What drives memory T cell inflation during MCMV infection?

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1. Summary

Cytomegaloviruses (CMVs), members of the β-herpesvirus family, infect between 60 to 90% of the human population throughout all geographic locations and socioeconomic groups. Acute CMV infection in humans and in mice is often clinically silent and is controlled by multiple arms of innate and adaptive immunity. However, complete clearance is never achieved, and similar to other herpesviruses, CMVs persist in their hosts lifelong in a latent form. Latent infection is believed to be a dynamic state, characterized by sporadic viral reactivation events which are readily controlled by the immune system. The importance of immune surveillance of CMV latency is evidenced by the severe consequences that CMV reactivation from latency causes in immunocompromised hosts. In particular, CD8 T cells are believed to play a major role by directly sensing virus reactivation events and terminating them by virtue of their effector functions. Evidence in support of this hypothesis is the exceptionally large CD8 T cell response that is induced in all seropositive humans as well as in feral mice, reaching up 30% of the total memory CD8 T cell pool. The mechanism leading to this impressive accumulation of memory CD8 T cells is largely unknown, but their highly activated phenotype is indicative of antigen-driven maintenance. The strong analogies between human CMVs (HCMVs) and murine (MCMVs) with respect to their interplay with the host immune systems make the mouse model a very valuable system to investigate the mechanism of antigen presentation to CD8 T cells during latency. Detailed analyses performed in C57BL/6 mice revealed that only a limited subset of CD8 T cells, referred to as ‘inflationary CD8 T cells’, contribute to this large response by increasing in number upon resolution of the acute infection. The majority of the MCMV-specific CD8 T cells that dominated the acute infection in fact contracted upon control of the infection and was present at low numbers with a T\textsubscript{CM} phenotype during latency.

To investigate the molecular and cellular factors contributing to the development of two so diverse CD8 T cell response patterns within one host during MCMV infection, we have successfully generated MHC class I-restricted TCR transgenic mouse lines with specificity for the MCMV-derived epitope M38, representing an immunodominant epitope of the inflationary response. In addition, we are currently generating TCR transgenic mice specific for one epitope derived from the M45 protein of MCMV, which induces a conventional type of CD8 T cell response.
With the assumption that induction and maintenance of the inflationary response during MCMV latency is antigen dependent, we first aimed to identify the cell types responsible for antigen presentation to inflationary CD8 T cells. We approached this question by generating bone-marrow chimeras in which the M38 epitope could be presented on every cell type, or exclusively on hematopoietic cells, and normalized the initial population of M38-specific CD8 T cells by adoptively transferring M38-specific transgenic cells prior to infection. We found that antigen presentation on cells of non-hematopoietic origin is absolutely required for memory inflation, and that these cells are also the major reservoir of latent virus. In addition we observed that M38-specific CD8 T cells in the lymph nodes, which display predominantly a T_{CM} phenotype, but not those in the periphery, where they are terminally differentiated, proliferated during latency in response to antigen presentation on non-hematopoietic cells. Based on these findings, we concluded that the inflationary pool in peripheral tissues is fueled by lymph-node resident CD8 T cells which are locally reactivated by MCMV antigens presented on non-hematopoietic cells, inducing their local expansion and migration to peripheral tissues where they can guarantee constant immunosurveillance.

Our proposed model of antigen presentation to inflationary CD8 T cells during MCMV latency suggests that viral antigens are presented directly on infected non-hematopoietic cells. However it is not to exclude that DCs acquire viral antigens through phagocytosis of apoptotic infected cells, and present them to CD8 T cells in a process referred to as cross-presentation. We therefore aimed to assess the role of direct versus cross-presentation in the induction and maintenance of the CD8 T response during latency, and compared it to the responses induced during the acute phase of infection. Using of mouse model that selectively lacks the major subsets of cross-presenting DCs, we discovered that CD8 T cell inflation and priming rely on different antigen presentation pathways. While the first was largely independent on cross-presentation, confirming our original hypothesis of antigen sensing directly on latently infected non-hematopoietic cells, CD8 T cell priming was strongly reduced in the absence of cross-presenting DCs. This finding outlines a plausible mechanism of how the immune system could induce strong priming despite the numerous immune evasion strategies of CMV aimed at inhibiting antigen presentation to CD8 T cells.
2. Riassunto

Il Cytomegalovirus (CMV) appartiene alla famiglia degli Herpes virus, precisamente alla sottofamiglia beta (β), e infetta la maggior parte della popolazione in tutte le aree geografiche. Sia negli esseri umani sia nei topi, l’infezione primaria è spesso asintomatica ed è controllata da diverse componenti del sistema immunitario innato e specifico. Tuttavia l’eradicazione completa non avviene, e come gli altri membri della famiglia, il virus rimane latente nell’organismo per il resto della vita. La fase latente dell’infezione è da considerarsi piuttosto dinamica, caratterizzata da episodi ricorrenti di riattivazione i quali però sono prontamente controllati dal sistema immunitario. Ma se il sistema immunitario è compromesso, come ad esempio in soggetti che sono stati sottoposti ad un trapianto o in pazienti affetti da HIV, una riattivazione dell’infezione latente può causare serie complicazioni.

Una funzione particolarmente importante è svolta dai linfociti T CD8⁺, per la loro capacità di percepire, e immediatamente terminare, eventi di riattivazione del CMV latente. Una delle prove a sostegno di questa ipotesi è la presenza di una percentuale eccezionalmente alta (può raggiungere fino il 30% della totale popolazione di linfociti T CD8⁺ di memoria) di linfociti CD8⁺ di memoria specifici per il CMV in tutti i pazienti infetti, specialmente nelle persone anziane. Il meccanismo che porta a questo accumulo di linfociti T CD8⁺ di memoria è in gran parte sconosciuto, ma il loro fenotipo caratteristico di cellule T di memoria effettrici differenziate in maniera terminale (T_EM), suggerisce che il loro mantenimento è mediato da un’esposizione stocastica e costante all’antigene durante la fase cronica dell’infezione. Le forti analogie tra il CMV umano (HCMV) e murino (MCMV) fanno del modello murino un sistema appropriato per lo studio della risposta T CD8⁺ di memoria indotta durante la fase cronica dell’infezione. Analisi dettagliate compiute nei topi C57BL/6 hanno rivelato che solo una parte dei linfociti T CD8⁺, denominati ‘inflazionistici’, contribuisce a generare questa imponente risposta CD8⁺ durante la fase cronica continuando ad espandere anche dopo la risoluzione della fase acuta dell’infezione. Per contro la maggior parte dei linfociti T CD8⁺, denominati ‘convenzionali’, contrae a seguito del controllo dell’infezione acuta, dando origine a una piccola popolazione di linfociti T CD8⁺ della memoria centrali (T_CM).

Allo scopo di investigare i fattori molecolari e cellulari che contribuiscono allo sviluppo di due risposte CD8⁺ così differenti durante l’infezione con CMV, abbiamo generato dei topi transgenici in cui i linfociti T CD8⁺ esprimono tutti il recettore TCR specifico per un peptidi
derivato dalla proteina M38 che domina la risposta CD8+ inflazionaria. Al momento stiamo inoltre generando una linea di topi analoga ma con specificità per un peptide derivante dalla proteina M45 che induce una risposta CD8+ convenzionale.

Partendo dal presupposto che il mantenimento dei linfociti inflazionistici con un fenotipo attivato sia antigene-dipendente, volevamo identificare i tipi di cellule che presentano l’antigene ai linfociti T durante la fase cronica dell’infezione. Abbiamo perciò generato dei topi chimera in cui l’antigene M38 può essere presentato virtualmente da ogni tipo di cellula, oppure esclusivamente da cellule ematopoietiche, e seguito l’evoluzione della risposta CD8+ diretta contro M38 durante l’infezione con MCMV. Per normalizzare la popolazione iniziale di linfociti CD8+ specifici per M38, cellule esprimenti il TCR transgenico sono state trasferite in modo adottivo prima dell’infezione. Questo esperimento ha rilevato l’essenziale importanza delle cellule non-ematopoietiche, che sono anche la riserva principale del MCMV latente, nella generazione e il mantenimento della risposta CD8+ di tipo inflazionario. Abbiamo inoltre osservato che il classico fenotipo attivato dei linfociti T di tipo inflazionario è solo visibile negli organi periferici, ma non nei linfonodi, dove queste cellule esprimono un fenotipo di memoria centrale T<sub>CM</sub>. Come atteso sulla base del differente potenziale proliferativo, le cellule nei linfonodi, ma non negli organi periferici, proliferano in risposta dell’antigene presentato dalle cellule non-ematopoietiche. Questi dati supportano l’ipotesi che il pool di linfociti T CD8+ inflazionistici nei tessuti periferici è continuamente alimentato da linfociti che sono stati attivati localmente da cellule infette di tipo non-ematopoietico nei linfonodi. La presenza costante di un elevato numero di linfociti effettori nella periferia garantisce la sorveglianza contro un’eventuale riattivazione del virus latente.

Il nostro modello suggerisce che durante la fase cronica dell’infezione l’antigene viene presentato dalle cellule non-ematopoietiche in modo diretto. Tuttavia non è da escludere che le cellule dendritiche (DCs) acquisiscano l’antigene virale tramite fagocitosi di cellule apoptotiche per poi presentarlo ai linfociti T CD8+ in un processo denominato cross-presentazione. Abbiamo quindi valutato il contributo della cross-presentazione nell’induzione delle risposte CD8+ di tipo convenzionale e inflazionario durante l’infezione con MCMV usando dei topi che mancano dei principali sottoinsiemi di DCs capaci di cross-presentare. Abbiamo scoperto che il priming dei linfociti T vergini (naive) durante la fase acuta dell’infezione è in gran parte dipendente dalla cross-presentazione, mentre l’accumulazione dei linfociti T di tipo inflazionario avviene principalmente tramite presentazione diretta dell’antigene. Due diversi meccanismi di presentazione dell’antigene che permettono da una
parte il priming efficiente dei linfociti nonostante le strategie di evasione immunitaria del CMV, e dall’altra il mantenimento di una popolazione di linfociti T effettivi negli organi periferici che viene attivata prontamente in caso di riattivazione del virus latente.
3. General Introduction

3.1 Cytomegaloviruses (CMVs)

Herpesviruses are large, double-stranded DNA viruses which infect the majority of the human population. Based on biological criteria, such as host range and growth kinetics, herpes viruses have been assigned to three different families: α-, β-, and γ-herpesviruses [4]. All herpesviruses share the ability to establish latent infection, probably due to the very long co-evolution with their host. Prototypes of the β-herpesvirus family are Cytomegaloviruses (CMVs), which include human (HCMV), chimpanzee (CCMV), rhesus macaque (RhCMV), mouse (MCMV), and rat CMV (RCMV). Their genomes are the largest among herpesviruses, encompassing up to 230 kbps, and containing about 230 ORFs [5]. The central region of the CMVs genomes, which contains about 40-50 ORFs, also called public genes [6], are highly conserved among different strains and encode for core proteins required for replication of viral DNA and assembly into viral particles. At the terminal regions, so called private genes are highly species-specific. Some of them share similarities with host genes and have important immune-evasion functions (see chapter 'Immunevasion'). Private genes are believed to be the result of eternalness co-evolution between the host and the virus and to be the cause for CMV’s ability to avoid complete elimination and establish lifelong latency.

3.2 Pathogenesis of CMVs infection

Infection with CMV can occur at any age, but it is more likely to happen during early childhood. CMV can spread vertically via breast milk or transplacentally, or horizontally, mostly through secretions like saliva, semen and cervicovaginal fluids [7]. CMV infection encompasses three different phases: the acute phase of infection, characterized by extensive virus replication and dissemination to almost every organ of the host. Acute infection is efficiently controlled by the immune system quite rapidly in most of the organs. In the salivary glands, however, CMV is particularly virulent and productive infection can last for up to two months (in mice) before it is eventually controlled, terminating the so called persistent phase of the infection [8,9]. Virus persistence in salivary glands seems to be particularly relevant in acutely infected children, who tend to carry the virus in the saliva and in the urine for long periods, facilitating horizontal transmission [10]. Yet complete clearance never happens, as CMV establishes a latent phase of infection which lasts for the whole life of the host.
The entire course of the infection usually progresses asymptotically, and manifestations of overt disease or organ damage are avoided by a very effective immune control. This is consistent with the fact that the majority of the human population is not even aware of its CMV seropositivity. However, CMVs are important opportunistic viruses affecting immunodeficient hosts, as observed for immunosuppressed transplant recipients or late-stage immunocompromized HIV patients. Reactivation of the latent virus due to impaired immune control can lead to various clinical manifestations, including pneumonitis, hepatitis, retinitis and encephalitis, which are often lethal for the patients. [7,10,11,12].

### 3.3 CMV tropism

Precise characterization of CMV tropism is intricate: it is generally very broad, and most likely depends on the replication status of the virus. Acute productive infection is likely to happen in highly permissive cells like fibroblasts and smooth muscle cells [13,14], whereas virus spread to different organs is promoted by monocytes and DCs [15]. Persistence has been shown to occur in glandular epithelial cells of salivary glands, thereby favoring host to host transmission via saliva [8] (Walton, S.M. et al. article in press). There are many discrepancies on the identification of the cell type that harbor the latent CMV genome, mainly due to the absence of a very sensitive technique to detect very low numbers of latently infected cells. In human CMV infection, undifferentiated myeloid cells have been identified as the major site of virus latency [16,17], whereas in the Balb/c mouse model latent MCMV has been mainly detected in endothelial cells [18,19].

### 3.4 CMV latency

Viral latency has been defined as persistence of viral genomes in the absence of lytic viral replication. Establishment of and reactivation from CMV latency are regulated at the chromatin level, where a critical step is likely to be silencing/desilencing of the major immediate-early promoter (MIEP) [20,21]. Although total viral reactivation is controlled by the immune system, low-level of persistent infection likely co-exists with true latency. Unclear is, however, if target cell intrinsic factors regulate the establishment of a latent versus persistent infection, or whether rather external factors like inflammatory or maturation stimuli which modify the activation or differentiation state of the infected cell induce reactivation of the latent virus. The extrinsic regulation of viral reactivation is supported by the observation that LPS, TNF-α, or IL-1β trigger latent MCMV reactivation in Balb/c mice [22]. Notably, the MIEP contains binding sites for NF-kB and AP-1, suggesting that pro-inflammatory
mediators might ultimately lead to NF-kB and AP-1 binding to MIEP, inducing viral reactivation [23]. In a more physiological experiment, Grizmel et al. divided the lungs of latently infected mice into 18 pieces, and detected low levels of IE1 and IE2 transcripts in some but not all pieces, implying that MCMV reactivation might occurs stochastically in some latently infected cells, possibly due to local inflammatory stimuli, while remaining latent in other cells [24].

3.5 Immune control

When talking about immune control of CMV, it is important to first clarify that no single arm of the immune system is absolutely crucial for virus control, but is rather a concerted and redundant contribution from different components of the innate and adaptive immune system. Most of our knowledge on the role of single effector cell subsets in immunity to CMV comes from studies performed in mice.

Control of primary infection

Primary infection with MCMV is largely controlled by the innate arm of the immune system, mainly by the action of type I and type II IFN and by NK cells [25]. NK cells primarily act via the activating receptor Ly49H, which unlike most other NK activating receptors it does not uses MHCI as ligand, but recognize infected cells by binding to the m157 gene product of MCMV [26]. Upon activation, NK cells control viral infections by cytotoxic mechanisms and by secretion of proinflammatory cytokines. As Ly49H is expressed on C57BL/6 mice [27], but not on 129/J and BALB/c strains, the former are classified as ‘resistant strains’, whereas the latter are classified as ‘susceptible strains’. Less dramatic appears to be the role of T cells in the control of the primary infection: when C57BL/6 mice are depleted of CD8 T cells, no effects on virus control is observed, whereas depletion of CD4 T cells results in impaired control exclusively in the salivary glands [28,29]. However, CD8 T cells play a fundamental role in immunocompromised hosts, where adoptively transferred MCMV-specific CD8 T cells are able to control CMV infection and prevent CMV-mediated organ diseases [30] [31]. Antibodies are likely to be dispensable during primary infection (Jonjic 1994, Lawson CM 1988) but might contribute to restriction of viral dissemination during primary infection and during reactivation events. Similar to adoptive transfer of CMV-primed CD8 T cells, transfer of CMV-specific antibodies were also shown to confer at least partial protection upon MCMV challenge [29].
Control of latent infection

The strongest evidence for a direct involvement of the immune system in controlling CMV latency comes from the severe consequences that CMV reactivation causes in immunocompromized patients.

