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# Blocking Virus Replication during Acute Murine Cytomegalovirus Infection Paradoxically Prolongs Antigen Presentation and Increases the CD8$^+$ T Cell Response by Preventing Type I IFN–Dependent Depletion of Dendritic Cells

**Christopher P. Loo,* Christopher M. Snyder,† and Ann B. Hill***

**Increasing amounts of pathogen replication usually lead to a proportionate increase in size and effector differentiation of the CD8$^+$ T cell response, which is attributed to increased Ag and inflammation. Using a murine CMV that is highly sensitive to the antiviral drug famciclovir to modulate virus replication, we found that increased virus replication drove increased effector CD8$^+$ T cell differentiation, as expected. Paradoxically, however, increased virus replication dramatically decreased the size of the CD8$^+$ T cell response to two immunodominant epitopes. The decreased response was due to type I IFN–dependent depletion of conventional dendritic cells and could be reproduced by specific depletion of dendritic cells from day 2 postinfection or by sterile induction of type I IFN. Increased virus replication and type I IFN specifically inhibited the response to two immunodominant epitopes that are known to be dependent on Ag cross-presented by DCs, but they did not inhibit the response to "inflationary" epitopes whose responses can be sustained by infected nonhematopoietic cells. Our results show that type I IFN can suppress CD8$^+$ T cell responses to cross-presented Ag by depleting cross-presenting conventional dendritic cells.** ***The Journal of Immunology*****, 2017, 198: 383–393.**

Cytomegalovirus is a ubiquitous virus, infecting most mammalian species with species-specific CMV strains. As a member of the herpesvirus family, it infects its host for a lifelong persistent/latent infection, which is usually asymptomatic. One of the most remarkable aspects of CMV immunobiology is the size of the CD8$^+$ T cell response to it, particularly during the chronic phase of infection. To understand the role of chronic virus replication in driving the response, we generated a recombinant murine CMV (MCMV) for which virus replication could be completely inhibited by drug treatment (1). During our initial characterization of acute infection with that reagent, we observed a striking phenomenon: when virus replication was completely inhibited by commencing drug treatment prior to infection, there was a paradoxical dramatic increase in the peak size of the CD8$^+$ T cell response. This article describes our investigation of that phenomenon.

Larger amounts of pathogen during acute infection generally elicit larger CD8$^+$ T cell responses, triggered by more Ag and more inflammation (1, 2). This was clearly dissected in studies of the CD8$^+$ T cell response to *Listeria monocytogenes*: increasing bacterial dose increased the response (2), and, conversely, antibiotic treatment shortly postinfection (pi) severely inhibited the response (3).

For MCMV, the relationship between virus load and the acute CD8$^+$ T cell response has primarily been investigated in the context of NK cells, where altered efficacy of NK cells led to a difference in virus load during acute infection. The results have been mixed, with positive or negative impacts of NK cells on the kinetics and peak CD8$^+$ T cell response (4–7, reviewed in Ref. 8). These studies are complicated by the use of virus strains that differ in virulence, as well as by the use of mouse strains that differ in susceptibility to MCMV. BALB/c and C57BL/6 mice differ at the Ly49H (*cmv1*) locus, which determines the ability of NK cells to control MCMV, as well as in other poorly understood ways that impact CMV control and the T cell response. More importantly, the complex cross-talk between NK cells and dendritic cells (DCs) that occurs during infection, with each cell type supporting the other, makes it difficult to determine whether NK cells impact the T cell response through immunological mechanisms or via their effect on virus titers (6, 7, 9). In addition to profoundly impacting virus titers, NK cells can influence the CD8$^+$ T cell response in multiple ways, including killing virus-infected APCs (10) while promoting the survival of uninfected DCs for effective T cell stimulation (6). We decided to remove NK cell control as a variable by using a virus lacking the NK-stimulatory protein encoded by *m157* and to vary virus titer by pharmacological inhibition of virus replication, to cleanly dissect the relationship between virus replication and the CD8$^+$ T cell response to MCMV.

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Abbreviations used in this article: cDC, conventional DC; DC, dendritic cell; dm157, delta m157; DT, diphtheria toxin; DTR, diphtheria toxin receptor; LCMV, lymphocytic choriomeningitis; MCMV, murine CMV; MFI, mean fluorescence intensity; MPEC, memory precursor effector cell; OHSU, Oregon Health and Science University; pDC, plasmacytoid DC; pi, postinfection; poly(I:C), polyinosinic-polycytidylic acid; SLEC, short-lived effector cell; TK, thymidine kinase; WT, wild-type.



