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The Dynamics of Mouse Cytomegalovirus-Specific CD4 T Cell Responses during Acute and Latent Infection¹

Senta M. Walton,* Philippe Wyrsh,* Michael W. Munks,[†] Albert Zimmermann,[‡] Hartmut Hengel,[‡] Ann B. Hill,[†] and Annette Oxenius^{2*}

The dynamics of mouse cytomegalovirus (MCMV)-specific CD4 T cell responses and the mechanisms by which these cells contribute to viral control are not well understood, mainly due to lack of appropriate tools to characterize MCMV-specific CD4 T cells. We therefore generated MCMV-specific CD4 T cell hybridomas, then used an MCMV expression library and overlapping peptides to identify CD4 T cell epitopes. We used these novel tools to study the long-term kinetics and organ distribution of MCMV-specific CD4 T cells in comparison to MCMV-specific CD8 T cell responses. We demonstrate that the overall MCMV-specific CD4 T cell response stabilizes during the latent stage, which stands in contrast to subpopulations of MCMV-specific CD8 T cells and HCMV-specific CD4 T cells which accumulate over the course of CMV latency. Furthermore, MCMV-specific CD4 T cells displayed a Th1 phenotype, secreting high levels of IFN- γ and TNF- α and to some extent IL-2, cytokines which are involved in protection from CMV disease. *The Journal of Immunology*, 2008, 181: 1128–1134.

Cytomegaloviruses belong to the β subfamily of herpesviruses. The mouse CMV (MCMV)³ genome consists of >230 kb and has ~170 open reading frames (ORFs) (1). Despite vigorous immune activation after primary infection, human CMV (HCMV) as well as MCMV persist in their host and establish latency.

NK cells play a major role in limiting viral replication and reducing viral load, especially at early time points during acute MCMV infection (2). Although primed B cells and T cells exert antiviral effects and are able to reduce viral replication after adoptive transfer in acutely infected recipient mice (2, 3), mice deprived of either CD8 T cell or B cell functions are able to control production of infectious virus with similar kinetics as their wild-type (wt) counterparts (2). However, in the absence of CD4 T cells, control of lytic virus is impaired in various organs, in particular in the salivary glands where shedding of infectious virus persists (2). It is believed that CD4 T cells inhibit MCMV replication at least to a certain extent by local secretion of TNF- α and IFN- γ (2). However, the exact mechanisms of how CD4 T cells exert their protective functions remain elusive. Evidence that CD4 T cells are crucial to control of CMV infection also come from

human studies; in HIV-1-infected patients, CMV end-organ disease correlates with the loss of HCMV-specific CD4 T cells (4). Furthermore, in transplant recipients, early expansion of HCMV-specific CD4 T cells protected from symptomatic disease (5) and impaired induction of HCMV-specific CD4 T cells in children is believed to be the cause of prolonged virus shedding (6). In humans, direct antiviral effects such as cytokine secretion and cytotoxicity (7) as well as helper functions (8) of CD4 T cells are proposed to contribute to control of viral replication.

Analysis of effector CD4 T cells during MCMV infection has been hampered because no virus-specific CD4 T cell epitopes have been described so far. Although HCMV-specific CD4 T cell epitopes were described previously (9), longitudinal analysis of CD4 T cell responses in humans is often limited to patients undergoing organ transplantation (5, 10), due to the fact that primary infection is clinically silent in healthy individuals and hence usually undiagnosed.

To assess the CD4 T cell response during acute and persistent MCMV infection, we first identified virus-specific CD4 T cell epitopes with the help of newly generated MCMV-specific CD4 T cell hybridomas. Using the identified MCMV-specific CD4 T cell epitopes, we were able to study for the first time long-term kinetics, organ distribution, and functional characteristics of MCMV-specific CD4 T cells.

Materials and Methods

Mice, in vivo CD4 depletion, and peptides

C57BL/6 mice were kept under specific pathogen-free conditions and were infected i.v. with 10⁷ PFU of MCMV. Animal experiments were performed according to the regulations of the cantonal veterinary office.

When indicated, mice were injected i.p. with 0.2 mg of purified anti-mouse CD4 YTS 191.1 mAb (11). For continuous depletion, mice were injected 3 and 1 days before immunization and then weekly. Depletion was analyzed by flow cytometry and was >95%.

The H-2K^b-restricted M45_{aa985–993} and M38_{aa316–323} peptides were purchased from NeoMPS. Overlapping peptides used to identify CD4 T cell epitopes recognized by MCMV-specific CD4 T cell hybridomas were 15-aa long with a 10-aa overlap and were purchased from EMC Microcollections.

Viruses

Recombinant MCMV- Δ m157 (lacking the *m157* gene of MCMV) was generated according to a previously published procedure (12) using bacterial artificial chromosome (BAC) plasmid pSM3fr (13). Specifically, for

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³ Abbreviations used in this paper: MCMV, mouse CMV; ORF, open reading frame; HCMV, human CMV; BAC, bacterial artificial chromosome; MEF, mouse embryonic fibroblast; wt, wild type; *FLP*, protein encoded by the *FLP* gene of the 2-micron plasmid of yeast.

