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Priming of CD8+ T Cells against Cytomegalovirus Encoded Antigens Is Dominated by Cross Presentation

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CMV can infect dendritic cells (DCs), and direct Ag presentation could, therefore, lead to the priming of CMV-specific CD8+ T cells. However, CMV-encoded immune evasins severely impair Ag presentation in the MHC class I pathway; thus, it is widely assumed that cross presentation drives the priming of antiviral T cells. We assessed the contribution of direct versus cross priming in mouse CMV (MCMV) infection using recombinant viruses. DCs infected with an MCMV strain encoding the gB498 epitope from HSV-1 were unable to stimulate in vitro naive gB498-specific CD8+ T cells from TCR transgenic mice. Infection of C57BL/6 mice with this recombinant virus led, however, to the generation of abundant numbers of gB498-specific T cells in vivo. Of the DC subsets isolated from infected mice, only CD8a+ DCs were able to stimulate naive T cells, suggesting that this DC subset cross-presents MCMV-encoded Ag in vivo. Upon infection of mice with MCMV mutants encoding Ag that can either be well or hardly cross-presented, mainly CD8+ T cells specific for cross-presented epitopes were generated. Moreover, even in the absence of immune evasion genes interfering with MHC class I–mediated Ag presentation, priming of T cells to Ag that can only be presented directly was not observed. We conclude that the host uses mainly DCs capable of cross presentation to induce the CMV-specific CD8+ T cell response during primary, acute infection and discuss the implications for the development of a CMV vaccine. The Journal of Immunology, 2013, 190: 000–000.

Dendritic cells (DCs) are professional APCs with the unique ability to induce primary T cell responses (1), for instance, against pathogens such as viruses, thereby permitting the establishment of immunological memory, which usually provides protection against second encounter with the same pathogen (2). The DC network consists of different DC subtypes with distinct functional properties that work together to mount an effective immune response (3–5). Migratory DCs in the peripheral tissues take up Ag, mature upon encounter with danger signals sensed by pattern recognition receptors, and migrate to the draining lymph nodes (LN), where they process the Ag to process naive T cells to activate them (6). According to this scenario, generation of an antiviral CD8+ CTL response would only be possible if these DCs can be infected and if they process endogenously produced viral Ag by the proteasome and present the resulting peptides on MHC class I (MHC I) molecules at the cell surface. However, other DC subsets, for instance, the CD8a+ DCs residing in lymphoid tissues, can present peptides in the MHC I pathway from Ag that are acquired from exogenous sources (7, 8), such as apoptotic bodies (9) remaining from virally infected cells. This process, termed cross presentation, explains how CTLs are generated against viruses (10) that either do not infect DCs or efficiently interfere with Ag presentation in the MHC I pathway (11–15).

Human and mouse CMV (HCMV and MCMV, respectively) are capable of infecting DCs in vitro and in vivo (16–20), and could therefore, in principle, induce a CTL response by direct priming. After CMV infection, a vigorous T cell response is observed, and a substantial fraction of all CD8+ memory T cells in chronically infected humans or mice is directed against CMV (21–23). Experimental immunotherapy and clinical trials provided evidence that these CD8+ T cells are protective against CMV viremia and disease (24–28). Because CMVs interfere with Ag presentation in the MHC I pathway by several mechanisms (reviewed in Refs. 29–32), it is not clear whether infected DCs can contribute to the priming process. The viral proteins interfering with Ag presentation, also called viral regulators of Ag presentation (vRAPS) (33), seem to be highly effective, because recognition or cytolysis of MCMV-infected fibroblasts, macrophages, or bone marrow–derived DCs by CTL lines was almost completely blocked in vitro (33, 34). If we assume that the CMV vRAPS display similar efﬁcacy in infected DCs in vivo, then we have to conclude that priming of CMV-speciﬁc T cells occurs primarily by cross presentation. This hypothesis is supported by the observation that the MCMV-speciﬁc T cell response was almost identical in mice infected with MCMV wild-type (wt) or a vRAP mutant (35, 36). Cross priming, however, leaves the host with the dilemma that
effector T cells may be generated that cannot detect infected cells because of failure in presenting the respective viral Ag, and these T cells may therefore be useless for protection against CMV. Experiments performed with HCMV mutants that lack the vRAP proteins US2, US3, US6, and US11 indicated that CMV-specific T cells exist in seropositive individuals, which are directed against viral epitopes that are not presented by fibroblasts infected with wt HCMV (37, 38). For MCMV, at least one study provided experimental evidence that T cells with such specificity are generated in vivo (39). On the contrary, there are results that indicated some leakiness in the functions of vRAPS (40–42). However, even if infected DCs retain a residual capacity for Ag presentation, a series of additional viral mechanisms ranging from downregulation of costimulatory molecules over changes in the secretion of chemokines and cytokines to the inhibition of maturation and maturation may severely impair DC functions (17, 18, 43–50). Yet again, there are studies that report on host countermeasures that again, there are studies that report on host countermeasures that retain the function of DCs after CMV infection (51–53). Although many hints point to an important role for cross presentation in the priming of the CMV-specific T cell response, experimental research on the contribution of cross presentation versus direct presentation is still in an early stage.