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In this study, we used a recombinant MCMV expressing the thymidine kinase (TK) gene from HSV (MCMV-TK) (1). This rendered the MCMV-TK exquisitely sensitive to the antiviral drug famciclovir in mice, enabling us to block viral replication without relying on NK cell–mediated resistance. Surprisingly, we found that completely blocking MCMV replication during acute infection had the paradoxical effect of augmenting certain CD8 T cell responses. Specifically, the already large responses to two immunodominant epitopes were augmented, whereas other responses were unaffected. This effect was directly attributable to a reduction in type I IFN and the consequent increased survival of conventional DCs (cDCs) when viral replication was blocked. Importantly, the magnitude of the antiviral CD8 T cell populations could be enhanced by blocking type I IFN signaling or reduced by the depletion of DCs or induction of type I IFN. These data show that type I IFN–dependent loss of cDCs during acute viral replication can limit the magnitude of some antiviral CD8 T cell populations.

## Materials and Methods

All studies were performed under the approval of the Institutional Biosafety Committee. All animal work was performed in accordance with the National Institutes of Health guidelines and the Oregon Health and Science University (OHSU) Institutional Animal Care and Use Committee. The OHSU Department of Comparative Medicine and Division of Animal Resources have accreditation from the Association for Assessment and Accreditation of Laboratory Animal Care.

### *Mice and famciclovir treatment*

C57BL/6, CD45.1 (B6.SJL-*Ptprc*$^{a}$ *Pepc*$^{b}$*/BoyJ*), BALB/c, and CD11c–diphtheria toxin receptor (DTR) [B6.FVB-Tg(Itgax-DTR/EGFP)57Lan/J] mice were purchased from the Jackson Laboratory. C57BL/6 mice were bred to CD45.1 mice in-house to generate the CD45.2 × CD45.1 F1 mice that were used for adoptive-transfer experiments. Mice were used between the ages of 6 and 36 wk. Famciclovir (250 mg; Roxane Laboratories) pills were crushed and dissolved in water at a 2 mg/ml concentration. Famciclovir treatment was administered continuously starting at 3 d prior to infection.

### *Virus strains*

All mice were inoculated i.p. with $2 \times 10^5$ PFU of MCMV-TK or MCMV-delta m157 (dm157) virus. Preparation of MCMV-TK and MCMV-dm157 mice was described by Snyder et al. (1). In short, the TK gene was amplified from a plasmid (derived from HSV-1) and subcloned into a plasmid containing kanamycin and flanked with FRT recombination sites. The TK-Kan construct was PCR amplified with MCMV flanking sequences of the *m157* region and homologous recombined with wild-type (WT) MCMV artificial bacterial chromosome (strain MW9701). The result was replacement of the *m157* gene with TK.

### *Detection of Ag-specific CD8 T cells*

Ag-specific CD8 T cells were detected by peptide stimulation or through staining by epitope specific tetramers (made by the National Institutes of Health Tetramer Core). Peptides used to stimulate or bound to tetramers were M45 (HGIRNASFI), M57 (SCLEFWQRV), m139 (TVYGFCLL), and M38 (SSPPMFRV).

For intracellular cytokine staining, cells were cultured for 6 h at 37°C with peptides and brefeldin A (10 μg/ml). DMSO was cultured with cells as an experimental negative control. Cells were stained for cell surface markers, followed by cell fixation and permeabilization with a BD Cytofix/Cytoperm Kit (according to the manufacturer's protocol). Following permeabilization, intracellular markers were stained with fluorochrome-conjugated Abs.

Tetramer and surface phenotype staining of CD8 T cells were performed at 4°C, 1 h. The following fluorescently labeled Abs were used in this study: KLRG1 (clone 2F1/KLRG1; BioLegend), CD127 (clone A7R34; BioLegend), PD-1 (clone RMP1-30; BioLegend), CD8α (clone 53-6.7; BioLegend), CD3 (clone 145-2C11; BioLegend), CD11b (clone M1/70; eBioscience), CD11c (clone N418; eBioscience), and LIVE/DEAD Fixable Aqua Dead Cell Stain Kit 405 (Invitrogen). All populations were of singlet cells from a gate positioned on the axis of forward scatter area versus forward scatter height. All samples were measured on a BD LSR II flow cytometer. Data were analyzed by FlowJo software (TreeStar, Ashland, OR).

### *DC isolation*

Spleens were harvested and minced. Samples were incubated at 37° with collagenase D and DNase for 30 min, with mixing every 10 min. Tissue was smashed and filtered through a 70-μm mesh filter and washed with 10% FBS RPMI 1640.

DC populations were identified as CD3$^{-}$, CD11c$^{+}$, and MHCII$^{+}$. cDCs were identified as CD11c$^{hi}$MHCII$^{+}$, and plasmacytoid DCs (pDCs) were identified as CD11c$^{int}$PDCA-1$^{+}$.

### *Adoptive transfer*

CD45.1 × CD45.2 F1 mice were bred in-house. CD8 T cells were purified from spleens using a magnetic negative selection kit (EasySep Mouse CD8+ T Cell Isolation Kit; STEMCELL Technologies), and $3 \times 10^6$ pure CD8 T cells (>95%) were transferred by i.v. injection.