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construction of the $\Delta m157$ -MCMV mutant, a PCR fragment was generated from the contiguous primers AZ-M157-1 (5'-CAGGAGAATCGAACC CCGATATTTGAGAAAGTACCCCGATATTCAGTACCTCTTGACC CCAAGTGAATTCGAGCTCGGTAC-3') and AZ-M157-2 (5'-AGATCGT GACCATTATCACCAAGATAGTTCACCACATAATTCATCGTCA CTAGAGTCGGACCATGATTACGCCAAGCTCC-3') using the plasmid pSLFRTKn (14) as template DNA. The PCR fragment containing a kanamycin resistance gene (Kn^r) was inserted into pSM3fr by homologous recombination in *Escherichia coli* replacing the *m157* gene region. The Kn^r was excised by *FLP*-mediated recombination (12) generating pSM3fr- $\Delta m157$. Correct mutagenesis of the recombinant MCMV-BAC was confirmed by restriction analysis and sequencing of the *m157* genome region (data not shown). Recombinant virus $\Delta m157$ -MCMV was reconstituted by transfection of mouse embryonic fibroblasts (MEF) using Superfect reagent (Qiagen). Residual BAC sequences were removed by sequential cell culture passages as described elsewhere (13).

BAC-derived MCMV MW97.01 was provided by Prof. U. H. Koszinowski (Max von Pettenkofer-Institute, Ludwig-Maximilians University, Munich, Germany) and is here referred to as MCMV.

MCMV was propagated on MEFs and viral titers were determined using plaque-forming assays as described previously (15).

Generation of CD4 T cell hybridomas

C57BL/6 mice were immunized s.c. with UV-inactivated MCMV (3×10^7 PFU) in IFA (Sigma-Aldrich) supplemented with 10 nmol of CpG oligonucleotide 1668 (5'-TCCATGACGTTCCCTGAATAAT-3', PTO-bonds). At the earliest 2 wk later, mice were challenged with 10^7 live wt MCMV i.v. and, 7 days later, spleen cells were isolated and activated for 3 days with 10 μ g/ml crude MCMV lysate before cells were fused with the BW5147 $\alpha^- \beta^-$ cell line (provided by Dr. R. M. Zinkernagel, University Hospital, Zurich, Switzerland) according to the protocol described by Kruisbeek (16).

To test the specificity of CD4 T cell hybridomas, cells were stimulated with thioglycolate-induced peritoneal C57BL/6 macrophages that were pulsed with either crude MCMV lysate or overlapping peptides. Twenty-four hours later, IL-2 production by the hybridomas was evaluated by an IL-2 bioassay (incubating supernatant with the IL-2-sensitive cell line CTLL-2 (ATCC TIB-214)). Survival of CTLL-2 cells was determined the following day by microscopy or using the AlamarBlue cell viability assay (Lucerna Chem).

Cell transfection and lysate production

MCMV lysate production. Crude MCMV lysate production was adapted from Refs. 17 and 18. MEFs were infected with MCMV and once cells started to round up, they were harvested, centrifuged, and the supernatant was discarded. A pellet of one 150-cm² tissue culture flask was resuspended in 0.5 ml of glycine buffer (0.1 M glycine and 0.9% NaCl, pH 9.75). After an incubation period of 20 min at 4°C, the cell lysate was sonicated four times for 10 s.

Lysate production of MCMV protein expression library. HEK cells propagated in DMEM/10% FCS were transfected with individual plasmids of a MCMV ORF library (19) using JetPEI transfection reagent (Chemie Brunschwig) according to the manufacturer's instructions. Two days later, transfected HEK cells were harvested and the cell lysate was prepared as described above.

Antibodies

The following mAbs were purchased from BD Pharmingen and used for stainings: anti-CD8 (FITC, PerCP, allophycocyanin), anti-CD4 (PE, PerCP), anti-IFN- γ (allophycocyanin or FITC), anti-TNF- α (FITC), anti-IL-2 (allophycocyanin), anti-IL-4 (PE), anti-IL-10 (PE), anti-IL-17 (PE).

Cell stimulation, immunofluorescent staining, and analysis

Lymphocytes were isolated from spleen, lung, liver, and salivary glands as previously described (20) and single-cell suspensions were used for 6 h in *in vitro* restimulations. CD8 T cells were stimulated with 1 μ g/ml peptide and CD4 T cells with 5 μ g/ml peptides or with cell lysates. In case cell lysates were used for CD4 T cell stimulations, the isolated lymphocytes were first rested overnight at 37°C before CD4 T cells were purified by magnetic cell sorting according to the manufacturer's instruction (Miltenyi Biotec). Purified cell fractions were stimulated in the presence of thioglycolate-elicited peritoneal macrophages that had been pulsed with 20 μ g/ml cell lysate overnight before adding CD4 T cells. For the final 6 h of stimulation, monensin A or brefeldin A was added to the cultures. Cells were harvested and first stained at 4°C for 20 min for cell surface markers before fixation and permeabilization (using 500 μ l of Fix/Perm Solution (FACSLyse;

BD Biosciences) diluted to 2 \times concentration with H₂O and 0.05% Tween 20 (Sigma-Aldrich)), followed by intracellular staining at room temperature for 20 min. Cells were washed and resuspended in PBS containing 1% paraformaldehyde (Sigma-Aldrich). Multiparameter flow cytometric analysis was performed using a FACS LSRII flow cytometer (BD Biosciences) with FACSDiva software (BD Biosciences). List mode data were analyzed using FlowJo software (Tree Star).

