of the IFN-γ $CD8^+$ T cell response. Our hypothesis is that a larger latent HCMV reservoir may result in more frequent HCMV re-activation and dissemination events, which consequently lead to expansion of CMV specific T cell responses (and potentially re-seeding of the latent pool). We did not detect HCMV DNA in the blood of 104/105 HCMV sero-positive donors included in the study; interestingly the one donor who did have low levels of detectable HCMV DNA (274 copies/ml whole blood) was an aged male donor who had an inverted CD4:CD8 ratio and an above average number of highly differentiated $CD8^+$ T cells. This individual result is supportive of the hypothesis discussed in the introductory section, i.e. the elderly may not be as able to control CMV replication as adequately as the young, possibly due to manipulation of the immune response by the virus. This donor cohort was recruited on the basis of HCMV sero-status and those suffering from immune altering illnesses or treatment for these conditions (e.g. cancer, autoimmune disorders, and systemic steroid treatment) were excluded from the study. This stringent recruitment criterion may have resulted in less healthy older donors, who may have lost control of CMV replication to some extent resulting in low level viral dissemination, not being included in our study and potentially skewing our observations (83).

It is also important to assess the functional capacity of the CMV specific T cells in response to the virus directly; again this approach may reveal defects in the immune response to CMV in the elderly which are not apparent in other population studies. In order to address this problem, we have developed a viral dissemination assay which we have used to interrogate the capacity of HCMV specific $CD8^+$ (84) and $CD4^+$ T cells (57) to control the spread of HCMV *in vitro*. We have demonstrated that purified $CD8^+$ and $CD4^+$ T cell populations when isolated directly *ex vivo* are able to prevent the spread

of the a GFP tagged clinical CMV isolate. To date, we have only been able to use this methodology with a limited number of donors aged over 65 years, however in the $CD8^+$ T cell study the evidence we do have suggests that the older donors were capable of controlling viral spread, but less efficiently compared to younger donors (84). This observation is also supported by our $CD4^+$ T cell work, where the older donor included in the study demonstrated a distinct loss of control of viral dissemination at low T cell to infected cell ratios, which was not seen in the younger donors (83). These preliminary results suggest that the viral dissemination assay will enable a more comprehensive insight in to understanding if there is a diminution in the effectiveness of CMV specific T cells in the elderly in the context of an active viral infection, where the full armoury of the virus immune evasion and manipulation mechanisms are expressed.

Fibroblast-adapted HCMV vaccines elicit conventional $CD8^+$ T cell responses in contrast to RhCMV vaccines (Ann Hill)

Studies using CMV as a vaccine vector in the monkey model of AIDS have uncovered a highly unusual aspect of CMV immunology. After serial passage in vitro, HCMV isolates become adapted to the fibroblasts they are grown in by losing the ability to form a functional pentameric complex of glycoproteins that is needed for entry into most non-fibroblast cells. When Louis Picker and colleagues developed their rhesus (Rh) CMV vaccine vector, they used Peter Barry's RhCMV BAC, which was derived from a fibroblast-adapted RhCMV (strain 68.1), and was pentameric complex-defective (85, 86). RhCMV expressing proteins from simian immunodeficiency virus (SIV) has provided impressive protection from a virulent SIV challenge, and the strategy is being very actively pursued to develop a vaccine for human AIDS (87). Studies to elucidate the immunological mechanism of the RhCMV-vectored vaccine's efficacy have yielded startling results: most of the antigen-specific $CD8^+$ T cells elicited by the vaccine are completely unconventional, being restricted either by MHC II- or the rhesus equivalent of HLA-E (88). Further studies showed that the unconventional responses were only elicited by RhCMV lacking a functional pentameric complex: restoring the pentameric complex led to the generation of conventional, i.e. classical MHC1-restricted, responses. These unconventional responses are believed to be responsible for the vaccine's efficacy against SIV. If that is the case, it is important to know whether the strategy could be translated into a human vaccine.

CMV's strict species specificity means that translating the monkey results to humans requires a shift in not only SIV to HIV antigens, but also of the vector, from RhCMV to HCMV. Additionally, rhesus and human immune systems have some differences. Most remarkable is the rhesus MHC complex, which is extremely polymorphic and polygenic. Up to 22 separate classical MHC I isoforms are simultaneously expressed, contrasting with up to 6 in humans or mice (89). The MHCII loci are similarly polygenic

and polymorphic in monkeys. It could be that the unconventional responses to RhCMV result from a distorted $CD8^+$ T cell repertoire that has arisen because of excessive negative selection by this highly diverse MHC. However, it should be noted that all other T cell responses studied in macaques have been conventional, including those elicited by wildtype, i.e. pentameric complex sufficient, RhCMV. Hence, it is likely that immune system manipulation by CMV itself is responsible for these responses, and that they would also be seen in humans.

As a first attempt to look at this question, we studied the CD8+ T cell responses to fibroblast-adapted HCMV vaccines (90). Stuart Adler's group had conducted a dose escalation phase I clinical study of 4 vaccines that are chimeras between Towne and Toledo strains (91). All share the same defect in the pentameric complex genes, and have impaired cellular tropism in vitro for epithelial, endothelial and macrophage cells. We were able to study the CD8+ T cell responses in 6 subjects. All subjects responded to HCMV IE1, and the typical pattern of immunodominant responses to 1 to 2 epitopes was seen. This contrasts with the responses reported in monkeys, which responded to an average of 36 epitopes in RhCMV IE1. Most responses in the human subjects were mapped to a minimal epitope, and HLA restriction was mapped for 8 epitopes. Each epitope that could be mapped was conventionally MHC I restricted, and two subjects responded to previously identified HCMV epitopes. Antibody blockade and use of HLA-E transfectants provided no evidence to suggest that the remaining responses were unconventional.

The response to these pentameric complex deficient HCMV vaccines thus appears to be predominantly or completely conventional in human subjects. This could be a result of differences in human and monkey immune systems. However, it should be noted that although both are pentameric complex deficient, the Towne-Toledo chimeras and RhCMV 68-1 differ in the genetic mutations that result in the functional deficiency. It will be important to understand the viral mechanism for eliciting the unconventional responses to have the best chance of eliciting the same responses in humans, should they prove to be important for the vaccine's efficacy.

The effect of CMV on the response to influenza virus vaccination (Debbie Van Baarle and Sara van den Berg)

Immunosenescence is the age-related deterioration of immunocompetence which is reflected in a poorer response to (new) antigens and leads to increased susceptibility of elderly to infection and lower response to vaccination. HCMV infection has been shown to enhance deleterious age-associated changes in immunity and may thereby contribute to poor responses to vaccinations. Indeed it has been shown that HCMV is part of the Immune Risk Profile (IRP), which is associated with all cause death (73). Based on a sys-

tematic review of studies performed between 2004 and 2014 on the role of HCMV persistence on T-cell immunosenescence in people aged fifty and older, we observed that CMV mainly seems to enhance immunosenescence through increasing the levels of the highly differentiated Effector Memory (TEM) and CD45RA expressing effector memory (TEMRA) cells in the $CD8^+$ and $CD4^+$ T cell pools (54). Although an elegant study (92) suggested also a decline in $CD4^+$ T cells in HCMV-positive individuals. Furthermore, CMV-infection was also shown to be associated with intrinsic B cell defects (93). How these changes may affect responses to other infections or vaccinations and whether these shifts within the T-cell compartments in HCMV-seropositive elderly are related to susceptibility to infectious diseases remains to be fully investigated.

In humans, in whom the effect of HCMV has especially been studied in the context of influenza virus vaccination which is shown to lead to less protection in older individuals, the data is less clear (see box/figure 1). Four studies suggest a negative effect of HCMV to antibody responses to flu vaccination in elderly (93-96). This negative effect of HCMV was also found to be present in young individuals in 2 additional publications (97, 98). However, two studies observed no effect of HCMV on influenza antibody responses in elderly (99, 100), one of which observed a positive correlation between IgG HCMV levels and the influenza antibody titer, suggesting a beneficial effect of HCMV. Moreover, a recent paper by Furman et al observed an enhancing effect of HCMV on the antibody response to influenza in young, but not in old individuals (77). These discrepancies in the literature may result from differences in pre-existing immunity to the seasonal influenza vaccination in participants. We hypothesize therefore, as shown in HCMV models in mice (101, 102), that CMV infection will have a larger impact on/impair the antibody response to neo-antigen influenza vaccinations.

In our study, focusing on middle aged individuals (n=287), of whom 60% were HCMV-seropositive and who were vaccinated with the novel pandemic influenza A strain (pH1N1) vaccine during the 2009 influenza pandemic, we found no effect of HCMV infection on antibody response to influenza vaccination, despite the fact that we do observe an age-related effect on influenza antibody responses in this group. (S. P. H. van den Berg, manuscript in preparation). As detectable titres to the novel pandemic influenza strain were present before vaccination in 23% of the individuals, this still may have contributed to the lack of effect of CMV on influenza antibody responses. However, correction for pre-titers and other potential confounding factors like age, sex and previous vaccination did not change the observed result. Furthermore, our data may even suggest a beneficial/positive effect of CMV infection on the protection rate after influenza vaccination in these adults. These data add to the discussion on the role of CMV infection on vaccination responses and suggests a scenario in which CMV infection may be beneficial during the first decades of life, but this effect may be lost upon ageing.

In mouse models of MCMV infection in which MCMV infected mice are challenged with neo-antigens, data are clear and specific defects in immunity to newly incoming pathogens have been reported leading to lower protection against these new infections (see Ramon Arens and Anke Redeker below). In these studies MCMV was shown to have effect mainly on the T cell compartment but effects on the antibody response were also observed. Prior MCMV infection was shown to result in significantly reduced (101) or remodeled (103) $CD8^+$ T cell responses to challenging infections and elevated viral loads (102). As HCMV mainly impacts the T cell repertoire and the T cell response plays a bigger role in cross-reactive immunity towards different influenza strains as well as vaccine-induced protection in later life, the question is whether we can find evidence for decreased cellular immune responses and whether previous HCMV infection would affect immune protection after vaccination in humans of different ages. A recent study shed first light on this by showing that CMV sero-positivity was associated with a decline in Granzyme B responses to influenza and may predict increased susceptibility to influenza in older adults (100). In contrast, Theeten et al show a positive relation between cellular IFN-γ and Granzyme B responses to CMV-pp65 antigen and influenza N1 antigen (104). Thus, with regard to the effect of CMV on cellular immune responses to influenza in humans the jury is still out.

Impact of the infectious dose of CMV on the severity of immune senescence (Ramon Arens and Anke Redeker)

The controversies with respect to the role of CMV in immunosenescence including the impact of CMV infection on vaccines and encountering other pathogens as discussed by Van Baarle and van den Berg above, might relate to the differences in the immunological response rate to HCMV, which varies significantly. In the human population the percentages of CMV-specific T cells occupying the memory T cell compartment is highly variable (ranges from 0.01 – 50%) (105). The average HCMV-specific memory T cell response is ~10% of the total memory T cell compartment, and is compared to other infections, extraordinarily high (70). In old age it has been estimated that the HCMV-specific memory T cell pool can occupy up to 50% of the total memory compartment (106). The high variation in the T cell response to CMV is likely related to the significant difference in the quantity of CMV found in bodily fluids such as breast milk, saliva and urine (10^1 - 10^5 copies/µl), causing horizontal transmission of CMV (107, 108) .