A study performed in B-cell deficient mice revealed that CD8, CD4 T cells and NK cells play a hierarchical and redundant role in controlling latent infection, meaning that depletion of one of these subsets did not induce virus reactivation, but was evident upon removal of an additional subset [32]. Neutralization of IFN-γ also gave rise to recurrent infection if combined with CD8 or CD4 T cell depletion, indicating that viral gene expression in reactivating cells might be sensed by these subsets of lymphocytes and immediately arrested through IFN-γ secretion. Evidences for a protective role of CD8 T cells during MCMV latency has been provided by Simon et al. [33] In this study, a MCMV mutant strain carrying a mutation which abrogates MHCI binding of an immunodominant epitope (pp89) derived from the immediate-early gene IE1 was generated. Mice infected with this virus showed a 5-fold increase in IE1 transcripts in the lungs compared to mice infected with WT MCMV, with an evident transition to IE3 transcription.

3.6 Memory inflation

More than for their function, CD8 T cells have been, and still are under extensive investigation due to their peculiar kinetics that are induced during the course of CMV infection. Longitudinal analysis performed in mice revealed that whereas most of the MCMV-specific CD8 T cells that expand during the acute phase of infection contract as the primary infection is resolved (referred to as conventional CD8 T cells), few others, the so called ‘inflationary’ CD8 T cells, expand further and are maintained at high percentages lifelong [34,35,36]. Conventional and inflationary CD8 T cells differ not only in their kinetics, but also in their activation state during latency, the former possessing a central-memory like phenotype (CD62L+, IL7R+, KLRG-1), and the latter an effector-memory like (CD62L-, IL7R-, KLRG-1+) phenotype. The activated phenotype of inflationary CD8 T cells suggests that their maintenance might be driven by low-level of antigen that is presented during CMV reactivation events. In a model called immune sensing hypothesis of latency control [33] immediate recognition of infected cells by MCMV-specific CD8 T cells will then terminate the reactivation events by exerting effector functions. Besides being necessary for effective immune surveillance of CMV reactivation, the inflationary pool might also
contribute to immune senescence, especially for elderly individuals, where CMV-specific CD8 T cells occupy 20-50% of the total CD8 T cell repertoire, thereby impairing immunity to heterologous viruses [37] [38].

3.7 Immunevasion

CMV is a master of immune evasion with strategies to evade both the innate and the adaptive immune response. In order to prevent NK-cell mediated lysis, MCMV encodes 4 genes aimed to downregulate NKG2D ligand expression on infected cells (m138, m145, m152, m155, discussed in [39]) contributing to effective downregulation of innate immunity to MCMV. m138 has also been shown to interfere with B7-1 costimulation in infected DCs, leading to impaired T cell responses in vitro [40]. The main mechanism of escape from CD4 and CD8 T cell recognition is however through direct targeting of MHCII and MHCI expression. MCMV inhibits both the constitutive and the interferon-γ induced MHCII expression on APCs [41,42], although the genes responsible for these functions have not yet been identified. In contrast, a lot of work has been done aimed to understand MCMV-mediated MHCI downregulation and the relative inhibition of the induction of CD8 T cell responses [43]. Three MCMV-encoded genes, namely m04, m06 and m152, are involved in the regulation of MHCI expression. Proteins encoded by m06 and m152 directly prevent surface expression of MHCI-peptide complex in infected cells, the first by redirecting MHCI complexes to lysosomes the second by arresting MHCI in the Golgi compartment [44] [45]. In contrast the m04-encoded gp34 does not inhibit MHCI surface expression but binds to it in the ER, forming a complex that is expressed on the cell surface. It was initially proposed that this protein acted together with m06 and m152 gene products to inhibit antigen presentation to CD8 T cells [43] However, recent studies showed that m04’s primary function was to block NK cell activation by allowing engagement of the inhibitory Ly49 receptor. Therefore, complete downregulation of MHCI due to the action of m06 and m152, which would ultimately lead to NK cell activation, would be prevented by the expression of m04. This was indeed the case, as an MCMV virus lacking Δm04 was attenuated in vivo and in vitro in an NK cell-dependent manner [46]. Still contradictory, however, is the evidence for in vivo efficacy of these immunevasins in downregulating MCMV-specific CD8 T cell responses. In vitro, they are highly effective, as CD8 T cells are not able to recognize and kill target cells infected with WT MCMV, but they do so when Δm04Δm06Δm152MCMV is used. However in vivo MHCI downregulation seems to be dispensable as CD8 T cell responses in the presence or in the absence of these MCMV-encoded genes are almost identical [47]. This led
to the assumption that the immune system is likely to counteract MCMV-mediated inhibition of CD8 T response by cross-priming. (see chapter 4.3).

3.8 Aim of the Ph.D. thesis

The overall aim of my PhD thesis focused on the investigation of the mechanism of antigen presentation to CD8 T cells during MCMV infection. In particular we were interested in understanding how two so different kinetic patterns of CD8 T cell responses, namely the conventional and the inflationary CD8 T cell responses, are induced during the course of the infection. Our first goal was therefore to generate two T cell receptor (TCR) transgenic mouse lines, one specific for an immunodominant epitope of the conventional response and the second for an immunodominant epitope of the inflationary response. These mice will serve as a source of monoclonal populations of CD8 T cells that can be tracked in vivo during the entire course of the infection. With the help of this newly generated tool, we investigated the cellular and anatomical requirements for the generation and maintenance of the inflationary response. Finally we assessed the role of cross-presentation for priming of the CMV-specific CD8 T cell response and for the induction of memory inflation.
4. Results

4.1 Generation of MHCI-restricted TCR transgenic mice with specificities for the MCMV-derived epitopes M38316-323 and M45985-993

Introduction

The rearrangement of germ-line encoded TCRα-, β-, γ-, and δ-chain gene segments in humans and in mice gives rise to an extremely diverse and polymorphic T-cell repertoire (Fig. 1).

Fig. 1 Germ-line organization of the mouse TCRα-, β-, γ-, δ chain gene segments.

Mouse TCRα-chain and β-chain DNA (chromosome 14)

Mouse TCRβ-chain DNA (chromosome 6)

Mouse TCRγ-chain DNA (chromosome 15)

It has been estimated that the naïve peripheral mouse TCRαβ repertoire consists of ~2x10^6 different TCRs, and around a 10-fold higher number has been estimated for the human repertoire. This high diversity provides the immune system with the ability to recognize virtually every pathogen in a highly specific manner. The diversification process occurs in the thymus by stochastic recombination of V, D and J segments of the TCRβ-chain first, and of V and J segments of the TCRα-chain after, generating a potential diversity of ~10^15 different TCR receptors. Thymocytes which have succeeded in generating a functional TCR are subjected to positive and negative selection processes which results in less than 10% survival. These surviving cells will further develop into CD4+ or CD8+ single positive thymocytes before they enter in the periphery as mature T cells. Calculations on the TCRαβ diversity in the periphery of a mouse estimated the size of a CD8 T cell clone expressing the same TCR.
being about 20-50 (from \( \approx 10^8 \) total CD8 T cells) [48]. This heterogeneity, although being vital for the host, represents a major obstacle for scientists interested in studying specific T cell responses against single antigens of single pathogens, in particular to study these responses at early time points of infection prior to clonal expansion. Therefore, an invaluable tool in the T cell field is the availability of TCR transgenic mice, which are engineered to possess a monoclonal pool of T cells expressing a TCR with the desired specificity. In optimal cases, one can recover up to \( 10^7 \) identical naïve T cells from a TCR transgenic mouse, which can be used for a variety of \textit{in vivo} as well as \textit{in vitro} studies. The rationale behind the generation of a TCR transgenic mouse is based on the principle of allelic exclusion, a mechanism that is operative in the thymus during TCRα- and TCRβ-chain rearrangements. This ensures that once a productive rearrangement of a TCR-chain allele is achieved, the rearrangement of the other allele is inhibited, ensuring that all the TCRs expressed by a single T cell have the same specificity. As a consequence, mice that carry productively rearranged TCRα- and TCRβ-chain transgenes will not rearrange endogenous TCR genes to substantial levels and every T cell will express the TCR chains encoded by the transgenes. However, one should mention that the penetrance of the transgene (i.e. the resulting frequency of TCR transgenic T cells) varies between expression constructs and between founder lines. Moreover, allelic exclusion is more stringent of the TCRβ chain locus and thereby leads to substantial co-expression of transgenic and endogenous TCRα chains.

In this section, we describe the generation of MHC-I-restricted TCR transgenic mice with specificity for two epitopes of Murine Cytomegalovirus (MCMV), namely M45\textsubscript{985-993} and M38\textsubscript{316-323}. These two epitopes have been shown to induce very strong but highly diverse CD8 T cell responses upon infection of C57BL/6 mice with MCMV. While CD8 T cells specific for M45 (and other epitopes, altogether classified as ‘conventional’ CD8 T cells) undergo a classical expansion-contraction CD8 T response kinetics, reminiscent of a CD8 T cell response during a resolved viral infection, CD8 T cells specific for the M38 epitope of MCMV (and other epitopes, altogether classified as ‘inflationary’ CD8 T cells), do not contract upon resolution of the primary infection but further proliferate and are maintained at high percentage with permanent effector memory phenotype for the whole life of the infected host. Our current understanding on the reasons why two different kinetic patterns of CD8 T cell responses are induced during MCMV infection are still limited. Different functional avidities of the TCR towards the respective peptides, or different kinetics/contexts of antigen presentation during the course of the infection are plausible reasons for this phenomenon. The
availability of TCR receptor transgenic mice as source of monoclonal populations of M45-specific and M38-specific CD8 T cells will represent an important tool to investigate and understand the mode and role of antigen presentation for conventional and inflationary CD8 T cell responses during MCMV infection.

Material and methods

Mice and infections

C57BL/6N mice were bred in the local animal facility under specific pathogen-free conditions. Co-injection of the TCR transgenes into C57BL/6N oocytes was performed by Dr. Thomas Rülicke (University of Veterinary Medicine Vienna, Austria) and was performed according to the standard method [49]. Recombinant MCMV-Δm157 (m157 deletion mutant) was described previously [50] and was grown on C57BL/6 embryonic fibroblasts (MEFs) and titrated by standard plaque-forming assays as described in [51]. Infection was performed intravenously (i.v.) with $10^7$ plaque forming units (PFU) of MCMV-Δm157.

Peptides, cell lines and culture Media

MCMV-derived M38$_{316-323}$ and M45$_{985-993}$ peptides were purchased by NeoMPS (Strasbourg, France) and had a purity of >95%. The T cell hybridoma fusion partner BWZ36.1/CD8α (αβ-BW5147 mouse lymphoma cells transfected with NF-AT-lacZ reporter and CD8α expression constructs), [52] were kindly provided by Dr. Marcus Groettrup (University of Konstanz, Germany) and were grown on IMDM supplemented with 10% FCS, 100 U/ml penicillin/streptomycin, 2mM L-glutamine (PSG) and 0.4 mg/ml Hygromycin B. B16-Flt3L were grown in DMEM containing 10% FCS and PSG. Thioglycolate-induced peritoneal macrophages were obtained by injecting 1.5 ml of a 4% Thioglycolate solution (Chemie Brunschwig AG, Basel, Switzerland) in the peritoneum of C57BL/6 mice which have been treated with the analgesic Temgesic (10 µg/l in drinking water) for at least 14 hours before injection. 3 days later, the abdominal cavity was washed with PBS. Isolated cells could be frozen or directly cultured in RPMI containing 10% FCS and PSG. The IL-2 sensitive cell line CTLL-2 was cultured in RPMI supplemented with 10% FCS, PSG and 100U/ml rIL-2 (R&D Systems, Abingdon, UK).
Generation of M38- and M45-specific CD8 T cell hybridomas

**Antigen presenting cells:** Plasmacytoid DC-enriched splenocytes were obtained by injecting 10^6 B16-Ft3L cells in the nuchal fold of C57BL/6 mice. 12 days later spleens were isolated, digested for 30 min at 37°C in RMPI containing 2.4 mg/ml Collagenase I (Gibco Invitrogen, Basel, Switzerland) and finally smashed to obtain single cell suspensions. Cells were loaded with 10^{-8}M M38 or M45 peptides for 1 h at 37°C, followed by extensive washing.

**Responding cells:** MCMV-specific memory CD8 T cells were obtained by infecting C57BL/6 mice with MCMV and at the earliest two months later spleen cells were isolated. 4x10^6 memory cells were mixed together with 1x10^6 antigen-loaded pDCs-enriched splenocytes in each well of a 24-well plate in RPMI medium supplemented with 10% FCS, PSG and 50μM β-MeOH. After two days, half of the medium was removed and replaced with new RMPI medium additionally supplemented with rIL-2 (final concentration 80 U/ml). After 14-20 days, when activated CD8 T cells regained a round shape, restimulation was performed in a round-bottom 96-well plate by adding 10^4 CD8 T cells to 10^3 thioglycolate-induced peritoneal macrophages which have been previously loaded with 10^{-8}M M38 or M45 peptide for 1 h at 37°C. Three days after restimulation, CD8 T cells were harvested and mixed with equal numbers of BWZ36.1/CD8α cells to generate CD8 T cell hybridomas according to the protocol described in [53]. The resulting fused cells were seeded into 96-round bottom well plates with a density of ~1 cell/well and grown in selective IMDM medium containing 10% FCS supplemented with HAT (100μM hypoxanthine, 0.4 μM aminopterin, 16 μM thymidine; Invitrogen AG, Basel, Switzerland).

**Testing Hybridoma specificity**

10^5 thioglycolate-induced macrophages/well were seeded in flat-bottom 96-well plates and loaded with 10^{-6} M M38 or M45 peptide or with Medium. 5x10^4 T cell hybridomas were added to each well followed by 24 h incubation at 37°C. To test hybridoma activation, 50 ul of the co-culture supernatant were incubated with 10^4 CTLL-2 cells overnight. Viability of the CTLL-2 cells was quantified using the Alamar blue cell viability assay (Lucerna Chem AG, Luzern, Switzerland) according to the manufacturer’s instructions.

**Characterization of the T Cell Receptor (TCR) expressed by the CD8 T cell hybridomas**

To identify the V, D and J components of the TCR expressed by the selected hybridomas, RNA was isolated from hybridomas using TRIzol reagent (Invitrogen, Basel, Switzerland) according to the manufacturer’s instructions, and cDNA was generated using M-MLV
RESULTS

Reverse Transcriptase RNase, H Minus (Promega, Dübendorf, Switzerland). cDNA was used as a template for a PCR-based screening, where each primer of a TCRα- or TCRβ-specific primer set was used as forward primer, and a unique constant region-binding primer served as reverse primer (Table I). Sequencing of the PCR products was done by Microsynth (Zürich, Switzerland) and then aligned to the mouse genome using the Ensemble database (http://www.ensembl.org/Mus_musculus). To assess the surface expression of the identified TCRVα and TCRVβ regions, surface staining was performed using the following antibodies: anti-TCRβ-chain [H57 597], anti-Vα2 [B20.1], anti-Vα8.3 [B21.14], anti-Vα11.1,11.2 [RR8-1] (BD Biosciences), and with selected antibodies of the anti-mouse Vβ TCR Screening Panel (BD Biosciences). Multiparameter flow cytometric analysis was performed using an LSRII flow cytometer (BD Biosciences) and analyzed with FlowJo software (Tree Star, San Carlos, CA). To amplify the rearranged TCRVα and TCRVβ variable regions from genomic DNA, primers were designed as indicated in Fig. 2

A

TCRVα amplification:

\[
\text{Fwd: } 5' - \text{NNN} \text{CCCGG} - 18 \text{nt} \text{ATG} - 3' \\
\text{Rev: } 5' - \text{NNNN} \text{GCGGCCG} - 15 \text{nt} - 3'
\]

B

TCRVβ amplification:

\[
\text{Fwd: } 5' - \text{NNN} \text{CTCGAG} - 27 \text{nt} \text{ATG} - 3' \\
\text{Rev: } 5' - \text{NNNN} \text{CCGCGG} - 31 \text{nt} - 3'
\]

**Fig. 2** Primer design for the amplification of the rearranged TCRVα and TCRVβ variable regions from T cell hybridomas. Forwad and reverse primers for TCRVα- and TCRVβ- chain amplifications were designed based on the criteria shown in A respectively B. Nucleotide sequences and graphic illustrations are shown.

Amplification was performed using the TripleMaster PCR System (Eppendorf) using genomic DNA of the selected hybridomas as template. PCR products were first cloned into the pGEM-T Easy vector (Promega), and after verification of the sequences, inserts were cut with the respective restriction enzymes and ligated into TCR expression cosmids pTRISα and
pTRISβ.[3] These vectors have been deliberately designed for the expression of T cell receptor genes in transgenic mice. They allow proper expression of the cloned T cell receptor genes by the presence of natural TCRα- and TCRβ- promoter/enhancer elements. In addition, they contain the entire gene locus of the constant regions Cα respectively Cβ (Fig. 3), as well as a prokaryotic region for propagation in E.coli.

Fig. 3 Vector maps of pTαcass and pTβcass [3].