Results

Generation of MCMV-specific CD4 T cell hybridomas for the identification of MCMV-derived CD4 T cell epitopes

With the aim to identify MCMV-derived CD4 T cell protein Ags and epitopes, we generated MCMV-specific CD4 T cell hybridomas. To this end, we immunized C57BL/6 (B6) mice with UV-inactivated MCMV (3×10^7 PFU) in IFA plus CpG oligonucleotide followed by infection with live MCMV. Spleen cells from these mice were fused with the BW $\alpha^- \beta^-$ lymphoma cell line and resulting CD4⁺TCR β^+ hybridoma clones were screened for MCMV specificity. We used lysates of MCMV-infected MEFs as Ags in combination with thioglycolate-induced macrophages from B6 mice to test for activation of the respective T cell hybridoma clones. Activation was assessed by measuring induced IL-2 secretion. Twenty-six CD4 T cell hybridomas tested positive for recognition of MCMV lysates. Next, we analyzed the MCMV protein specificity of these hybridomas. To this end, we used a recently published MCMV protein expression library which includes 170 ORFs of MCMV and hence most of the so far known viral ORFs (19). All 170 plasmids were individually transfected into 293 HEK cells and 2 days after transfection crude lysates were generated. These lysates were used individually as Ags for the stimulation of the 26 MCMV-specific CD4 T cell hybridomas. For 9 of the 26 MCMV-specific hybridomas, the MCMV protein recognized was identified (Table I). These nine hybridomas were specific for four different protein Ags. For the remaining 17 clones, the MCMV protein specificity could not be determined with this approach, either because the ORF they are specific for is not present in the library or the protein they are specific for is expressed poorly or unstable in the transfected HEK cells. Furthermore, not all Ags may be presented as well by thioglycolate-elicited macrophages.

Next, we mapped the antigenic peptides recognized by the nine hybridomas for which the specific MCMV protein could be identified. For this purpose, we stimulated them with overlapping peptides (15-mer peptides, overlapping by 10 aa with the consecutive peptide) that spanned the entire identified MCMV proteins. Using this approach, we were able to identify five MCMV-specific CD4 T cell epitopes within four different MCMV proteins, namely, m14_{aa136-150}, M25_{aa411-425}, M25_{aa721-735}, M112_{aa36-50}, and m142_{aa26-40} (Table I).

Increased MCMV-specific CD4 T cell response after infection with the recombinant MCMV- $\Delta m157$ strain compared with wt MCMV

Infection with wt MCMV induces modest frequencies of MCMV-specific CD4 T cells, assessed by IFN- γ -secretion after stimulation with crude MCMV lysate (Fig. 1). With the aim of enhancing MCMV-specific CD4 T cell responses, we infected B6 mice with a recombinant MCMV that lacks the *m157* gene (MCMV- $\Delta m157$). The gene product of *m157* encoded on the viral genome interacts with the NK-activating receptor Ly49H present on a subset of NK cells of B6 mice. Bubic et al. (21) showed that B6 mice infected with MCMV- $\Delta m157$ have increased virus titers in various organs due to impaired viral activation of Ly49H⁺ NK cells. When we infected B6 mice with either MCMV or MCMV- $\Delta m157$, MCMV-specific CD4 T cell responses were substantially increased in mice

Table I. *MCMV-specific CD4 T cell hybridomas*

Clone	ORF	Peptide Position	Peptide Sequence	Family or Function
5.33	<i>m14</i>	m14 ₁₃₆₋₁₅₀ ^a m14 ₁₄₁₋₁₅₅	VTDNIYISSQVSSNQ YISSQVSSNQLGLIC	Glycoprotein family m02
5.81	<i>M25</i>	M25 ₄₁₁₋₄₂₅	LYETPISATAMVIDI	Viral tegument protein
6.37 B2 ^b	<i>M25</i>	M25 ₄₁₁₋₄₂₅	LYETPISATAMVIDI	
10.12	<i>M25</i>	M25 ₄₁₁₋₄₂₅	LYETPISATAMVIDI	
10.18	<i>M25</i>	M25 ₄₁₁₋₄₂₅	LYETPISATAMVIDI	
10.42	<i>M25</i>	M25 ₄₁₁₋₄₂₅	LYETPISATAMVIDI	
6.38	<i>M25</i>	M25 ₇₂₁₋₇₃₅ M25 ₇₂₆₋₇₄₀	SLGNFVYNFSRALEE VYNFSRALEELRGM	Nucleoprotein E1
10.45	<i>M112</i>	M112 ₃₆₋₅₀ ^a M112 ₄₁₋₅₅	DLRQWTYESARVLDC TYESARVLDC ^c TNGEG	
5.03 H8 ^b	<i>m142</i>	m142 ₂₆₋₄₀	RYLTAAAVTAVLQDF	

^a Respective peptides are recognized at lower concentrations.

^b Respective hybridoma cell lines were subcloned to generate clonal populations.

infected with MCMV- Δ m157 (Fig. 1). Accordingly, MCMV-specific CD8 T cells were present at higher frequencies after MCMV- Δ m157 compared with MCMV infection. Although the CD4 T cell response increased severalfold, the CD8 T cell response only increased slightly (data not shown). However, the overall kinetics of T cell responses were comparable after infection with both virus strains (data not shown). The increase of MCMV-specific T cell response is most likely due to the higher Ag burden seen after infection with MCMV- Δ m157 (Fig. 1B).