The mouse studies that have been performed previously, wherein the impact of long-term CMV infection on host immunity was investigated (101-103), showed a relationship between CMV positivity and immune senescence. Specifically, these studies demonstrated that long-term latent CMV-infection induces high numbers of effector-type like memory $CD8^+$ T cells reactive to CMV, while newly generated $CD8^+$ T cell responses to

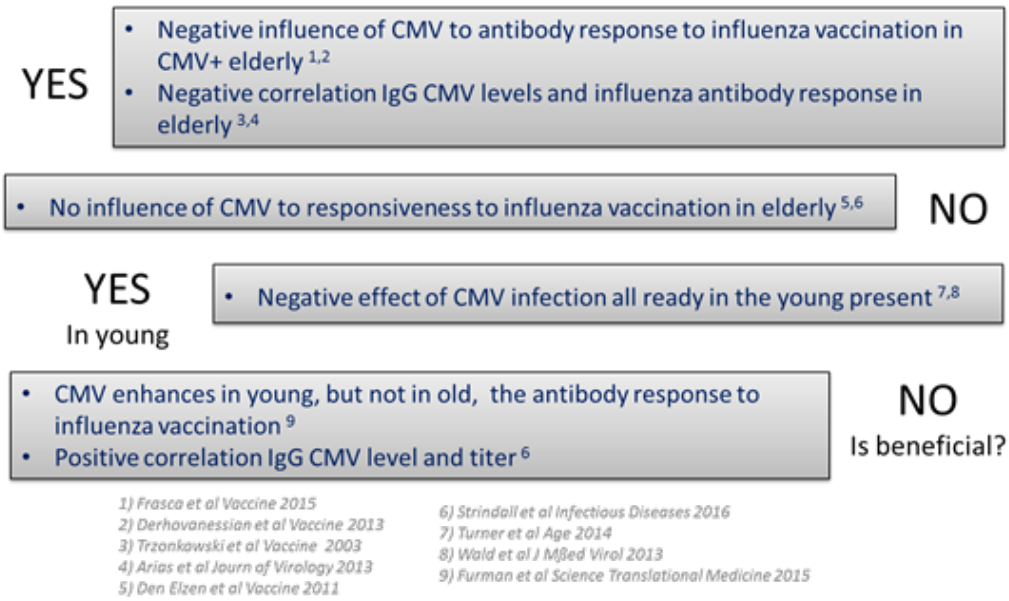

Figure 1: Overview of studies performed and their findings on the role of CMV-infection on the immune/ antibody response to influenza vaccination.
Overview of (77, 93, 95-99, 146, 147).

heterologous viral infections were diminished. However, this was not followed by an increase in mortality from heterologous viral infection (109). This is in contrast with the controversial data observed in humans. However, these mouse studies used relatively high doses of virus for infection leading to high CMV-specific T cell responses. Hence, the diversity in the HCMV-specific immune response as seen in humans was not taken into account. Previously, we have shown that the degree of accumulation and phenotype of inflationary CMV-specific $CD8^+$ T cells corresponds to the size of the initial infectious dose (110). To gain insight into the controversial results with respect to the possible contribution of CMV to immune senescence in humans, and whether this is related to the large variance in the frequency, phenotype and accumulation of CMV-specific memory T cells, we performed a highly controlled prospective study. We infected mice with different inoculum dosages of MCMV and investigated longitudinally the influence of life-long CMV infection on alterations within the peripheral T cell pool. Our results show that the initial viral inoculum determines the degree of CMV-induced immune alterations in life-long infection. A heightened MCMV infection resulted in reduced frequencies of naive circulating $CD8^+$ T cells ($CD44^{low}CD62L^+KLRG1^-$), and on the other hand, increased the accumulation of effector-memory (EM; $CD44^{high}CD62L^-KLRG1^+$) $CD8^+$ T cells. A remarkable correlation becomes apparent between the magnitude of the CMV-specific $CD8^+$ T cell response and the EM-phenotype of these cells. Basically, the higher the magnitude of response, the higher the frequency of the EM-like $CD8^+$ T cells. Importantly, these correlations between the CMV specific $CD8^+$ T cell response

and memory phenotype, were also apparent in CMV infected humans.

To specifically assess the role of the CMV inoculum size on the development of heterologous anti-viral immunity, aged MCMV-infected mice received a challenge with lymphocytic choriomeningitis virus (LCMV). Strikingly, mainly in the mice that were infected with the highest dose of MCMV, the LCMV-specific $CD8^+$ T cell expansion was impaired. The latter correlated with a reduced activation status of the LCMV-specific $CD8^+$ T cells, and importantly the control of LCMV infection was hampered. This detrimental effect of high dose infection was not observed in young mice, where even a positive effect was apparent. Thus, only in the high dose infected aged mouse the control of heterologous infection was found to be impaired. This suggests that predominantly in aged individuals with pronounced HCMV-induced pertubations, detrimental effects for these persons can be contributed to CMV while in persons with low HCMV-specific responses negligible effects of CMV are present. Instead, in young persons, HCMV may even have beneficial effects. To settle these statements additional prospective longitudinal studies are necessary that incorporate stratification based on the quantity and quality of the HCMV response in each individual.

CD4 T cell control of CMV persistence during initial infection: How does it impact viral-shaping of the immune system over a lifetime? (Chris Benedict)

As discussed above, HCMV-specific $CD4^+$ T cells can secrete a variety of cytokines, producing some more frequently when they encounter their cognate antigens expressed by latently infected cells (e.g. IL-10). However, it is very challenging to assemble cohorts to assess T cell effector and memory responses during primary HCMV infection, given it is largely asymptomatic in healthy people. Therefore, most studies have measured the function and phenotype of these cells in persons already infected for many years/decades, long after the acute and high-level persistent replication phases of infection have been controlled. As the mouse provides a tractable model to study all 3 phases of CMV infection (acute, persistence and latency), and T cells induced by MCMV show similar phenotypes and functions to that of HCMV, we set out several years ago to identify the first MCMV epitope-specific $CD4^+$ T cells, characterizing fifteen in C57BL/6 (B6) mice (I-Ab restricted) (111) and two in BALB/c (I-Ad restricted) (112). Our recent construction of several MHC-II tetramers has allowed the enrichment and characterization of these cells from lymphoid and non-lymphoid tissues, a necessity given they are present at markedly lower numbers than their $CD8^+$ T cell counterparts (113).

There is no identifiable IL-10 sequence orthologue encoded by MCMV, unlike the primate CMVs, however various host immune cell types produce IL-10 during both the acute and persistent phases of infection. $CD4^+$ T cells secrete the majority of this IL-10 during MCMV persistence in the salivary glands of B6 mice (56), where high-level viral

replication continues for several months. Studies performed a decade ago showed that signalling by the IL-10 receptor sustains MCMV replication at this mucosal site (41) and restricts the expansion of $CD8^+$ and $CD4^+$ T effector cells (40), with a similar role also reported for RhCMV vIL-10 in monkeys (114). In turn, $IFN\gamma^+$ $CD4^+$ Th1-like cells play a key role in the eventual resolution of MCMV persistence (115, 116), while their production of TNF appears unnecessary for this (116, 117). Consequently, the relative numbers of $CD4^+$ T effector and memory cells that produce IFNγ and IL-10 during the various phases of CMV infection likely form an axis that regulates the magnitude, duration and reactivation of CMV in both mice and men.

It has been shown that the ability to mounting a robust, $IFN\gamma^+$ secreting HCMV-specific $CD4^+$ T cell response, correlates with reduced duration of persistent CMV replication in the kidneys of young children (118). Initial MCMV infection induces a strong IFNγ+ $CD4^+$ T cell response, and these cells are present in the salivary gland within one week. However, they are not sufficient to resolve viral replication at this time of 'early-persistence', due at least in part to the high levels of IL-10 that are commensurately produced. Intriguingly, our past studies identifying MCMV epitope-specific $CD4^+$ T cells found that some responses did not expand until much later times following infection, after systemic replication was controlled and persistence had been established. It is interesting to postulate that these 'late-rising' $CD4^+$ T cells might display distinct anti-viral effector functions during viral persistence compared to their conventional counterparts, as they would likely differentiate in a unique inflammatory environment. In turn, it is possible that the duration of CMV persistence prior to latency establishment may influence the viral impact on immune aging. For example, a longer persistent replication phase may result in a higher initial 'set-point' of CMV-specific cellular responses and latent virus load, with these differences being amplified over a subsequent lifetime. Perhaps this contributes to the dose-dependent impact of initial MCMV infection on the T cell compartment in old mice described by Arens and Redeker. Finally, how these two distinct populations of conventional and late-rising $CD4^+$ T cells induced by MCMV might equate to the HCMV-specific $CD4^+$ T memory cells seen by Wills and colleagues that preferentially recognize viral antigens expressed during the lytic and latent phase of infection is unclear, if they do at all, but it is an intriguing question.

Immune evasion of memory inflation in the murine model (Luka Čičin-Šain)

The mouse model (MCMV) reflects the balance between the latent virus and the host immune system, where a hierarchical and redundant cellular immune response keeps CMV from reactivating (119). The continuous low-level transcription of viral genes (120) results in the persistence of T-cell infiltrates in the lungs of latently infected mice (121), where immunodominant effector or effector-memory T-cells against epitopes transcribed in latency prevail (122). The expansion and persistence of T-cells against

immunodominant CMV-antigens has been aptly named memory inflation (65) and it was shown that it reflects T-cell responses to HCMV (69). Importantly, memory inflation is an expansion of effector T-cells against defined MCMV-specific epitopes, but the size of the effector T-cell pool remains unaltered (101), implying that memory inflation is a focusing of $CD8^+$ T cell responses on few immunodominant epitopes at the expense of subdominant ones, rather than a general expansion of T-cell responses to cytomegalovirus. The size of the inflationary response depends on the latent transcriptional activity of the viral genes encoding the epitope (123) and on its availability for processing by the constitutive proteasome (124, 125), implying that $CD8^+$ T cell re-stimulation may occur by antigens presented directly on latently infected cells. In fact, the expansion of MCMV-specific $CD8^+$ T cells in memory inflation requires antigen presentation on non-hematopoietic cells (126, 127), likely from within the latently infected cell. It has remained somewhat controversial if this phenomenon depends on the viral evasion of the immune system, or if it occurred independently of it.

Akin to the HCMV situation, MCMV devotes a substantial part of its genome to the evasion of the immune system. It has developed multiple strategies to downregulate MHC-I molecules from the surface of infected cells and thus avoid detection by $CD8^+$ T-cells (128). However, an MCMV mutant lacking all viral genes known to interfere with MHC-I surface presentation induced similar hierarchies of $CD8^+$ T cell immunodominance as the wild-type MCMV, both in primary and in latent infection (129). This observation might be anticipated if one considers that the known MHC-I downregulators m04, m06 and m152 are lytic viral genes that are unlikely to be expressed during virus latency. Surprisingly, another study showed that the MCMV lacking these three genes induces a weaker $CD8^+$ T cell response (130), arguing for a paradoxically improved priming and inflation state in the presence of immune evasion. The latter study showed that immune evasive genes protect the virus from the antiviral activity of CD8+ T-cells, allowing it to proliferate longer and to higher titers in draining lymph nodes during primary infection (130). Therefore, it was proposed that immune evasion enhances viral replication, antigen availability for priming and consequently the size of the inflationary response. The conditions of primary infection and inoculum define the size of the latent reservoir (131) and of the inflationary response (110). In particular, the latent MCMV burden in the spleen, but not in in the lungs, is associated with a more pronounced inflationary response to MCMV (132). Consequently, by improving viral fitness in the primary infection and by increasing the number of cells carrying latent viral genomes, immune evasive genes may indirectly affect the size of the inflationary response, although they are not expressed at the time of latency and T-cell expansion.

This does not exclude possible additional effects of viral genes at the time of latency. The CMV genome is the largest known among mammalian viruses and the function of nu-

merous viral genes remains unknown (133). Beyond the viral genes that downregulate MHC-I from cell surface, CMV expresses numerous additional genes which may interfere with $CD8^+$ T cell priming, for instance those interfering with costimulatory signals 2 or 3 (134, 135). Therefore, it is likely that numerous additional genes interfere with the immune response and it cannot be excluded that their activity may influence the size and the quality of the inflationary T-cell response. A shotgun approach to the study of immune evasion by targeted deletion of large genomic regions rich in immune evasive genes yielded an MCMV variant lacking 34 viral genes in regions rich in immune evasive genes (136). While this mutant induced inflationary responses that could not be distinguished from wild-type MCMV in size (136), it cannot be excluded that other viral genes – including the essential ones – may affect the T-cell responses. Therefore, future studies in models of *in vivo* infection may inform us if immune evasion acts actively during viral latency and if it defines the size and the functionality of the responding T-cell pool.

FUTURE PERSPECTIVES

The preceding summaries of current research work in both humans and mouse models go some way to assessing the impact that CMV infection and viral immune evasion may have on the immune response to CMV and the immune response more generally. However, there are still many unanswered questions, and the real impact of CMV manipulation of the immune response in the elderly remains to be fully ascertained. We propose that future studies may wish to consider the following issues:

To what extent is the association of CMV infection with detrimental changes to the immune response in ageing a co-incidence or a real phenomenon, and, if so, is the virus the direct causative agent or are the changes to the immune response a bystander effect of CMV infection? Whilst this question is not straightforward to address in humans, due to the many unknowns in the CMV infection history of an individual subject, designing clearly defined population studies which measure multiple parameters, (e.g. pertinent medical history, socio-economic background, country or region of birth, absolute immune cell counts, CMV specific immune responses and quality/quantity of these – both T cell and immunoglobulins, CMV DNA quantification), will help going forward. Additionally, the adoption of similar strategies and measurements in geographically distinct donor cohorts will enable comprehensive intra-study comparison, thus increasing the overall power of the conclusions that can be derived.

Is CMV infection an important co-morbidity factor in human ageing? To address this question will require comparison of the impact of CMV infection in less healthy elderly donor cohorts, e.g. does CMV infection in donors with cardiovascular related health

problems lead to less control viral reactivation and dissemination, compared to age matched healthy people. This question could be asked in a number of different disease cohorts, however in cases of cancer or auto-immune diseases (e.g. Rheumatoid Arthritis) the impact of these conditions on the immune response will have to be accounted for when interpreting the results.