The resulting pTαVαJα and pTβVβJβ were digested with SalI respectively KpnI to excise prokaryotic vector DNA. The linearized fragments were co-injected in equimolar ratios into fertilized C57BL/6N oocytes.
RESULTS

Results and Discussion

*Generation of M38- and M45- specific CD8 T cell hybridomas*

With the aim to identify and clone the rearranged TCRα- and TCRβ-chains specific for the M38- and M45- epitopes of MCMV, the first step was to generate CD8 T cell lines specific for these epitopes. To this end, we infected C57BL/6 mice with MCMV and two months later we isolated memory CD8 T cells from the spleen and restimulated them *ex vivo* with M38- and M45-peptides. By day 8 after the first restimulation, up to 40% of CD8 T cells were specific for the respective antigen as shown by tetramer staining (Fig. 4).

![Fig. 4: Generation of CD8 T cell lines](image)

**Fig. 4: Generation of CD8 T cell lines** M45- and M38- specific CD8 T cell lines were generated by *ex-vivo* restimulating splenocytes derived from a latently infected mouse with M45 or M38 peptide. As control no peptide was added. 8 days later, CD8 T cell expansion was assessed by co-staining with M45-Tetramer and M38-Tetramer. Plots are pre-gated on CD8 T cells.

After 14 days, CD8 T cells were restimulated with M38 respectively M45 peptide, and 3 days later they were harvested and fused with BWZ36.1/CD8α cells to generate CD8 T cell hybridomas. Different clones were first screened for their TCR surface expression with an antibody directed against the TCRβ chain. Positive clones were then tested for their specificity towards the M38 respectively M45 peptides by CTLL-2 assay. From the fusion with the M38-specific CD8 T cell line we obtained 47 different T cell hybridomas, 30 of which were specific for M38. Based on the TCR expression, staining with M38-tetramer and affinity towards the M38 peptide (assessed by peptide titration and quantified by CTLL-2 assay, not shown), we selected the clone number 8 (Fig. 5A). In contrast, fusion with M45-specific CD8 T cell line generated 54 different hybridomas, from which only 1 was specific for M45 and was the clone number 13 (Fig. 5B).
Fig. 5: Screening of CD8 T cell hybridomas CD8 T cell hybridomas were generated by fusing M38-specific (A) and M45-specific (B) CD8 T cell lines with BWZ36.1/CD8α cells. The obtained hybridomas were left unstained (dotted lines), stained with TCRβ (upper panels, filled curves) or with the respective tetramers (lower panels, filled curves). Two clones from each of the two fusions are shown as representative.

Characterization of the TCR Variable regions segments expressed by M38- and M45-specific CD8 T cell hybridomas

The TCR variable region of the α-chain is composed of a V and J segment, whereas the β-chain contains in addition a D segment (Fig. 1). To identify the gene segments used by the M38- and M45- T cell hybridomas, we made use of primer libraries specific for all possible TCRVα segments and TCRVβ segments expressed in the mouse (see Table I), which were used in combination with reverse primers binding to the unique constant regions Cα and Cβ to amplify the cDNA from the hybridomas as illustrated in Fig. 9. The resulting PCR products were sequenced and aligned with the mouse genome sequence, revealing that the TCR expressed by the M38-hybridoma was composed of Vα4Jα13 respectively Vβ10Jβ2.1 gene segments, whereas the M45-hybridoma expressed a TCR composed of Vα16Jα11 and Vβ8.1Jβ2.5. The expression of the TCRVβ10 on the M38-hybridoma and TCRVβ8.1 on the M45-hybridoma was confirmed by FACS staining using monoclonal antibodies directed against these particular Vβ genes (Fig. 6). Due to the unavailability of antibodies directed against TCRVα4 and TCRVα16, we were not able to prove their surface expression on M38- respectively M45-hybridomas.
RESULTS

![Fig. 6: TCRVβ expression by M38- and M45-specific CD8 T cell hybridomas. CD8 T cell hybridomas were left unstained (dashed lines) or stained with anti-Vβ10 or anti-Vβ8.1 antibodies (filled areas).]

The rearranged genomic variable regions TCRVαJα and TCRVβDβJβ were amplified from the genomic DNA of M38-specific and M45-specific CD8 T cell hybridomas with primers (amplification primers, table II) that included the XmaI/NotI restriction sites for the α-chain, respectively XhoI/SacII restriction sites for the β-chain as illustrated in Fig. 2. The amplification products (Appendix I) were digested and cloned into pTαcass respectively pTβcass and both were digested with the appropriate restriction enzymes. After linearization and removal of the prokaryotic regions, the two fragments containing the rearranged TCRVα and TCRVβ regions of the M38-hybridomas or M45-hybridomas were coinjected into fertilized C57BL/6 oocytes, which were transferred into the uterus of pseudopregnant mothers. TCRVα and TCRVβ transgene integration by the Founders was assessed by PCR using amplification primers (Table II). At the current stage, we have screened and characterized the Founders from the M38-specific TCRVαVβ transgene microinjection, while we are waiting for the Founders of the M45-specific TCRVαVβ transgene microinjection.

M38-specific TCR transgenic mouse: screening of the Founders

From the microinjection of the TCRVαJα13 and TCRVβ10Jβ2-1 transgenes into C57BL/6 oocytes 8 offsprings were born, 2 of which contained both transgenes (Nr. 4 and Nr. 8), and 1 contained only the TCRVβ10Jβ2-1 (Nr. 1) (Fig. 7).
**Fig. 7: PCR-based screening of the M38-specific TCR transgenic Founders**
Tail biopsies from Founders 1-8 were digested and lysates were directly amplified with amplification primers M38TCRαfwd and M38TCRαrev for TCRVα4Jα13 amplification and M38TCRβfwd and M38TCRβrev for TCRVβ10Jβ2-1 amplification. Amplification products were loaded on a 1% agarose gel and run at 80 V for 1 h. Numbers indicate Founder numbers.

Analysis of the TCRVβ10 expression from the blood of the Founders revealed that Founder Nr.1 expressed TCRVβ10 on nearly 100% of CD8 T cells, but only ~10% of them were binding to the M38-tetramer, likely due to fact that this mouse did not contain the TCRVα4Jα13 transgene. In contrast, the double transgenic Founders had a less efficient expression of the TCRVβ10 on CD8 T cells, however had a higher specificity towards M38, especially for the Founder Nr.8 where ~30% of the total CD8 T cells stained positive for M38-tetramer. Interestingly, upon backcrossing of the Founder Nr.8 with a WT C57BL/6 mouse, we obtained offsprings with nearly 100% of the CD8 T cells expressing the TCRVβ10 and binding to the M38-tetramer (Fig. 8).

**Fig. 8: Transgene expression by the M38-specific TCR transgenic Founders**
(A) Vβ10 expression and binding of M38-Tetramer is shown for the Founders Nr. 1, 4 and 8. Staining was performed from fresh blood samples. (B) Founders Nr.1 and Nr.8 were backcrossed to C57BL/6 WT mice. Blood samples were collected from the offsprings and tested for Vβ10 expression and binding of M38-Tetramer. 1 offspring of each breeding is shown as representative.

The mouse line derived from the Founder Nr 1 was called Mini, whereas the mouse line derived from the Founder Nr 8 was called Maxi.
RESULTS

In conclusion, we have successfully generated two TCR transgenic mouse lines with specificities for the M38 epitope of MCMV. Mini mice are highly enriched in M38-specific CD8 T cells due to transgenic expression of the TCRβ-chain specific for M38. As a consequence of the expression of the endogenous TCRα-chains, the transgenic CD8 T cells present in these mice express TCRs with diverse affinities towards the M38 peptide, as reflected from the broad range of MFIs of tetramer binding in Founder Nr. 1 (Fig. 8). In contrast, the Maxi mouse contains both the TCRα- and TCRβ-chain transgenes, though the penetrance of expression was rather low in the Founders. This is not surprising, as so called 'genetic mosaics' (i.e. transgene expression in only a fraction of the cells) are frequently observed in the Founders and are the result of transgene integration after the first round of DNA replication. Thereby the transgenes are unequally distributed to the daughter cells upon each cell division. However, backcrossing to a WT C57BL/6 mouse generated offspring with almost 100% of the CD8 T cells being specific for M38, suggesting that the transgenes were transmitted to germ line cells and that they were efficiently transcribed and translated in T cells.
Figure 9 Experimental strategy for the generation of MHCI-restricted TCR transgenic mice: cDNA was generated from CD8 T cell hybridomas and used as template for PCR amplification with TCRVα and TCRVβ screening primers. Amplification products were sequenced to determine the composition of the TCRα and TCRβ variable region segments. Based on the sequences, primers were designed in order to amplify the rearranged genomic regions from the hybridomas and subsequently clone them into multiple cloning sites of pTαCass and pTβCass. After digestion of the prokaryotic regions, linearized fragments were co-injected into fertilized C57BL/6N oocytes. Transfer into pseudopregnant mothers and screening of the founders.
## RESULTS

Table I TCRVα and TCRVβ screening primers Adapted from [1,2]

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Table II TCR_{\alpha} and TCR_{\beta} amplification primers

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Appendix I: sequences of rearranged TCR\(\alpha\) and TCR\(\beta\) genomic regions of M38- and M45-hybridomas

**M38-TCRV\(\alpha\)16J\(\alpha\)11 647 bps**

M38-TCRV\(\alpha\)4J\(\alpha\)13 623 bps

**M38-TCRV\(\beta\)8.1J\(\beta\)2.1 660 bps**

**M45-TCRV\(\alpha\)10J\(\alpha\)13 647 bps**

**M45-TCRV\(\beta\)10J\(\beta\)2.1 573 bps**
4.2 Non-hematopoietic cells in lymph nodes drive memory CD8 T cell inflation during murine cytomegalovirus infection

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Abstract

During human and murine cytomegalovirus (MCMV) infection an exceptionally large virus-specific CD8 T cell pool is maintained in the periphery lifelong. This anomalous response is only seen for specific subsets of MCMV-specific CD8 T cells which are referred to as 'inflationary T cells'. How memory CD8 T cell inflation is induced and maintained is unclear, though their activated phenotype strongly suggests an involvement of persistent antigen encounter during MCMV latency.

To dissect the cellular and molecular requirements for memory CD8 T cell inflation, we have generated a transgenic mouse expressing an MHC class I-restricted T cell receptor specific for an immunodominant inflationary epitope of MCMV. Through a series of adoptive transfer experiments we found that memory inflation was completely dependent on antigen presentation by non-hematopoietic cells, which are also the predominant site of MCMV latency. In particular, non-hematopoietic cells selectively induced robust proliferation of inflationary CD8 T cells in lymph nodes, where a majority of the inflationary CD8 T cells exhibit a central-memory phenotype, but not in peripheral tissues, where terminally differentiated inflationary T cells accumulate. These results indicate that continuous restimulation of central-memory CD8 T cells in the lymph nodes by infected non-hematopoietic cells ensures the maintenance of a functional effector CD8 T pool in the periphery, providing protection against viral reactivation events.
RESULTS

Introduction

Memory CD8 T cells develop following primary encounter with an infectious agent and provide protection against subsequent infections. Depending on their phenotype and anatomical location, memory CD8 T cells have been categorized into central-memory CD8 T cells (T_{CM}) and effector-memory CD8 T cells [54] [55,56]. T_{CM} localize to secondary lymphoid organs due to expression of the lymph nodes homing markers CD62L and CCR7, and are maintained by homeostatic proliferation responding to the cytokines IL-7 and IL-15 [57,58]. Because of their self-renewal properties and their exquisite ability to proliferate and exert effector function upon re-encounter with the original pathogen, T_{CM} are able to provide long-term protective immunity. T_{EM}, in contrast, reside predominantly in the periphery, usually at the site of primary infection, and possess a terminally differentiated phenotype, characterized by low expression of IL7Rα and CD62L and high expression of killer cell lectin-like receptor G1 (KLRG1). T_{EM} are believed to form the front line of defense against re-infections due to their immediate activation upon secondary infection at peripheral sites and due to their ready performance of effector functions [59,60]. In absence of antigen, their long-term maintenance in the periphery is generally believed to be unstable because of their poor responsiveness to IL-7 and IL-15. However, this notion has recently been challenged by the demonstration of long-term maintenance of effector memory cells in the skin, gut and brain in absence of detectable antigen persistence [61,62,63]. Studying the development and function of memory CD8 T cells should always take into account the nature of the infectious agent, as the duration of the initial antigen stimulation, the level of inflammation, the persistence of the pathogen, the cell tropism as well as the ability to interfere with the antigen presentation machinery of the host, are all factors influencing the development of the memory CD8 T cell response [64,65,66]. In particular, generation of memory responses are known to be highly perturbed during chronic or latent viral infections, where persistent antigen stimulation has often been shown to lead to functional impairment and antigen-dependent maintenance of CD8 T cells [67,68,69].

Infection with the β-herpes virus cytomegalovirus (CMV) leads to disseminated acute lytic replication which is controlled by various immune effector cells [9,26,31,70], followed by life-long latency with presumably sporadic and low-level viral reactivation events [33,71]. Thus, CMV infection allows studying memory CD8 T cell responses in the context of a latent/persistent viral infection. Both humans and mice are natural hosts of CMV and both elicit an impressively large virus-specific memory CD8 T cell response during latency which
increases with time, a phenomenon referred to as ‘memory inflation’ [34,72,73,74,75]. Longitudinal analysis performed in mice revealed that upon infection with MCMV, two major kinetic patterns of CD8 T cell responses emerge: the majority of CD8 T cells, referred to as ‘conventional CD8 T cells’, undergo expansion during the acute phase of infection followed by rapid contraction, eventually resulting in low numbers of T_{CM} cells which are stably maintained during latency by homeostatic proliferation. In contrast, so called ‘inflationary CD8 T cells’, continue to expand even after control of acute infection, and are maintained at high percentages in the absence of overt viral replication [35,36]. However, these cells display a T_{EM} phenotype, strongly suggesting that their accumulation and maintenance is driven by viral antigen [35,36,76]. Therefore, it has been postulated that during MCMV latency, low levels of viral gene expression/antigen presentation in latently infected cells, undergoing viral reactivation, would constantly stimulate inflationary CD8 T cells, which in turn would prematurely terminate such viral reactivation attempts [33,77]. However, Snyder et al. showed that inflationary CD8 T cells divide only sporadically during latency and that they are not maintained in an antigen-dependent manner, thereby favoring a model where the inflationary pool is maintained by continuous replacement of newly activated effector CD8 T cells [76].

However, critical aspects regarding the mechanism of antigen presentation during MCMV latency are still unknown. We recently showed that contrary to the primary expansion during the acute phase of infection, which was largely driven by cross-presenting dendritic cells (DCs), accumulation and maintenance of the inflationary CD8 T cell pool occurred in absence of cross-presenting DCs, arguing for different antigen presentation requirements during memory inflation [78]. In addition, priming and inflation also differ in their costimulatory requirements, the latter being independent of B7-CD28 interaction [79]. Taking together, these data suggest that antigen presentation during latency might not depend on DCs, but on other cell types which are presumably hosting latent viral genomes and directly presenting viral antigens to the inflationary CD8 T cells.

A major obstacle in studying memory inflation is the lack of a monoclonal population of inflationary CD8 T cells that can be tracked in vivo during the entire course of MCMV infection. Therefore, we generated MHC class I-restricted TCR transgenic mouse lines with specificity for the MCMV-derived epitope M38, representing an immunodominant epitope of the inflationary response in C57BL/6 mice. Through a series of adoptive transfer experiments, we discovered that memory inflation was completely dependent on antigen
presentation by non-hematopoietic cells. Consistent with this, we identified non-hematopoietic cells as major carriers of MCMV genomes during the whole course of the infection, especially during latency. While inflationary CD8 T cells with an effector phenotype accumulated during latency in the periphery without increased local proliferation, a small proportion of inflationary CD8 T cells in lymph nodes, displaying a T\textsubscript{CM} phenotype, proliferated extensively during latency. We propose a mechanism where latently infected non-hematopoietic cells stochastically present viral antigens to T\textsubscript{CM} CD8 T cells in lymph nodes, which differentiate into effector cells and migrate to peripheral tissues where they do not further proliferate but instead facilitate the control of local viral reactivation events.