A wide range of MCMV proteins are recognized by CD4 T cells during acute MCMV infection

To verify that CD4 T cell responses against the MCMV proteins m14, M25, M112, and m142, recognized by at least one MCMV-specific CD4 T cell hybridoma, are indeed induced in vivo during MCMV infection, and to further characterize the overall specificities of the MCMV-specific CD4 T cell response, we incubated CD4 T cells from acutely infected mice (day 7) individually with the protein lysates of the MCMV expression library and assessed IFN- γ production within CD4 T cells after short-term stimulation. Already in the absence of Ag exposure, 0.4% of CD4 T cells of acutely infected mice secreted IFN- γ ex vivo (Fig. 2, medium), indicating that these cells were highly activated, most likely by exposure to MCMV Ag in vivo. Incubation with lysates from untransfected HEK cells (Fig. 2, mock) did not increase the IFN- γ production above this level, indicating that the human origin of the

HEK cell lysates was not perturbing our analysis. However, incubation of CD4 T cells from acutely infected mice with lysates from HEK cells that had been transfected with an irrelevant Ag (such as β -galactosidase) increased the background slightly to \sim 0.9% IFN- γ -producing CD4 T cells. The reason for this increase in unspecific IFN- γ production remains unclear at the moment. Due to the high activation status of CD4 T cells at early stages of MCMV infection, we set a threshold at 1.2% IFN- γ -secreting CD4 T cells (Fig. 2A, solid line) as the lower limit of a positive response toward the recombinantly expressed MCMV proteins in the HEK cell lysates. Using this threshold, we could verify that all of the MCMV proteins recognized by the CD4 T cell hybridomas (m14, M25, m112, m142) elicited CD4 T cell responses ex vivo above background levels (Fig. 2, red bars). Furthermore, we were able to identify

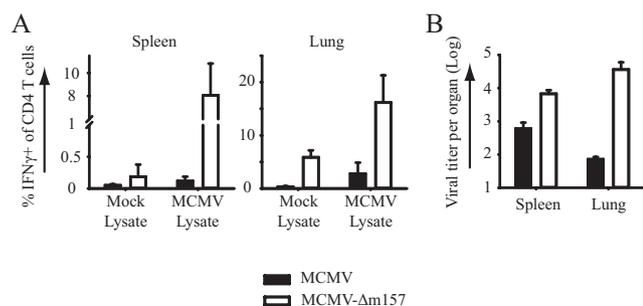


FIGURE 1. Enhanced MCMV-specific CD4 T cell responses after infection with MCMV- Δ m157. B6 mice were infected with either MCMV wt (■) or MCMV- Δ m157 (□) 6 days before isolation of spleen (*left diagram*) and lung (*center diagram*) lymphocytes. *A*, IFN- γ -secretion by CD4 T cells was analyzed after stimulation with crude lysate of mock-infected (Mock lysate) or MCMV-infected (MCMV lysate) MEFs. Frequencies of IFN- γ -producing CD4 T cells are depicted. *B*, Virus titers were determined in the respective organs using plaque-forming assays. Bars show averages of three mice per group and data of one representative experiment are shown.

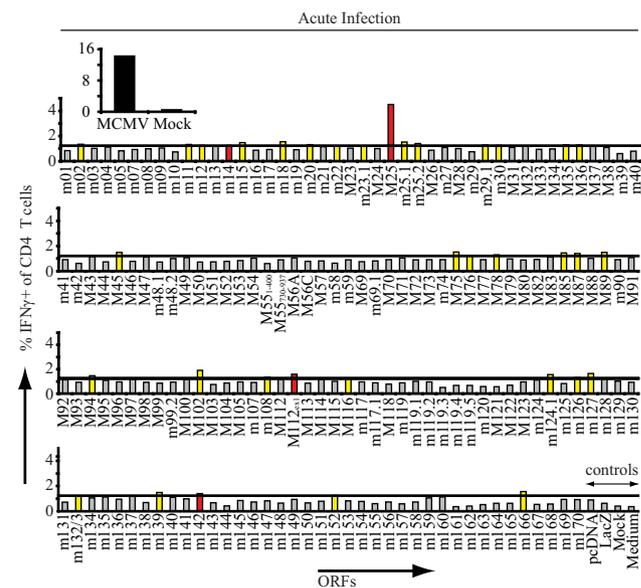


FIGURE 2. MCMV-specific CD4 T cells are specific for a wide range of MCMV proteins. At day 7 postinfection, lung lymphocytes were isolated and purified. CD4 T cells were stimulated with peritoneal macrophages pulsed with a panel of crude HEK cell lysates (generated after transfection with plasmids, each expressing one individual ORF of MCMV) and specific IFN- γ production by CD4 T cells was determined by intracellular cytokine staining. ORFs stimulating a CD4 T cell response above background levels (solid line) are depicted (yellow bars). All ORFs recognized by MCMV-specific CD4 T cell hybridomas (red bars) elicit a CD4 T cell response above background. The *inset* in the *top left corner* depicts the overall MCMV-specific CD4 T cell response (using a lysate of MCMV-infected MEFs for stimulation).

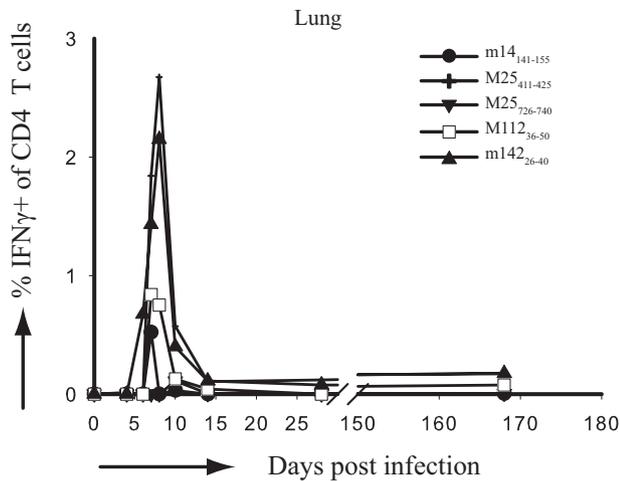


FIGURE 3. CD4 T cell responses specific for MCMV-derived CD4 T cell epitopes. B6 mice were infected with MCMV- Δ m157. Lymphocytes of the lung were isolated during the course of infection and stimulated *ex vivo* with the identified MCMV-specific CD4 T cell epitopes. Frequencies of IFN- γ -producing CD4 T cells are shown after subtraction of background IFN- γ production in the absence of Ag. Symbols show averages of three to four mice per group and data of one representative experiment are shown.

additional 32 MCMV proteins (Fig. 2, yellow bars) that were recognized by CD4 T cells during acute virus infection. Interestingly, the M25 protein was clearly the immunodominant protein in our analysis with over 4% of CD4 T cells being specific for this protein. This nicely correlates with the high abundance of M25-specific CD4 T cell hybridomas in our panel of MCMV-specific CD4 T cell hybridomas (Table I). Aside from the M25 protein, CD4 T cells broadly target MCMV proteins but no other MCMV protein by itself reached values comparable to the overall MCMV-specific CD4 T cell response of 14% (Fig. 2, *inset*) or even comparable to the M25 protein (4%).