Two recent studies that relied on a replication-defective MCMV mutant or on a mouse strain with an impaired ability for cross presentation (54, 55) provided evidence that priming occurs mainly by cross presentation. If cross presentation prevails, a bias in the antiviral T cell response is expected, because cross presentation favors Ag with certain characteristics (56–60), one being the stability of an antigenic protein (61). Wolkers et al. (62) reported that epitopes derived from signal sequences of proteins can be presented directly but are poor substrates for cross presentation, whereas epitopes derived from a stable protein can be presented by either pathway.

In this study, we made use of this difference in antigenic substrates to evaluate the contribution of the different Ag presentation pathways to the generation of MCMV-specific CD8+ T cells. We found that CD8+ T cells were predominately generated to such MCMV-encoded model epitopes that could be cross-presented, but not to those epitopes that could only be presented directly. Moreover, the same T cell pattern was observed when mice were infected with MCMV mutants that lacked the vRAPS, which principally allowed direct presentation of Ag. These results suggest that cross presentation is dominating in priming of the T cell response against MCMV.

Materials and Methods

Mice and MCMV infection

Six- to 8-wk-old female C57BL/6 (B6) mice (Charles River, Sulzfeld, Germany) were intraperitoneally infected with 10⁶ PFU of the MCMV strains in 50 µl PBS. Mice were housed in ventilated cages in an area otherwise free of murine pathogens as assessed by microbiological monitoring, in accordance with European Laboratory Animal Science Associations recommendations (63). All experiments were carried out in accordance with national and institutional guidelines.

MCMV strains, propagation, and plaque assay

The MCMV strain MW97.01, referred to as MCMV-wt in this study, was previously generated from the bacterial artificial chromosome (BAC) pSM3fr (64), as was the MCMV-GFP mutant (20). The genomes of the MCMV-IE1gB and MCMV-noX strains were constructed by Red recombineering in Escherichia coli as described previously (65). The viruses were propagated on MEFs and virus stocks produced as described previously (66). For analysis of growth kinetics, MEFs were infected with recombinant viruses at a multiplicity of infection (MOI) of 0.1. After a 2-h adsorption period, cells were washed with PBS and further cultivated in DMEM supplemented with 5% FCS. The supernatants of the infected cells were collected at different times postinfection (p.i.) and stored at −80°C until titration was performed. Virus titers were determined by plaque assays performed on subconfluent MEF monolayers in triplicate. The inocula were replaced after 2 h of incubation by 500 µl of 0.75% (w/v) carboxymethylcellulose (Sigma) in growth medium per well. On day 6 p.i., plaques were counted and viral titers calculated.

Isolation of splenic DC subsets

DCs were isolated essentially as described previously (68) from untreated or infected mice. In brief, spleen fragments were digested for 20 min at room temperature with collagenase/DNase and then treated for 5 min with 0.1 M EDTA to disrupt T cell–DC complexes. Cells not of DC lineage were depleted by incubation in predetermined optimal concentrations of purified rat mAbs: anti-CD3 (KT3), anti-Thy-1 (T24/31.7), anti-CD19 (ID3), anti–Gr-1 (RB6-8C5), and anti-erythrocyte (TER-119). Ab-binding cells were then removed with anti-rat Ig-coupled magnetic beads (Miltenyi Biotech). The DCs were enriched by plastic adherence, CD11c (rat anti-mouse FITC), CD8 (rat-anti-mouse allophycocyanin), and CD4 (rat anti-mouse PE; all from BD Biosciences), and total CD11c+ cells or specific subsets were sorted on an MoFlo (Dako Cytomation) or FACSAria (BD Biosciences).

Analysis of T cell proliferation

CD8+ gBT-I T lymphocytes that express a Vα2/Vβ8.1+ TCR recognizing the H-2Kk−restricted gB 498–505 epitope from HSV-1 were purified from single-cell suspensions of pooled LNs (inguinal, axillary, sacral, cervical, and mesenteric) of the TCR transgenic mouse strain gBT-I.1 (69) by depletion of non-CD8+ T cells using a CD8 enrichment kit (BD Biosciences). These cells were labeled by incubating 10⁵ purified cells/ml with CFSE (1 µM; Molecular Probes) for 10 min at 37°C. Cells were then washed three times in HEPES-modified Eagle’s medium containing 2.5% FCS. The T cell preparations were routinely 85–95% pure, as determined by flow cytometry. A total of 5 × 10⁴ CFSE-labeled gBT-I cells were added to graded numbers of DCs ranging from 5 × 10³ to 2 × 10⁴, isolated either from naive or MCMV-infected mice, in 200 µl RPMI 1640 containing 10% FCS and 50 µg 2-ME in 96-well U-bottom plates (BD Biosciences). Proliferation of gBT-I T cells was determined after 60 h as described previously (15). In brief, transgenic gBT-I T cells were labeled with anti-CD8 and anti-Vα2, and samples were analyzed using LSR II flow cytometer (BD Biosciences). Total number of live dividing lymphocytes (CD8+ and CD4+ cells) was determined and analysis was done using FlowJo software (Tree Star, Ashland, OR).