### *Cytokine array*

Circulating cytokines and chemokines were quantified in the plasma of infected mice. Plasma was collected on multiple days and frozen at −80°C until all specimens were collected. Plasma was thawed and analyzed by a multiplex ELISA assay. (Mouse Cytokine Screen, catalog number 110951MS; Quansys Biosciences or Cytokine Mouse Magnetic 10-PLEX Kit, catalog number LMC001M; Life Technologies). IFN-α was measured by a single-plex ELISA (Life Technologies).

### *TLR agonist, diphtheria toxin, and blocking Ab administration*

Diphtheria toxin (DT; Sigma) was given in a single i.p. injection at a concentration of 3 ng/g of body weight. Polyinosinic-polycytidylic acid [poly(I:C)] was a gift from Dr. Jadeep S. Obrahi (OHSU) (catalog number tlrl-pic; InvivoGen) and was administered i.p. (100 μg) to mice on days 1 and 2 pi. MAR1-5A3 mAb and GIR-2308 isotype-control IgG1 were purchased from Bio X Cell and administered i.p. (1 mg per mouse) 12 h pi. PD-L1–blocking Ab (10F.9G2) was purchased from Bio X Cell. Anti–PD-L1 treatment started at day 0 pi (administered at 200 μg per mouse i.p., every 3 d).

### *Statistics*

Prism software (GraphPad) was used for statistical analyses.

## Results

### *Blocking viral replication enhances the acute CD8$^{+}$ T cell response to MCMV*

Native MCMV is only moderately sensitive to inhibition by nucleoside analog antivirals. To investigate the impact of virus replication on the ensuing CD8$^{+}$ T cell response, we generated an MCMV that is sensitive to nucleoside analog antiviral drugs by inserting the TK gene from HSV into the *m157* locus of MCMV. The *m157* locus is the preferred locus for insertion of transgenes because it eliminates the ability of C57BL/6 mice to sense MCMV through the activating NK receptor Ly49H and, consequently, makes handling of MCMV by C57BL/6 mice similar to most wild mouse strains (11). HSV-TK renders MCMV sensitive to the nucleoside analog famciclovir, and we showed previously that MCMV-TK replication is completely inhibited by famciclovir in vitro and in vivo (1).

We examined the impact of blocking MCMV replication on the CD8$^{+}$ T cell response to immunodominant epitopes, expecting that the much lower antigenic load would lead to a reduction in the CD8$^{+}$ T cell response. Indeed, previous work showed that blocking replication of *L. monocytogenes* leads to a truncated effector CD8$^{+}$ T cell response and early formation of memory T cells (3). Strikingly, however, treating mice with famciclovir before MCMV-TK infection resulted in an increase in the number of CD8 T cells and the frequency of MCMV-specific CD8$^{+}$ T cells in the spleen and blood at day 7 pi (Fig. 1A, 1B). The increase was particularly dramatic in response to M45 and M57, two responses that are immunodominant at the peak of the acute response