Does CMV mediated modulation of the immune response in the elderly result in low level viral reactivation and dissemination, that is below the level required to present as overt clinical disease but nevertheless may drive inflammatory pathologies? This will require that future studies incorporate a virological assessment of subjects, quantification of virions present (or not) in blood, saliva and urine alongside measuring immunological parameters could be highly informative.

As illustrated in this review the use of the Mouse model has been very important in understanding the multiple consequences of CMV infection, immune evasion and manipulation on the host immune response. However, this does not replace the need for studies to be performed in humans, particularly in the context of immune ageing research. Extrapolating results from murine ageing studies to humans requires consideration of a number of key differences; (i) the contribution from thymic output replenishing the peripheral T cell compartment is higher in mice than men (137), (ii) mouse telomeres are proportionally longer than humans, therefore it is less likely that immune cells from aged mice will exhibit a telomere mediated replicative senescence phenotype compared to humans (138) and (iii) are genetically similar mouse breeds sterile housed for 600 days or more equivalent to human subjects with wide genetic variability and decades of exposure to multiple antigens from many different environments. These important differences between mice and man have led to the use of longer lived animal models, including baboons (139) and the rhesus macaque model (140, 141), to interrogate the interaction of CMV and ageing. Again, there can be issues with cross-species comparisons, as discussed in this paper by Ann Hill, the T cell responses generated to vaccines for RhCMV and HCMV in the respective hosts, show distinct differences in the breadth and type of $CD8^+$ T cell responses produced.

In order to determine if the effect of CMV infection on global immune responses in ageing is truly detrimental and requiring of medical intervention in older people, will require far stronger evidence in order to convince the wider medical community of its need. For many physicians, CMV infection is not perceived as a major problem, because unless the patient is immune compromised or immune naïve, CMV mediated disease is not commonly observed. Therefore, to provide a truly convincing argument in the future will require studies which also consider whether ageing or putative life-long CMV infection alters the quality of the immune response to the virus and then consid-

ers whether there are consequence for overall health. Many of the historical population studies performed to interrogate whether CMV is detrimental in ageing in humans, have relied on comparing total immune cell phenotypes with CMV serology relied on as a measure of determining infection history. Reliance on CMV IgG/IgM serology to identify CMV infectious history may be problematic, as anecdotal evidence has shown that there are discrepancies in the results seen from CMV serology test kits from different manufacturers, and CMV specific T cells have been detected in CMV sero-negative donors.

Lastly, it is clear from murine studies that not only does CMV infection persist in different tissues, but also, the CMV specific T cell response that is resident in these tissues can have distinct phenotype and functions (112). Within humans, the peripheral blood provides a mere snapshot of the immune response, comprising only 2% of the bodies total lymphocyte numbers (142). A recent study has identified CMV specific $CD8^+$ T cells in many different tissue sites in humans (via a collaboration with an organ transplantation body), and the results further suggest that CMV specific T cells isolated from different tissues can also have distinct phenotype and functionality (143). Whilst it is not possible to access multiple tissue sites at one time in living human donors, there are possibilities through surgical collaborations to access immune cells from disparate tissue sites enabling comparison with immune cells isolated from peripheral blood. Studies have been published investigating the presence of CMV infection or CMV immune responses in the mucosal tissue (56), the lung via bronchoalveolar lavage fluid (144) and the bone marrow (145). Interrogating the function and quality of tissue resident CMV specific immune cells may help to inform our understanding of the frequency of CMV reactivation and dissemination and whether this is detrimental to an individual's health.

Overall, it is clear there are still many questions regarding the impact of CMV mediated immune evasion and manipulation of the immune system with ageing that remain incompletely answered and that require further research.

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CHAPTER 9

Murine cytomegalovirus infection via the intranasal route offers a robust model of immunity upon mucosal CMV infection

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ABSTRACT

Typically, during viral infections, T cells encounter antigen, undergo proliferative expansion and ultimately contract into a pool of memory cells. However, after infection with cytomegalovirus, a ubiquitous ß-herpesvirus, T cell populations specific for certain epitopes do not contract but instead are maintained and/or accumulate at high frequencies with a characteristic effector-memory phenotype. This feature has also been noted after other infections, for example, by parvoviruses. We discuss this so-called memory T cell inflation and the factors involved in this phenomenon. Also, we consider the potential therapeutic use of memory T cell inflation as a vaccine strategy and the associated implications for immune senescence.

INTRODUCTION

Cytomegalovirus (CMV) infection is the most common congenital viral infection in developed countries (1, 2). Congenital HCMV infections occur upon infection or reinfection of adult women during pregnancy (3, 4). Infection can also occur via blood transfusion and organ transplantation, which is especially problematic in immunosuppressed sero-negative recipients, where such an infection may cause severe disease. The exact natural route of transmission and entry into the body of most hosts infected with CMV post-partum remains, however, uncertain. While it is reasonable to assume that infection occurs via mucosal surfaces exposed to infectious secretions, the exact route(s) are difficult to identify by epidemiological studies. HCMV can be shed for months, if not years, upon infection by numerous body fluids (5), arguing that CMV may naturally transmit itself by more than one means of infection. Numerous studies identified that HCMV shedding by breast milk (6) may be a way of transmission (7, 8), especially in pre-term infants (9).

Sexual activity may present a risk factor for HCMV infection in adult women (10) and CMV infection is common in day-care workers attending very young children (11). However, multiple body fluids are likely to be simultaneously exchanged during close contacts that are required for infection, and the lack of pathognomonic symptoms complicates the epidemiological study of HCMV transmission in adults.

Experimental studies of transmission in animal models, on the other hand, are limited to HCMV-orthologue viruses, such as the rhesus CMV (RhCMV) or the murine CMV (MCMV), due to the strict species specificity of cytomegaloviruses. Nevertheless, the mouse model of CMV infection has provided deep insight in CMV pathogenesis and immunity and reflects numerous aspects of HCMV biology (12). Surprisingly, little is known about the natural route of infection by MCMV. While intranasal MCMV infection of adult mice has been occasionally used in previous reports (13-15), the only effort to systematically compare it to other routes of infection relied on low infection doses and was limited in its interpretation because of wide variations of outcomes between cages (16). A study in the model of neonate mouse infection has reported that a transgenic MCMV, expressing mCherry as a reporter gene may be found in the lungs of neonates infected by the laryngopharyngeal route and adult mice infected intranasally (i.n.) (17). This study used a molecular clone of MCMV lacking the viral protein Mck-2 (18), and a subsequent study by the same group showed that Mck-2 is a determinant of viral pathogenicity in lungs of neonate mice infected by the laryngopharyngeal route (19). Therefore, the replication and immune response to mucosal infections by a wild-type MCMV remain unknown.

On the other hand, the well-studied model of adult mouse infection has relied on systemic infection of mice by the intraperitoneal (i.p.) (20) or intravenous (i.v.) (21) route, or by injection of the virus into the footpad, assuming that this may mimic a putative

transmission by mouse bites (22). Parenteral infection results in MCMV dissemination to multiple organs during a primary viremia phase, and a secondary viremia delivering the virus to the salivary glands (22). This also results in a remarkably robust CD8 T cell response (23), where virus specific CD8 T cells accumulate at the time of virus latency (24), a phenomenon aptly named memory inflation (21, 25). Inflationary responses were shown to consist predominantly of $CD62L^-$ effector memory cells (21, 26), implying recent antigenic encounter, and similar types of responses were observed in natural and experimental rhesus monkey infection with RhCMV (27-29), or in people carrying latent HCMV (30). However, it has remained unclear if mucosal infection would elicit such responses.

To define the mucosal route reflecting the natural infection of an adult host, and whether this would result in CD8 T cell responses matching those upon systemic infection, we compared the kinetics of virus replication, the latent viral load, and CD8 T cell responses in mice infected by the intranasal, the gastric lavage (gavage) and the intraperitoneal route. We observed that i.n. infection results in robust virus replication during primary infection and secondary dissemination to salivary glands, as well as elevated latent loads in the lungs, whereas gavage infection was either abortive or resulted in poor virus replication and very low latent loads in the tested organs. Inflationary responses were less pronounced upon mucosal infection than upon the systemic one, but clearly noticeable in all tested compartments upon i.n. infection. Therefore, the data presented here suggest that intranasal MCMV infection may be a suitable model of infection of adult mice, and potentially a model of natural mucosal CMV infection.

MATERIALS AND METHODS

Mice

BALB/c mice were purchased from Janvier, 129/Sv mice were purchased from Charles River. Animals were kept in SPF conditions at the animal facility of the HZI Braunschweig. Housing and handling was performed in agreement with good animal practice defined by Federation of Laboratory Animal Science Associations (FELASA). All animal experiments were performed in accordance with the German animal protection law (TierSchG BGBI S. 1105; 25.05.1998) and were approved by the responsible state office (Lower Saxony State Office of Consumer Protection and Food Safety) under permit number 33.9-42502-04-10/0109.

Cells

The murine bone marrow stromal cell line M2-10B4 (CRL-1972) (31, 32) was purchased from ATCC and maintained in DMEM supplemented with 10% FCS, 1% glutamine and 1% penicillin/streptomycin. C57BL/6 murine embryonic fibroblasts (MEFs) were generated and cultured as previously described (22).

Virus

The BAC-derived mouse cytomegalovirus clone pSM3fr 3.3 (33) was used as $MCMV^{wt}$, it was propagated on M2-10B4 cells and titrated on MEFs as previously described (22).

Infection

Six- to ten-weeks-old female BALB/c or 129/Sv mice were infected with $2x10^5$ PFU $MCMV^{wt}$ using different infection routes. Virus diluted in 200µl PBS was i.p. injected. 20µl of virus solution (in PBS) were administered into both nostrils of mice anaesthetized with 10 µl/g bodyweight of ketamine (10 mg/ml) + xylazine (1 mg/ml) in 0.9% NaCl. We used ketamine, because isoflurane anaesthesia resulted in less efficient infection (detected by virus titers in the lungs on day 4 p.i. – Fig. S2). As a second mucosal route, mice were infected by gavage. Mice were fasted for 5 hrs and stomach acid was neutralized with 100µl of a 5% $NaHCO_3$ solution injected into the stomach by a feeder needle prior to infection with virus in 200µl PBS by the same route.

Determination of infectious virus in organs

Organs were sterilely harvested from infected mice and stored in DMEM at -70°C until titration. Thawed organs were homogenised on 70 µm-pore-size cell strainers in 5 mL DMEM (supplemented with 5% FBS, 1% glutamine and 1% penicillin/streptomycin) and homogenates were titrated on MEFs with centrifugal enhancement as described previously (34).

Blood and organ collection, processing and counting

Blood was collected from the retrobulbar venous plexus of isoflurane anaesthetized mice. 50µl of blood was transferred into EDTA reaction tubes and used for lymphocyte counting at the VetScan HM5 Hematocytometer (Abaxis). The remaining blood (approximately 100µl) was mixed with 300µl of heparin in HBSS (2U/ml) to prevent clotting. Erythrocytes were lysed and cell pellets were stained for flow-cytometric analysis.

Mediastinal and inguinal lymph nodes as well as spleens were harvested in ice cold RPMI (supplemented with 10% FBS, 1% glutamine, 1% penicillin/streptomycin, 1% sodium pyruvate and 0.1% β-mercaptoethanol) before further processing. Organs were homogenized on 70µm-pore-size cell strainers and pelleted by centrifugation. Lymph node samples were resuspended in 300 µl RPMI; 60 µl were used for counting of lymphocytes, 240 or 80 µl (detailed kinetic) were used for immunofluorescence staining. Erythrocytes were removed from spleen samples by 1 min incubation in 5 ml ACK buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 1 mM ETDA; pH = 7.3), which stopped by addition of 10 ml RPMI, centrifugation, a PBS wash and resuspension in 1 ml RPMI. 6 µl of each sample was mixed with 54 µl RPMI (1:10 dilution) for counting. Additionally, one sample of each group was counted using a Neubauer chamber and approximately 10^6 cells/sample were used for immunofluorescence staining.

Immunofluorescence staining and flow cytometry analysis

Cells were stained for surface markers and multimers recognizing MCMV-specific CD8 T cells. In this study, MHC-Streptamers recognizing T cells specific for $M38_{316-323}$ (SSPPMFRV) were used in case of samples from 129/Sv mice and a conventional tetramer binding T cells specific for $IE1/pp89_{168-176}$ (YPHFMPTNL) was used for samples from BALB/c mice. All multimers were generated in house as described previously (MHC-Streptamer (35); tetramer (36)).