To confirm that CD4 T cells specific for the identified epitopes were primed during MCMV infection, lymphocytes from the lung, liver, salivary gland, and spleen were isolated and stimulated *ex vivo* with the antigenic peptides m14_{aa136-150}, M25_{aa411-425}, M25_{aa721-735}, M112_{aa36-50}, and m142_{aa26-40}. As shown in Fig. 3 for lung lymphocytes, CD4 T cell specific for any of the identified epitope were present during acute MCMV infection although in various frequencies. Cell numbers of M25_{aa410-425}-specific CD4 T cells were highest in all organs analyzed. Frequencies of all epitope specificities declined and remained at low levels during the latent stage of infection. Considering that the M25 was the most antigenic ORF of MCMV during acute infection (Fig. 2), these data define M25_{aa410-425} as the immunodominant MCMV CD4 T cell epitope.

Different kinetics of MCMV-specific CD4 and CD8 T cell responses during latent stages of infection

During latent MCMV infection, CD8 T cells specific for some epitopes such as M38_{aa316-323} accumulate over time, whereas frequencies of CD8 T cells specific for other epitopes such as M45_{aa985-993} stay stable over long periods of time. To directly compare CD4 and CD8 T cell kinetics during MCMV infection, overall MCMV-specific CD4 T cell responses, CD8 T cell responses against the epitopes M45_{aa985-993} and M38_{aa316-323}, representing major CD8 T cell epitopes in B6 mice (22), as well as viral titers were measured at various time points after MCMV infection. MCMV-specific CD4 T cell responses and MCMV-specific CD8 T cell responses were determined using intracellular IFN- γ staining after short-term *ex vivo* stimulation with crude MCMV lysate (Fig. 4,

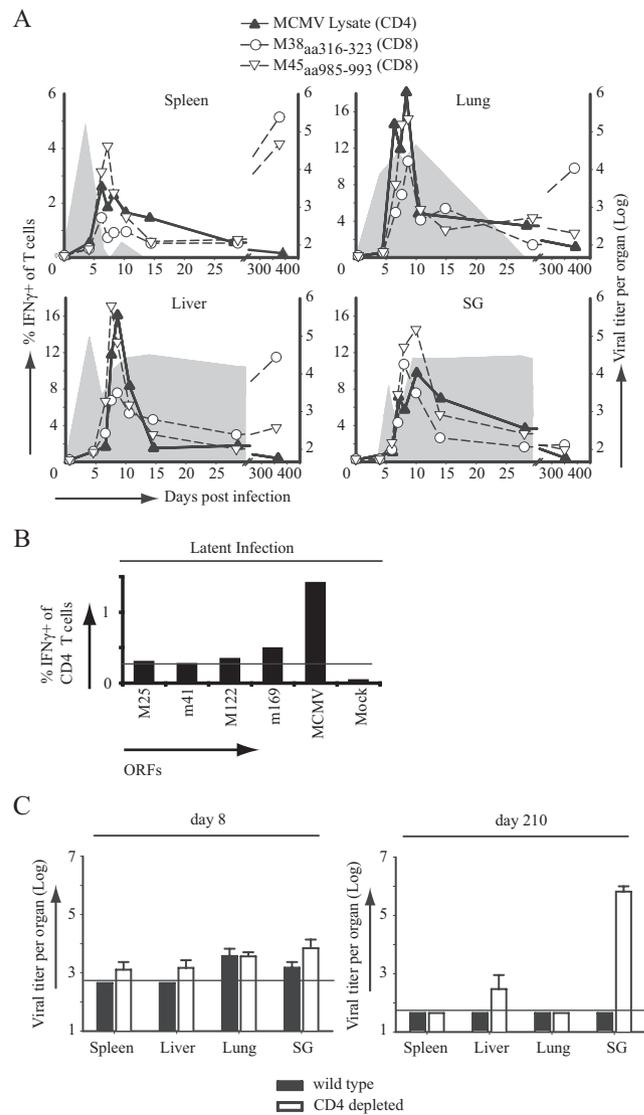


FIGURE 4. Long-term kinetics of MCMV-specific CD4 and CD8 T cells in comparison to viral loads in peripheral and lymphoid tissues. *A*, B6 mice were infected with MCMV- Δ m157 and at various time points overall MCMV-specific CD4 T cell responses were measured after stimulation with MCMV-infected MEF cell lysate (solid line, \blacktriangle). Concomitantly, frequencies of IFN- γ -secreting CD8 T cell response after short-term *ex vivo* stimulation with the epitopes M45_{aa985-993} (∇) and M38_{aa316-323} (\circ) as well as viral titers (shaded areas) were evaluated in the spleen, lung, liver, and salivary glands (SG) during acute and latent infection. Frequencies of IFN- γ -secreting CD4 and CD8 T cells are depicted. *B*, Lung CD4 T cells were isolated at day 351 after MCMV- Δ m157 infection and stimulated with the same panel of HEK cell lysates as in Fig. 2 or with a lysate of MCMV-infected MEFs and secretion of IFN- γ by CD4 T cells was measured. Only responses above background (0.25% solid line) are shown. *C*, B6 mice were depleted of CD4 T cells by administration of anti-CD4 Abs (\square) or left untreated (\blacksquare) before MCMV- Δ m157 infection. Ab administration was repeated weekly over the course of infection. At day 8 (*left diagram*) or day 210 (*right diagram*) after infection, viral titers were analyzed in different organs. Bars show averages of at least three mice per group and one of two independent experiments is shown.