Quantification of MCMV-specific T cells

At designated times p.i., blood was taken, or mice were sacrificed and spleens were excised and subsequently homogenized by passing through a 0.55-mm wire mesh (Roth, Karlshruhe, Germany) to isolate blood cells and splenocytes, respectively. DC2.4 cells (70) were pulsed for 2 h at 37°C with
of MCMV-encoded Ags that drive expansion of memory CD8+ MHC-I–restricted T cell epitope (73), which belongs to the class epitope because it contains a well-characterized immunodominant (Fig. 1A). The IE1 protein seemed to be an ideal carrier for the gB contrasted by efficient generation of gB 498-specific CD8+ T cells MCMV (18, 20, 44, 45). The result of the in vitro experiment was be a contribution of other immunomodulatory mechanisms of most likely explanation for this observation, although there might in vitro (Fig. 2B). Because peptide-pulsed, MCMV-infected DCs failed, however, to induce proliferation of naive gBT-I T cells expression of the gB 498–505 epitope in a suitable context, we fused the HSV-1 glycoprotein B, for which specific T cells of the TCR expression, the well-characterized model epitope gB 498–505 from the HSV-1 glycoprotein B, from which specific T cells of the TCR transgenic mouse strain gBT-I are available (69). To ensure ex-pression of the gB 498–505 epitope in a suitable context, we fused the coding sequence for it to the 3’ end of the MCMV IE1 ORF (Fig. 1A). The IE1 protein seemed to be an ideal carrier for the gB epitope because it contains a well-characterized immunodominant MHC-I–restricted T cell epitope (73), which belongs to the class of MCMV-encoded Ags that drive expansion of memory CD8+ T cells (memory inflation) during chronic latent infection (22, 74). The IE1gB protein maintained the same nuclear localization as the native IE1 protein (Fig. 1B) and also its function, as demonstrated by the ability to disperse promyelocytic leukemia nuclear bodies in infected cells (Fig. 1C) (75). The growth properties of the re-combinant and the parental virus on fibroblasts were basically identical (Fig. 1D). After the initial characterization of the mutant, we tested whether the gB 498 epitope could be presented by infected DCs and stimulate specific T cells. Splenic DCs were in-oculated with different doses of MCMV-IE1gB, and at an MOI of 5, ~90% of the DCs became infected as assessed by flow cytometric detection of GFP expression (Fig. 2A). The infected DCs failed, however, to induce proliferation of naive gBT-I T cells in vitro (Fig. 2B). Because peptide-pulsed, MCMV-infected DCs were able to rescue proliferation of specific T cells (data not shown), prevention of adequate presentation of the endogenously generated epitope by viral MHC I immune evasion proteins is the most likely explanation for this observation, although there might be a contribution of other immunomodulatory mechanisms of MCMV (18, 20, 44, 45). The result of the in vitro experiment was contrasted by efficient generation of gB 498–specific CD8+ T cells in vivo after infection of B6 mice with MCMV-IE1gB (Fig. 2C, 2D). The acute T cell response against the genuine Ag M45 and

**In vivo cytotoxicity assay**

Splenocytes from naive mice were first uniformly labeled with the red dye PKH-26 (Sigma) or with different amounts of CFSE (100 nM, 500 nM, and 2 μM), and loaded with 10^8 M of the indicated peptides. Eighteen hours after i.v. injection into infected B6 mice, the fraction of remaining target cells was assessed by flow cytometry.

**Results**

MCMV-infected DCs are incapable of inducing proliferation of naive Ag-specific CD8+ T cells in vitro

The MHC I immune evasion genes of MCMV appear to be highly effective in preventing Ag presentation in infected cells (reviewed in Refs. 31, 32). However, many studies addressing the function of vRAPs were done in fibroblasts or macrophages using CTL clones or memory T cells, which may not reflect the priming of naive T cells by APCs. Naive CD8+ T cells specific for a defined virally encoded Ag were therefore required to analyze the capacity of MCMV-infected APCs for their activation. Because TCR transgenic mouse strains recognizing CMV Ag were not available when we began the study, we decided to generate a recombinant MCMV expressing the well-characterized model epitope gB 498–505 from the HSV-1 glycoprotein B, for which specific T cells of the TCR transgenic mouse strain gBT-I are available (69). To ensure expression of the gB 498–505 epitope in a suitable context, we fused the coding sequence for it to the 3’ end of the MCMV IE1 ORF (Fig. 1A). The IE1 protein seemed to be an ideal carrier for the gB epitope because it contains a well-characterized immunodominant MHC-I–restricted T cell epitope (73), which belongs to the class of MCMV-encoded Ags that drive expansion of memory CD8+ T cells (memory inflation) during chronic latent infection (22, 74). The IE1gB protein maintained the same nuclear localization as the native IE1 protein (Fig. 1B) and also its function, as demonstrated by the ability to disperse promyelocytic leukemia nuclear bodies in infected cells (Fig. 1C) (75). The growth properties of the recombinant and the parental virus on fibroblasts were basically identical (Fig. 1D). After the initial characterization of the mutant, we tested whether the gB 498 epitope could be presented by infected DCs and stimulate specific T cells. Splenic DCs were inoculated with different doses of MCMV-IE1gB, and at an MOI of 5, ~90% of the DCs became infected as assessed by flow cytometric detection of GFP expression (Fig. 2A). The infected DCs failed, however, to induce proliferation of naive gBT-I T cells in vitro (Fig. 2B). Because peptide-pulsed, MCMV-infected DCs were able to rescue proliferation of specific T cells (data not shown), prevention of adequate presentation of the endogenously generated epitope by viral MHC I immune evasion proteins is the most likely explanation for this observation, although there might be a contribution of other immunomodulatory mechanisms of MCMV (18, 20, 44, 45). The result of the in vitro experiment was contrasted by efficient generation of gB 498–specific CD8+ T cells in vivo after infection of B6 mice with MCMV-IE1gB (Fig. 2C, 2D). The acute T cell response against the genuine Ag M45 and