MHC multimer staining was initiated for 15 min at 4°C; subsequently, cell surface marker antibodies were added and staining was continued for 30 min at 4°C. IE1-specific tetramer-APC was directly used with surface marker antibodies to stain cells for 30 min at 4°C. The following surface marker antibodies were used: αCD3-APC-eFluor 780 (clone 17A2; eBioscience), αCD4-Pacific blue (clone GK1.5; Biolegend), αCD8-PerCp-Cy5.5 (clone 53-6.7; BD Biosciences), αCD11a-PE-Cy7 (clone 2D7; BD Biosciences), αCD44-Alexa Fluor 700 (clone IM7; Biolegend / BD Biosciences), αKLRG1-FITC (clone 2F1/KLRG1; Biolegend), αCD62L-eFluor605NC (clone MEL-14; eBioscience), α-KLRG1-Biotin (clone 2F1/KLRG1; Biolegend). For the detection of KLRG1-Biotin a secondary staining with streptavidin-Brilliant Violet 510 (Biolegend) was performed.

After washing cells twice with FACS buffer (2% FBS in 1x PBS), samples were acquired using an LSR-Fortessa (BD Biosciences). Results were analysed using FlowJo software version 9.7.6 (Treestar).

Quantification of latent viral genomes

For quantification of MCMV genomes, DNA was isolated from spleens or lungs using the DNeasy Blood & Tissue Kit (QIAGEN) and quantitated by M55 (encoding gB)- specific qPCR normalized to cell number by pthrp specific qPCR as previously described (37).

RESULTS

Intranasal MCMV infection results in robust virus replication in the lungs, whereas infection by gavage is inefficient

Viral replication upon mucosal infection was compared to systemic infection via the i.p. route by determining infectious virus titers in spleens and lungs of BALB/c mice at 5 and 14 days post infection (dpi), as well as in salivary glands at 14 dpi. In mice infected with MCMV by gavage, infectious virus in spleen and lungs was almost undetectable. In half of these mice, salivary gland MCMV titers were substantially lower than in i.p. infected controls (Fig. 1a). In contrast, i.n. infection induced higher viral titers in the lungs than i.p. injection. On the other hand, titers in spleens were reduced after i.n. infection. Spread to the salivary glands had nearly the same efficiency in both infection routes (Fig.

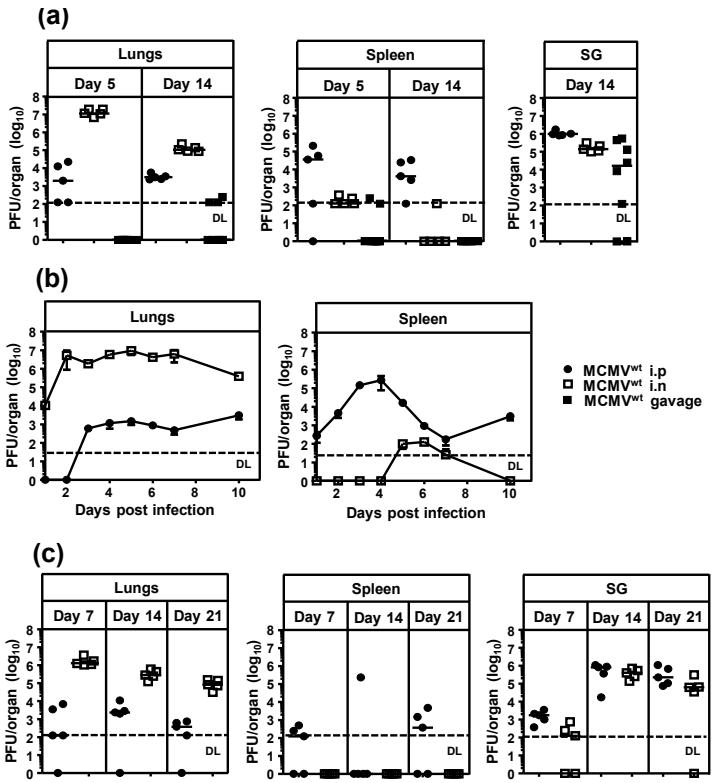

Fig. 1. Intranasal MCMV infection results in robust virus replication in the lungs and efficient spread to the salivary glands. BALB/c mice were infected with $2x10^5$ PFU MCMVwt using the indicated infection route. (a) Virus titers were determined in the lungs and spleen 5 and 14 dpi and in the salivary glands 14 dpi by plaque assay. (b) Lungs and spleens were harvested daily from 1 to 7 days p.i. and on day 10 p.i. Virus titers were analysed in organ homogenates. The group means ± SEM are displayed (n=5), dashed lines show the detection limit. (c) Infectious virus was detected in lungs, spleen and salivary glands on day 7, 14 and 21 p.i.. (a+c) Each symbol represents one mouse, solid lines display the median and dashed lines show the detection limit. (a-c) SG = salivary glands, DL = detection limit

1a). Since high virus titers in the salivary glands are deemed important for transmission to the next host, our results may suggest that transmission of CMV in mice occurs via the respiratory rather than the gastrointestinal tract. Hence, we analysed MCMV replication after infection via this route in detail.

Virus titers were determined for a week in spleen and lung samples harvested daily from mice infected i.n. or i.p., and at 10 dpi. Virus was detected in lungs of i.n. infected mice as soon as 1 dpi, and titers were increased several orders of magnitude above those in mice infected by the i.p. route at all tested time points. It is important to note that i.n. infected mice showed no obvious symptoms of disease at any time point, despite the persistence of very high virus titers in their lungs. In a separate experiment, spleen, lung and salivary glands were analysed for infectious virus titers at 7, 14 and 21 dpi. Elevated titers in the lungs could be detected even by 21 dpi (Fig. 1c), and some infectious virus could be detected in some mice as late as 42 dpi (not shown). In contrast, low titers of

infectious virus were detectable in the spleen only from 5 to 7 dpi after i.n. infection, whereas i.p. infection induced substantially higher infectious titers which peaked by 4 dpi and could still be detected by 21 dpi (Fig. 1b, 1c). Virus titers in salivary glands of i.n. or i.p. infected mice were comparable at all tested time points (Fig. 1c).

IE1-specific CD8 T cells inflate in the blood after intranasal infection

After systemic MCMV infection, CD8 T cells recognizing defined immunodominant peptides have been shown to accumulate after clearance of lytic viral replication, a phenomenon known as memory inflation (20, 21, 24, 25, 38). In order to test whether memory inflation is induced upon mucosal MCMV infection, we compared CD8 T cell kinetics after i.n. infection and infection by gavage to systemic infection by the i.p. route. We monitored CD8 T cells for their response to MCMV infection in the peripheral blood up to six months p.i.. Mice that showed no CD8 T cell response above background at any tested time point were excluded from the CD8 T cell analysis, to censor false negative events due to inefficient primary infection. One should note that these events occurred only in gavage infection, consistent with data showing the absence of infectious virus titers in organs (see Fig. 1a).

First, the percentage and absolute numbers of primed CD8 T cells (defined by $CD11^{high}$/$CD44^{high}$) were monitored. Primed cells displayed a strong initial increase in mice infected via the i.p. route by 1 week after infection, which slightly contracted by week 4 and remained rather stable in relative and absolute terms later on. The initial increase was delayed for a week after mucosal infection and the obtained values were clearly lower than after i.p. infection. After a contraction around week 4, the frequency of primed CD8 T cells remained rather stable in i.n. infected mice, but decreased to background level in the gavage-infected group (Fig. 2a, left panel). The absolute numbers of primed cells upon mucosal infection was hardly elevated over levels observed in uninfected mice (Fig. 2a, right panel). While the difference was small, mice infected via the i.n. route showed a higher frequency and number of primed CD8 T cells than mice infected by gavage at all time points (Fig. 2a). Next, we analysed the CD8 T cell response to an immunodominant MCMV-derived epitope; the $IE1_{168\text{-}176}$ epitope, which induces CD8 T cells with an inflationary phenotype in mice with the $H\text{-}2^{d}$ haplotype (e.g. BALB/c) (21, 24, 38). We used a MHC class I tetramer (IE1-tetramer) to stain IE1-specific CD8 T cells and detect them via flow cytometry. In mice infected by the i.p. route, we observed the previously reported inflationary kinetic profile of IE1-specific CD8 T cells, with highly elevated relative and absolute counts throughout the time course of the experiment (Fig. 2b). Consistent with the kinetics of primed cells, the initial peak of IE1-specific responses occurred later (in week 2 p.i.) upon both mucosal infection routes. Whereas the frequency and counts of IE1-specific CD8 T cells declined in mice infected by gavage by week 4 p.i. and remained low thereupon, in i.n. infected mice the percentage and absolute number increased from week 4 to week 13 and stayed constant afterwards (Fig. 2b).

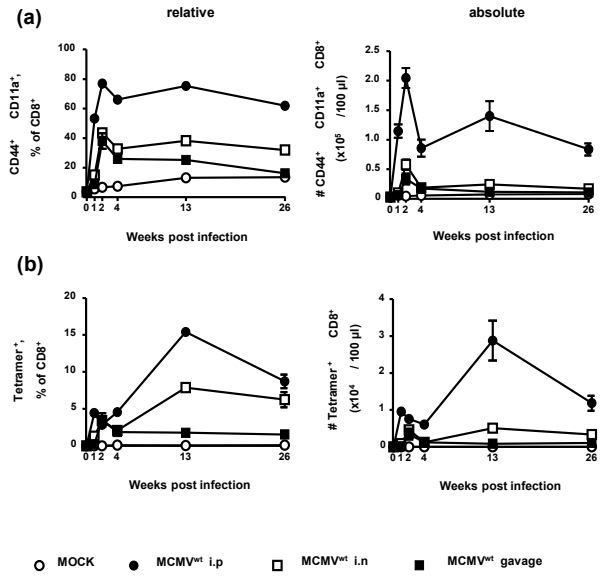

Fig. 2. IE1-specific CD8 T cells inflate in BALB/c mice upon intranasal MCMV infection. BALB/c mice were left untreated (MOCK) or were infected with $2x10^5$ PFU MCMVwt using the indicated infection route. Each group consisted of at least 12 mice. At least 5 mice per group were sacrificed and used for detailed analysis at 2 weeks p.i. The rest (at least 7 mice per group) were analysed at indicated time points p.i. Blood from 5 mice was analysed prior to infection, to define the basis of the CD8 T cell fractions. For the analysis of CD8 T cell subsets, blood leukocytes were stained with an IE1-tetramer and with antibodies against CD3, CD4, CD8a, CD11a and CD44, and analysed by flow cytometry. (a) Percentage and absolute counts of antigen experienced $CD11a^+$ $CD44^+$ CD8 T cells. (b) Percentage and absolute counts of $CD11a^+$ IE1-$tetramer^+$ CD8 T cells. (a+b) Displayed are the group means ± SEM ($n \geq 5$) at indicated time points.

IE1-specific responses after i.n. infection were weaker than after i.p. infection and this difference was more obvious for absolute (Fig. 2b, right panel) than for relative numbers (Fig. 2b, left panel). These data suggest a hierarchy of CD8 T cell responses upon the three infection routes tested: Responses are clearly strongest after i.p. infection, followed by i.n. infection and only then by gavage inoculation.

IE1-specific CD8 T cells inflate in lymphoid organs after intranasal infection

The robust T cell response in mice infected via the i.n. route prompted us to examine it in more detail. Therefore, we investigated the virus-specific responses in several lymphoid compartments in addition to the blood by determining the kinetics of primed and MCMV-specific CD8 T cells in the spleen, the mediastinal lymph nodes (MLN) and the inguinal lymph nodes (ILN) of BALB/c mice. The MLNs are the draining lymph nodes of the lungs and have been shown to also drain substances administered via the i.p. route (39-42), while the inguinal lymph nodes were used as non-draining lymph nodes.

The kinetics of primed and IE1-specific CD8 T cells (Fig. 3a) in the blood was in essence comparable to the previous experiment (Fig. 2), although the absolute numbers in i.p.