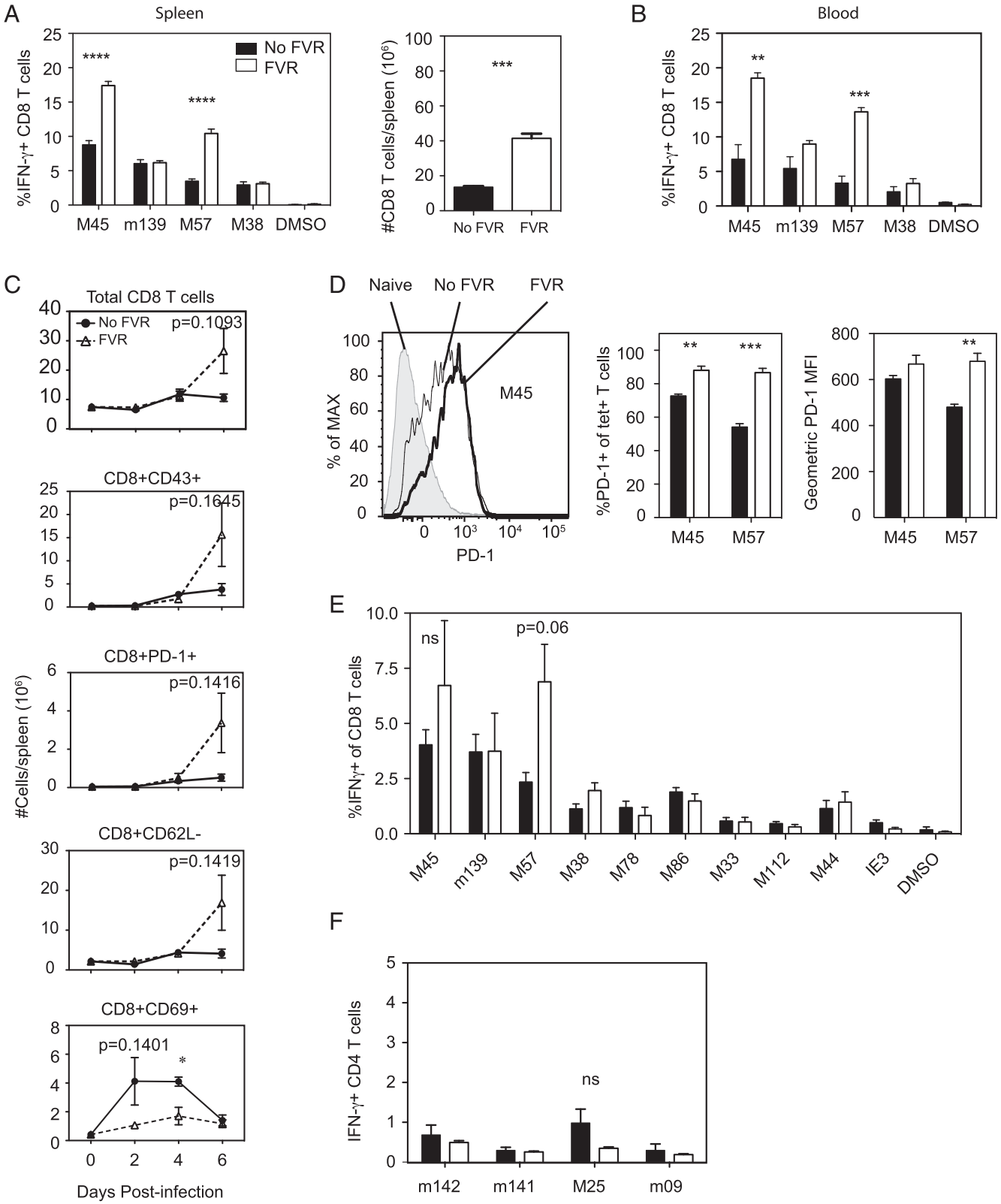


**FIGURE 1.** Enhanced Ag-specific CD8 T cells in C57BL/6 mice with replication-deficient MCMV-TK. C57BL/6 mice were treated with famciclovir-fortified water, starting 3 d prior to infection. Mice were infected i.p. with $2 \times 10^5$ PFU of virus, and splenocytes and blood were analyzed 7 d later for CD8 T cell activation and the frequency of Ag-specific cells by flow cytometry. (**A**) The IFN-γ response to peptide stimulation identifies Ag-specific CD8 T cells and total numbers of CD8 T cells from the spleen. (**B**) The IFN-γ response to peptide stimulation identifies Ag-specific CD8 T cells in the blood. (**C**) Frequency of CD8 T cells from the total lymphocyte population, as well as the expression of CD43, PD-1, CD62L, and CD69 on CD8 T cells. Data points were measured at 2, 4, and 6 d pi. (**D**) PD-1 expression on tetramer$^+$ M45-specific cells. The representative graphs show the frequency of PD-1 on M45- and M57-specific T cells and the geometric MFI from tetramer$^+$ PD-1 CD8 T cells. MCMV produces a wide breadth of antigenic peptides. (**E**) IFN-γ response after peptide stimulation to dominant and subdominant peptide epitopes. (**F**) To identify whether famciclovir treatment enhances the CD4 T cell response during acute infection, splenocytes were stimulated with known CD4-specific peptides. Graphs represent the average + SEM. Bars represent three mice per group. Experiments in (C) and (E) were performed once; all other experiments were repeated at least two times. *$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$, Student $t$ test. ns, not significant.

(12, 13). This increase in the frequency of Ag-specific T cells was accompanied by a marked expansion of total $CD8^+$ T cells in the spleen as early as day 6 pi (Fig. 1C), consistent with the known ability of uncontrolled MCMV infection to deplete lymphocytes from the spleen (14). Moreover, significantly larger numbers of splenic $CD8^+$ T expressed the activation markers CD43 and PD-1 while losing CD62L expression (Fig. 1C). These phenotypic changes are characteristic of Ag-driven activation and T cell clonal expansion. Notably, the frequency and intensity of expression of the inhibitory receptor PD-1 were not reduced by famciclovir treatment; in fact, it was as high or higher on M45- and M57-specific cells in famciclovir-treated mice compared with untreated mice (Fig. 1D). Thus, inhibition of clonal expansion by PD-1–mediated T cell suppression cannot explain the difference in Ag-specific $CD8^+$ T cell numbers. Interestingly, CD69 expression was affected by famciclovir in the opposite direction to the other activation markers (Fig. 1C). CD69 expression peaked earlier and was more marked on T cells in untreated animals. Although expression of CD69 is frequently interpreted as indicating Ag-driven T cell activation because it is transiently induced on T cells by TCR signaling, it is also known to be upregulated in an Ag-independent manner by cytokines on bystander cells (15). Thus, collectively, these data are consistent with the notion that, when virus replication is inhibited there is less nonspecific cytokine stimulation and, hence, less CD69 expression. However, activation markers associated with TCR signaling (CD43, PD-1, CD62L downregulation), as well as numbers of virus-specific $CD8^+$ T cells were increased, which suggests that when virus replication is inhibited, there is a paradoxical increase in Ag-driven T cell stimulation.