**FIGURE 1.** Generation of an MCMV recombinant expressing the gB 498 model epitope. (A) The genome of the MCMV Smith strain with the HindIII fragments is shown at the top, and the modifications carried by the indicated MCMV recombinants are schematically represented below. (B) Nuclear localization of the modified IE1 protein is maintained in cells infected with MCMV-IE1gB. Expression of IE1 and GFP was visualized in MEF cells 24 h p.i. with the indicated viruses (MOI of 1) by immunolabeling and fluorescence microscopy. The dashed lines mark the nuclei as assessed by DAPI staining. (C) The viral IE1 protein with the fused gB epitope preserved its function. Expression of PML proteins and GFP was visualized by fluorescence microscopy in MEF cells 8 h p.i. with MCMV-IE1gB (MOI of 0.5). Nuclei were marked as described in (B). (D) Comparable growth properties of MCMV-IE1gB and MCMV-GFP. Virus titers in the supernatants of MEF cell cultures infected with MCMV-IE1gB or MCMV-GFP (MOI of 0.1) were measured at the indicated time points p.i. by plaque assay. Dashed line indicates the detection limit of the assay. Scale bars, 10 μm (B), 20 μm (C). gB, HSVgB498–505 epitope; IE1 and IE2, immediate-early genes iel and ie2, respectively; mMIEP and hMIEP, major immediate-early promoters of MCMV and HCMV, respectively.

IE3 of MCMV was comparable for the recombinant MCMV and the parental virus, displaying a pattern described previously (23, 71). Notably, at the peak of the T cell response at 1 wk p.i., the frequency of gB 498–specific CD8+ T cells was in a comparable range as the frequency of the CD8+ T cells recognizing the M45 epitope, which are immunodominant during the acute infection phase in B6 mice (23). Altogether, these results indicated that
mechanisms are operational in vivo that can overcome the observed deficiency in T cell stimulation of infected DCs in vitro.

**CD8α+ DCs can cross-present in vivo acquired Ags to CD8+ T cells in vitro**

Next, we asked which subtypes of splenic DCs mediate the presentation of viral Ags after MCMV infection. Conventional murine splenic DCs can be divided into different subsets based on the expression of distinct surface markers (76). Although both CD8α+ and CD8α− DCs are capable of direct presentation of endoge-
nously produced Ag, predominantly the CD8α+ DCs cross-present exogenously acquired Ag (77). We infected mice with the recombinant MCMV-IE1gB or the parental virus and isolated splenic DC subsets after 18 h. All DC populations were >95% pure as assessed by flow cytometric analysis of CD11c, MHC II, and CD8α surface staining (data not shown), and MCMV infection did not generally affect their capacity to stimulate T cell proliferation as demonstrated after exogenous peptide loading (Fig. 3A). Notably, only CD8α+ DCs isolated from MCMV-IE1gB-infected mice were capable of stimulating gBT-I T cells in vitro (Fig. 3B), indicating that either cross presentation is dominating or that these cells were preferentially infected in vivo. The total spleen cell population or the CD8α+ and CD8α− DC subsets were cocultivated with MEF indicator cells and plaques indicating productively infected cells were counted 7 d p.i. to distinguish between these options. A fraction of the CD8α− DCs was productively infected, whereas cocultivation of CD8α+ DCs never led to plaque formation (Fig. 3C). Because the CD8 α− DCs were not able to induce T cell proliferation upon ex vivo cultivation (Fig. 3B), this suggested that the MCMV immune evasion mechanisms are acting in the infected cells of this DC subset, preventing Ag presentation. The absence of productively infected CD8α+ DCs implies that priming by these cells resulted from cross presentation of exogenously acquired Ags. Furthermore, the number of infected splenocytes exceeded the number of infected CD8α− DCs, indicating that cells other than DCs constitute a major source of viral Ag in spleen.

**Cross presentation is predominant in the priming of CD8+ T cells in vivo**

To evaluate the contribution of cross presentation and direct presentation to T cell priming after MCMV infection, we made use of the finding by Wolkers et al. (62) that epitopes located in signal peptides are poor substrates for cross presentation and can therefore only be presented directly, whereas epitopes connected to stable proteins can be presented by both pathways. We generated two reporter mutants encoding the T cell epitope E749 from human papillomavirus and NP366 from influenza virus (Fig. 4A). The two mutants, termed noX E7 and noX NP (noX stands for no cross presentation of the E7 or NP epitope), differ by the order of the epitopes within the GFP reporter protein. One of the epitopes is expressed within the signal peptide, whereas the other epitope is part of the mature GFP. Both viruses were found to replicate with comparable kinetics as MCMV-wt in fibroblasts (Fig. 4B). Infection of B6 mice with MCMV-noX NP led to the generation of a high number of E749-specific CD8+ T cells, whereas at best few NP366-specific T cells were detected (Fig. 4C). Likewise, infection with MCMV-noX E7 resulted in abundant generation of NP366-specific CD8+ T cells and a low number of E749-specific T cells (Fig. 4D), indicating that cross presentation is the predominating mechanism for generation of CD8+ T cells during acute MCMV infection.