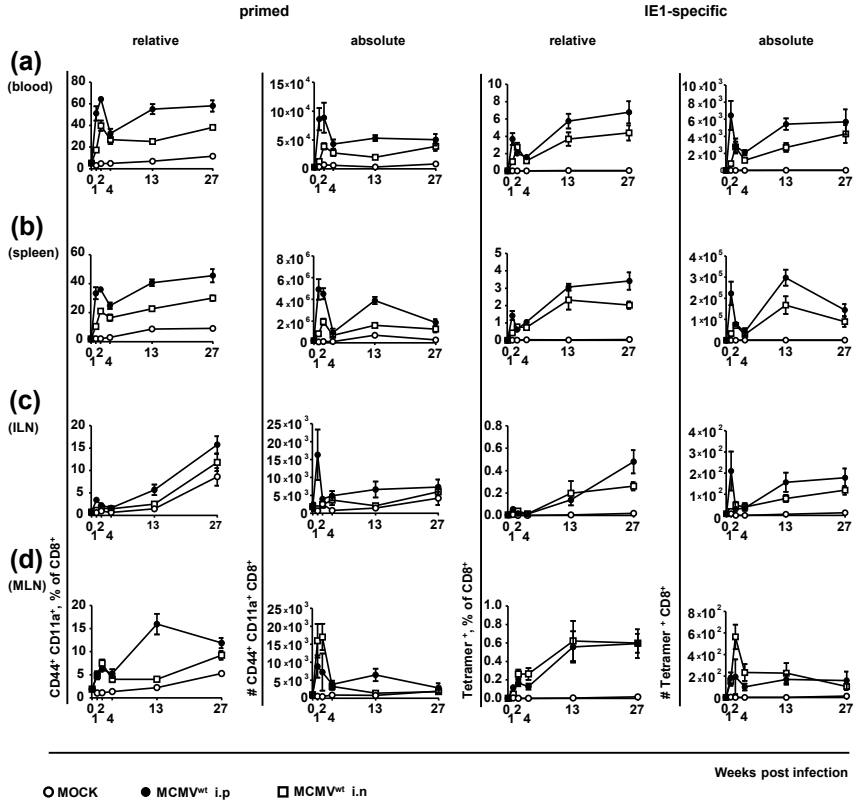

Fig. 3 Memory inflation in the spleen and lymph nodes upon systemic or intranasal MCMV infection. Two cohorts of BALB/c mice were infected with $2x10^5$ PFU MCMVwt by the intraperitoneal or the intranasal route. 20 µl PBS were administered via the intranasal route to MOCK controls. The first cohort consisted of 25 mice per group, sacrificed at week 1, 2, 4, 13 and 27, and the second cohort of 10 mice per group analysed at week 1 or 27 only. Samples from 5 mice were collected prior to infection to set the baseline of CD8 T cell subsets (time point 0). At indicated time points p.i., the frequency and the absolute counts of primed ($CD11a^+CD44^+$) or IE1-tetramer$^+$ CD8 T cells were determined in (a) the blood, (b) the spleen, (c) the inguinal and (d) the mediastinal lymph nodes. Leukocytes were stained with an IE1-tetramer and antibodies against CD3, CD4, CD8a, CD11a and CD44 and analysed by flow cytometry. Displayed are the group means ± SEM ($n \geq 3$) at indicated time points; combined data from both cohorts are shown at 1 and 27 weeks p.i. Absolute counts are shown per 100 µl blood, whole spleen or one lymph node. ILN = inguinal lymph nodes; MLN = mediastinal lymph nodes

infected mice were lower in this experiment, resulting in a smaller difference to mice infected via the i.n. route. In both infection routes, the kinetics of CD8 T cells in the spleen (Fig. 3b) mimicked closely those in the blood (Fig. 3a). Both the percentages and the numbers of primed and IE1-specific CD8 T cells were much lower in the lymph nodes in general (Fig. 3c, 3d). Percentages of both primed and MCMV-specific CD8 T cells increased in the non-draining ILNs only at late time points, but remained lower as in the blood or spleen (Fig. 3c, first and third panel). Notably, the increase in the frequency of primed CD8 T cells was also detected in uninfected controls, suggesting that it may be a function of exposure to environmental antigens in aging mice, rather than caused by MCMV infection. The numbers of primed CD8 T cells remained very low throughout

the experiment, with the exception of a transient increase in i.p. infected mice 1 week p.i. (Fig. 3c, second panel), which was reflected as an early peak in the count of IE1-specific cells as well (Fig. 3c, fourth panel). Furthermore, total counts of IE1-specific cells increased in both infection groups at later time points, but again much less than in blood or spleen (Fig. 3c, fourth panel). In the MLNs of i.n. infected mice, the absolute count of primed and IE1-specific CD8 T cells exceeded those after i.p. infection early upon infection (Fig. 3d, second and fourth panel), but this reversed for primed cells by 13 weeks p.i. (Fig. 3d, second panel). On the other hand, the percentage and number of IE1-specific CD8 T cells was comparable in the MLN of i.p. and i.n. infected mice at later time points (Fig. 3d, third and fourth panel).
In sum, these data suggest that i.n. infection initiates a robust MCMV-specific T cell response in the draining lymph node and induces memory inflation, not only in the blood, but also in the spleen and lymph nodes.

MCMV-specific CD8 T cells inflate after intranasal infection of 129/Sv mice
To exclude that these results are restricted to the BALB/c mouse strain, we analysed the kinetics of the CD8 T cell response in the blood upon mucosal MCMV infection in mice of the 129/Sv strain. Like BALB/c mice, these mice do not express the resistant Cmv1 allele (43), and thus display similar levels of CMV control by NK cells. We analysed the kinetics of primed CD8 T cells and CD8 T cells specific for an inflationary K^b-restricted epitope corresponding to the amino acid position 316-323 of the M38 protein of MCMV (20).
The largest discrepancy between BALB/c and 129/Sv mice was observed upon gavage infection. Namely, 23 out of 35 129/Sv mice showed no MCMV-specific response to infection at any time point, whereas this was the case in only 2 out of 20 BALB/c mice. Among the mice showing responses, the kinetics of primed and M38-specific CD8 T cells displayed the same hierarchy as observed for primed and IE1-specific cells in the BALB/c model (Fig. 2). Systemic i.p. infection induced the strongest inflationary response, followed by i.n. infection, which still induced inflating M38-specific CD8 T cells, but at a lower level than i.p. infection. Infection by gavage resulted in the weakest response and no increase of antigen-specific CD8 T cells after 4 weeks of infection (Fig. 4a, 4b).

Most primed CD8 T cells display an effector memory phenotype upon intranasal infection
Most inflationary CD8 T cells show an effector memory (EM) phenotype after systemic i.p. infection, characterised by low expression of CD62L and high expression of KLRG1. In contrast, non-inflationary cells usually assume a central memory (CM) phenotype (38, 44), defined here as $CD62L^{hi}$ $KLRG1^{lo}$. We analysed the phenotype of primed CD8 T cells upon MCMV infection in 129/Sv mice and observed that most primed CD8 T cells of i.p. infected mice assumed an EM phenotype ($KLRG1^+/CD62L^-$) throughout the infec

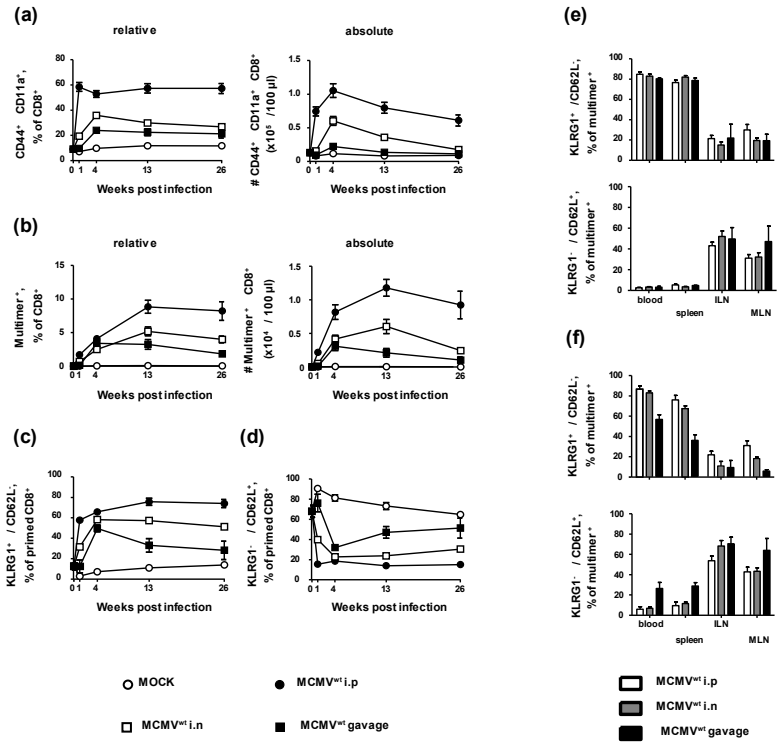

Fig. 4 Intranasal MCMV infection of 129/Sv mice results in inflation of M38-specific CD8 T cells with an effector memory phenotype. Two cohorts of 129/Sv mice were left untreated (MOCK) or were infected with $2x10^5$ PFU MCMVwt using the indicated infection route. Each group in each cohort contained at least 5 mice. Blood from 5 mice was analysed prior to infection to define the basis of the CD8 T cell fractions. Infected mice were bled at indicated time points p.i., isolated leukocytes were stained with an M38-multimer and with antibodies against CD3, CD4, CD8a, CD11a, CD44, CD62L and KLRG1 and subsequently analysed by flow cytometry. (a) Percentage and absolute counts of primed ($CD11a^+CD44^+$) cells in the CD8 T cell pool. (b) Percentage and absolute counts of M38-specific ($CD11a^+$M38-multimer$^+$) in the CD8 T cell pool. (c) Percentage of EM ($KLRG1^+CD62L^-$) cells in the primed CD8 T cell pool. (d) Percentage of CM ($KLRG1^-CD62L^+$) in the primed CD8 T cell pool. (a-d) Displayed are the group means ± SEM ($n \geq 3$) at indicated time points. (e) Frequencies of M38-multimer$^+$ CD8 T cells with EM (KLRG1+CD62L-) and CM ($KLRG1^-CD62L^+$) phenotypes in indicated compartments at 2 weeks post systemic or mucosal infection. (f) Phenotypes as in panel E at 26 weeks p.i. (e+f) Displayed are the group means + SEM ($n \geq 5$) in indicated compartments.

tion. This percentage was a bit lower in mice infected via the i.n. route, but the lowest percentage was observed upon gavage or in uninfected mice (Fig. 4c). For cells displaying a CM phenotype, the hierarchy was inverted (Fig. 4d). Kinetics of CM and EM phenotypes were similar in BALB/c mice, with most EM cells upon systemic infection, the least in uninfected mice, and intermediate phenotypes in the two mucosal infections (data not shown).

The phenotype of M38-specific CD8 T cells was also analysed in different compartments (blood, spleen, ILN, MLN) at an early (week 2) and a late time point (week 26) after infection. Early after infection, the phenotype was comparable between all infection routes, with more $KLRG1^+/CD62L^-$ M38-specific cells in the blood and spleen (Fig. 4e, upper panel) and with more $KLRG1^-/CD62L^+$ M38-specific cells in the lymph nodes (Fig.

4e, lower panel). Later after infection, this distribution remained the same, but mice infected by gavage had clearly less EM cells and more CM cells in M38-specific CD8 T cells of the blood and spleen than seen upon i.p. or i.n. infection (Fig. 4f).
In combination, our data argue that i.n. MCMV infection induces inflationary CD8 T cell responses and that these inflationary cells retain the EM phenotype, although at slightly lower frequencies than upon i.p. infection.

Latent virus is elevated in the lungs and low in the spleen after intranasal infection
It was recently suggested that the spleen might be an important site for the maintenance of memory inflation (45, 46). In that case, the attenuated memory inflation upon intranasal infection (Fig. 2-4) may be a reflection of lower splenic viral genome load upon mucosal infection. Hence, we determined the latent load in spleens of 129/Sv mice infected by the different infection routes at 26 weeks p.i.. I.p. infection resulted in the highest latent load in the spleen (Fig. 5a). This pattern was not maintained in lungs, where i.n. infection led to the highest latent viral genomes (Fig. 5b). The latent genome load was lowest after infection by gavage in both organs (a more detailed comparison of latent loads is provided in Fig. S1). We repeated this analysis in BALB/c mice with similar results (data not shown).
In sum, our results indicate that the latent MCMV load in an organ depends on the route of infection. Considering the results shown in Fig. 1, we observed an association between the magnitude of viral replication in an organ during primary infection, and the latent genome load during latency.

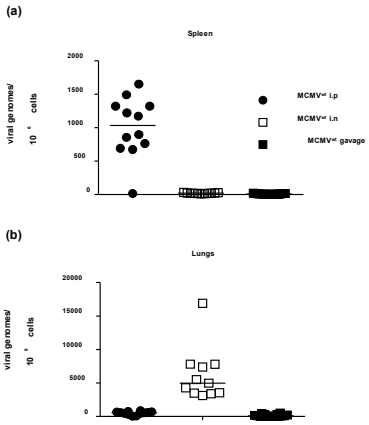

Fig. 5: Latent virus is elevated in the lungs of intranasally infected mice. 129/Sv mice were infected with $2x10^5$ PFU MCMVwt using the indicated infection route. Lungs and spleens were harvested 26 weeks p.i. and latent viral genomes were determined in these organs by real-time PCR. Latent viral genomes per million host cells in (a) spleen and (b) lungs are shown. Each symbol represents one mouse, horizontal lines show the medians.