Results

*Tissue-specific pattern of MCMV-specific CD8 T cell responses*

Infection of C57BL/6 mice with MCMV results in viral replication which is controlled by the immune system with an organ-dependent kinetics: clearance of infectious virus was first observed in the spleen, followed by the lungs, liver and lastly salivary glands, where lytic replication persisted for at least two months (Fig. 1A). By day 150 post infection infectious virus was no longer detected in any organ, despite the presence of the latent viral genomes [80]. MCMV infection induces two distinct patterns of CD8 T cell responses: a conventional and an inflationary response. Conventional CD8 T cells, which are dominated by M45-specific CD8 T cells, contract after the resolution of the acute phase of infection to eventually generate low numbers of T\textsubscript{CM} CD8 T cells (Fig. 1B). Inflationary CD8 T cells, exemplified by M38-specific CD8 T cells, accumulate until a stable plateau is reached. These two kinetic patterns were observed in the spleen and in peripheral organs such as lungs and liver (Fig. 1B, liver not shown), where M45-specific CD8 T cells contracted between day 7 and day 14 post infection while M38-specific CD8 T cells increased until day 14-28 post infection to eventually stabilize at high percentages. In these organs, the majority of M45-specific CD8 T cells were IL7R\textsuperscript{a\textsubscript{high}}KLRG1\textsubscript{low} and expressed high levels of CD62L (not shown) by day 150 after infection (Fig. 1C, right panels), reminiscent of a T\textsubscript{CM} phenotype. In contrast, M38-specific CD8 T cells displayed largely an effector phenotype (IL7R\textsubscript{low}KLRG1\textsubscript{high}CD62L\textsubscript{low}), especially in the lungs (Fig 1 C, left panels) and in the liver (not shown). Interestingly, in the lymph nodes (here the inguinal LN is shown) M38-specific CD8 T cells did not inflate and were present at much lower percentages than in spleen and peripheral tissues (Fig. 1B).
Moreover, about 50% of M38-specific CD8 T cells displayed a $T_{CM}$ phenotype, independent of the anatomical location of the LNs analyzed (Fig. 1C and D).

Thus, during MCMV infection two different CD8 T cell response patterns were induced, whose kinetics and phenotype were strongly influenced by the anatomical location.

Figure 1: Tissue-specific pattern of MCMV-specific CD8 T cell responses (A) C57BL/6 mice were infected with MCMV$^{\Delta m157}$ and virus titers were determined in the spleen, lungs, liver and salivary glands (SG) at days 7, 14, 28 and 150. The horizontal lines represent the respective detection limits. (B) The percentage of M38- (black bars) and M45-specific CD8 T cells (white bars) were measured by tetramer staining in the spleen, lungs and inguinal lymph nodes at the indicated time points post MCMV infection. For spleen and lungs, bars show averages of three mice per group ± SEM, whereas for lymph nodes bars represent cells pooled from three mice. (C) Representative plots showing KLRG-1 and IL7Rα expression on M38- and M45-specific CD8 T cells in the spleen, lungs and inguinal lymph nodes on day 150 post infection. All data are representative of at least three independent experiments. (D) Mediastinal, axillary, cervical and inguinal lymph nodes were pooled from three individual C57BL/6 mice which had been infected with MCMV-$\Delta m157$ for 80 days. Representative plots showing the percentages of M38-specific CD8 T cells among total lymphocytes are shown for each lymph node (upper plots), and the lower plots show the expression of KLRG-1 and IL7Rα on M38-specific CD8 T cells.
RESULTS

**Generation of MHC class I-restricted TCR transgenic mice with specificity for the M38 epitope of MCMV**

To dissect the cellular and molecular requirements for memory inflation, adoptive transfer experiments of MCMV-specific CD8 T cell populations at various differentiation statuses and at various time points of infection are required, optimally using monoclonal T cell populations to normalize the heterogeneity inherent within total populations. Therefore, we generated TCR transgenic mouse lines with specificity for the MCMV-derived M38 epitope. For this, we first generated T cell hybridomas specific for the M38-derived epitope and focused on one high affinity hybridoma whose TCR variable regions were identified as $\text{V}_{\alpha}4\text{J}_{\alpha}13$ and $\text{V}_{\beta}10\text{J}_{\beta}2.1$ (nomenclature according to Wilson et al., [81]). The rearranged genomic variable regions were amplified and cloned into TCR cosmid expression vectors which have been previously shown to allow efficient expression of transgenic TCRs [3]. Coinjection of the linearized transgenes into fertilized C57BL/6 oocytes gave rise to two different mouse lines, which we called Mini and Maxi (Fig. 2A). The Mini mouse was transgenic for the $\text{V}_{\beta}10\text{J}_{\beta}2.1$ chain only, which was expressed by nearly 100% of the CD8 T cells, while the TCR$\alpha$ chain was of endogenous origin (not shown). This increased the percentage of M38-specific CD8 T cells from undetectable levels in C57BL/6 mice to ~10%. The Maxi mouse was transgenic for both $\text{V}_{\alpha}4\text{J}_{\alpha}13$ and $\text{V}_{\beta}10\text{J}_{\beta}2.1$ variable regions, resulting in a percentage of M38-specific CD8 T cells among total CD8 T cells of around 90% (Fig. 2A).

To compare the relative affinities of the TCRs expressed by the Mini and Maxi CD8 T cells, we first analyzed their dose response to graded amounts of M38 peptide *in vitro*. Both Mini and Maxi CD8 T cells were specific for the M38 peptide, though Maxi CD8 T cells had a 10'000-fold higher affinity compared to Mini CD8 T cells as identified by *in vitro* proliferation (Fig. 2B). To determine the ability of transgenic CD8 T cells to proliferate *in vivo* in response to MCMV infection, we adoptively transferred $10^5$ Mini or $10^4$ Maxi CD8 T cells into C57BL/6 mice one day prior to infection and compared endogenous and transgenic M38-specific CD8 T cell responses in the blood at different time points after infection (Fig. 2C). Adoptively transferred cells were distinguished from endogenous cells by expression of the CD45.1 congenic marker. Mini CD8 T cells responded to MCMV infection comparable to the endogenous M38-specific CD8 T cells over the whole course of the infection, showing inflation and maintenance at high percentages during latency. The much higher affinity of the TCR expressed by Maxi CD8 T cells resulted in a 10-fold higher expansion during the acute phase of infection compared to equivalent starting numbers of transferred Mini CD8 T cells.
Interestingly, the classical inflationary behavior was not observed for the Maxi CD8 T cells, as a contraction phase followed the expansion phase. Nevertheless, Maxi CD8 T cells were still maintained at high percentages during latency and displayed a $T_{EM}$ phenotype, indicative of the inflationary response (not shown).

Thus, the Maxi mouse is a source of a monoclonal population of CD8 T cells that expresses a TCR with an exquisitely high affinity for the M38-derived antigen. In contrast, CD8 T cells that originate in the Mini mouse are highly enriched in M38-specific CD8 T cells and are rather polyclonal, which renders them well suited for adoptive transfer experiments mimicking the endogenous response that is induced in vivo upon MCMV infection.

**Figure 2:** Generation of MHC class I-restricted TCR transgenic mice with specificity for the M38 epitope of MCMV (A) Splenocytes from WT C57BL/6 mice and from the transgenic mouse lines Mini and Maxi were stained with M38-tetramer (top panels) and with anti-Vβ10 antibody (lower panels). Percentages of CD8$^+$ M38$^+$ and CD8$^+$ Vβ10$^+$ are depicted. (B) 6x10$^5$ Mini CD8 T cells and 6x10$^5$ Maxi CD8 T cells were CFSE labeled and incubated with DCs loaded with the indicated concentration of M38 peptide or with medium for three days. The percentages of CFSE$^{low}$ cells are depicted. Bars show averages of three mice per group ± SEM. (C) 10$^5$ Mini CD8 T cells or 10$^4$ Maxi CD8 T cells expressing the CD45.1 congenic marker were adoptively transferred into CD45.2 recipient C57BL/6 mice one day prior to infection with MCMV. The percentages of endogenous (black circles) and transferred (white circles) M38-specific CD8 T cells were measured by tetramer staining from blood samples collected at the indicated time points. Each point shows the average of three to five mice per group ± SEM.
Antigen presentation by non-hematopoietic cells is essential for M38-specific CD8 T cell inflation

With the assumption that memory inflation is dependent on MHC class I-antigen complex recognition and with the availability of the TCR transgenic CD8 T cells, we sought to determine which cell types are responsible for antigen presentation to inflationary CD8 T cells. In particular, we addressed the question whether H-2Kb restricted M38-specific CD8 T cells require antigen presentation by hematopoietic or non-hematopoietic cells for their inflation. To this end, we generated bone-marrow chimeras in which the MHC class I H-2Kb molecule was expressed on every cell type (WT→WT) or selectively removed on non-hematopoietic cells (WT→H-2Kb−/−), and compared these two groups of mice for their kinetics of M38- and M45-specific (H-2Db-restricted) CD8 T cell responses. To overcome the absence of positive selection of H-2Kb restricted CD8 T cells in H-2Kb−/− recipient mice, we adoptively transferred congenically marked Mini CD8 T cells the day prior to infection. We first compared the kinetics of M38- and M45-specific CD8 T cell responses in the blood (Fig. 3A). As expected, in WT→WT mice M45-specific CD8 T cells underwent the classical expansion-contraction kinetics typical of a conventional response, while M38-specific CD8 T cells expanded until day 15 post infection, after which they stabilized at high percentages (Fig. 3A, left graph). Strikingly, memory inflation was not at all induced in WT→H-2Kb−/− mice (Fig. 3A, right graph) in the absence of antigen presentation by non-hematopoietic cells. Instead, M38-specific CD8 T cells underwent classical expansion and contraction kinetics as observed for M45-specific CD8 T cells. Next, we investigated whether antigen presentation by non-hematopoietic cells was also a prerequisite for memory inflation in peripheral tissues. Consistent with the blood data, we measured a 20-fold reduction of M38-specific CD8 T cells in WT→H-2Kb−/− compared to WT→WT mice (Fig. 3B and C) in the lungs of latently infected mice. Similar results were obtained in the spleen and in the liver (not shown). A hallmark of inflationary CD8 T cells is their activated phenotype, which has been suggested to be a consequence of presentation of viral-derived antigens during latency. If this is true, then absence of antigen sensing on non-hematopoietic cells would favor the formation of TCM M38-specific CD8 T cells during latency. This was indeed the case: in both the spleen and the lungs of latently infected WT→ H-2Kb−/− mice, M38-specific CD8 T cells up-regulated the expression of IL7Rα and CD62L to a similar extent as in M45-specific CD8 T cells. M38-specific CD8 T cells also displayed high expression of CD122 in WT→H-2Kb−/− mice, allowing for IL-15-mediated homeostatic proliferation (not shown). Importantly, this was the
case despite comparable abundance of viral genomes in the spleen and lungs of WT→WT and WT→H-2K\(^{b/-}\) mice on day 60 post infection (Fig. 3E), indicating that latency is established in WT→H-2K\(^{b/-}\) mice and that absence of memory inflation is due to impaired/missing antigen presentation. Moreover, virus titers were comparable on day 7 post infection, and by day 60 post infection virus control was almost complete in every organ analyzed in both WT→WT and WT→H-2K\(^{b/-}\) chimeras (Fig. 3F).

These data show that antigen presentation by non-hematopoietic cells is a prerequisite for memory inflation during MCMV infection. In its absence, inflationary CD8 T cells are indistinguishable from the conventional CD8 T cells with respect to their kinetics and phenotypes.
RESULTS

Figure 3: Antigen presentation by non-hematopoietic cells is essential for M38-specific CD8 T cell inflation

WT→WT and WT→H-2Kb-/- chimeric mice were generated by irradiating WT and H-2Kb-/- mice followed by reconstitution with WT bone-marrow. One day prior infection with MCMV-Δm157, 10⁶ CD45.1⁺ Mini CD8 T cells were adoptively transferred. (A) The percentage of M38- (pregated on CD45.1⁺ cells) and M45-specific CD8 T cells among total CD8 T cells were measured in the blood on days 7, 12, 15, 28 and 65 post infection. Every point represents the average of four individual mice ± SEM. The mice shown in (A) were sacrificed on day 100 post infection and the percentage of M38- (pregated on CD45.1⁺ cells) and M45-specific CD8 T cells among total CD8 T cells (B) and their total numbers (C) were determined in the lungs. Bars indicate averages of four mice per group ± SEM. The relative expression of IL7Rα and CD62L on M38- and M45- specific CD8 T cells is indicated in (D) in the same groups of mice shown in (A) for lungs and spleen. (E) MCMV latent genomes were quantified in the spleen (upper panel) and in the lungs (lower panel) of WT→WT (black circles) and WT→H-2Kb-/- mice (white circles) on day 60 post infection or from naïve mice by qPCRs using primers specific for β-actin and the MCMV-encoded M38 gene. The relative amount of viral genome was calculated using the delta-delta Ct method, where 10⁻⁶ was the highest value detected for naïve mice and used as detection limit. (F) Virus titers were determined in spleen, lungs, liver and salivary glands on day 7 (top panel) and day 60 (bottom panel) in WT→WT (black circles) and WT→H-2Kb-/- (white circles) mice. Lines represent the geometric means of three individual mice. One of at least four independent experiments are shown for (A to D) and of two independent experiments for (E to F). Significances were analyzed by Student’s t test. *, P < 0.05; **, P < 0.01; *** P < 0.001; ****, P < 0.0001.

Restriction of antigen presentation to DCs abrogates memory inflation

Memory inflation is not solely restricted to CD8 T cells specific for the M38-derived antigen. In C57BL/6 mice, at least three other MCMV-derived antigens have been identified that induce CD8 T cell inflation, namely IE3416-425, IE3461-475, and m139. To investigate whether antigen presentation by non-hematopoietic cells is a general requirement for memory inflation, we took advantage of Tg(CD11c-β2m) x Tg(K14-β2m) x β2m-/- mice (DC-MHCI) [82,83]. These triple transgenic mice are β2-microglobulin-/- mice with a tissue specific expression of trasngenic β2-microglobulin in dendritic cells (DCs), on keratinocytes and on the thymic cortical epithelium, ensuring positive selection of CD8 T cells in the thymus. As a result, only DCs (and keratinocytes) are able to present antigens in secondary lymphoid organs and peripheral tissues in the context of MHC class I molecules in DC-MHCI mice. The presence of a normal peripheral CD8 T cell compartment in these mice [82] allows the assessment of endogenous H-2Kb- and H-2Db-restricted CD8 T cell responses. Similarly as described above, WT and DC-MHCI mice were lethally irradiated and reconstituted with bone-marrow derived from WT mice (WT→WT and WT→ DC-MHCI) or from DC-MHCI mice (DC-MHCI →WT and DC-MHCI → DC-MHCI). We first analyzed the M38-specific CD8 T cell response in the blood, which confirmed the previous results and verified the validity of this model (Fig. 4A). In fact, memory inflation was only induced in the two groups of mice where the recipients were WT, irrespective of the origin of the hematopoietic cells,
but not in DC-MHCI recipient mice. Notably, when DCs were the only cell type capable of presenting viral antigens (DC-MHCI → DC-MHCI), this had no effect on the expansion of M45-specific CD8 T cells during acute infection and showed only a minor reduction for M38-specific CD8 T cells (Fig. 4C), implying that DCs alone are sufficient for priming and expansion of MCMV-specific CD8 T cell responses, but not for their inflation. To investigate the consequences of selective depletion of antigen presentation by non-hematopoietic cells for inflation of CD8 T cells specific for other antigens, we performed tetramer staining for M38-specific and IE3-specific CD8 T cells in the lungs of mice which had been infected for 120 days (Fig. 4B). We found a similar pattern of responses for the two inflators, namely a 5-10-fold reduction in DC-MHCI compared to WT recipient mice, suggesting that inflation of IE3-specific CD8 T cells is also dependent on antigen presentation by non-hematopoietic cells. Consistent with the data shown above, absence of antigen-presentation by non-hematopoietic cells resulted in up-regulation of IL-7Rα on M38- and IE3-specific CD8 T cells during latency (not shown). Thus, DCs are sufficient for priming of both the conventional and inflationary response, whereas inflation requires antigen presentation by cells of non-hematopoietic origin.