solid line) or MCMV-specific CD8 T cell epitopes M45_{aa985-993} (\triangle) and M38_{aa316-323} (\circ), respectively. In addition to lymphoid organs such as the spleen, we included also peripheral tissues where virus replicates for extended periods of time (Fig. 4). During acute infection, MCMV-specific CD4 T cells and MCMV-specific CD8 T cell responses greatly expanded during the first 5–6 days

after infection, peaking at day 8 after infection and sharply contracting thereafter. At day 14 after infection, MCMV-specific CD4 T cells were already at very low levels, although virus replication had not ceased in most of the organs analyzed. Interestingly, the kinetics of MCMV-specific CD4 T cell responses were delayed in the salivary glands, reaching detectable levels only at day 7 after infection and peaking of the response was at day 10. These delayed kinetics might reflect the lag period in appearance of MCMV replication in this tissue, suggesting that activated T cells are only recruited to infected organs secondary to expansion in secondary lymphoid organs. Expansion, contraction, and early memory CD4 T cell responses to lysates of MCMV-infected MEFs were similar to responses directed against the identified CD4 T cell epitopes (Fig. 3). Intriguingly, overall MCMV-specific CD4 T cell responses as well as CD4 T cell responses specific for the identified epitopes did not accumulate over time, although a subpopulation of their CD8 T cell counterparts did accumulate in the spleen, lung, and liver.

To exclude that CD4 T cell responses specific for MCMV proteins accumulate over time, we repeated the experiment shown in Fig. 2 (during the acute phase) in mice having been infected with MCMV for almost 1 year (Fig. 4B and data not shown). CD4 T cells specific for only a few ORFs of MCMV were detectable at this late time point and frequencies were low. ORFs stimulating frequencies of CD4 T cells above background levels are depicted in Fig. 4B. This further argues against a significant accumulation of overall MCMV-specific CD4 T cell populations during the latent phase of MCMV infection, although a slight increase of CD4 T cells directed against some MCMV proteins cannot be excluded. In particular, m169-specific CD4 T cells seem to be more abundant in relation to other protein specificity during latent (Fig. 2) than during acute infection (Fig. 4B). Gamadia et al. (23) reported that during HCMV infection in humans, virus-specific CD4 T cells were only detectable shortly after acute infection and absent for up to 1 year before they became measurable again. To exclude that MCMV-specific CD4 T cell accumulation could only be seen at very late stages postinfection, mice infected for >1.5 years were analyzed for MCMV-specific CD4 as well as CD8 T cell responses in various organs. Similar to results seen at day 350 postinfection, 1.5 years after primary infection M38_{aa316–323}-specific CD8 T cell responses were present at inflating levels, whereas overall MCMV-specific CD4 T cell responses remained at low levels (data not shown).

MCMV-specific CD4 T cells are required for control of MCMV-Δm157 infection

Jonjic et al. (24) showed that CD4 T cells are crucial in BALB/c mice to control infectious virus production after MCMV wt infection, especially in the salivary glands. We showed in Fig. 4A that MCMV-specific CD4 T cell responses peak early and contract sharply to very low levels during acute infection in B6 mice. Within 2 wk of infection, CD4 T cells specific for MCMV are barely detectable anymore even though virus replication persists in various organs. To demonstrate that MCMV-specific CD4 T cells, despite their low level, are crucial to control viremia in B6 after MCMV-Δm157 infection, we depleted CD4 T cells during the course of infection by administration of CD4-depleting Abs. Indeed, in the absence of CD4 T cells, control of virus replication was impaired, especially in the salivary glands but also in other organs such as the lung (Fig. 4C). This demonstrates that although present at low numbers 2 wk after MCMV infection, virus-specific CD4 T cells exert antiviral effector functions that are essential for viral control.

Cytokine profile of MCMV-specific CD4 T cells

To get deeper insight into how MCMV-specific CD4 T cells protect the host from infectious virus replication, we analyzed the