DISCUSSION

In this study we have systematically compared different routes of MCMV infection. We show that the intranasal route results in robust and long-lasting virus replication in the

lungs and salivary glands, consistent with a previous report (15), and is accompanied with memory CD8 T cell inflation, yet this is impaired upon intragastric infection, even when we optimize pH conditions to avoid virus neutralization prior to its entry into cells. This implies that the respiratory mucosa, rather than the gastrointestinal one, may serve as a major site of viral entry into the host. Previous studies by the Förster group showed similar results in newborn mice (17), but they used transgenic viruses containing three separate mutations, and it remained unclear if the poor replication in the gut mucosa upon oral infection reflected the biology of the wild-type MCMV infection. This is in stern contrast with data indicating that intragastric infection of newborn mice with wild-type MCMV can result in viral dissemination (47). However, that study was based on qualitative PCR of viral nucleic acids, while we measured the replicating virus by a quantitative assay, allowing us not only to identify its presence, but also to assess its abundance. Interestingly, the large majority of mice infected by gavage had no detectable viral titers in the tested primary organs (spleen, lungs), but a distinct number of those mice had detectable viral replication in the salivary glands. As the salivary glands are generally thought to be targeted only upon secondary viremia (48), this may be either explained by virus replicationin a primary organ that was not tested by us, or by the possibility that virus might disseminate without a major primary expansion.

Our results suggest that the wild-type MCMV infection by the intragastric route is substantially less efficient than intranasal infection in adult mice, arguing that the respiratory rather than the gastrointestinal mucosa, may be a more dominant natural site of entry of this virus. One needs to note, however, that our study focused only on the respiratory and gastrointestinal epithelium, whereas other mucosal surfaces, such as the genitourinary mucosa or the epithelium of the oral cavity, need to be addressed in future studies. Surprisingly, the high viral titers observed upon i.n. infection resulted in no overt disease. Since low pathology is also a feature of primary HCMV infections of adults, one may speculate that i.n. infection reflects a natural infection condition. However, more studies are required to validate this hypothesis.

Most evidence for memory T cell inflation has rested on systemic experimental infection with a high dose of the virus (20, 21). Recently, it was shown that low dose inoculum of MCMV results in a severely hampered inflation of memory T cells (46) suggesting that the amount of virus is also a determinant of memory inflation. While it is generally accepted that systemic infection occurs during congenital HCMV infection by transfer of the virus through placenta, infections after birth most likely occur via mucosal routes. Therefore, it was not clear if infection of adult hosts by a mucosal route would result in a sufficient level of acutely replicating virus to elicit memory inflation. We showed here that inflation of MCMV-specific T cells can occur upon i.n. infection, but is less pronounced than in mice infected by the i.p. route (Fig. 3).

It has been proposed that intermittent low-level antigen expression during latent MCMV infection induces inflationary CD8 T cell responses (49), and it has been recently report-

ed that inflationary responses occur due to the proliferation of T cells in contact with the blood circulation, rather than in organ parenchyma (45), probably because endothelial cells lining blood vessels are a major site of MCMV latency (50). If this model is accurate, and considering that mice infected by the i.n. route showed substantially higher latent loads in the lungs, but lower ones in the spleen (Fig. 5), our data imply that latent viral load in the spleen, rather than the lungs, predicts the magnitude of memory inflation, consistent with the recent observation that CMV-specific T cells proliferate in the spleen of latently infected mice upon i.p. infection (46). We focused in our study on prototypical inflationary epitopes. While it is likely that non-inflationary epitopes will exhibit a similar hierarchy of responses upon various infection routes, this aspect needs to be formally confirmed in future studies.

Taken together, our results argue that i.p. infection drives inflationary CD8 T cell responses that are more pronounced than the responses observed upon i.n. infection, but essentially both infection routes induce responses with similar kinetics and phenotypes of responding T cells. Since CMV induced pneumonia is a major pathology caused by this virus and mucosal infection is likely to reflect the natural infection better than intraperitoneal injection, we propose that the i.n. infection route offers a robust model for the study of CMV replication and latency and mucosal immune responses in a highly relevant site of infection.

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SUPPLEMENTARY FIGURES

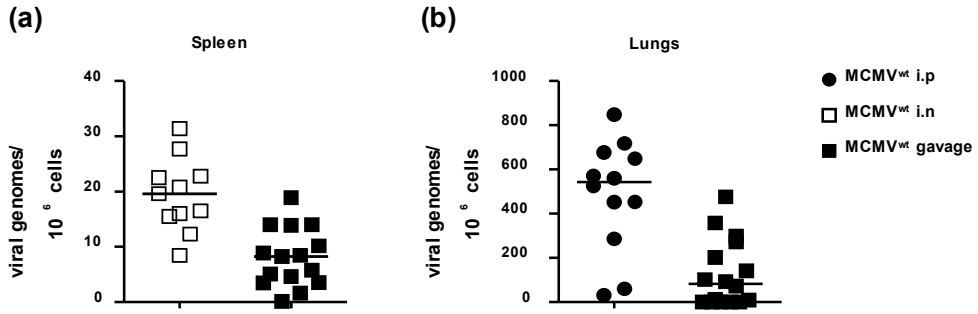

Figure S1. Latent virus in spleen and lungs is lowest after intragastric infection. 129/Sv mice were infected with $2x10^5$ PFU MCMVwt using the indicated infection route. Lungs and spleens were harvested 26 weeks p.i. and latent viral genomes were determined in these organs by real-time PCR. Latent viral genomes per million host cells in (a) spleen and (b) lungs are shown. To optimize presentation, the infection routes with the highest latent viral genome loads are omitted. Each symbol represents one mouse, horizontal lines show the medians.

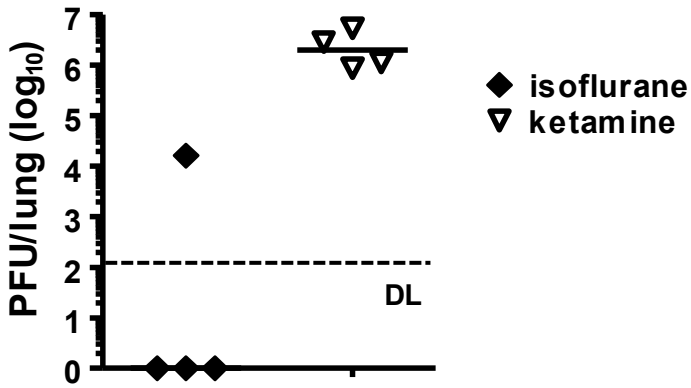

Figure S2: Isoflurane anaesthesia results in inefficient intranasal MCMV infection. BALB/c mice were anaesthetized with either isoflurane or ketamine/xylazine (10mg/ml / 1mg/ml in 0.9% NaCl) and subsequently infected with $2x10^5$ PFU MCMVwt intranasally. Infectious virus was determined in lungs 4 days p.i.. Each symbol represents one mouse, solid lines display the median and the dashed line shows the detection limit (DL). This analysis was performed once.

CHAPTER 10

General discussion

10

GENERAL DISCUSSION

Elimination of intracellular bacteria, viruses and malignant cells is highly dependent on the adaptive immune system. However, T and B cells are not always successful in eradicating pathogens or malignant cells. Hence, immunotherapeutic strategies are being developed that aim to induce or enhance specific T and B cell responses. In this thesis we focused specifically on CD8$^+$ T cells. For rational development of strategies aiming to improve T cell responses, the preferred features of the therapy-induced CD8$^+$ T cells should be identified. Subsequently, immunotherapy can be tailored to induce CD8$^+$ T cells harboring the preferred phenotype and function as well as the capacity to expand these cells upon re-challenge. However, to skew T cells towards a specific phenotype, factors that control T cell differentiation and expansion need to be identified. In this thesis we have investigated how IL-2 production, costimulatory signals and different antigenic inoculums differentially shape CD8+ T cells.

Enhanced autocrine IL-2 production by CD8+ T cells

One of the factors that are known to be crucial for CD8$^+$ T cell expansion and maintenance is IL-2 signaling, with IL-2 being produced by the CD8$^+$ T cells themselves (autocrine manner) (1). Corroborating on this, we showed that the relative number of IL-2-producing CD8$^+$ T cells within an antigen-specific population is predictive of the expansion potential of the total population. Moreover, enhancement of autocrine IL-2 production results in improved CD8$^+$ T cell expansion and maintenance, so the quantity of IL-2 that is produced on a per cell basis dictates the expansion capacity (chapter 2 and figure 1). Therefore in adoptive T cell therapy (ACT), where transferred T cells often fail to expand and persist, enhancing the autocrine IL-2 production by CD8$^+$ T cells could be a novel strategy to advance clinical response rates (chapter 4). Approaches to enhance autocrine IL-2 production include retroviral transduction of constructs harboring an IL-2 gene (chapter 2). Retroviral vectors have already been proven safe for use in patients (2); however whether introduction of an additional IL-2 gene would be save, remains to be elucidated. We have observed that in mice that were transplanted with IL-2-transduced CD8$^+$ T cells, the maintenance of these cells is increased compared to control CD8$^+$ T cells. However, we cannot exclude the possibility that cells with improved autocrine IL-2 production undergo continuous proliferation leading to immunopathology. In that case it is questionable if engineering CD8$^+$ T cells to express additional IL-2 genes is feasible in clinical settings. Additionally, high accumulation of CD8$^+$ T cells is suggested to have also negative effects on heterologous immunity later in life (chapter 7 and 8).

CD70 and CD80/86 (B7.1/2)-mediated costimulatory interactions are crucial for the induction of IL-2 production in CD8$^+$ T cells (3, 4). However dual costimulation via CD70 and CD80/86 is required for optimal autocrine IL-2 expression. The mechanism by

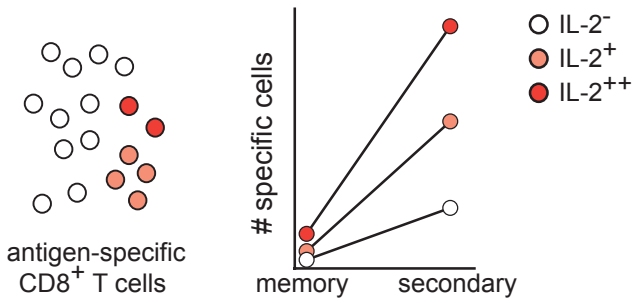

Figure 1 The role of autocrine IL-2 in CD8⁺ T cell expansion capacity. CD8⁺ T cells that are void of the capacity to produce IL-2 possess very limited capacity to expand upon secondary challenge compared to IL-2 proficient CD8⁺ T cells. Importantly, the quantity of autocrine IL-2 that is produced in a CD8⁺ T cell determines the degree to which it can expand. Together the relative number of IL-2 producing CD8⁺ T cells and the amount of IL-2 that is produced on a per cell basis dictate the expansion potential of an antigen-specific CD8⁺ T cell population

which these costimulatory signals regulate IL-2 expression in CD8⁺ T cells depends on the transcription factor c-rel. Upon signaling through CD27 and CD28 c-rel is translocated to the nucleus where it binds to the specific DNA-binding site (κB site) and thereby initiates IL-2 gene transcription. Abrogation of CD70 or CD80/86-mediated signaling decreases the nuclear translocation of c-rel. This decrease is more pronounced in the absence of CD80/86 than CD70 mediated signaling and is further diminished when both costimulatory molecules are absent. Importantly, the CD70 and CD80/86 costimulatory molecules that are required for the induction of IL-2 by CD8⁺ T cells are provided by CD11c⁺ APCs (chapter 3). However, to improve ACT, implementation of agonistic CD27 and blocking CTLA-4 antibodies during the ex vivo culture procedure to enhance signaling through the CD27 and CD28 pathways respectively might also be effective in improving autocrine IL-2 production of CD8⁺ T cells (chapter 4). In addition to signaling through CD27 and CD28, also 4-1BB and OX40-mediated signaling are known to induce IL-2 expression in CD8⁺ T cells, so agonistic antibodies targeting these molecules can also be considered as a strategy to enhance ACT protocols (5, 6). Alternatively immunotherapeutic strategies can be designed in a way that the preferred costimulatory ligands are upregulated on APCs in vivo. This can be achieved by inclusion of the appropriate adjuvants, including TLR ligands, Montanide, CD40 agonists or stimulator of IFN genes (STING) agonists (7-9).

Besides costimulation (signal 2), also TCR triggering (signal 1) and inflammatory cytokines (signal 3) contribute to the autocrine IL-2/IL-2 receptor signaling of CD8⁺ T cells . Upon TCR triggering IL-2 production in CD8⁺ T cells is initiated, which is as discussed above further increased upon costimulation-mediated signaling. Exposure to the inflammatory cytokines IL-12 and IFNα sustains expression of CD25, the high affinity IL-2 receptor, thereby prolonging sensitivity to IL-2. Signals mediated via IL-2 – IL-2R

interactions are sometimes referred to as signal 2 (10), however costimulatory signals involve reciprocal and sequential receptor – ligand interactions between T cells and APCs (11). As IL-2 needs to signal in an autocrine fashion (1), this does not support the idea of IL-2 as a mediator of signal 2. Instead it seems more reasonable to regard IL-2 mediated signaling in the context of CD8+ T cell priming as distinct signal (signal 4).