Figure 4: Restriction of antigen presentation to DCs abrogates memory inflation  WT→WT, DC-MHCI→DC-MHCI, WT→DC-MHCI, DC-MHCI→WT chimeric mice were generated by reconstituting WT and DC-MHCI irradiated mice with WT and DC-MHCI bone marrow. All groups (3-5 mice per group) were infected with MCMV-Δm157 and the M38-specific CD8 T cell response was measured by tetramer staining from blood samples obtained on days 7, 9, 13, 20 and 45 post infection (A). The experiment was terminated on day 120 post infection and M38-, IE3-, and M45-specific CD8 T cell responses were quantified in the lungs by tetramer staining (B). M38- and M45-specific CD8 T cells responses on day 7 post infection in the blood are shown (C). One of two independent experiments is shown. Significances were analyzed by Student’s t test. *, P < 0.05; **, P < 0.01; *** P < 0.001.
Non-hematopoietic cells promote extensive and systemic cell proliferation in the early phase of MCMV infection, but only in lymph nodes during latency

By careful kinetic analysis, the inflationary M38-specific CD8 T cell response can be divided into three consecutive phases: the primary expansion between day 0 and 9 post infection (Fig. 5A, yellow area), a first and most pronounced inflation (secondary expansion) from day 11 and day 15 post infection (Fig. 5A, pink area) followed by a maintenance phase (Fig. 5A, blue area) (Fig. 5A). In contrast, in WT→H-2Kb+ mice primary expansion was followed by contraction instead of secondary expansion, suggesting that antigen presentation by non-hematopoietic cells promotes either survival of the effector cells or prolongs cell proliferation. To distinguish between these two possibilities, we first analyzed the expression profiles of the anti-apoptotic molecule Bcl-2 in adoptively transferred Mini CD8 T cells in WT→WT and WT→H-2Kb+/− mice over the course of MCMV infection. At the peak of primary expansion, Mini CD8 T cells downregulated Bcl-2 expression in both groups of mice, consistent with their highly activated phenotype (Fig. 5B, yellow area). However, contraction of Mini CD8 T cells in WT→H-2Kb+/− mice resulted in a continuous upregulation of Bcl-2, comparable to conventional M45-specific CD8 T cells, whereas Mini CD8 T cells remained Bcl-2low in WT→WT mice during secondary expansion (Fig. 5B, pink area). These data are in line with the notion that absence of antigen presentation by non-hematopoietic cells abrogates inflation and results in generation of long-lived M38-specific TCM CD8 T cells during latency. Unexpectedly, even in WT→WT mice where contraction never happens, Mini CD8 T cells started to upregulate the expression Bcl-2 at the onset of the maintenance phase, despite their phenotype remaining activated (Fig. 5B, blue area). Likewise, we observed a constant up-regulation of Bcl-2 expression by the endogenous M38-specific CD8 T cells (and as expected by M45-specific CD8 T cells) in the lungs (Not shown). From these data we exclude that non-hematopoietic cells provide surviving stimuli to inflationary CD8 T cells at the peak of the primary expansion preventing their contraction, instead they might deliver additional antigen stimulation resulting in prolonged cell activation and proliferation. This was confirmed by the expression profile of the proliferative marker Ki67 which was concomitantly analyzed with Bcl-2 expression. Indeed, Mini CD8 T cells underwent a second round of proliferation in WT→WT mice exactly at the time when secondary expansion was induced (Fig. 5C, pink area), but not in WT→H-2Kb+/− mice, where Ki67+ Mini cells sharply decreased during the contraction phase and remained low for the rest of the infection. Consistent with the results by Snyder et al. who showed that inflationary CD8 T cells were
dividing only sporadically during latency [76], we observed a decrease of proliferating Mini CD8 T cells at the onset of the maintenance phase in WT→WT mice (Fig. 5C, blue area) to eventually reach the low level of proliferation measured in WT→H-2Kb/- as latency was established (D60: WT→WT: 7%; WT→H-2Kb/-: 3%). Next, we analyzed whether the proliferation pattern observed in the blood was also apparent in lymphoid and peripheral tissues. We therefore analyzed Ki67/Bcl-2 expression in M38-specific and M45-specific CD8 T cells isolated from lungs or lymph nodes on day 12 post infection and during latency (Fig. 5D). Analogous to the blood, M38-specific CD8 T cells in WT→WT mice showed increased proliferation compared to WT→H-2Kb/- mice on day 12 post infection, especially in the lymph nodes (Fig. 5E). Strikingly, by 4 months post infection (Fig. 5D and F) M38-specific cell proliferation had ceased almost completely in the lungs as was the case for M45-specific cells, while it was still ongoing at high levels in the lymph nodes. Importantly, while the few Ki67+ M45-specific CD8 T cells in the lymph nodes were Bcl-2high, indicative of cytokine-driven homeostatic proliferation, proliferating M38-specific CD8 T cells were Bcl-2low, suggesting that their proliferation was antigen driven. Since M38-specific CD8 T cells display largely a TCM phenotype in lymph nodes, our data strongly suggest that central-memory cells in the lymph node, and not effector memory cells in peripheral tissues, respond to antigen presentation on non-hematopoietic cells during latency by proliferation. To test this hypothesis, we adoptively transferred in vivo generated TEM or TCM M38-specific CD8 T cells into latently infected mice or naïve mice, and assessed their proliferative history 30 days later. As expected, TCM M38-specific CD8 T cells proliferated to a much higher extent compared to TEM M38-specific CD8 T cells in latently infected recipients (Fig. 5G and H).

Together, these data suggest that antigen presentation by non-hematopoietic cells promote extensive and systemic proliferation of M38-specific CD8 T cells during the early phase of the infection, resulting in their secondary expansion. However, subsequent maintenance of the peripheral inflationary pool at high percentage during latency cannot be attributed to extensive systemic cell proliferation, but instead might be facilitated by prolonged survival of the inflationary cells, facilitated by Bcl-2 upregulation, and by continuous recruitment of newly activated central memory CD8 T cells from lymph nodes.
RESULTS

Figure 5 Non-hematopoietic cells promote extensive and systemic cell proliferation in the early phase of MCMV infection, but only local during latency

10^5 Mini-CD8 T cells were transferred in WT→WT and WT→H-2K^{b,c} chimeric mice one day prior to infection with MCMV→m157. (A) The percentage of M38-specific CD8 T cell response was measured in the blood on days 7, 9, 11, 13, 17, 21, 27, 42 and 60 post infection. Colors indicate different phases of the M38-specific response: primary expansion in yellow; secondary expansion in pink; maintenance phase in blue. Bcl-2 (B) and Ki67 (C) expression levels were measured on M38-specific CD8 T cells depicted in (A). One of two independent experiments is shown. (D) Representative FACS plots showing Bcl-2 and Ki67 expression on M38- and M45-specific CD8 T cells in the lungs and inguinal lymph nodes of WT→WT chimeric mice on day 12 post infection (upper panels) and day 130 post infection (lower panels). (E) The percentage of ki67^{+} cells in WT→WT and WT→H-2K^{b,c} chimeric mice is shown for M38- and M45-specific CD8 T cells in the lungs (left graph) and in the lymph nodes (right graph). One of three independent experiments is shown. (F) The percentage of M38-specific cells expressing Ki67 during latency is shown for the lungs and the lymph nodes. Data pooled from three independent experiments ± SEM are shown. (G) Preferential antigen-driven proliferation of T_{CM} M38-specific CD8 T cells during latency. Experimental layout: CD45.2^{+} C57BL/6 mice were adoptively transferred with CD45.1^{+} Maxi CD8 T cells and during latency T_{CM} Maxi CD8 T were isolated from the LNs and the spleen, whereas T_{EM} Maxi CD8 T cells were isolated from the lungs. Isolated cells were labeled with CFSE and transferred into naive or latently infected CD45.2^{+} C57BL/6 mice. CFSE dilution was measured 30 days later in the lungs. (H) Graphical summary showing percentage of CFSE_{low} cells among total transferred Maxi CD8 T cells.
MCMV genomes preferentially localize in non-hematopoietic cells

Given that priming and memory inflation rely on antigen presentation by different cell types, the first being dependent on DCs and the latter on non-hematopoietic cells, it is conceivable that productive infection during the acute phase of infection and establishment of latency occurred in different cell types. We aimed to analyze the target cells of MCMV distinguishing between hematopoietic cells or non-hematopoietic cells in secondary lymphoid organs and in peripheral tissues at different time points of the infection. To this end, we isolated spleen and lungs from WT mice on day 7, 14 and 28 after infection and stained the isolated cells with anti-CD45 antibody, a known marker for hematopoietic cells. The proportion of cells that stained positive for CD45 was roughly ~80% in the spleen and ~60% in the lungs (not shown). We then sorted CD45-positive and CD45-negative cell populations and measured the relative MCMV genome content by quantitative RT-PCR. In both the spleen and the lungs, we found a clear bias towards the CD45-negative population at every time point analyzed (Fig 6). While MCMV genomes were also found in the CD45-positive population on day 7 and 14 post infection, when productive infection was still ongoing, they were hardly detectable on day 28, a time point when the virus had already established latency in both the spleen and the lungs (Fig. 1A). Thus, during the acute phase of the infection, when CD8 T cells of both the conventional and inflationary type are primed, MCMV genomes are found in both hematopoietic and non-hematopoietic cell compartments. At later time points, when the inflationary memory CD8 T cell response is induced, MCMV localizes predominantly in non-hematopoietic cells, strengthening the hypothesis that latently infected non-hematopoietic cells directly present viral-derived antigens during latency, leading to memory inflation.
RESULTS

Figure 6 MCMV genome localizes preferentially in non-hematopoietic cells
C57BL/6 mice were infected with MCMV-Δm157 and on day 7, 14 and 28 post infection total cells from the spleen and the lungs were sorted into CD45⁺ (black circles) and CD45⁻ (white circles) cell populations. MCMV latent genomes were quantified by qPCR using primers specific for β-actin and the MCMV-encoded M38 gene. One of two independent experiments is shown.
Discussion

MCMV infection induces accumulation and life-long peripheral maintenance of a population of virus-specific CD8 T cells with an activated phenotype, a phenomenon referred to as ‘memory inflation’ [34]. A major population of these inflationary CD8 T cells in the C57BL/6 background is specific for the MCMV-derived epitope M38 [35]. The cellular and molecular requirements leading to memory inflation are still poorly understood, mainly due to lack of monoclonal populations of MCMV-specific CD8 T cells that can be traced in vivo during the course of the infection. In the present study we describe the generation of two MHC class I-restricted mouse lines with specificities for the M38-epitope of MCMV, which differ in their affinity towards the peptide. Through a series of adoptive transfer experiments we could demonstrate that i) memory inflation is completely dependent on antigen presentation by cells of non-hematopoietic origin, ii) only in lymph nodes inflationary CD8 T cells respond to continued antigen presentation by proliferation whereas antigen presentation by non-hematopoietic cells in peripheral tissues might contribute to their long term effector phenotype in these tissues during latency, and iii) non-hematopoietic cells are the major reservoir of MCMV latent genomes after resolution of acute infection. We propose a model where memory inflation is induced and maintained by latently infected non-hematopoietic cells that directly restimulate inflationary CD8 T cells with a T\text{CM} phenotype in lymph nodes, inducing secondary expansion and migration in the periphery.

This model also provides an attractive explanation to why two patterns of CD8 T cell responses are induced during MCMV infection, if combined with a recent finding by Hutchinson et al, who showed that conventional CD8 T cells displayed a higher dependence on the immunoproteasome subunit LMP7 compared to inflationary CD8 T cells [84]. The immunoproteaseome is constitutively expressed by immune cells like DCs and macrophages, whereas expression in non-immune cells is only induced by IFN\text{γ} [85]. Thus, in non-inflammatory conditions as during MCMV latency, infected non-hematopoietic cells would only process proteins through the conventional immunoproteasome.

Inflationary CD8 T cells, in contrast to conventional CD8 T cells, do not contract upon control of lytic MCMV replication but rather continuously accumulate even in the absence of overt viral replication [35,36]. Systematic longitudinal proliferation analyses allowed the subdivision of the M38-specific CD8 T cell response into three consecutive phases: a primary expansion characterized by extensive cell proliferation, immediately followed by a secondary
expansion with reduced but substantial proliferation, and a maintenance phase, where M38-specific CD8 T cells stabilized in number and underwent very little proliferation - with the notable exception of lymph nodes. Primary expansion of M38-specific CD8 T cells occurred in response to acute infection and showed high similarities to the conventional M45-specific CD8 T cell response. In fact, both responses almost completely relied on antigen presentation by DCs and generated effector cells with similar effector functions and activation state [35,86]. The two responses diverged at the peak of the primary expansion, as conventional CD8 T cells contracted and inflationary CD8 T cells underwent secondary expansion. Strikingly, secondary expansion was promoted by robust proliferation which was entirely dependent on non-hematopoietic cells. Two possible mechanisms could account for the ability of non-hematopoietic cells to induce proliferation of inflationary CD8 T cells: one is that infected non-hematopoietic cells in the periphery perpetuate clonal expansion that is initiated in the secondary lymphoid organs, as described for acute LCMV infection [87]. The second is that secondary expansion is induced in lymph nodes by infected non-hematopoietic cells, re-stimulating inflationary CD8 T cells with a T<sub>CM</sub> phenotype, thereby inducing reactivation and migration to the periphery. Our data support the second hypothesis as the highest proliferation levels during secondary expansion were not detected in peripheral organs such as lungs or liver where massive accumulation of inflationary CD8 T cells was observed, but in the lymph nodes, where M38-specific CD8 T cells did not inflate and possessed largely a T<sub>CM</sub> phenotype. However, since the two hypotheses are not mutually exclusive, we do not formally rule out that a certain degree of proliferation might also be induced in peripheral tissues. The proposed scenario where non-hematopoietic cells directly restimulate T<sub>CM</sub> CD8 T cells in lymph nodes and induce their secondary expansion is reasonable, as it is well-known that memory CD8 T cells are less stringent in terms of costimulatory requirements compared to their naïve counterparts and that T<sub>CM</sub> CD8 T cells are by far superior in proliferative recall responses as opposed to T<sub>EM</sub> CD8 T cells [60,88,89]. Moreover, we recently showed that memory inflation, in contrast to priming, was largely independent of CD8α<sup>+</sup> and CD103<sup>+</sup> DCs [78], which are the major subsets of cross-presenting DCs described so far, suggesting that memory inflation is largely driven by direct antigen presentation. Last but not least, non-hematopoietic cells are the major reservoir of MCMV latent genomes after resolution of acute infection in both peripheral and secondary lymphoid organs.
While it seems evident that secondary expansion is antigen-dependent and characterized by extensive proliferation, it is still unclear what the requirements are for maintenance of the inflationary pool in the periphery once that true latency is established. By five months post infection, proliferation of inflationary CD8 T cells is almost undetectable in the lungs, although they are maintained at high numbers in this peripheral tissue. A number of recent reports are consolidating the notion that so called tissue-resident memory T cells with an effector memory phenotype can persist in the periphery for long time periods in the absence of the cognate antigen \[61,62,90,91\]. Whether this also applies for MCMV infection remains to be shown. However, long-term maintenance of virus-specific CD8 T cells are likely to play an important role during latent infections, where they would provide a key protective role in active immune surveillance controlling virus reactivation events, as demonstrated for HSV infection \[62\]. The cellular requirements ensuring survival of these effector cells are still undefined; CD103 expression on CD8 T cells seems to play a critical role for HSV and other infections \[61,62\], but probably not during MCMV as inflationary CD8 T cells do not express CD103 (data not shown). However, inflationary CD8 T cells upregulate Bcl-2, also in peripheral tissues and despite exhibiting a T_{EM} phenotype, suggesting that cytokines or other stimuli might promote their survival. Yet, it is unlikely that the inflationary pool is maintained lifelong without being replenished, as already suggested by Snyder et al \[76\]. In line with this, we found a high percentage of proliferating M38-specific CD8 T cells in the lymph nodes during latency. Moreover, T_{CM} M38-specific CD8 T cells as opposed to T_{EM} CD8 T cells proliferated in an antigen-dependent manner when transferred into a latently infected host. Together, these data indicate that re-stimulation of T_{CM} memory cells by non-hematopoietic cells in the lymph nodes might continuously supply the inflationary pool in the periphery with new and functional effector cells.
Materials and Methods

Ethics statement

This study was carried out in strict accordance to the guidelines of the animal experimentation law (SR 455.163; TVV) of the Swiss Federal Government. The protocol was approved by Cantonal Veterinary Office of the canton of Zurich, Switzerland (Permit number 145/2008). All surgery was performed under isoflurane anesthesia and all efforts were made to minimize suffering.

Mice, viruses and immunization

C57BL/6N, H-2K\(^{b/\nu}\) and Tg(CD11c-\(\beta_2m\)) x Tg(K14-\(\beta_2m\)) x \(\beta_2m^{+/+}\) (DC-MHCI) mice were bred in the local animal facility under specific pathogen-free conditions. KM14 mice were kindly provided by Prof. T. Brocker (Ludwig-Maximilians-University, Munich). Recombinant MCMV-\(\Delta m157\) (m157 deletion mutant) was described previously [50] and was grown on C57BL/6 embryonic fibroblasts (MEFs) and titrated by standard plaque-forming assays as described in [51]. Infection was performed intravenously (i.v.) with \(10^7\) plaque forming units (PFU) of MCMV-\(\Delta m157\).

Generation of Mini and Maxi transgenic mice

A) Generation of M38\(_{316-323}\)-specific CD8 T cell hybridomas

C57BL/6N mice were infected intravenously with MCMV-\(\Delta m157\) and at least 60 days later spleen cells were isolated and activated for 8 days with \(10^{-8}\) M of M38\(_{316-323}\) peptide (NeoMPS, Strasbourg, France) in presence of 80U/ml of recombinant IL-2 (BD Biosciences, Basel, Switzerland). Another restimulation followed, and three days later cells were fused with BW2 36.1 CD8\(\alpha\) cell line (kindly provided by Prof. Marcus Groettrup, University of Konstanz, Germany) according to the protocol described in [53]. Specificity of the CD8 T cell hybridomas was assessed as described in [50]. The hybridoma with the highest peptide affinity was selected for further characterization.