**Restoring MHC I expression on infected cells does not rescue direct priming in vivo**

Because the downmodulation of MHC I surface expression on MCMV infected cells is well established and offers an explanation for the observed lack of direct priming, we generated reporter mutants lacking the vR APs m06 and m152. DC2.4 cells infected with the deletion mutants ΔvRAP-noX E7 and ΔvRAP-noX NP preserved the MHC I surface expression, in contrast with noX E7 and noX NP infected cells expressing the viral immune evasins (Fig. 5A). Moreover, deletion of the vR A P genes enabled infected cells to directly present the E749 epitope and the NP366 epitope,
inducing proliferation of E7<sub>49</sub>-specific and of NP<sub>366</sub>-specific T cells in vitro, respectively, no matter whether the respective epitope was located within the signal peptide (Fig. 5B, lane 8, D<sub>vRAP-noX</sub> E7; and Fig. 5C, lane 7, D<sub>vRAP-noX</sub> NP) or within the mature protein (Fig. 5B, lane 7, D<sub>vRAP-noX</sub> NP; and Fig. 5C, lane 8, D<sub>vRAP-noX</sub> E7). This finding is in accordance with the data of Wolkers et al. (62), who demonstrated for the NP<sub>366</sub> and the E7<sub>49</sub> epitope that after transfection of the reporter constructs, direct presentation occurs with comparable efficiency independent of the localization of the respective epitope within the antigenic protein. As expected, cells infected with the reporter viruses expressing the vRAPs m06 and m152 were not able or displayed strongly diminished capacity to induce proliferation of specific T cells (Fig. 5B, lanes 4, 5, and Fig. 5C, lanes 4, 5), most likely because of reduced MHC I surface expression and insufficient Ag presentation.

Infection of B6 mice with the reporter mutants lacking m06 and m152 still led to the generation of CD8<sup>+</sup> T cells specific for the epitopes present within the mature GFP protein, whereas basically no T cells could be detected that were specific for the epitopes contained within the signal peptide (Fig. 6A, B). This result was confirmed by in vivo cytotoxicity assays (Fig. 6C, D). When cells loaded with those peptides that cannot be cross-presented were injected into mice infected before with the respective reporter viruses, no cytotoxicity was observed, indicating absence of CTLs specific for the respective Ag. In contrast, cells loaded with the peptides that can be cross-presented were eliminated quite efficiently. These results indicated that restoration of direct presentation was not sufficient for direct priming of T cells to virally encoded Ag. Moreover, this corroborates the important role of cross presentation for CD8<sup>+</sup> T cell responses during MCMV infection, even in the case of the D<sub>vRAP</sub> mutants.

**Predominance of CD8<sup>+</sup> T cells generated via cross presentation is not altered during chronic infection**

The abundance of CD8<sup>+</sup> T cell populations directed against distinct MCMV-encoded epitopes increases during the latent, chronic infection phase (designated as inflammatory response), which is believed to result from stochastic transcriptional reactivation events and frequent encounter of the T cells with virally expressed Ag (78). A previous study suggested that differentiation of T cells primed early in infection, as well as de novo priming of naive T cells during chronic infection, contribute to the expansion and maintenance of certain effector memory T cells at high levels (79). Recent results indicated moreover that cells of nonhematopoietic origin are involved in this process (55, 80, 81). We hypothesized, therefore, that stochastic expression of the major immediate early promoter-driven reporter genes by latently infected host cells may also give rise to T cells recognizing the signal peptide–encoded epitopes. In mice infected with the mutants D<sub>vRAP-noX</sub> NP and D<sub>vRAP-noX</sub> E7, we found a dramatically increased number of MCMV IE3-specific T cells (Fig. 7A, B, IE3 column) when compared with acutely infected mice (cf. Fig. 2D, IE3 column), whereas the number of M45-specific T cells was rather decreased.