We analyzed the T cell response in the spleen to a broader spectrum of epitopes (Fig. 1E). This revealed that the increased response in famciclovir-treated mice was largely restricted to the M45 and M57 epitopes, two of the most immunodominant responses during acute infection. Moreover, the frequency of Ag-specific $CD4^+$ T cell responses was largely unaffected, and certainly not increased, by famciclovir treatment (Fig. 1F). In sum, our data show that inhibiting virus replication resulted in the selective increase in certain $CD8^+$ T cell responses. The responses that were increased (M45 and M57) are immunodominant during acute infection (12); subdominant responses were not enhanced by famciclovir. However, the frequency of $CD8^+$ T cells responding to m139, which is also an immunodominant epitope, was not increased by famciclovir. At this point, the basis for the selective impact of famciclovir was quite unclear. We decided to focus our efforts on understanding the mechanism by which the M45 and M57 responses were increased by famciclovir.

### *Famciclovir does not increase the CD8 T cell response to MCMV-TK in the presence of simultaneous uninhibited viral replication*

There are two basic ways in which famciclovir treatment could result in an enhanced $CD8^+$ T cell response to MCMV-TK: it (in the presence of TK) could specifically boost the response (e.g., viral DNA incorporating famciclovir could act as a previously unappreciated adjuvant), or, in untreated animals, uninhibited viral replication could suppress the potential response. If the first explanation were true, famciclovir+TK should boost the response, even in the presence of uninhibited (WT MCMV) viral replication. Conversely, if the second explanation were true, the presence of active virus replication should prevent famciclovir from augmenting the $CD8^+$ T cell response to MCMV-TK. To distinguish these possibilities, we performed a coinfection experiment in which mice were infected with the virus that could be inhibited by famciclovir (MCMV-TK), which lacks *m157*, at the same time as a WT virus that was similarly deficient in *m157* but resistant to famciclovir (MCMV-dm157). As shown in Fig. 2, famciclovir treatment had only a very small impact on the frequency of M45- or M57-specific T cell populations in mice infected with MCMV-dm157 lacking HSV TK. WT MCMV replication is inhibited, although only modestly, by famciclovir, suggesting that a modest inhibition of virus replication resulted in a small increase in T cell responses. In contrast, as shown previously, famciclovir treatment strongly boosted the frequency of M45- and M57-specific T cells when mice were infected with the drug-sensitive MCMV-TK. Strikingly, coinfection with both viruses prevented famciclovir from boosting the frequency of M45- and M57-specific populations above the level seen in famciclovir-treated WT-infected mice. Therefore, these data argue that famciclovir is not acting as an adjuvant and instead suggest that viral replication directly or indirectly suppresses the CD8 T cell response to MCMV infection.

### *Impact of famciclovir on $CD8^+$ T cell expansion, effector phenotype, contraction, and memory*

The primary $CD8^+$ T cell response to acute infection results in two populations of cells: short-lived effector cells (SLECs) that are destined to die and memory precursor effector cells (MPECs) that can form long-lived memory cells (16). These populations are distinguished by the markers KLRG-1 and CD127 (16). Previous work showed that truncating acute pathogen replication reduces inflammation, which, in turn, results in accelerated T cell memory formation (17). In line with this, famciclovir treatment increased the proportion of M45- and M57-specific cells that displayed an MPEC phenotype and decreased the percentage of cells with an SLEC phenotype (Fig. 3A). We predicted that this increase in MPECs at acute time points would result in significantly higher numbers of memory cells at late times pi. Indeed, by 25 wk pi, famciclovir-treated mice had significantly more M45- and