B) Identification of TCR\(\alpha\) and TCR\(\beta\) variable region genes and generation of TCR transgenic mouse lines

RNA was isolated form the selected hybridoma using TRIzol reagent (Invitrogen, Basel, Switzerland) according to the manufacturer’s instructions, and cDNA was generated using
M-MLV Reverse Transcriptase RNase, H Minus (Promega, Dübendorf, Switzerland). cDNA was amplified with a TCRα-specific primer set [2] and a TCRβ-specific primer set [1]. Sequencing of the PCR products was done by Microsynth (Zürich, Switzerland) and then aligned to the mouse genome using Ensembl database (http://www.ensembl.org/Mus_musculus). The identified Vα4Jα13 and Vβ10Jβ2.1 gene segments were amplified from the genomic DNA using the following primers: for Vα4Jα13: fwd (5'-TGA CCC GGG TTC TAG ATG ACA CTA AAG ATG G-3'), TCRα rev (5'-ATA TGC GGC CGC ACA ATT CAG ACA TGG ACT TAC-3'), for Vβ10Jβ2.1: fwd (5'-TAA CTC GAG GCT TAT TTG CCC TGC CTT GAC CCA ACT ATG-3'), rev (5'-TTT CCG CGG CTC CCA CCT GTA TGG CCT CTG CCT TCT TAC CTA-3').

PCR products containing Vα4Jα13 and Vβ10Jβ2.1 gene segments were digested with XmaI and NotI respectively with XhoI and SacII and cloned into previously described TCR expression vectors [3]. The resulting pTαVα4Jα13 and pTβVβ10Jβ2.1 were digested with SalI respectively KpnI to excise the transgenes from prokaryotic vector DNA. The isolated linearized fragments were co-injected in equimolar ratios into fertilized C57BL/6N oocytes according to the standard method [49]. The resulting two TCR transgenic mouse lines were designated according to the standardized genetic nomenclature for mice: C57BL/6N-Tg(Tcrb)330Biat [92] and C57BL/6N-Tg(Tcra,Tcrb)329Biat (Maxi) [93].

*Generation of bone-marrow chimeras*

Chimeric mice were generated by transferring 2-5 x 10^6 bone marrow cells derived from C57BL/6N or DC-MHCl mouse 6 to 8 hours after lethal irradiation (950 rad) of recipient mice (C57BL/6N, H-2K^b/- or DC-MHCl mice). During the first two weeks of reconstitution, mice were treated with the antibacterial Borgal 24% (Intervet, Boxmeer, Netherlands). Bone marrow chimeric mice were used for experiments 8-10 weeks after reconstitution.

*Antibodies and tetramers*

APC-conjugated peptide-MHC class I tetramers were generated as described in [94]. The following conjugated antibodies were purchased either from Biolegend (Lucerna Chem AG, Luzern, Switzerland) or from BD Biosciences (Allschwil, Switzerland): anti-CD8α [53-6.7], anti-CD127 [SB/199], anti-CD62L [MEL-14], anti-KLRG-1 [2F1], anti-Vβ10 [B21.5], anti-CD45.1 [A20], anti-CD45.2 [104], anti-CD44 [IM7], anti-Ki67 [B56], anti-Bcl-2 [3F11].
RESULTS

Lymphocyte isolation, cell surface and intracellular staining

Lymphocytes were isolated from spleen, lymph nodes, lungs and liver as described in [95]. Fresh blood was obtained from the tail vein. For surface staining, cells were incubated for 20 min at 4°C or at 37°C when using tetramers before lysis with 1X BD lysis buffer (BD Biosciences) for 5 min at room temperature. For intracellular staining of Ki67 and Bcl-2, cells were first permeabilized by incubation in 1X wash/perm buffer (BD Biosciences) for 15 minutes at 4°C. Staining was then performed in 1X wash/perm buffer containing the appropriate antibody dilutions for 30 min at 4°C. Multiparameter flow cytometric analysis was performed using a LSRII flow cytometer (BD Biosciences) and analyzed with FlowJo software (Tree Star, San Carlos, CA).

Adoptive transfer experiments

CD8 T cells were purified from Mini- or Maxi- derived splenocytes with anti-CD8α MACS beads (Miltenyi Biotech, Bergisch Gladbach, Germany) according to the manufacturer’s instructions and $10^5$ Mini- or $10^4$ Maxi-CD8 T cells were adoptively transferred into recipient mice one day prior to infection. For transfer of different CD8 T cell memory subsets, $10^4$ MAXI CD8 T cells were first adoptively transferred into recipient mice one day prior to infection MCMV-Δm157 as described above. After at least 60 days post infection, mice were sacrificed and Maxi CD8 T cells were separated into $T_{CM}$ (CD62L$^{high}$ IL7Rα$^{high}$, cells isolated from pooled lymph nodes and spleen) and $T_{EM}$ (CD62L$^{low}$IL7Rα$^{low}$, cells isolated from the lungs) using a BD FACSARia™ sorter. Sorted populations of cells were labeled with 0.5 µM CFSE (Invitrogen, Basel, Switzerland) for 6 minutes and equal numbers of cells (varying inter-experimentally between $0.5x10^5$ to $1x10^5$) were transferred into latently infected mice or naïve mice. CFSE dilution analysis was assessed 30 days later in the lymph nodes and in the lungs.

In vitro CD8 T cell proliferation assay

Mini- and Maxi- CD8 T cells were obtained from splenocytes by MACS positive selection with anti-CD8α microbeads. Cells were labeled with 0.5 µM CFSE for 6 minutes. DCs were isolated from the spleen of naïve C57BL/6 mice by MACS positive selection with anti-CD11c microbeads. $1x10^4$ DCs were loaded with serial dilutions of the M38 peptide epitope and were co-cultured with $6x10^5$ Mini- or $6x10^4$ Maxi-CD8 T cells. At day 3 after stimulation, proliferation was measured by CFSE dilution by flow cytometry.
Quantification of MCMV genomes by qPCR

Spleen and lungs were isolated from mice which have been infected with MCMV-Δm157 for the indicated time, and single cell suspensions were obtained as described in [95], except that no Percoll gradient was performed. Hematopoietic and non-hematopoietic cell populations were sorted based on the expression of CD45. Total genomic DNA was isolated using the Biophenol/Chloroform/Isoamyl alcohol 25:24:1 reagent (Biosolve Ltd, Valkenswaard, Netherlands) and isopropanol-based DNA precipitation. For qPCR, SYBR green incorporation (Qiagen, Hombrechtikon, Switzerland) was measured using a Rotorgene 3000 machine (Corbett Research, Eight Miles Plains, Australia). The reaction was carried out using 100 ng of genomic DNA and 5µM of primers specific for β-actin (fwd: 5’-CCC TGA AGT ACC CCA TTG AAC-3’, rev: 5’-CTT TTC ACG GTT GGC CTT AG-3’) or M38 (fwd: 5’-AGTCCAGGGTGAGGTCTATG-3’, rev: 5’-CCTTCAACTCGGTGCGATTC-3’). The relative amount of MCMV genome was calculated using the delta-delta Ct method. Results from different runs were normalized using an internal β-actin standard.
4.3 Cross-presenting DCs in CMV infection: important for priming but dispensable for inflation

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Abstract

Priming of CD₈⁺ T cells specific for viruses that interfere with MHC class I presentation pathway is a challenge for the immune system and is believed to rely on cross-presentation. Cytomegalovirus (CMV) infection induces vigorous CD₈⁺ T cell responses despite its potent immune evasion strategies. Furthermore, CD₈⁺ T cells specific for a subset of viral epitopes accumulate and are maintained at high levels exhibiting an activated phenotype - referred to as ‘inflationary T cells’. Taking advantage of a mouse model in which the development of cross-presenting CD₈α⁺ and CD₁₀₃⁺ DCs is severely compromised, we analyzed their role in induction and inflation of MCMV-specific CD₈⁺ T cell responses. We found that priming of MCMV-specific CD₈⁺ T cells was almost completely abolished in absence of cross-presenting DCs. However, MCMV-specific CD₈⁺ T cell inflation during latency was largely dependent on direct antigen presentation, highlighting differential antigen presentation requirements during acute and latent MCMV infection.

Footnotes

A shortened version of this work has been accepted for publication in the European Journal of Immunology with the title:
Introduction

Priming of an antiviral CD8\(^+\) T cell response is a tightly regulated process which requires the interaction between antigen-specific naïve T cells and mature antigen-bearing dendritic cells (DCs). Antigen presentation to CD8\(^+\) T cells by DCs can occur in two ways: by direct presentation of endogenously synthesized viral antigens or by cross-presentation of exogenously acquired antigens [96,97]. The ability to cross-present antigens \textit{in vivo} has been attributed to two distinct subsets of conventional DCs, namely to lymphoid organ-resident CD8\(\alpha^+\) DCs and more recently to migratory CD103\(^+\)CD11b\(^-\) DCs [98,99,100]. Although cross-presentation of various cellular and pathogen-derived antigens has been widely demonstrated \textit{in vitro} [101], evidence for a requirement of cross-presentation \textit{in vivo} in the context of viral infections is still limited and largely based on \textit{in vitro} antigen loading [102]. In particular, in case of viruses which directly infect DCs, cross-presentation may not be required \textit{in vivo}. However, cross-presentation may play a fundamental role in the induction of antiviral CD8\(^+\) T cell responses in situations where viruses encode for proteins that directly interfere with the antigen presentation machinery of an antigen-presenting cell (APC), thereby inhibiting direct antigen presentation.

The mouse cytomegalovirus MCMV is a well-established experimental model to study immune evasion. Viral proteins that directly target the expression of major histocompatibility complex molecules and costimulatory molecules on infected APCs have been described and characterized [42,103,104,105]. The fact that, despite impressive immune evasion, strong CD8\(^+\) T cell responses are induced upon MCMV infection, strongly suggests that cross-presentation may play an important role [47]. Unraveling the mechanism of antigen presentation to CD8\(^+\) T cells during MCMV infection is particularly interesting since this infection drives distinct patterns of CD8\(^+\) T cell responses [35,36]. During the peak of the acute infection a large number of CD8\(^+\) T cells with different specificities are induced, most of which contract to eventually form central-memory cells as the primary infection is controlled (here referred to as ‘conventional’ response). In contrast, ‘inflationary’ CD8\(^+\) T cells do not contract substantially, but instead continue to increase in numbers and percentages during the latent phase of infection, presumably driven by repetitive antigen encounter [33,36,76]. Whether these different patterns of CD8\(^+\) T cell responses rely on different antigen processing/presentation pathways is unclear.
RESULTS

Here we assessed the requirement of cross-presentation for the induction of conventional and inflationary CD$^+$ T cell responses during MCMV infection. We made use of Batf3$^{-/-}$ mice which have a severe defect in the development of conventional CD$^+$ DCs and migratory CD103$^+$CD11b$^-$ DCs, which renders them unable to cross-present antigens [106,107]. We compared CD$^+$ T cell responses against a panel of MCMV epitopes in Batf3$^{-/-}$ mice and wild type (WT) at different stages of the infection. Absence of cross-presenting CD$^+$ and CD103$^+$ DCs severely compromised the priming of CD$^+$ T cells with all specificities, but particularly the conventional ones. Despite reduced priming, memory CD$^+$ T cell inflation during viral latency was largely independent on cross-presenting DCs, indicating that memory CD$^+$ T cell inflation is promoted by direct antigen presentation and implying that priming and inflation rely on different antigen presentation pathways.

Results and Discussion

Batf3$^{-/-}$ mice: a model to assess the role of cross-presenting DCs in MCMV infection

MCMV infection is controlled by different arms of the innate and adaptive immune system, primarily by NK cells, and CD$^+$ T cells, CD$^+$ T cells and B cells which are all also participating in long-term control of MCMV infection [32,108]. To address the question whether short and long-term MCMV control was affected in Batf3$^{-/-}$ mice, viral titers were assessed over 110 days in various organs of WT and Batf3$^{-/-}$ mice. We found that Batf3$^{-/-}$ mice were able to clear the virus with the same efficiency as WT mice from all organs analyzed (Fig. 1 A). As DCs are direct targets of MCMV, the possibility that CD$^+$ T cells are directly primed by infected DCs cannot be excluded a priori. Importantly, absence of CD$^+$ and CD103$^+$ DCs in Batf3$^{-/-}$ mice did not alter the susceptibility of the remaining DCs to get infected (Fig. 1 B). Furthermore, the total number of CD11c$^{\text{high}}$MHCII$^{\text{high}}$ cells was comparable in WT and Batf3$^{-/-}$ mice (Fig. 1 C). Since absence of CD$^+$ and CD103$^+$ DCs does not alter the amount of viral antigen and hence does not impede direct presentation of MCMV-derived antigens, we conclude that Batf3$^{-/-}$ mice are an appropriate model to study the role of cross-presentation in the induction of CD$^+$ T cell responses during MCMV infection.
ROLE OF CROSS-PRESENTING DCs DURING CMV INFECTION

Figure 1 Batf3−/− mice: a model to the role of cross-presenting DCs during MCMV infection
(A) WT and Batf3−/− mice were infected with MCMV-Δm157 and the viral titers were evaluated in the spleen, liver, lungs and salivary glands at day 7, 28 and 110 post infection by virus plaque assay. (B) The percentage of infected DCs was assessed by isolating CD11c+ cells from splenocytes of WT or Batf3−/− mice that had been infected for 2 days or left naïve followed by staining with the CROMA 229 antibody. Numbers indicates percentage of infected cells. (C) Total number (left panel) and percentage (right panel) of CD11c+MHCIIhigh cells among splenocytes of WT (■) or Batf3−/− (□) mice were analyzed at day 0 (naive) and 7 post infection. Bars represent the mean + SEM. One of 2 representative experiments is shown

Priming of MCMV-specific CD8+ T cell responses is highly impaired in the absence of cross-presenting DCs

As the CD8+ T cell response against MCMV has been previously described in C57BL/6 (H-2b) and Balb/c (H-2d) mice, but not in the 129SvEv (H-2b) mouse strain [35,75], we compared the kinetics of the M45-specific CD8+ T cell response (conventional type) and the M38-specific CD8+ T cell response (inflationary type) in C57BL/6 and 129SvEv mice. Although reduced in magnitude, the kinetics of the responses was comparable in both strains (Fig. 2A). To assess the role of CD8α+ and CD103+ cross-presenting DCs for priming of MCMV-specific CD8+ T cell responses, we compared the expansion of both conventional and inflationary CD8+ T cell responses in acutely infected WT and Batf3−/− mice. For this, we restimulated lung-derived lymphocytes with an array of CD8+ T cell epitopes that have been previously described for C57BL/6 mice to induce either conventional or inflationary CD8+ T cell responses [35] and measured IFNγ production by intracellular cytokine staining (ICS). We found that priming of all conventional CD8+ T cell responses, with the exception of the
one specific for the M86 epitope, was severely impaired in the absence of cross-presenting DCs (Fig. 2 B and D). Similar results were obtained in the spleen (data not shown). A reduced primary response was also observed for the inflationary M38- and m139-specific CD8+ T cells, although less pronounced in comparison to most conventional responses (Fig. 2 C and D). The inflationary IE3-specific response was barely detectable at day 7 post infection in both WT and Batf3−/− mice. A similar pattern was observed when we measured M38-, IE3-, and M45-specific CD8+ T cell responses in the lungs and spleen by tetramer staining, excluding the possibility of decreased functionality of these cells (Fig. 2 E and F). Previous work showed that CD8α+ DCs are specialized in peptide presentation and priming of CD8+ T cells, whereas CD4+ T cells are preferentially activated by CD8α- DCs [109]. Consistent with this, Batf3−/− mice were not impaired in the induction MCMV-specific CD4+ T cell responses (not shown).