Influence of viral inoculum size on CD8+ T cell phenotype and function

The outcome of the T cell response upon viral infections is prominently influenced by the nature of the invading pathogen (12). Akin, we found that the height of the infectious dose also impacts the outcome of the T cell response significantly (chapter 5 and chapter 7). The accumulation of CD8+ T cells, considered as one of the hallmarks of CMV infection, was impaired when mice were infected with a low MCMV dose. Also, a high MCMV inoculum resulted in the accumulation of CD8+ T cells preferentially displaying an effector-memory phenotype, whereas in a low inoculum size these T cells are skewed towards a central-memory phenotype. So the height of the viral inoculum determines the degree of T cell accumulation as well as the ratio of CD8+ T cells displaying an effector versus central-memory phenotype. In a model of acute LCMV infection we also observed that the infectious dose determines the balance of central-memory and effector-memory CD8+ T cells that comprise the LCMV-specific memory pool (unpublished observations). Underlying causes are likely to be related to differences in the quantity of antigen and the degree of inflammation that is induced upon infection with either low or high viral inoculum (13). Importantly, the viral inoculum size does not only determine the phenotype of CMV-specific T cells. Later in life it can also determine the capacity to eliminate invading pathogens. After heterologous infection with LCMV in mice that had experienced long-standing latent MCMV infection the phenotype of the LCMV-specific CD8+ T cells reveals impaired priming when MCMV infection was initiated with a high dose. This was evidenced by impaired upregulation of CD27 and a decreased potential to produce IL-2. Thus, the development of immunity against certain previously encountered viruses, especially CMV, can influence the immune response against new viruses encountered later in life (chapter 7). This is in agreement with other studies that have been performed, though these studies did not assess the influence of the viral inoculum (14-16). Appreciating the influence of the viral inoculum size, we refined the statement that CMV per definition aggravates immune ageing by demonstrating that a high viral inoculum is a prerequisite for this phenomenon to occur and that upon infection with lower doses no signs of impaired viral immunity were observed (chapter 7).

It has been postulated that the accumulating CMV-specific CD8+ T cells are causative for the CMV-induced impaired heterologous immunity by competing with newly primed T cells and accelerating age-associated loss of naive CD8+ T cells (14-16). In addition to impaired CD8+ T cell-mediated immunity also heterologous B cells responses can be

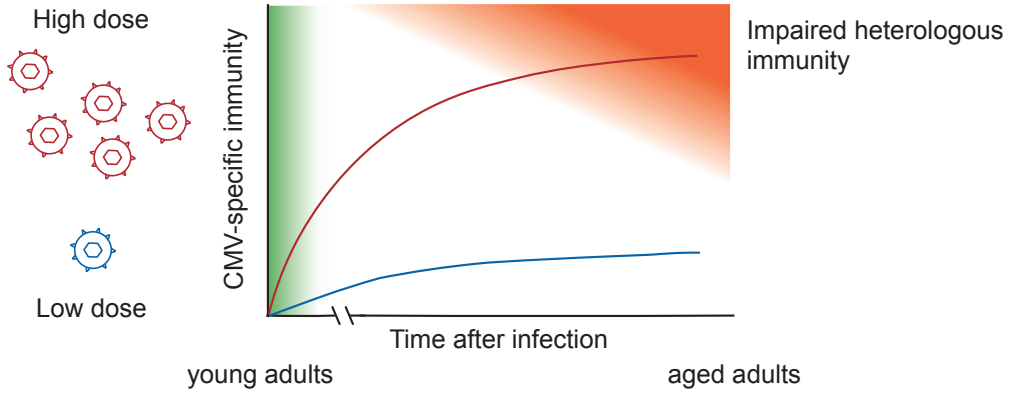

Figure 2. The infectious dose dictates the degree of CMV-associated immune perturbations in aged hosts. CMV elicits the accumulation of viral-specific EM CD8 T cells and antibodies. When the degree of CMV-induced responses reaches a certain threshold (dark red shading) heterologous immunity is substantially impaired. The requirements for reaching this threshold are high dose CMV infection and an aged host. In contrast, primary infection in young adults is beneficial for heterologous immunity, albeit temporarily.

hampered as a result of latent CMV infection (chapter 7). In mouse and human CMV infection inflation of CMV-specific antibodies has been reported to occur (chapter 5 and 6) (17) , this is possibly the result of accumulation of CMV-specific B cells. These B cells might be able to compete with newly activated B cells that are induced upon heterologous infection, thereby impairing the humoral arm of the immune system.

Another mechanism by which CMV possibly compromises immune responses against new pathogens involves cells of the myeloid compartment. Myeloid cells can serve as latent CMV reservoirs and differentiation of these cells harboring latent CMV results in reactivation of the virus and even production of new virus particles (18, 19). Upon encounter of a new infection, myeloid cell differentiation and thereby CMV-reactivation can be induced. Especially in the elderly, where immune surveillance is decreased, it is easy to envision that reactivation caused by heterologous infection results in considerable CMV lytic cycles. During lytic infection a wide range of immune modulatory strategies are being exploited by CMV. For example, CMV-derived proteins can cause local inflammatory cytokine sinks by acting as cytokine receptors. CMV also encodes genes that can modulate IFN responses, and encodes cytokine homologues like the immune suppressive cytokine IL-10 (chapter 8). Since the height of the initial viral inoculum correlates with the amount of latent virus reservoirs (chapter 5), reactivation may thus only be substantial enough to exert negative effects in individuals in which CMV infection occurred with a high infectious dose.

Intriguingly, in young mice latent CMV infection does not impair heterologous immunity, but responses against LCMV infection even seem to be strengthened in these mice

(chapter 7). The same phenomenon was observed after heterologous infection with influenza virus and this cross-protection was shown to be dependent on IFN-γ, which is likely to be derived from CMV-specific $CD4^+$ and $CD8^+$ T cells (20). However, the improved response against influenza wanes in time, so only early and established CMV latency seems to favor heterologous immunity and not long-standing latency. Importantly, in these experiments CMV latency was established using a high viral inoculum. It would be interesting to assess if cross-protection would also occur if the CMV infectious dose is low.

Models of CMV infection

Murine CMV (MCMV) is accepted widely to model CMV infection in humans as it parallels many aspects of HCMV infection (21). However, the intraperitoneal route of infection that is predominantly used to establish infection may not equal the natural routes of infection in humans. Natural infections are expected to mostly occur by exposure of mucosal surfaces to infectious bodily fluids from virus-shedding individuals. Hence, the mucosa might represent one of the major sites of virus entry in the host. Although comparison of intranasal with intraperitoneal CMV infection revealed no overt differences in kinetics and phenotype of the responding $CD8^+$ T cells, accumulation of CMV-specific $CD8^+$ T cells was decreased upon intranasal infection (chapter 9). Important to note is that accumulation of $CD8^+$ T cells in the lungs is likely to be increased after intranasal infection, due to high latent genome load in the lungs. Through the accumulation of specific memory $CD8^+$ T cells, CMV-based vaccines are thought to be promising tools for vaccination strategies (22-24). If the route of infection indeed determines in which organs CMV-specific $CD8^+$ T cells specially accumulate then the route of vaccine administration should depend on the site where the vaccine-induced $CD8^+$ T cells should exert their effects. In this regard it is also important to take into account that the $CD8^+$ T cell response can likely be further modulated by varying the dose of CMV-based vaccines (chapter 5). Depending on the preferred effector-memory / central-memory $CD8^+$ T cell ratio the vaccine dose should either by increased or decreased.

CONCLUDING REMARKS

The phenotype and functionality that $CD8^+$ T cells acquire upon encountering their cognate antigen depend on many factors that are controlled by the nature of the pathogen The acquired phenotypical and functional characteristics determine the potential of the $CD8^+$ T cells to form bona fide memory populations that are able to expand upon secondary challenge. In this thesis we investigated factors that control $CD8^+$ T cell differentiation. We have shown that the height of the viral dose greatly impacts the immunological outcome by affecting the magnitude of the response and the phenotype and function

of the (memory) CD8+ T cells that are induced. The acquired phenotype and functions determine the capacity to expand upon re-encounter with the same pathogen and we have shown that the IL-2 producing capacity of CD8+ T cells functions as a crucial determinant of CD8+ T cell expansion potential. Although we addressed only a small fraction of all variables involved in the induction and maintenance of antigen-specific T cell populations (25) (chapter 4) our observations and insights gathered by others already provide a basis for rational immunotherapeutic treatment design, however, for fine-tuning of therapy-induced immune responses aspects about e.g. the timing and cooperating signals still need to be understood in more detail.

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10

APPENDIX

English summary
Dutch summary
Curriculum Vitae
List of publications
Acknowledgements

ENGLISH SUMMARY

The immune system is a defense mechanism that protects the body against disease-causing microorganisms such as viruses and bacteria, but also identifies and eliminates tumor cells. Within the immune system two branches can be distinguished: the innate (non-specific) and the adaptive (specific) system. The innate system forms the first line of host defense and is indispensable for activation of the adaptive system. Recognition of pathogens or tumor cell fragments by cells of the adaptive immune system results in activation of these cells. Thereby the number of immune cells that can recognize the antigens (of pathogens and tumor cells) increases and subsequently, elimination of pathogens or tumor cells can be achieved. After clearance, memory for the recognized antigens is formed and this enables the immune system to respond faster upon secondary encounter with the same antigen. The two main cell types that are involved in the adaptive system are B cells and T cells; of which B cells mediate their effects through the production of antigen-specific antibodies. T cells can be divided into two types: T helper cells ($CD4^+$ T cells) and cytotoxic T cells ($CD8^+$ T cells). $CD4^+$ T cells are important for the regulation of the immune response and $CD8^+$ T cells can directly eliminate virus-infected cells and tumor cells. However, not always does the immune system succeed in eliminating virus-infected cells or tumor cells. To induce or improve antigen-specific immune responses in such circumstances, immune activating strategies are being developed (immunotherapy). For rational design or improvement of current immune therapeutic strategies a better understanding of the factors controlling the adaptive immune responses is required. In this thesis we investigated factors that are important for shaping immune responses and more specifically, the $CD8^+$ T cell response.

One of the factors that are known to be important for a potent $CD8^+$ T cell response is the cytokine IL-2 (an important growth factor for $CD8^+$ T cells), which needs to be produced by the $CD8^+$ T cells themselves. In chapter 2 we show that predominantly the memory $CD8^+$ T cells that produce IL-2 are the cells that divide the most after antigen recognition and that the frequency of these cells within an antigen-specific population predicts the degree to which this population can expand upon secondary challenge. By enhancing IL-2 production in $CD8^+$ T cells we showed that the extent to which a single $CD8^+$ T cell can divide depends on the amount of IL-2 that the cell can produce. For proper activation and subsequent IL-2 producing capacity, $CD8^+$ T cells need, in addition to antigen recognition, extra signals (costimulation). These costimulatory signals are provided to the T cells by other cells of the immune system; antigen presenting cells (APCs). The costimulatory receptors are expressed on the cell-surface of T cells (e.g. CD27 and CD28), while the costimulatory ligands are expressed on APCs after activation (e.g. CD70 and CD80/86). In chapter 3 we report about the role of CD27-CD70 and CD28-CD80/86 interactions in the induction of IL-2 production by $CD8^+$ T cells. We show that these interactions are jointly required for induction of optimal IL-2 production. Also we demonstrate

that IL-2 gene transcription that is initiated upon CD27 and/or CD28 costimulation is dependent on the NF-κB member c-rel. Next, in chapter 4, we discuss how this information can be used to improve a form of immunotherapy called adoptive cell therapy (ACT). We provide an overview of cytokine-mediated signals and costimulatory signals that have the potency to enhance the therapeutic effect of ACT. Combinations of these stimulatory signals with blocking antibodies to inhibitory molecules and vaccination are probably necessary to improve clinical responses of ACT.

Viral infections often elicit strong T cell responses; therefore virus infection models are often used to study T cell responses as well as their requirements. In chapter 5 we use Cytomegalovirus (CMV) infection as a model to gain more insight into the regulation of T cells. We found that the viral dose with which the infection is initiated determines which characteristics the T cells acquire during infection. A low viral dose predominantly induces memory $CD8^+$ T cells with high IL-2 producing capacity and proper potential to divide upon secondary encounter with the same antigen (central memory T cells), whereas in memory T cells that arise upon high dose infection these functions are decreased while cytotoxic functions are increased (effector memory T cells). One of the characteristics of these CMV-specific effector memory $CD8^+$ T cells is that their numbers increase over time (inflation), therefore they are often referred to as inflationary T cells. However, we have shown that $CD8^+$ T cell inflation is impaired when infection occurred with a low CMV dose. In addition, we show that B cell responses are also differentially affected by the infectious dose; heightening of the infectious dose results in an increased degree of accumulation of CMV-specific antibodies (chapter 5 and 6).