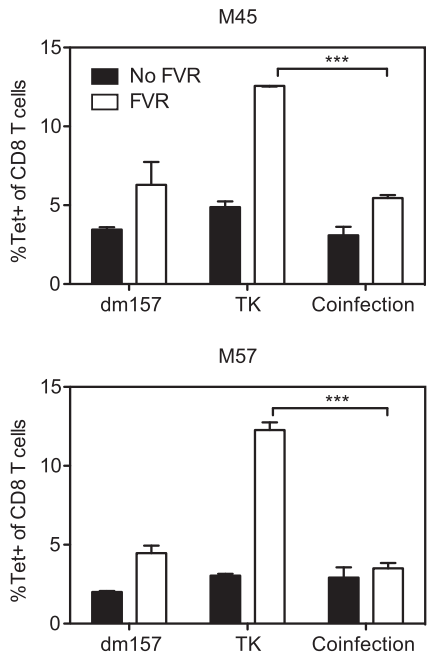


**FIGURE 2.** Mice coinfected with replicating dm157-TK lack enhanced M45- and M57-specific responses with famciclovir treatment. At day 7 pi, the frequency of splenic M45- and M57-tetramer specific CD8 T cells was measured from a single or coinfection of mice with dm157-TK ($2 \times 10^5$ PFU) and MCMV-TK ($2 \times 10^5$ PFU). Graphs represent the average + SEM. Bars represent three mice per group. Experiments were done three times. ***$p < 0.0005$, Student $t$ test.