We next asked if the reduced population of virus-specific CD8+ T cells observed at day 7 was a result of reduced proliferation of CD8+ T cells that were primed by directly infected CD8α- DCs in Batf3−/− mice, or due to decreased cell survival. We therefore stained total and MCMV-specific CD8+ T cells for the proliferation marker Ki67 and the anti-apoptotic marker Bcl-2. We found that CD8+ T cells that were primed in Batf3−/− mice displayed a highly reduced proliferation compared to CD8+ T cells primed in WT mice, whereas the expression of the Bcl-2 marker was comparable (Fig. 2 G and H). The reduced proliferation observed in Batf3−/− mice might be a consequence of impaired antigen presentation on directly infected DCs due to MCMV-driven immune-evasion or due to an inferior capacity of CD8α+ DCs for CD8+ T cell priming [105,109]. We excluded, however, that Batf3 deficiency leads to an overall defect in CD8+ T cell priming, as infection of Batf3−/− mice with recombinant VV expressing the M45 protein of MCMV (VV-M45) induced comparable frequencies of CD8+ T cells specific for the VV-derived B8R peptide in WT and Batf3−/− mice, while CD8+ T cell responses specific for the recombinant MCMV-derived M45 peptide were again strongly reduced in Batf3−/− compared to WT mice (Fig. 2I). This latter observation is of interest, as it suggests that the nature of a particular protein / epitope seems to effectively contribute to the dependence on CD8α+ and CD103+ DCs for effective CD8+ T cell priming and that this might be more relevant than the actual nature of a viral infection including the capacity of certain viruses to down-modulate MHC class I antigen presentation as is the case for CMVs. Finally, to address the question whether CD8α+ DCs are indeed responsible for the majority of MCMV-specific CD8+ T cell priming, we have tried, albeit without success, to functionally reconstitute Batf3−/− mice with CD8α+ DCs prior to MCMV infection. The main
obstacle with these experiments was the inefficiency of reconstitution; even when transferring $6 \times 10^5$ purified CD8α+ DCs prior to infection, only very small numbers of those will end up in the spleen, their longevity of DCs is very limited – in particular in the context of an inflammatory setting as is the case during MCMV infection - and these few cells have to be exposed in vivo to non-infectious MCMV antigens, phagocytose them and cross-present them. Altogether, the combination of all of these prerequisites renders the success of such an experiment rather unlikely. In previous attempts to reconstitute CD8α+ DCs in Batf3−/− mice, these cells had been preloaded in vitro with the antigen of interest prior to transfer [107], suggesting that this forced enrichment of antigen presentation on the reconstituting DCs facilitated an in vivo effect upon reconstitution.

Hence, based on the failure to reconstitute the wiltype phenotype in Batf3−/− mice by transfer of CD8α+ DCs prior to MCMV infection, we cannot formally exclude that other factors than absence of CD8α+ DCs were responsible for the reduced priming of CD8+ T cells in Batf3−/− mice. However, this seems rather unlikely, as we have observed normal priming of VV-specific CD8+ T cell responses in Batf3−/− mice.
**RESULTS**

CD8+ T cell inflation during latent MCMV infection occurs largely in the absence of cross-presenting DCs

After the resolution of the acute phase of infection, MCMV is maintained life-long in a latent state which is controlled by MCMV-specific CD8+ T cell immunity [33]. The phenomenon of CD8+ T cell inflation, leading to massive CD8+ T cell accumulation during MCMV latency, is likely to be driven by MCMV antigens which are continuously or sporadically expressed at low levels[33,36,76]. So far it is unknown why only several subsets of CD8+ T cells inflate...
and which cells present MCMV-derived antigens to inflationary CD8+ T cells. The likely involvement of repeated antigen stimulation in the inflation process is also supported by the effector-memory phenotype of the inflationary CD8+ T cells, characterized by low expression of CD62L and IL7Rα and high expression of KLRG-1 [36,76]. In contrast, conventional MCMV-specific CD8+ T cells display a central-memory phenotype during latent MCMV infection. We showed above that priming of the MCMV-specific CD8+ T cell response was impaired in absence of CD8α+ and CD103+ DCs. To investigate the role of these subsets of DCs for CD8+ T cell inflation during latency, we infected WT and Batf3−/− mice and analyzed CD8+ T cell responses on day 110 post infection when infectious virus was cleared from all the organs (Fig. 1 A). We found that IFNγ-producing m139- and M38-specific CD8+ T cells were only slightly decreased in Batf3−/− compared to WT mice. In contrast, the response to IE3 was 4-fold decreased in the absence of cross-presenting DCs, but was still higher than the responses observed for all conventional CD8+ T cells (Fig. 3A). Similar results were obtained when we assessed the MCMV-specific CD8+ T cell responses by tetramer staining (Fig. 3 B and C, data from the lungs and the spleen are shown). Furthermore, memory CD8+ T cell inflation in Batf3−/− mice due to repeated antigen encounter is supported by the effector memory phenotype of M38- and IE3- positive CD8+ T cells (Fig. 3 D). Together, these data suggest that accumulation of the inflationary m139- and M38-specific CD8+ T cells occurs largely in absence of cross-presenting DCs while IE3-specific CD8+ T cell inflation was significantly promoted in their presence.
RESULTS

Figure 3 CD8+ T cell inflation during latent MCMV infection occurs largely in the absence of cross-presenting DCs

WT (■) and Batf3−/− (□) mice were infected with MCMV-Δm157 and lymphocytes were isolated from the lungs 110 days later. (A) The responses to the indicated peptides were measured by intracellular staining for IFNγ. Bars indicate the percentage of IFNγ producing cells among total CD8+ T cells. M38-, IE3-, and M45-specific CD8+ T cells were measured by tetramer staining (B) in the lungs and (C) in the spleen and plotted as percentage among total CD8+ T cells. (D) The expression of IL7Rα, CD62L and KLRG1 was assessed on tetramer positive cells isolated from the lungs. Bars show average of three mice per group ± SEM, and one experiment out of two independent experiments is shown. Significance was measured by unpaired 2-tailed Student’s t test. *, P < 0.05; ***, P < 0.001

Kinetics of inflationary CD8+ T cell responses in absence or presence of cross-presenting DCs

Amongst the inflationary MCMV-specific CD8+ T cell responses in C57BL/6 mice, two major patterns can be distinguished: early versus late inflation [35], the M38- and m139-specific CD8+ T cell response representing the former and the IE3-specific response representing the latter. We addressed the question whether this different inflation kinetics might be the result of different antigen presentation requirements. For this purpose, we followed the M38-, IE3-, and M45-specific CD8+ T cell responses over an extended period of time in the blood of infected WT and Batf3−/− mice. The first burst of inflation was observed between day 7 and day 10 post infection, when CD8+ T cell responses to M38 and IE3 increased two- to three-fold in both WT and Batf3−/− mice (Fig. 4 A and B). M38-positive CD8+ T cells were subsequently maintained at a high frequency, independent of presence or absence of cross-presenting DCs (Fig. 4 C). Similarly, m139-specific CD8+ T cell responses were comparable in WT and Batf3−/− mice on days 28 and 110 post infection (Fig. 3 A and not shown).

In case of the IE3-specific response, a secondary inflation boost was observed after day 23 post infection, but only in WT mice, confirming that the IE3-specific response during latency is partially dependent on cross-presenting DCs (Fig. 4 A). Consistent with this, only a very small percentage of M38-specific CD8+ T cells was proliferating at day 28 post infection (3.8% in WT, 1.5% in Batf3−/−), whereas IE3-specific CD8+ T cells were extensively dividing in WT, but not in Batf3−/− mice (17% in WT, 5% in Batf3−/−; Fig. 4 D).

These data are reminiscent of observations in MHCII−/− mice where M38- and m139-specific CD8+ T cell responses, including their inflation, were comparable to those in WT mice, whereas the IE3-specific response was strongly reduced [110]. Based on these similarities
one might speculate that CD4+ T cells might interact with cross-presenting DCs to facilitate activation and expansion of IE3-specific CD8+ T cells during later stages of infection.

In conclusion, inflation of CD8+ T cell responses, as opposed to priming, is largely independent of the presence of cross-presenting DCs and is likely being driven by direct presentation of a limited subset of viral antigens, probably by cells hosting latent, reactivating viral genomes [33]. While inflation and maintenance of M38-and m139 CD8+ T cells was mainly independent of CD8α+ and CD103+ DCs, IE3-specific CD8+ T cell inflation was
substantially promoted by their presence. Our results clearly demonstrate that CD8α+ and CD103+ DCs are critical for the expansion of MCMV-specific CD8+ T cell responses at early time points after the infection, strongly suggesting that priming occurs mainly via cross-presentation. The relative need of the presence of cross-presenting DCs for priming of MCMV-specific CD8+ T cell responses was dependent on the antigen specificity of the responding CD8+ T cells and overall was stronger for CD8+ T cells of the conventional type. The ability of certain CD8+ T cells, in particular the inflationary ones, to be primed in the absence of CD8α+ and CD103+ DCs might be due to a reduced susceptibility to viral-mediated immune escape mechanisms, or to different stability/cellular localization or expression kinetics of some viral proteins in the directly infected DCs which might favor their direct presentation. In addition, APCs other than CD8α+ and CD103+ DCs have been recently described to cross-present antigens in vivo [111], implying that cross-presentation might not be completely abolished in Batf3−/− mice. Finally, the population of CD8+ DCs is not completely eliminated in Batf3−/− mice but severely reduced; hence, the residual population of CD8+ DCs might also contribute to the remaining CD8+ T cell priming observed in Batf3−/− mice. Thus, with this work we provide evidence that in case of MCMV infection, presence of CD8+ DCs strongly promotes priming of CD8+ T cell responses, which is in line with recently published results where MHC class I-deficient fibroblasts, infected with a spread-defective mutant of MCMV was used to assess the role of cross-presentation in priming of MCMV-specific CD8+ T cell responses [112]. However, subsequent shaping of the virus-specific CD8+ T cell response in presence of latent / reactivating infection is largely independent of cross-presenting DCs, at least for two of the three inflating specificities in C57BL/6 mice, and probably relies on antigen presentation on directly infected cells. This division of labor in CD8+ T cell activation between cross-presenting DCs during the acute infection process and directly infected cells during viral latency seems sensible as viral reactivation events should be controlled instantaneously and in a targeted manner focusing on actually infected cells in an immune host.
Material and Methods

Mice, viruses and immunization

Batf3<sup>−/−</sup> mice [107] (H-2<sup>b</sup>), 129SvEv (H-2<sup>b</sup>) and C57BL/6 (H-2<sup>b</sup>) control mice were bred in the local animal facility under specific pathogen-free conditions. Recombinant MCMV-Δm157 (m157 deletion mutant) was described previously [50]. The virus was grown on C57BL/6 mouse embryonic fibroblasts and titrated by plaque-forming assays as described previously [51]. Recombinant Vaccina Virus expressing the M45 protein of MCMV was kindly provided by Prof. P. Klenerman (Oxford University, Oxford, UK), and was grown on BSC40 cells. Infection was performed intravenously (i.v.) with 10<sup>7</sup> plaque-forming units (PFU) of MCMV-Δm157 and with 10<sup>7</sup> PFU of VV-M45. All animal experiments were in accordance with institutional policies and have been reviewed by the cantonal veterinary office.

Peptides and MCMV crude lysate

The following peptides have been used in this work; MHC class I-restricted: MCMV-derived: m139<sub>419-426</sub>, M38<sub>316-323</sub>, IE3<sub>416-423</sub>, M57<sub>816-824</sub>, m141<sub>15-23</sub>, M86<sub>1062-1070</sub>, M45<sub>985-993</sub>, M33<sub>47-55</sub>; VV-derived: B8R<sub>20-27</sub>; MHC class II-restricted: MCMV-derived: M25<sub>411-425</sub>, m141<sub>181-195</sub>, m18<sub>872-886</sub>, m139<sub>560-574</sub> M112<sub>36-50</sub> and m14<sub>141-155</sub>. All the peptides were purchased by NeoMPS. A crude lysate of MCMV infected embryonic fibroblasts was prepared as described in [50].

Antibodies and Tetramers

The following fluorescently conjugated antibodies were purchased from Biolegend (Lucerna Chem AG, Luzern, Switzerland): anti-CD8α [53-6.7], anti-CD4 [GK1.5], anti-IFNγ [XMG1.2], anti CD127α [SB/199], anti-CD11c [N418], anti-I-A/I-E [M5/114.15.2]. The following fluorescently conjugated antibodies were purchased by BD Biosciences (Allschwil, Switzerland): anti-Ki67 [B56], anti-Bcl-2 [3F11]. The CROMA 229 antibody (mouse IgG<sub>1</sub>), which recognizes the MCMV gp48 antigen, was kindly provided by S. Jonjic (University of Rijeka, Croatia). For staining with the CROMA 229 antibody, anti-mouse IgG<sub>1</sub>-FITC was used as a secondary reagent (purchased from Jackson Immunoresearch Europe, Suffolk, UK). All peptide/MHC class I tetramers were generated as previously described [94].
RESULTS

*Lymphocyte isolation, cell surface staining and intracellular cytokine staining*

Splenocytes and lung-derived leucocytes were isolated from perfused mice. Fresh blood was obtained from the tail vein. Dendritic cells were purified with CD11c MACS beads according to the manufacturer’s instructions (Miltenyi Biotech, Bergisch Gladbach, Germany). [50]For surface staining, cells were incubated for 30 min at 4°C or at room-temperature when using tetramers before they were lysed with 1X BD lysis buffer (BD Biosciences). For measurement of intracellular IFNγ, the cells were stimulated with 1 µg ml⁻¹ peptide in the presence of Monensin A (2 µM; Sigma Aldrich) for 6 h at 37°C. The cells were permeabilized in 2X BD lysis buffer containing 0.05% Tween 20 (Sigma-Aldrich) for 10 min, and the intracellular staining was performed for 30 min at 4°C. For intracellular staining of Ki67 and Bcl-2, the cells were first fixed in 1X Cytofix/Cytoperm buffer (BD Biosciences) for 15 min, and subsequently permeabilized by incubation in 1X wash/perm buffer (BD Biosciences) for 15 min. Antibodies were diluted in wash/perm buffer and cells were stained for 30 min at 4°C. CROMA 229 intracellular staining was performed as the Ki67/Bcl-2 staining with an additional wash step followed by incubation with anti-mouse IgG1-FITC (30 min at 4°C). Samples were acquired on an LSRII flow cytometer (BD Biosciences, Allschwil, Switzerland) and analyzed with FlowJo software (Tree Star).
5. General discussion

The generation of a functional memory T cell pool upon primary encounter with an infectious pathogen is in combination with humoral immunity, an essential process to confer immunity against possible reencounters with the same pathogen. A prerequisite for the generation of long-lived and sustainable memory T cells is the absence of antigen, and this is usually fulfilled upon resolution of acute viral infections. However, memory T cells play a fundamental role also during persistent viral infections, yet the mechanisms how protection is conferred during these two types of infections are substantially different.

This section will focus on memory CD8 T cells which are elicited during persistent/latent viral infections, with major emphasis on the induction, maintenance and function of virus-specific memory CD8 T cells during the chronic/latent phase of the infection.

5.1 T cell memory

In a rather reductionist way, memory T cells have been subdivided into two major categories: central-memory (T<sub>CM</sub>) T cells, and effector-memory T cells (T<sub>EM</sub>) [55] [113]. This distinction has been made originally according to the different anatomical localization of memory T cells, with T<sub>CM</sub> localizing to secondary lymphoid organs and T<sub>EM</sub> localizing to peripheral tissues and the spleen. In addition, T<sub>CM</sub> and T<sub>EM</sub> populations differ in their longevity, self-renewal ability and rapidity to proliferate and exert effector functions upon reactivation [57,58,59]. T<sub>CM</sub> reside in the secondary lymphoid organs, where their survival and homeostatic self-renewal are supported by local secretion of cytokines, mainly IL-15 and IL-7. High expression of IL7Rα and CD122 (part of the IL-15 receptor), together with the expression of the homing markers CD62L and the chemokine receptor CCR7, are indicative of a T<sub>CM</sub> phenotype in mice. In humans, T<sub>CM</sub> are identified based on the expression of costimulatory receptors CD27 and CD28 and are CD45RA<sup>-</sup> and CCR7<sup>+</sup> [88,114]. The cytokine-driven persistence and their extraordinary potential to proliferate and become effector cells upon secondary stimulation render T<sub>CM</sub> highly efficient for a rapid replenishment of an effector cell population and they are believed to be an integral part in protection against secondary infection by providing long-term immunity. T<sub>EM</sub>, in contrast, reside in the periphery, usually at the site of primary infection, where their maintenance is believed to require interaction with the cognate antigen [55]. Phenotypically, they resemble effector CD8 T cells (CD62L<sup>-</sup>, CCR7<sup>-</sup>) with the exception of being IL7Rα<sup>+</sup>, so they have the ability to sense IL-7 as a survival signal. In humans, T<sub>EM</sub> are CD27 CD28 CCR7 CD45RA<sup>-/+</sup>. 
After restimulation by antigen, $T_{EM}$ proliferate poorly but are extremely rapid in producing effector cytokines such as IFN$\gamma$ and TNF$\alpha$. Because of these properties, $T_{EM}$ are involved in immediate protection in peripheral organs and at mucosal surfaces, which are often portals of entry for invading pathogens. But more importantly, in recent years it has become apparent that $T_{EM}$ play key roles in immune surveillance during latent viral infections, by sensing and immediately terminating viral reactivation events from latently infected cells, which could be fatal for the host [33] [62] [91]. Therefore, it is of interest for the immune system to maintain a pool of $T_{EM}$ cells in close proximity to latently infected cells, which are readily activated and terminate recurrent viral reactivation events. To be able to perform these tasks, $T_{EM}$ must encompass the following criteria: i) they need to be maintained at a stable number at the site of latency; ii) $T_{EM}$ need survival stimuli which likely include IL-7, the presence of cognate antigen and/or other organ-dependent factors; iii) they must exert immediate effector functions and in a way without causing immunopathology.