**FIGURE 3.** CD8α<sup>+</sup> DCs from MCMV-infected mice activate specific naive T cells. (A) DCs isolated from spleens of MCMV-infected B6 mice induce T cell proliferation after exogenous peptide loading. CFSE<sup>+</sup>-labeled gBT-I T cells were either cultured without DCs (upper panel) or with the indicated DC populations exogenously loaded with the gB<sub>49</sub> peptide. CD8α<sup>+</sup> and CD8α<sup>+</sup> DCs were purified from mice 18 h after i.p. infection with 10<sup>6</sup> PFU of the indicated viruses. CFSE dilution was analyzed by flow cytometry. One experiment out of two is shown. (B) Presentation of in vivo acquired, virus-encoded Ag. CD8α<sup>+</sup> and CD8α<sup>+</sup> DCs were purified as described in (A). The number of proliferated, CFSE<sup>+</sup> gBT-I T cells obtained after 60 h at the indicated T cell/DC ratios was measured by flow cytometry in triplicates. One of two experiments is shown. (C) Quantification of productively infected spleen cells isolated from B6 mice infected as described in (A). A total of 5 × 10<sup>5</sup> of the indicated cells were cocultured with permissive fibroblasts. Plaques were counted 7 d after plating. One of two independent experiments is shown. nd, Not detectable.
in comparison with acute infection. Furthermore, a high level of m139- and M38-specific T cells (data not shown) was observed, which in agreement with previous reports is a pattern of the T cell response typical for latently infected B6 mice (23). In mice latently infected with DvRAP-noX NP, we found E7 49-specific T cells at a frequency that was even higher than the frequency of the T cells specific for the inflationary IE3 epitope, whereas basically no T cells were detectable that were specific for the NP366 epitope, which was contained within the signal peptide of the reporter protein. In DvRAP-noX E7 infected mice, we found a substantial number of T cells specific for the NP 366 epitope in only one of five mice analyzed, and notably, no CD8+ T cells specific for the E7 epitope expressed within the signal peptide were detectable. We conclude from these results that direct priming of naive CD8+ T cells does not seem to play a role during the latent infection phase. Moreover, the E7 49 epitope appears to possess characteristics similar to those of inflating epitopes of MCMV, leading to an increase of this specific T cell population during the latent infection, whereas the NP 366 epitope may either lack this property or, alternatively, the observed result can be explained by the lower capacity of the NP366 epitope for priming an efficient T cell response, as was already seen during acute infection (cf. Fig. 4C, 4D and Fig. 6A, 6B).

Discussion

CMV infection is the leading cause of virally induced malformation in newborns (82), with the risk for CMV transmission to the fetus being especially increased upon primary infection of pregnant women. The other main group at risk for CMV disease is immunocompromised patients such as transplant recipients. Inducing or restoring protective immunity to CMV in these individuals is, therefore, of utmost clinical importance. The development of a CMV vaccine would greatly benefit from a detailed understanding of the induction of T cell immunity after natural infection with CMV.

The results of a series of studies (see the introduction of this article) suggested that priming of the CD8+ T cell response to HCMV in humans, as well as to the closely related MCMV in mice, results from cross presentation of viral Ag by APCs that are not directly infected. Although CMVs can infect DCs and this could lead to direct priming of naive CD8+ T cells, there is abundant evidence that viral immune evasion proteins almost completely block Ag presentation in various cell types, including infected DCs, thereby preventing T cell activation. Most of these studies were done with either T cell clones or CD8+ memory T cells that may have a lower threshold or fewer requirements for costimulation to get activated than naive CD8+ T cells. In this study, we established a system that allowed us to measure the capacity of MCMV-infected DCs to induce proliferation of naive CD8+ T cells that express a TCR specific for an epitope encoded by an MCMV recombinant. In agreement with the mentioned studies, we observed a complete incompetence of the MCMV-infected DCs to activate the naive T cells, confirming that immunomodulatory mechanisms of MCMV effectively prevent direct