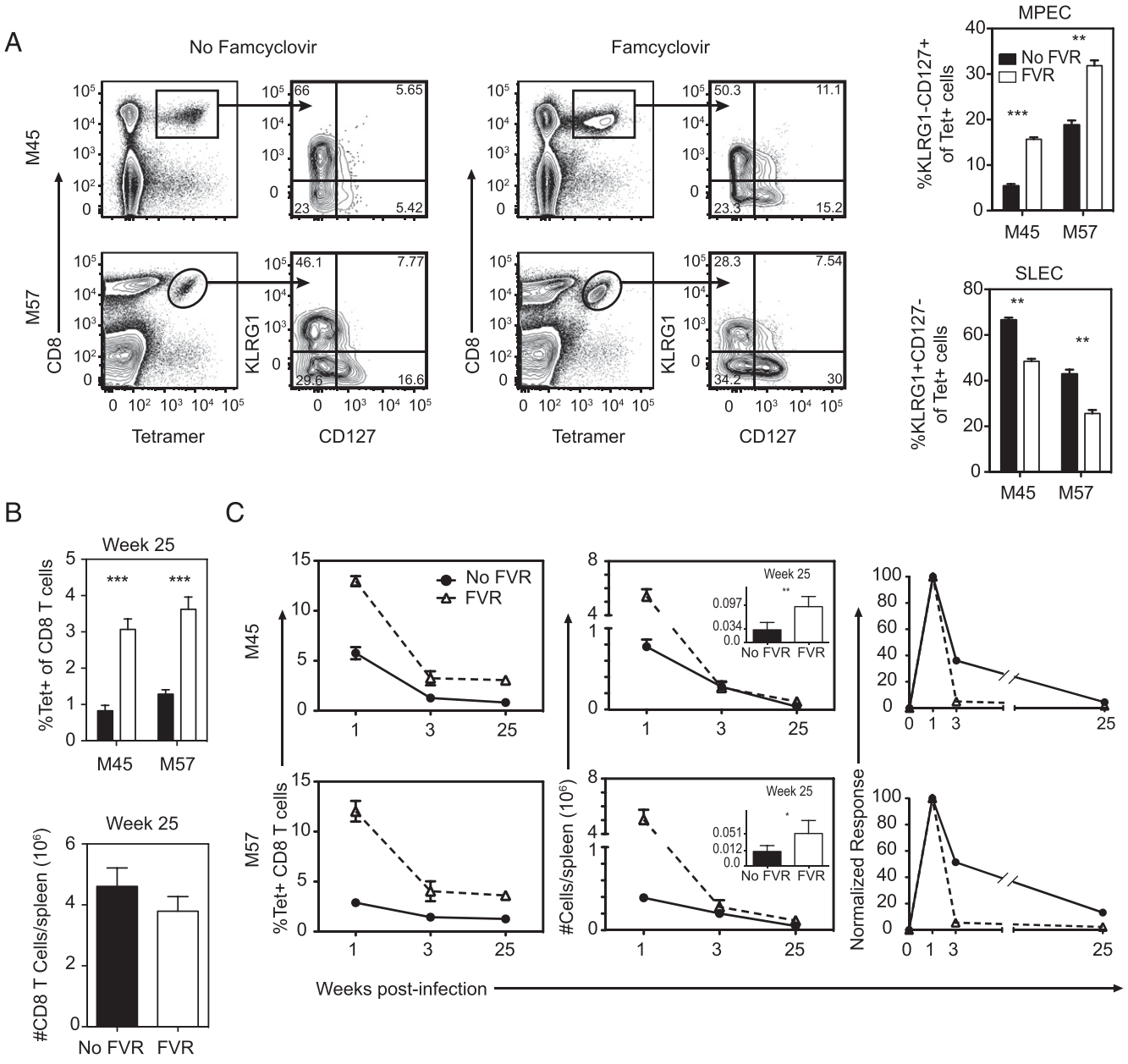


**FIGURE 3.** Impact of virus replication on expansion and contraction of Ag-specific populations. Mice were infected with MCMV-TK and monitored for expansion, contraction, and memory-effector phenotype over time. (**A**) Dot plots and bar graphs show the frequency of SLECs ($CD127^{lo}$, $KLRG1^{hi}$) and MPECs ($CD127^{hi}$, $KLRG1^{lo}$) of $tetramer^{+}$ cells at day 7 pi. (**B**) The frequency of M45- and M57-specifc and total number of CD8 T cells in the spleen were quantified at week 25 pi. (**C**) Line graphs show the frequency and absolute number kinetics of M45- and M57-specific cells at weeks 1, 3, and 25 pi (inset, week-25 data). Absolute numbers of M45 and M57-tetramer+ cells were normalized to week 1 data. Graphs represent the average plus SEM. Bars represent three mice per group. Experiments were done twice. $*p < 0.05$, $**p < 0.005$, $***p < 0.0005$, Student $t$ test.

M57-specific $CD8^{+}$ T cells compared with untreated mice (Fig. 3B). Interestingly, however, the differential between the two conditions was not as high as expected from their difference at the peak of the response, particularly taking into account the disparity in MPEC frequency. Analyses of the kinetics revealed that M45- and M57-specific T cells underwent a more rapid rate of contraction in famciclovir-treated mice (Fig. 3C). In sum, however, these data indicate that famciclovir treatment enabled a larger primary T cell response and also increased memory T cell numbers.

*Famciclovir suppresses the inflammatory response*

In published models of other infections, inflammatory cytokines increase the magnitude of the $CD8^{+}$ T cell response (18). However, at the same time, inflammatory cytokines drive the responding T cells toward a more differentiated SLEC phenotype (16). Because famciclovir inhibits viral replication, it would be expected to limit the acute inflammatory response stimulated by MCMV infection. Thus, the results reported above are discordant with the expected relationship among inflammation, $CD8^{+}$ T cell population size, and the production of SLECs. To investigate the impact of famciclovir on inflammation, we measured key inflammatory cytokines over the first few days of infection (Fig. 4). As expected, the very earliest cytokine response to MCMV, an increase in type I IFN at 8 h pi (19, 20), was identical in the presence or absence of drug. However, subsequent cytokine responses were absent or severely muted in the drug-treated animals. Type I IFN and IL-12 act on $CD8^{+}$ T cells to promote proliferation (signal 3), yet these cytokines were expressed at much higher

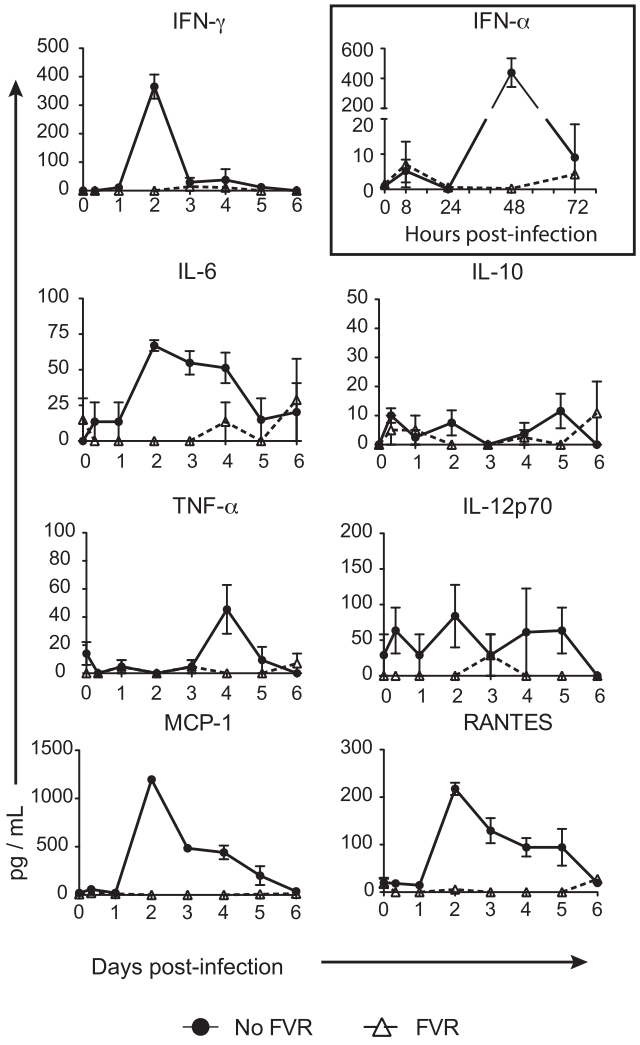


**FIGURE 4.** Famciclovir (FVR) treatment abrogates acute inflammatory cytokines. Mice were infected with MCMV-TK, and plasma was collected at multiple time points pi and subjected to a single or multiplex cytokine detection assay. Line graphs show the quantity of peripheral cytokines and chemokines (IFN-γ, IL-6, IL-10, IL-12p70, MCP-1, and RANTES) on days 0–6 pi. The graph in the box shows the peripheral IFN-α response measured at times 0, 8, 24, 48, and 72 h pi (upper right panel). Each time point represents three mice per group. Experiments were done twice.

levels in animals that did not receive famciclovir and in which the CD8$^+$ T cell responses were lower. IL-10 can suppress immune responses, yet this cytokine was not found in significant amounts in either infection condition; thus, it is unlikely to account for the difference. Overall, inflammatory responses were similar to those described in the literature for the infected animals that did not receive famciclovir and were, as expected, very low in the drug-treated animals.