It is suggested that long-standing CMV infection is harmful for the host by accelerating the (natural occurring) aging of the immune system, resulting in a decreased capacity to clear new infections. In chapter 7 we show that CMV does not per definition causes intensified immune ageing, but that it solely occurs after infection with a high dose. We demonstrate that high dose CMV infection impairs $CD8^+$ T cell and antibody responses against newly encountered viruses. How CMV manages to decrease immune responses to newly encountered antigens is a matter of great interest and being investigated by many researchers in the field. In chapter 8 we discuss some recent insights regarding the manners by which CMV compromises immune responses to new antigens.

In addition to addressing the effect of different infectious doses of CMV, we also investigated the influence of the administration route on the $CD8^+$ T cell response. We compared the intraperitoneal route, which is not a natural route of infection with intra-nasal and intra-gastric infection. The latter two are both considered to represent a natural way of infection. However, comparison revealed that the route of infection does not differentially influence T cell phenotype and function (chapter 9). Important to note is that it

is likely that the location where CMV-specific CD8+ T cells accumulate differs between routes of infection, though this needs to be confirmed.

Finally, chapter 10 provides an overview and discussion regarding all research that is presented in this thesis. To summarize, we have studied factors that control the differentiation of $CD8^+$ T cells into the different types of memory cells. We have shown that the type of memory $CD8^+$ T cell that is induced upon viral infection is dependent on the height of the viral dose. The capacity of memory $CD8^+$ T cells to divide after secondary encounter with the same antigen is determined by their potential to produce IL-2. Although many more variables are involved in the shaping the $CD8^+$ T cell response, these findings already contribute to the understanding of how immunotherapeutic treatments can be improved.

NEDERLANDSE SAMENVATTING

Het immuunsysteem is een verdedigingsmechanisme dat het lichaam beschermt tegen ziekteverwekkende micro-organismen zoals virussen en bacteriën. Het systeem kan daarnaast ook tumorcellen identificeren en elimineren. Het immuunsysteem kan worden onderverdeeld in het aangeboren (aspecifieke) en het adaptieve (specifieke) systeem. Het aangeboren systeem vormt de eerstelijnsafweer van de gastheer én is essentieel voor het activeren van het adaptieve systeem. Specifiek voor cellen van het adaptieve immuunsysteem is dat ze kleine fragmenten van pathogenen en tumorcellen (antigenen) kunnen herkennen. De herkenning van een antigeen zorgt ervoor dat de immuun cel wordt geactiveerd en gaat delen. Hierdoor neemt het aantal actieve immuun cellen dat het antigeen kan herkennen toe (expansie) en kan het pathogeen of de tumor worden opgeruimd. Nadat de ziekteverwekker is geëlimineerd vormt zich geheugen voor het antigeen. Dit stelt het immuunsysteem in staat om sneller te reageren wanneer het opnieuw in aanraking komt met hetzelfde pathogeen of dezelfde tumor. De twee celtypen die samen het adaptieve immuunsysteem vormen zijn: B-cellen die antigeen-specifieke antilichamen produceren en T-cellen. T-cellen zijn onder te verdelen in twee categorieën: T-helpercellen ($CD4^+$ T-cellen) en cytotoxische T-cellen ($CD8^+$ T-cellen). CD4+ T-cellen zijn belangrijk voor de regulering van de immuunreactie en $CD8^+$ T-cellen kunnen direct virus-geïnfecteerde cellen en tumorcellen opruimen. Het immuunsysteem slaagt er echter niet altijd in om virus-geïnfecteerde cellen of tumorcellen te elimineren. Therapieën die zorgen voor verbeterde activatie van het immuunsysteem (immunotherapie) kunnen dan uitkomst bieden. Er wordt veel onderzoek gedaan om huidige therapieën te verbeteren en nieuwe therapieën te ontwikkelen. Een beter begrip van de factoren die de adaptieve immuunreactie reguleren kan hierbij helpen. In dit proefschrift hebben we aspecten onderzocht die belangrijk zijn voor goede activatie van het immuun systeem en in het bijzonder voor de ontwikkeling van $CD8^+$ T-cel reacties.
Voor een goede reactie van geheugen $CD8^+$ T-cellen is het cytokine IL-2 (een groei factor) zeer belangrijk. Een voorwaarde is wel dat IL-2 door de $CD8^+$ T-cellen zelf wordt geproduceerd. In hoofdstuk 2 tonen we aan dat vooral de geheugen $CD8^+$ T-cellen die IL-2 produceren, de cellen zijn die na antigeen herkenning gaan delen. Het percentage van deze cellen binnen een antigeen-specifieke populatie is voorspellend voor de mate waarin deze populatie kan expanderen. Door de hoeveelheid IL-2 die geproduceerd wordt te verhogen zagen we dat de mate waarin een $CD8^+$ T-cel kan delen, afhankelijk is van de hoeveelheid IL-2 die de cel kan produceren. Om IL-2 te kunnen produceren moeten $CD8^+$T-cellen goede activatie signalen krijgen. Dit houdt in dat naast antigeen herkenning, $CD8^+$ T-cellen extra signalen nodig hebben (costimulatie). Costimulatie komt tot stand door interacties tussen oppervlakte moleculen op T cellen en een ander type immuun cellen, namelijk antigen-presenterende cellen (APC's). De receptoren zitten op het celoppervlak van de T-cellen (bijvoorbeeld CD27 en CD28) en hun liganden

op APC's (bijvoorbeeld CD70 en CD80/86). In hoofdstuk 3 beschrijven we de invloed van CD27-CD70 en CD28-CD80/86 interacties op de IL-2 productie door $CD8^+$ T-cellen. We stellen hier vast dat CD27-CD70 en CD28-CD80/86 interacties gezamenlijk nodig zijn voor optimale IL-2 productie door $CD8^+$ T-cellen. Ook tonen we aan dat de afschrijving van het IL-2 gen die start na antigeen herkenning in combinatie met CD27 en/of CD28 costimulatie afhankelijk is van de transcriptie factor c-rel. Vervolgens (hoofdstuk 4) bespreken we hoe deze informatie kan helpen om adoptieve celtherapie (ACT) te verbeteren. Dit is een vorm van immunotherapie waarbij antigeen-specifieke cellen uit de patiënt worden gehaald en worden geëxpandeerd waarna ze in grote hoeveelheden worden terug gegeven aan de patiënt. We bespreken in dit hoofdstuk hoe cytokine-gemedieerde signalen en costimulerende signalen het therapeutische effect van ACT zouden kunnen verbeteren. Naast het toedienen van stimulerende signalen, kunnen immuun reacties ook worden verbeterd door remmende signalen te blokkeren met behulp van antilichamen, of door te vaccineren. Waarschijnlijk zal een combinatie van verschillende strategieën nodig zijn om de klinische resultaten van ACT duidelijk verbeteren.

Virus infecties wekken meestal sterke T-cel reacties op en daarom worden virus infectie modellen vaak gebruikt om T-cel reacties te bestuderen. In hoofdstuk 5 gebruiken we muis Cytomegalovirus (CMV) infectie als model om meer inzicht te krijgen in de regulering van de T-cel reactie. We laten zien dat de virale dosis waarmee de infectie wordt geïnitieerd bepaalt welke eigenschappen de T-cellen verwerven tijdens de infectie. Na een infectie met een lage dosis hebben de geheugen $CD8^+$ T-cellen die zijn ontstaan een hoog IL-2-producerend vermogen en een goede capaciteit om te delen als ze opnieuw in aanraking komen met hetzelfde antigeen (centrale geheugen T-cellen). Geheugen T-cellen die ontstaan als gevolg van een hoge dosis CMV daarentegen, hebben verminderde capaciteit om IL-2 te maken en te expanderen, terwijl de celdodende (cytotoxische) functies zijn verhoogd (effector geheugen T-cellen). Een belangrijk kenmerk van deze CMV-specifieke effector geheugen $CD8^+$ T-cellen is dat hun aantal toeneemt in de tijd (inflatie), daarom worden ze vaak inflatoire T-cellen genoemd. Wij hebben echter aangetoond dat de inflatie van $CD8^+$ T-cellen vooral optreedt na infectie met een hoge dosis en dat het sterk verminderd is na infectie met een lage dosis CMV. Daarnaast laten we zien dat de CMV infectie dosis ook invloed heeft op de B-cel reacties; een verhoging van de infectieuze dosis zorgt voor een verhoogde inflatie van CMV-specifieke antilichamen (hoofdstukken 5 en 6).

Verschillende onderzoeken hebben laten zien dat langdurige CMV-infectie schadelijk is voor de gastheer omdat het de (natuurlijke voorkomende) veroudering van het immuunsysteem versnelt. Sterke veroudering van het immuunsysteem heeft tot gevolg dat de capaciteit om nieuwe infecties op te ruimen is verlaagd. In hoofdstuk 7 laten we zien dat alleen een hoge dosis CMV de $CD8^+$ T-cel en B-cel reacties tegen nieuwe virussen kan

verzwakken; CMV versnelt immuun-veroudering dus niet per definitie. Dit fenomeen treedt alleen op na infectie met een hoge dosis. Naar de vraag hoe CMV immuunreacties verlaagt wordt veel onderzoek gedaan. In hoofdstuk 8 geven we een overzicht van enkele recente inzichten over de manieren waarop CMV immuunreacties tegen nieuwe antigenen vermindert. Naast het effect van de infectieuze doses, hebben we ook de invloed van de toedieningsroute van CMV op de $CD8^+$ T-cel reactie onderzocht (hoofdstuk 9). De intra-peritoneale (i.p. in de buikholte) route wordt veel gebruikt voor het initiëren van een muis CMV infectie, maar weerspiegelt geen natuurlijke manier van infectie. Wij hebben de i.p. route vergeleken met twee infectie routes die wel beschouwd worden als mogelijke modellen voor natuurlijke infectie: de intra-nasale (via de neus) en intra-gastrische (in de maag). Uit onze vergelijking bleek echter dat de T-cellen die werden geïnduceerd na infectie via de verschillende routes niet van elkaar verschilden. Wij verwachten echter dat de locaties in het lichaam waar de CMV-specifieke effector memory CD8+ T-cellen de sterkste inflatie laten zien waarschijnlijk wel verschillen tussen de verschillende routes.

In hoofdstuk 10 bediscussieer ik de resultaten uit alle voorgaande hoofdstukken. Kort samengevat hebben we factoren onderzocht die de ontwikkeling van $CD8^+$ T-cellen naar de verschillende typen geheugencellen beïnvloeden. We hebben aangetoond dat de verhouding centrale geheugen cellen / effector geheugen $CD8^+$ T-cellen in CMV infectie afhankelijk is van de hoogte van de infectieuze dosis. Verder laten we zien dat de capaciteit van de geheugen $CD8^+$ T-cellen om te delen wanneer ze opnieuw in aanraking komen met hetzelfde antigeen, afhankelijk is van de mate waarin ze IL-2 kunnen produceren. Ondanks het feit dat er veel meer variabelen betrokken zijn bij de ontwikkeling van de $CD8^+$ T-cel reactie dan we hier bespreken, dragen onze bevindingen bij aan de kennis die nodig is voor het ontwikkelen en verbeteren van immuun therapeutische behandelingen.

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CURRICULUM VITAE

Anneke Redeker was born on 17 February 1977 in Apeldoorn, the Netherlands. In 1995 she obtained her HAVO diploma and in the same year she started her bachelor immunology at the Hanze Hogeschool in Groningen. As part of this bachelor, she did a nine month internship at the Tumor Immunology group at the University Medical Center Groningen under supervision of Prof. Dr. Wijnand Helfrich. After obtaining her bachelor degree in 1999 she started working in 2000 as a research technician at the Immunohematology and Bloodtransfusion (IHB) department at the Leiden University Medical Center and she continued working there as a technician until 2013. In March 2013 she started with her PhD project at the IHB department under the supervision of Dr. Ramon Arens and Prof. Dr. Ferry Ossendorp. She currently works as a post-doctoral researcher in the group of Dr. Ramon Arens.

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LIST OF PUBLICATIONS

Human plasmacytoid dendritic cells acquire phagocytic capacity by TLR9 ligation in the presence of soluble factors produced by renal epithelial cells

Ruben JM, García-Romo GS, Breman E, van der Kooij S, **Redeker A**, Arens R, van Kooten C

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