A prototype of viruses causing latent infections is the herpes virus family, which include $\alpha$-, $\beta$-, and $\gamma$- subfamilies. All herpesviruses share a common viral structure, a generally high prevalence in humans and most importantly, they all establish a persistent latent infection which is clinically silent unless the host is immunocompromized.

The first focus will be on the memory CD8 T cell response that is induced during Murine Cytomegalovirus infection (MCMV), a member of the $\beta$-herpesvirus family, which was the topic of this PhD thesis. Afterwards CD8 T cell memory responses against selected members of the $\alpha$- and $\gamma$-herpesvirus families will be discussed in a comparative manner.

### 5.2 Memory response against $\beta$-herpesviruses

**Murine Cytomegalovirus (MCMV) infection**

CMV seropositivity in humans is associated with a massive pool of CMV-specific memory CD8 T cells, especially in the elderly, occupying ~10% of the total memory compartment ([Sylwester, 2005 #130]). Phenotypically, these cells are mostly CCR7$^+$, CD27$^-$ and CD28$^-$, all indicators of the $T_{EM}$ subset of memory cells, and the majority show high expression of CD57, which has been associated with replicative senescence [73] [115]. In addition, CMV-specific CD8 T cells might also re-express CD45RA, indicative of terminally differentiated effector cells. Functionally, CMV-specific CD8 T cells secrete MIP-1$\beta$ and TNF$\alpha$, and to a lower extent perforin upon *ex-vivo* peptide stimulation and they show immediate cytotoxicity.
Similarly, naturally infected mice as well as laboratory mouse strains latently infected with MCMV develop a very large population of CD8 T<sub>EM</sub> cells [34,36]. The identification of the whole spectrum of MHC-I restricted epitopes eliciting a CD8 T cell response in C57BL/6 mice made it possible to perform longitudinal analyses of the different responses [35,116]. This work revealed that two very distinct kinetic patterns of CD8 T cell responses are induced upon infection with MCMV. The majority of the CD8 T cells, referred to as ‘conventional CD8 T cells’, undergo expansion during the acute phase of infection followed by rapid contraction eventually resulting in low numbers of T<sub>CM</sub> cells which are stably maintained during latency by homeostatic proliferation. In contrast, five epitopes (M38<sub>316-323</sub>, m139<sub>419-426</sub>, IE3<sub>416-423</sub>, IE3<sub>461-475</sub>, M102<sub>486-500</sub>) induced a so called ‘inflationary response’, characterized by continuous expansion even after control of acute lytic infection, to eventually stabilize at high percentages during latency. As observed for HCMV-specific CD8 T cells, inflationary CD8 T cells in mice display the classical phenotype of terminally differentiated T<sub>EM</sub> cells in the periphery (CCR7<sup>−</sup> CD62L<sup>−</sup> IL7Rα<sup>−</sup> KLRG1<sup>+</sup>) and retain cytotoxic functions as well as the ability to secrete IFNγ and TNFα.

So far, it is unknown what processes determine which CD8 T cell specificities will inflate during latency and which will undergo a classical expansion-contraction kinetics. The T<sub>EM</sub> phenotype of the inflationary cells strongly suggests that accumulation and maintenance at high percentage during latency are antigen-driven [33]. One possibility is that some viral genes are more abundantly expressed than others during latency. Most of the work in this respect has been done in the Balb/c mouse strain, where highly sensitive RT-PCR detected immediate-early 1 (IE1) and IE2 transcripts [24], without detecting any early or late gene-products. This might explain the immunodominance of the IE1-derived pp89 epitope in latently infected Balb/c mice. According to a model called the “immune sensing hypothesis of latency control”, presentation of the pp89 epitope by infected cells will activate IE-1 specific CD8 T cells, which would in turn terminate the reactivation event. Consistent with this, mice infected with an MCMV mutant strain carrying a mutation in the pp89 epitope which abrogates MHC class I binding show a 5-fold increase in IE1 transcripts with transition to IE3 transcripts in latently infected lungs [33], though transcription does not proceed to late genes.

By now, nothing is known about the expression profile of M38, m139 and M102 genes in the C57BL/6 model. We have tried to detect differences in the expression levels of M38 and M45 genes during latency (the first one inducing an inflationary, the second one a conventional
response) by qRT-PCR, but we were unable to detect any viral transcripts during latency, and during the acute phase of infection the two genes were expressed at similar levels. While it remains an open question whether a preferential viral gene expression profile determines the specificities of inflating CD8 T cell populations during latency, it is possible that additional mechanisms are involved, as suggested by the observation that a second epitope contained in the M38 gene product, M38_{38\text{-}45}, induces a conventional CD8 T cell response [116]. Hence, it is possible that post-transcriptional mechanisms are involved.

In this line, it has recently been reported that conventional CD8 T cells display a higher dependence on the immunoproteasome subunit LMP7 compared to inflationary CD8 T cells [84]. The immunoproteasome is constitutively expressed by immune cells like DCs and macrophages, whereas expression in non-immune cells can be induced by IFNγ. As we and others [18,19] showed that MCMV latency is established predominantly in non-immune cells, it is an intriguing possibility that these cells would only be able to process and present those antigens whose processing is independent of the immunoproteasome. We could additionally show that accumulation of CD8 T cells during latency is independent of the major subsets of cross-presenting DCs, indicating that viral antigens are presented to the inflationary CD8 T cells directly by infected cells, presumably of non-hematopoietic origin [78]. To confirm this hypothesis, we analyzed the kinetics of the H-2K{b}-restricted inflationary M38-specific CD8 T cells in mice in which the expression of the H-2K{b} molecule was selectively removed on non-hematopoietic cells (Manuscript in revision). Indeed, memory inflation was completely abolished in these mice and the otherwise highly activated inflationary CD8 T cells displayed a T_{CM} phenotype during latency. Thus, based on these data, latently infected cells of non-hematopoietic origin, in which the reactivation program has been induced, would present viral-derived antigens (which are limited to antigen processing by the constitutive proteasome by virtue of their lack of the immunoproteasome during viral latency when IFNγ levels are low) to the specific CD8 T cells in the periphery, inducing their activation and proliferation and/or maintenance. However, we and others showed that inflationary CD8 T cells divide only sporadically during latency in peripheral tissues such as the lung and the liver, and they do not express CD69, suggesting that events of antigen encounters are either extremely rare in the periphery, or even absent [76]. Interestingly, a small proportion of inflationary CD8 T cells in the lymph nodes, displaying largely a T_{CM} phenotype, proliferate extensively in an antigen dependent manner during latency, indicating that the massive inflationary pool in the periphery is maintained by
continuous replenishment of newly activated effector CD8 T cells coming from the lymph nodes. Notably, local proliferation of inflationary T\textsubscript{CM} cells during viral latency in the lymph nodes was strictly dependent on antigen presentation by non-hematopoietic cells. This suggests that viral reactivation events in non-hematopoietic cells in the lymph nodes are sensed by locally resident T\textsubscript{CM} cells, resulting in their extensive proliferation and differentiation into T\textsubscript{EM} cells, which can consequently migrate and home to peripheral tissues. In such a scenario, the long-term maintenance of peripheral T\textsubscript{EM} cells being able to perform constant immunosurveillance duties is guaranteed as it is based on recurrent tapping of T\textsubscript{CM} resources from the lymph nodes.

Although some progresses have been done towards understanding the memory CD8 T cell response during MCMV infection, many unanswered questions remain. Primary among these is whether antigen exposure due to continued gene expression, or other factors, are required for maintenance of the inflationary pool in the periphery, or if is completely sustained by constant supply of newly activated cells from the lymph nodes. For this, a detailed turnover analysis of the inflationary cells would provide information about the life-span of these activated cells in the periphery. Interestingly, inflationary CD8 T cells upregulate Bcl-2 during latency, also in peripheral tissues and despite exhibiting a T\textsubscript{EM} phenotype, suggesting that cytokines or other stimuli might promote their survival in the periphery. Another important issue that requires further investigation is about the role that this massive CD8 T cell response has in the control of CMV reactivation from latency. As discussed above, evidence for a protective role of inflationary CD8 T cells has been provided in the Balb/c model [33], though both control of the primary infection and establishment/maintenance of latency are not impaired in CD8 T cell deficient mice [28,29]. Conversely, life-long maintenance of such a high number of CMV-specific CD8 T cells may significantly contribute to immune senescence [117,118].

5.3 Memory response against α-herpesviruses

Herpes Simplex Virus Type 1 (HSV-1) infection

Herpes-simplex virus 1 is a ubiquitous α-herpesvirus that establishes latency within sensory neurons. Lifelong infection in humans is associated with spontaneous events of virus reactivations characterized by persistent viral gene expression [119,120,121]. In mice, latency is established in sensory ganglia but reactivation does not occur spontaneously and requires
physical or emotional stresses [122]. Contrary to MCMV infection, where depletion of CD8 T cells does not impair virus clearance and establishment of latency, absence of this subset of lymphocytes during HSV-1 infection results in impaired termination of the replicative phase of the infection [123] [124]. Upon ocular HSV-1 infection of mice, both memory CD8 and CD4 T cells selectively accumulate in latently infected sensory ganglia [125] [126] (Theil, Am J Pathol 2003). Notably, the majority of the CD8 T cells found in the sensory ganglia are specific for a glycoprotein B-derived HSV-1 epitope (gB-498-505), and expresses CD69, a sign of recent antigen stimulation. gB498-505-specific CD8 T cells have been shown to block HSV-1 reactivation from latently infected sensory ganglia cultured ex-vivo [126], whereby a major mechanism of CD8 T cell-mediated protection is through IFN-γ secretion and release of non-cytotoxic lytic granules [127,128]. This non-cytotoxic mechanism of viral inactivation appears to be particularly important for the maintenance of neuronal integrity during HSV-1 infection, but could also be a general mechanism exploited by the immune system to avoid major organ damage due to persistent CD8 T cell activation during other latent viral infections. Whether long-term maintenance of these CD8 T cells in the sensory ganglia is dependent on prolonged antigen exposure is not known yet. In a model of HSV-1 infection of the skin, Gebhardt et al. showed that virus-specific CD8 T cells remain resident in the dermis upon resolution of the primary infection and confer protection against subsequent infections [62]. These cells, which are not in equilibrium with the circulating pool of CD8 T cells, show increased expression of CD103 which is important for interaction with epithelial cells. Notably, a key involvement of CD103 expression on CD8 T cells for their retention in the periphery (in this case the brain) has also been demonstrated upon intranasal infection with VSV. While initial upregulation of CD103 was dependent on interaction with their cognate antigens, long-term persistence occurs in the absence of any detectable viral antigen or viral genomic RNA [61].

While specific CD8 T cells accumulate in the sensory ganglia during latency upon local ocular HSV-1 infection, they don’t do so systemically, where they are present in low numbers with a resting TCM phenotype [129]. However, upon systemic HSV-1 administration gB-498-505-CD8 T cells show a very comparable behavior to inflationary CD8 T cells during MCMV infection [130], with a steady increase in numbers and display of a TEM phenotype during latency. The strong analogies in the CD8 T cell responses induced upon systemic infections with HSV-1 and CMV is indicative of similar mechanisms of latency establishment and antigen presentation patterns, and might be exploited to better understand the complex interactions between latent viruses and their hosts.
5.4 Memory response against γ-herpesviruses

Ebstein Bar Virus (EBV) infection

EBV is a ubiquitous γ-herpesvirus that infects about 90% of the human population. EBV primary infection, if delayed until adolescence, can cause acute infectious mononucleosis (IM). Also for this herpesvirus, establishment of latency is clinically silent in healthy individuals, whereas it can cause severe disease in immunosuppressed patients, including nasopharyngeal carcinoma, a subset of Hodgkin’s lymphomas and B cell lymphomas. Primary infection is characterized by extensive virus replication in permissive cells, most likely epithelial cells of the oropharynx. The lytic cycle involves sequential expression of immediate-early (IE), early (E) and late proteins (L), altogether referred to as lytic cycle proteins. At the same time, EBV infects B cells, inducing their transformation and proliferation which results in amplification of the latent virus reservoir [131]. This phase of infection involves, and requires, the expression of so called latent proteins. In a third phase, both lytic and latent infections are controlled by the immune system, but some infected B cells downregulate latent genes expression and become long-lived, latently infected memory B cells [132]. However their occasional reactivation into productive cycle is thought to occasionally occur, leading to persistent low-level of antigen stimulation [133,134]. The availability of specific HLA class I-peptide tetramers allowed the analysis, in terms of magnitude and phenotype, of CD8 T cell responses against lytic cycle and latent proteins in patients experiencing EBV infection causing acute IM [135,136]. This important work revealed that responses against these two classes of proteins are highly diverse. CD8 T cells specific for lytic antigens expanded massively and possessed a highly activated phenotype during primary infection. As IM symptoms resolved, the majority of these cells contracted, leaving behind a small population of cells, which nevertheless remained CCR7- CD62L- and partly CD45RA+, characteristic of TEm cells [137]. In contrast CD8 T cells specific for EBV latent proteins were present at low frequencies during the acute phase of infection, yet they were maintained or even increased in numbers upon establishment of latency. Interestingly, these cells remained uniformly CD45RO+/RA- and reexpressed CCR7 and CD62L, which allowed their migration into the tonsil where they accumulated to a much higher extent compared to the more activated CD8 T cells specific for lytic proteins [138]. In some circumstances, EBV-specific CD8 T cells can reach up to 20% of the total CD8 T cells in the tonsils. Interestingly, the majority of EBV-specific cells in the tonsillar tissue express CD103.
**Murine herpes virus-68 (MHV-68) infection**

An accepted murine model for EBV infection and in general for all γ-herpesviruses is MHV-68 infection [139]. Upon intranasal MHV-68 infection, virus replication is first observed in the lungs, before latency is established in B cells and other APCs [140]. As for EBV, MHV-68 specific CD8 T cells have been subdivided into lytic- and latent protein-specific CD8 T cells. CD8 T cells specific for lytic antigens peak in the lungs at ~day 10 post infection, whereas in the spleen these cells are barely detectable. In contrast CD8 T cells specific for latent antigens peak in the spleen and in the lymph nodes at day ~20, but not in the lungs [141]. Both populations of CD8 T cells contract during latency, although those specific for latent Ag are present at slightly higher percentages compared to cells specific for lytic Ag (~0.9% vs. ~0.3 % of CD8+). Consistent with the responses induced during EBV infection, CD8 T cells specific for latent Ag are predominantly CD62L+CD43+, reminiscent of a T<sub>CM</sub> phenotype, while CD8 T cells specific for lytic-specific Ag are significantly more activated. Nevertheless, T cells recognizing latent Ag (which display a T<sub>CM</sub> phenotype), but not lytic Ag (display a T<sub>EM</sub> phenotype), can rapidly kill target cells in vivo, a function that has usually been attributed to T<sub>EM</sub> type of cells.

Together, the studies performed in human patients infected with EBV and in mice infected with MHV-68 indicate that like CMV, these two members of the γ-herpesvirus family induce two very distinct CD8 T cell responses which differ in their kinetics, phenotype and function during latency. However, there are some important differences between these two infections: first of all, the responses that increase during the chronic phase of EBV infection display a T<sub>CM</sub> phenotype during latency, which is in strong contrast with the T<sub>EM</sub> phenotype of inflationary CD8 T cells during CMV latency. In addition, the kinetic patterns of the CD8 T cell responses strongly correlate with the gene expression program of EBV, which seems not to be the case for CMV. While it seems evident that expression of lytic genes versus latent genes in different target cells regulates the CD8 T cell responses during primary infection, it is not clear what determines the different phenotype of lytic- and latent Ag-specific CD8 T cells during latency. One explanation is that lytic Ag are expressed at much higher levels compared to latent Ag during latency. Another is that both classes of Ag are similarly expressed, but at different locations, rendering some CD8 T cells more exposed to Ag compared to others. Finally, it is possible that the massive expansion of CD8 T cells specific for lytic Ag during primary infection hampers the acquisition of typical T<sub>CM</sub> markers, even in the absence of cognate antigens.
Concluding remarks

A growing body of evidence indicates that memory T cells are important players in immune protection during latent viral infections. Their role is to mount an immediate response to viral reactivation events directly at the sites where these are likely to occur, often in peripheral tissues or secretory organs. To perform this task, the phenotype and function of these peripheral memory T cells need to adapt to both the particular anatomical locations and to the pathogen/antigen they are specific for. For each of the herpes virus infections discussed here, selected populations of memory CD8 T cells take up residence at specific sites, where they likely play key roles in effective immune surveillance. How these memory T cells are selected among all other memory T cells to become effector memory cells, what signals drive their migration to the selected sites and how their maintenance are guaranteed, are all factors which are likely to be determined by a very strong interplay between the infecting viruses and their host and represent important questions to be addressed in future studies.
6. References


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7. Curriculum Vitae

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