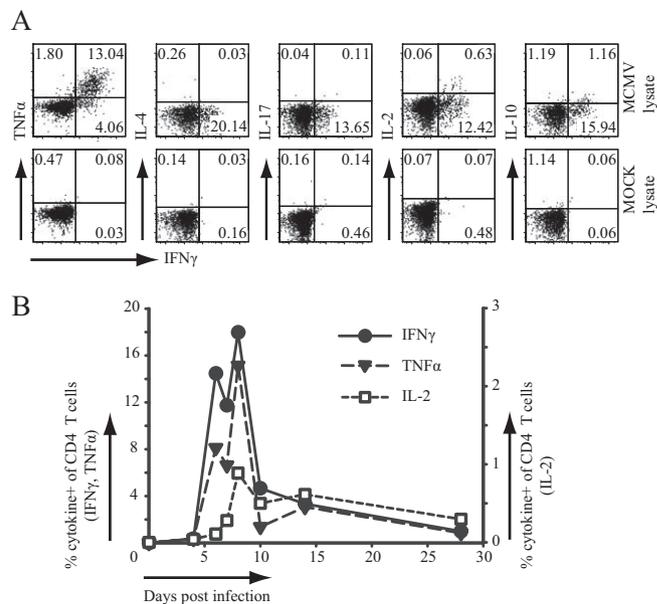


FIGURE 5. Cytokine secretion profile of MCMV-specific CD4 T cells. *A*, Lung lymphocytes (8 days after MCMV-Δm157 infection) were stimulated with lysates of MCMV-infected (*upper panel*, MCMV lysate) or mock-infected (*lower panel*, MOCK lysate) MEFs and were examined for production of various cytokines. Dot plots are gated on CD4 T cells. *B*, Dynamics of IFN-γ, TNF-α, and IL-2-secreting MCMV-specific CD4 T cells from the lung were analyzed during the first month of infection. Symbols show averages of three to four mice per group and data of one representative experiment are shown.

cytokine profile of virus-specific CD4 T cells during acute infection. Effector CD4 T cells can broadly be divided into three lineages: the Th1, Th2, and Th17 lineages. CD4 T cells of the Th1 lineage secrete high amounts of IFN-γ and are important for control of many intracellular pathogens, whereas Th2 effector CD4 T cells preferentially produce IL-4, IL-5, IL-13, and IL-25 and play a role in control of helminth infections. The more recently described Th17 lineage produces as its key cytokine IL-17 and is involved in protection against certain extracellular pathogens and fungi (25). To investigate the cytokine profile of MCMV-specific CD4 T cells during acute MCMV infection, CD4 T cells were isolated from different organs, restimulated with lysate from MCMV-infected MEFs, and production of IFN-γ, TNF-α, IL-4, and IL-17 was analyzed (Fig. 5). The MCMV-specific CD4 T cell response was dominated by the Th1 lineage, demonstrated by high frequencies of IFN-γ and TNF-α double-producing CD4 T cells. Significant frequencies of neither IL-4- nor IL-17- producing CD4 T cells were detected at any time point or organ analyzed. Effector CD4 T cells can also secrete IL-2 and IL-10 in response to certain pathogens. During MCMV infection, IL-2- secreting CD4 T cells were only detectable at later time points after (between days 8 and 14) infection (Fig. 5B) and were consistently double positive for IL-2 and IFN-γ (Fig. 5B). IL-10- secreting CD4 T cells were only detected at very low frequencies, which varied between experiments. However, in the experiments in which we were able to detect a small population of IL-10-producing MCMV-specific CD4 T cells (only seen at days 8–10 postinfection), they concomitantly secreted IFN-γ.

Discussion

There is growing evidence that CD4 T cells play a major role during control of chronic MCMV infection (2, 26, 27). However, a detailed analysis of the MCMV-specific CD4 T cell response has

so far been hampered by the lack of appropriate tools required to analyze the kinetics and the specificities of MCMV-specific CD4 T cells. With the help of newly generated MCMV-specific CD4 T cell hybridomas, we were able to identify four different proteins expressed by MCMV that contained five different MCMV-specific CD4 T cell epitopes, namely, $m14_{aa136-150}$, $M25_{aa411-425}$, $M25_{aa721-735}$, $M112_{aa36-50}$, $m142_{aa26-40}$. The MCMV $m14$ protein belongs to family of $m02$ glycoproteins (28). Proteins of the $M25$ ORF are part of the viral tegument and are expressed during early and late stages of the replication cycle (29). Interestingly, the $M112$ protein ($\epsilon 1$) is not associated with the virion and localizes predominantly to the nucleus of infected cells (30, 31). Finally, the transcripts of $m142$ appear with immediate-early kinetics, whereas the protein that is expressed with early kinetics, accumulates over time in the viral life cycle and is present in fully assembled virions (32). Thus, the varying expression kinetics and distribution patterns of these MCMV proteins that harbor CD4 T cell epitopes suggests that MCMV-specific CD4 T cell responses exhibit a wide specificity of proteins which are expressed with different kinetics and with different cellular as well as virion localization. Six of the nine hybridomas for which the MCMV protein specificity could be identified are specific for the $M25$ protein, suggesting that the $M25$ protein represents an immunodominant protein Ag in MCMV infection.

When we analyzed the overall MCMV protein specificities of CD4 T cells after acute MCMV infection, we identified 32 MCMV proteins which were able to stimulate IFN- γ production (Fig. 2). The functions and cellular localization of these viral proteins were also diverse, as was found for the proteins recognized by the CD4 T cell hybridomas: 16 proteins are present in the virion (31, 32), 5 proteins are members of the endoplasmic reticulum-Golgi network (1, 33), 1 protein is present mainly in the nucleus of infected cells (30), and for the remaining 14 proteins the function and cellular localization is unknown. This finding in murine CMV infection is reminiscent of HCMV infection, since also during HCMV infection the specificities of CD4 T cell responses are broad and diverse: 40 of the 213 examined ORFs were recognized by CD4 T cells of seropositive individuals *ex vivo* (9). In summary, many MCMV proteins independent of their expression kinetics or function are Ags for CD4 T cells at the early stage of MCMV infection.