FIGURE 4. MCMV reporter mutants induce mainly a T cell response against epitopes that can be cross-presented. (A) The indicated GFP gene constructs driven by the major immediate-early promoter (mMIEP) were inserted into the m157 locus of the MCMV genome (with its HindIII map indicated at the top). The resulting reporter viruses express either the E749 epitope (noX E7) or the NP366 epitope (noX NP) within a signal peptide, rendering them poor substrates for cross presentation, whereas the respective epitopes contained at the C-terminal end of GFP represent good substrates for cross presentation (62). (B) Comparable growth kinetics of noX E7 and noX NP mutants and MCMV-wt. MEFs were infected at an MOI of 0.1, and virus titers in the culture supernatants were determined at the indicated time points p.i. Dashed line indicates the detection limit. (C and D) Mice were infected with noX NP (C) or noX E7 (D), and 8 d p.i. blood was analyzed for the presence of T cells specific for the indicated peptides by IFN-γ-ELISPOT assay. Symbols indicate the results for individual mice; bars mark the medians; gray bars mark the response attributed to cross priming. One representative experiment of four is shown.
priming, at least in the in vitro setting. However, p.i. of mice with the recombinant virus, T cells directed to the gB498 epitope were active in these infected cells, preventing the presentation of MCMV-infected mice were functional when used as presenter cells for the gB peptide, suggesting that the infected DCs did not express soluble or membrane proteins interfering with T cell activation. Our data appear to differ in this respect from the findings of Andrews et al. (18), who reported functional paralysis of MCMV-infected conventional splenic DCs; however, the experimental conditions applied may not be completely comparable. There is some controversy about the DC subsets that are infected during CMV infection. It is long-known fact that despite the existence of effective viral immune evasins dedicated to impede MHC I–restricted Ag presentation (reviewed in Refs. 31, 83), a prominent CD8+ T cell response is generated after CMV infection, which is an essential component of the host immune response for controlling acute and latent CMV infection. The finding that CD8α+ DCs isolated from spleens of infected mice presented the gB peptide and induced proliferation of the naive gB-specific T cells (Fig. 3B) supports the hypothesis that cross presentation is the prevailing mechanism, not just because this DC subset displays this property, but especially because our data suggest that this cell type was not productively infected (Fig. 3C). Both the CD8α+ and the CD8α− DC subsets isolated from MCMV-infected mice were functional when used as presenter cells for the gB peptide, suggesting that the infected DCs did not express soluble or membrane proteins interfering with T cell activation. Our data appear to differ in this respect from the findings of Andrews et al. (18), who reported functional paralysis of MCMV-infected conventional splenic DCs; however, the experimental conditions applied may not be completely comparable. There is some controversy about the DC subsets that are infected by MCMV; Dalod et al. (19) found that ~0.9% of the splenic CD11c+ DCs were infected with MCMV 1.5 d p.i., and most of these DCs appeared to be CD8α−. It might be that this small cell fraction either escaped detection by the techniques that we applied or, alternatively, these DCs did not create infectious progeny. Because of the technical limitations of the plaque assay used, we cannot exclude that some CD8α+ DCs were infected with MCMV; however, if this happened, it is very likely that the vRAP proteins were active in these infected cells, preventing the presentation of endogenously produced Ag. The observation that CD8α− DCs, which included infected cells (Fig. 3C), did not stimulate gBT-I
showed that priming of MCMV-specific CD8+ T cells was specific T cell response, and that there is only a minor contribution that cross presentation predominates in priming of the CMV-ways, we observed predominant generation of T cells specific for with a differential capacity to enter the two Ag-presenting path-lymphoid organs with similar properties is needed to produce adaptive immune response (85). It will be interesting to see from type I IFNs, to provide sufficient Ags for activation of the pathic virus, vesicular stomatitis virus, in a manner uncontrolled metallophilic macrophages, allows replication of another cyto-pathic virus, vesicular stomatitis virus, in a manner uncontrolled A recent study revealed that a specialized cell type in spleen, metallophilic macrophages, allows replication of another cyto-pathic virus, vesicular stomatitis virus, in a manner uncontrolled from type I IFNs, to provide sufficient Ags for activation of the adaptive immune response (85). It will be interesting to see whether these macrophages or another cell type in secondary lymphoid organs with similar properties is needed to produce CMV Ag in amounts required for cross presentation. After infection with the recombinant viruses that express Ag with a differential capacity to enter the two Ag-presenting path-ways, we observed predominant generation of T cells specific for such epitopes that can be cross-presented, but at best, a small T cell population was found specific for the epitopes that can only be presented directly (Fig. 4C, 4D). Based on these data, we conclude that cross presentation predominates in priming of the CMV-specific T cell response, and that there is only a minor contribution of direct presentation. Our finding corroborates and extends the results of two other recently published studies. Torti et al. (55) showed that priming of MCMV-specific CD8+ T cells was severely impaired in Batf3 knockout mice, which are compromised in the development of CD8α+ and CD103+ DC subsets. This pointed to a prominent role for cross presentation, but left open whether the residual T cell priming observed resulted from direct priming or from other remaining APCs in these mice that are capable of cross presentation. Using a spread-defective MCMV mutant, Snyder et al. (54) reported that cross presentation is sufficient to generate an MCMV-specific CD8+ T cell response that is qualitatively similar to that observed after infection with replication-competent MCMV-wt. However, it remained unclear whether the quantitative difference in the T cell response was due to limiting amounts of viral Ag or to the absence of direct presentation. By using different approaches, these studies and our work come to similar conclusions, and thus nicely complement each other. One limitation of our approach is that we could ana-lyze only model epitopes; therefore, our results cannot be gener-alized to all genuine MCMV epitopes, which may differ in their characteristics. Already by comparing the response to the E7 and NP epitopes, differences became obvious, because a higher number of E7/N-specific than of NP-specific T cells was gener-ated during acute infection with the noX NP and noX E7 mutants, respectively (cf. Fig. 4C, 4D). This may be because of differences in the processing of the Ag and loading to MHC I molecules or in the frequency of specific T cell precursors and the avidity of the TCRs. Most important for the interpretation of the results, Ag presentation as assessed by proliferation of E7/N-specific or NP-specific T cells after in vitro infection with the mutants ΔvRAP noX NP and ΔvRAP noX E7 was very similar (Fig. 5B, 5C), regardless of at which position of the GFP protein the epitopes are located. Using presenter cells transfected with the same reporter constructs, Wolkers et al. (62) found comparable results for the presentation of the epitopes and also observed a slightly stronger T cell response against the E47 epitope. Altogether, we conclude, therefore, that the opposing trend in the frequency of E7/N and NP-specific T cells after infection of mice with the noX NP and noX E7 mutants cannot be explained by the intrinsic properties of the epitopes but must be because of the different presentation pathway. One of the main findings of this study is that similar patterns of T cell specificities were observed after infection with the vRAP mutants or the wt reporter viruses. This is in agreement with the results of Munks et al. (36), who found that the specificity of the MCMV CD8+ T cell response during acute and chronic infection with ΔvRAP mutants was essentially unchanged when compared with MCMV-wt infection. The magnitude of the T cell response to the epitope that can be cross-presented appeared to be slightly smaller than after infection with the respective reporter mutant expressing the vRAP genes (c.f. Fig. 4C, 4D and Fig. 6A, 6B), and we found virtually no T cells specific for the directly presented epitope (Fig. 6A–D). The latter finding was completely contrary to our expectation, because the experiments shown in Fig. 5B and 5C indicate that Ag presentation was effectively restored in the cells infected with the mutants lacking the vRAP mutants. We can think of three possible explanations. First, directly infected DCs play a minor role in providing the viral Ag, and thus in priming of the CD8+ T cells. This is in agreement with our finding that most of the infected cells in spleen do not appear to be of DC lineage (Fig. 3C). Furthermore, the CD8α− DCs may be rather important for stimulating CD4+ T cell help and play a minor role in priming of the CD8+ T cell response (4, 5). Second, Böhm et al. (84) reported that CD8+ T cell priming is less efficient after infection with ΔvRAP mutants and explained this with a negative feedback re-action of newly primed CD8+ T cells, leading to the rapid elim-ination of infected cells in the LN, limiting further supply of viral Ags. The somewhat smaller T cell response that we observed after infection with the ΔvRAP mutants can be explained by this “immune evasion paradox” described by Böhm et al. (84). Moreover, if we assume that the small fraction of infected CD8α− DCs (Fig. 3C) makes a minor contribution to the priming by direct presentation after infection with MCMV-wt (Fig. 4C, 4D), it is perhaps not surprising that these few infected DCs are eliminated by the first wave of activated CD8+ effector T cells because of lack of immune evasion in case of infection with the ΔvRAP mutants, thereby preventing the generation of a detectable number of T cells specific for the directly presented epitope (Fig. 6A, 6B). Third, because MCMV interferes not just with Ag presentation,
but also with costimulation by downregulating CD40, CD80, and CD86 on infected APCs (43, 44, 46), it is possible that a second layer of defense impairs direct priming even when Ag presentation is restored. Further research is required to investigate whether one or several of the proposed mechanisms can explain the observed data.