B6 mice infected with MCMV- $\Delta m157$ exhibit a decreased NK cell response (data not shown and Ref. 21) and consequently increased viral titers in various organs. MCMV-specific CD4 as well as CD8 T cell responses were enhanced in B6 mice infected with recombinant MCMV- $\Delta m157$ compared with mice infected with wt MCMV (Fig. 1 and data not shown), most likely due to the increased Ag burden. However, it is also conceivable that MCMV- $\Delta m157$ -infected APCs that do not express $m157$ on their cell surface are not recognized and killed by NK cells, which would result in higher numbers of APCs. Hence, Ag presentation and priming of virus-specific CD4 and CD8 T cells would be facilitated in the absence of the $m157$ gene which might lead to increased induction of MCMV-specific T cell responses. Interestingly, the enhanced adaptive immune response seen after MCMV- $\Delta m157$ cannot compensate for the decreased NK cell activation because viral burdens remain higher after MCMV- $\Delta m157$ infection compared with wt MCMV infection even at later time points when virus-specific CD4 and CD8 T cells are substantially augmented.

The long-term kinetics of MCMV-specific CD8 T cell responses are very peculiar, as the dynamics of MCMV-specific CD8 T cells differ substantially depending on the epitope specificity. The overall MCMV-specific CD8 T cell response can be subdivided into four different dynamic patterns in B6 mice (22). Although CD8 T cells specific for certain epitopes such as $M45_{aa985-993}$ stabilize in frequencies and numbers during the latent phase of infection, CD8

T cell subsets for other epitopes such as $M38_{aa316-323}$ increase over the course of infection (memory inflation). Similar divergent kinetics of CD8 T cells were also described in BALB/c mice (34) as well as in HCMV infection where HCMV-specific CD8 T cell frequencies are highly increased in elderly compared with young individuals (35, 36). With respect to CMV-specific CD4 T cell dynamics, data from human as well as rhesus macaque studies also indicated an "inflationary" behavior of CMV-specific CD4 T cells in seropositive individuals during latent stages of infection (37, 38).

Interestingly, overall MCMV-specific CD4 T cell frequencies stayed at stable low levels during the course of latent infection. However, at this point, we cannot exclude that there is some degree of accumulation of CD4 T cells specific for certain MCMV epitopes over time as our analysis might have missed small accumulations of CD4 T cells specific for individual epitopes. However, we can clearly show that there is no significant overall accumulation of MCMV-specific CD4 T cells over time in all organs analyzed. Furthermore, our studies were limited to the assessment of IFN- γ -secreting CD4 T cells after short-term *ex vivo* restimulation. It is possible that CD4 T cells are driven into partial exhaustion during the latent phase of MCMV infection and, although still present at the physical level, might not be able to secrete cytokines such as IFN- γ after *in vitro* stimulation, as was proposed for HCMV infection (39). With our present analysis, we cannot exclude such a scenario of induction of unresponsiveness; however, this seems unlikely because the Ag loads during latent infection will be considerably lower compared with acute viremic infection. The finding that overall MCMV-specific CD4 T cells do not substantially inflate during MCMV latency stands in contrast to studies with HCMV where it was shown that elderly people have higher frequencies of HCMV-specific CD4 T cell populations (37).

The discrepancy in kinetics of CD4 vs CD8 T cell responses may be explained by different requirements of these T cell subsets for priming and memory formation. CD4 T cells require a longer Ag exposure, are more dependent on costimulatory signals, proliferate less vigorously after Ag encounter, and need different cytokine environments for expansion (40). Furthermore, MHC class II molecules are present on fewer cells in the body than MHC class I molecules. During latency, when Ag is limiting, these factors may play a major role for the accumulation of MCMV-specific CD8 T cell responses in comparison to MCMV-specific CD4 T cell responses. Furthermore, it is also possible that immunosuppressive functions exerted by MCMV impair the CD4 T cell responses considerably more than the MCMV-specific CD8 T cell responses.

The MCMV-specific CD4 T cell response was dominated by the Th1 lineage demonstrated by high frequencies of IFN- γ and TNF- α double producing but no IL-4- or IL-17-secreting CD4 T cells. This phenotype reflects the cytokine profile of CD4 T cells seen during HCMV infection in humans (5, 7) and is most common during viral infections. The functional role of IFN- γ - and TNF- α -secreting CD4 T cells in control of MCMV infection is demonstrated by the observation that *in vivo* neutralization of IFN- γ as well as TNF- α reduced the antiviral effects of CD4 T cells during MCMV infection (2). Between days 8 and 14, a subset of virus-specific CD4 T cells secreted IL-2. This cytokine is crucial for T cell growth and differentiation but also for maintaining peripheral tolerance. Since IL-2R signaling was shown to be crucial for the continuous expansion of MCMV-specific CD8 T cell populations over time (41), it is tempting to hypothesize that CD4 T cells are the source of IL-2 required for the memory CD8 T cell inflation. At days 8 and 10 after infection, low frequencies of IL-10-secreting MCMV-specific CD4 T cells could be detected in some experiments. IL-10 is an anti-inflammatory cytokine that can be produced by a wide range of immune cells. Interestingly, *in vivo*

blocking of IL-10 signaling during MCMV infection increased numbers of CD4 T cells which could be triggered to produce IFN- γ after unspecific TCR stimulation. This was associated with reduced virus titers in the salivary glands but also with increased immunopathology (27, 42).

In summary, we identified five MCMV-specific CD4 T cell epitopes, including the immunodominant epitope M25_{aa411–425}. During acute infection, MCMV-specific CD4 T cell expansion peaked between days 7 and 8 and thereafter T cell frequencies rapidly declined. A longitudinal analysis of MCMV-specific CD4 T cell frequencies clearly argues against a marked accumulation of MCMV-specific CD4 T cells during latent infection in any organ examined. This stands in clear contrast to the accumulation of certain MCMV-specific CD8 T cell populations over time and to observations in humans where aged seropositive individuals had a significantly increased HCMV-specific CD4 T cell population compared with a younger control group.

Disclosures

The authors have no financial conflict of interest.

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