During chronic MCMV infection, CD8+ T cells with specificity to “inflating” epitopes are enriched or maintained at high levels (22, 23, 74, 86, 87). It was reported that memory T cells primed early during acute infection and, at least in part, de novo priming during chronic infection sustain this large memory T cell population (79). Our data suggest that those T cells that give rise to the highly enriched effector memory T cells originate from cross priming as well. A high number of E740-specific T cells was observed after infection with ΔvRAP noX NP, but not after infection with ΔvRAP noX E7 (cf. Fig. 7A, 7B). Interestingly, the NP366-epitope encoded by the ΔvRAP noX E7 mutant seemed to be less efficient in driving “memory inflation” because at best, few NP366-specific T cells were detected in these infected mice (Fig. 7B). It is currently not completely clear which properties qualify an epitope for inducing memory inflation (87). One requirement is the spродadic expression of the Ag during latent infection, which because of the use of the major immediate-early promoter (88) seemed to be guaranteed for the Ag encoded by our reporter mutants. Another criterion is the generation of the epitope by constitutive proteasome activity, rather than by the immune proteasome (89). It will be interesting to learn whether the differences observed between the E7 and NP epitopes of our reporter mutants in inducing the memory response depends on this property.

We want to point out that our results cannot offer an answer to the question of which cell types are providing the Ag for memory inflation during the chronic infection phase. Elegant studies by several colleagues, which relied on different approaches, indicated that nonhematopoietic cells play a central role in this process (55, 80). Several colleagues, which relied on different approaches, indicated that nonhematopoietic cells play a central role in this process (55, 80). However, as pointed out by Seckert et al. (80), priming of the CD8+ T cells by hematopoietic cells is one prerequisite for the subsequent enrichment of memory T cells. Our data are in line with this fact and indicate that for the initial priming of those T cells that later give rise to effector memory T cells, cross presentation is pivotal.

At first glance, one may conclude that cross priming overcomes the effects of the viral vRAP and, consequently, there may be no impact of the vRAPs on the antiviral T cell response. It is clear that vRAPs provide CMVs with other qualities such as the capability to reinfect the host in the presence of fully developed immunity (90); however, one should not ignore the consequences of cross priming for the host and its immune response. The host generates a very broad T cell response against CMV, and actually it may have to do so to guarantee the generation of protective CD8+ T cells, because in the case of cross presentation the immune system does not “know,” whether the cross-presented Ag is also presented by infected cells and, thus, whether the primed T cell will be able to provide protection. Accordingly, the host has to pay a considerable price and to devote a substantial part of the immune reaction to the control of CMV. Experimental evidence (39), as well as hints from the study of CMV-specific T cells (37, 38), indicates that a fraction of the CMV-specific T cells has no protective capacity. Furthermore, the T cell response will be biased toward such viral Ags that are good substrates for cross presentation. For the development of a CMV vaccine and for use of CMV as a vaccine vector against other pathogens (91–93), it is therefore recommended to use stable, long-lived Ags that can be cross-presented, as was proposed previously (54). The concern that deletion of vRAP genes reduces the efficacy of a live CMV vaccine (84) may be of less importance because this will probably lead to desired attenuation of a vaccine and, as we show in this study, will not affect the Ag presentation pathway, and hence in accordance with the experimental findings (36), will not influence the specificity of the induced T cell response.

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