*Famciclovir prevents depletion of cDCs*

In the absence of effective NK cell control, MCMV is known to deplete CD11c$^+$ cDCs from the spleen during acute infection (4, 19, 21). Because our virus lacks *m157*, it is not susceptible to control by Ly49H$^+$ NK cells. Therefore, we thought that MCMV-TK might deplete cDCs and that famciclovir might prevent this depletion. Therefore, we quantified splenic DC numbers on days 0, 2, 4, and 6 d pi, measuring two cDC subsets (CD11b$^+$ and CD8$^+$), as well as pDCs (Fig. 5A). Without famciclovir treatment, both cDC subsets decreased by day 2 pi and remained low. In contrast, in the presence of famciclovir, cDCs were not depleted; in fact, both subsets increased in number by day 4 pi. Consistent with previous reports, pDC numbers were not depleted by MCMV infection, and famciclovir had no additional effect. Thus, inhibiting viral replication rescued cDCs from MCMV-induced depletion. We also asked whether famciclovir altered the phenotype of DCs, because PD-L1–expressing DCs can inhibit CD8$^+$ T cell responses. Famciclovir-treated mice had a lower frequency of CD8$^+$ DCs expressing PD-L1. However, the mean fluorescence intensity (MFI) of PD-L1 cDCs did not differ between treated and untreated mice (Fig. 5B).

*Enhanced CD8$^+$ T cell responses are dependent on cDCs*

We postulated that preservation of cDCs in famciclovir-treated mice was important for the enhanced CD8$^+$ T cell response. Most available evidence indicates that, in common with other infections, initial priming of the CD8$^+$ T cell response to MCMV depends on DCs (22, 23). CD8$^+$ cDCs are critical for cross-presentation of pathogen Ags, and the acute response to MCMV is driven by cross-presented Ag (24–26). However, it is not known whether DCs are involved in further enhancing the extent of CD8$^+$ T cell proliferation (i.e., the clonal burst size) after the initial priming event. Because replicating virus depletes cDCs, we considered that this loss could be responsible for the muted CD8$^+$ T cell response to cross-presented epitopes. Indeed, Robbins et al. (4) reported that virus-induced DC loss reduced the very early (day 4) CD8$^+$ T cell responses to MCMV in BALB/mice, albeit without impacting the peak (day 7) response. To determine whether DC loss could be preventing a later amplification of the CD8$^+$ T cell response in C57/BL6 mice, we used CD11c-DTR–transgenic mice, in which DCs can be depleted by administration of DT. To allow for CD8$^+$ T cell priming but to remove DCs during the clonal expansion phase, CD11c-DTR–transgenic mice and littermate controls were infected with MCMV-TK, and DT was administered 2 d later (Fig. 5E). The CD8$^+$ T cell response to M57 and M45 was measured at 7 d pi (Fig. 5C, 5D). Because activated CD8$^+$ T cells express CD11c, they might also be depleted by DT, distorting any interpretation. To avoid this, we adoptively transferred WT CD8 T cells prior to infection. However, we found no difference in the frequency of T cells responding in the donor and recipient populations (data not shown); therefore, the data shown in Fig. 5D are for the total CD8$^+$ T cell population. As expected, littermate control mice developed enhanced responses to M45 and M57 when pretreated with famciclovir. Remarkably, however, when DCs were depleted from CD11c-DTR$^+$ mice by treatment with DT, the response to M45 and M57 was equivalent to that in mice that did not receive famciclovir. This result indicates that the ablation of DCs after initial priming has a suppressive effect on the peak acute CD8 T cell response of MCMV-TK infection. Together, these data strongly imply that the preservation of the CD11c APC compartment is necessary for the enhancement of the M45- and M57-specific CD8 T cell responses in replication-deficient MCMV infection.

*TLR agonist reduces CD8 T cell responses in famciclovir-treated mice*

It seemed likely that the vastly increased cytokine response to replicating virus would be responsible for the ability of replicating virus to inhibit the T cell response to M45 and M57. For this reason, we asked whether a sterile induction of cytokines would reproduce the impact of virus replication on the T cell response. MCMV is known to stimulate TLR3 (27). Therefore, to mimic an MCMV-mediated inflammatory response in famciclovir-treated mice, we

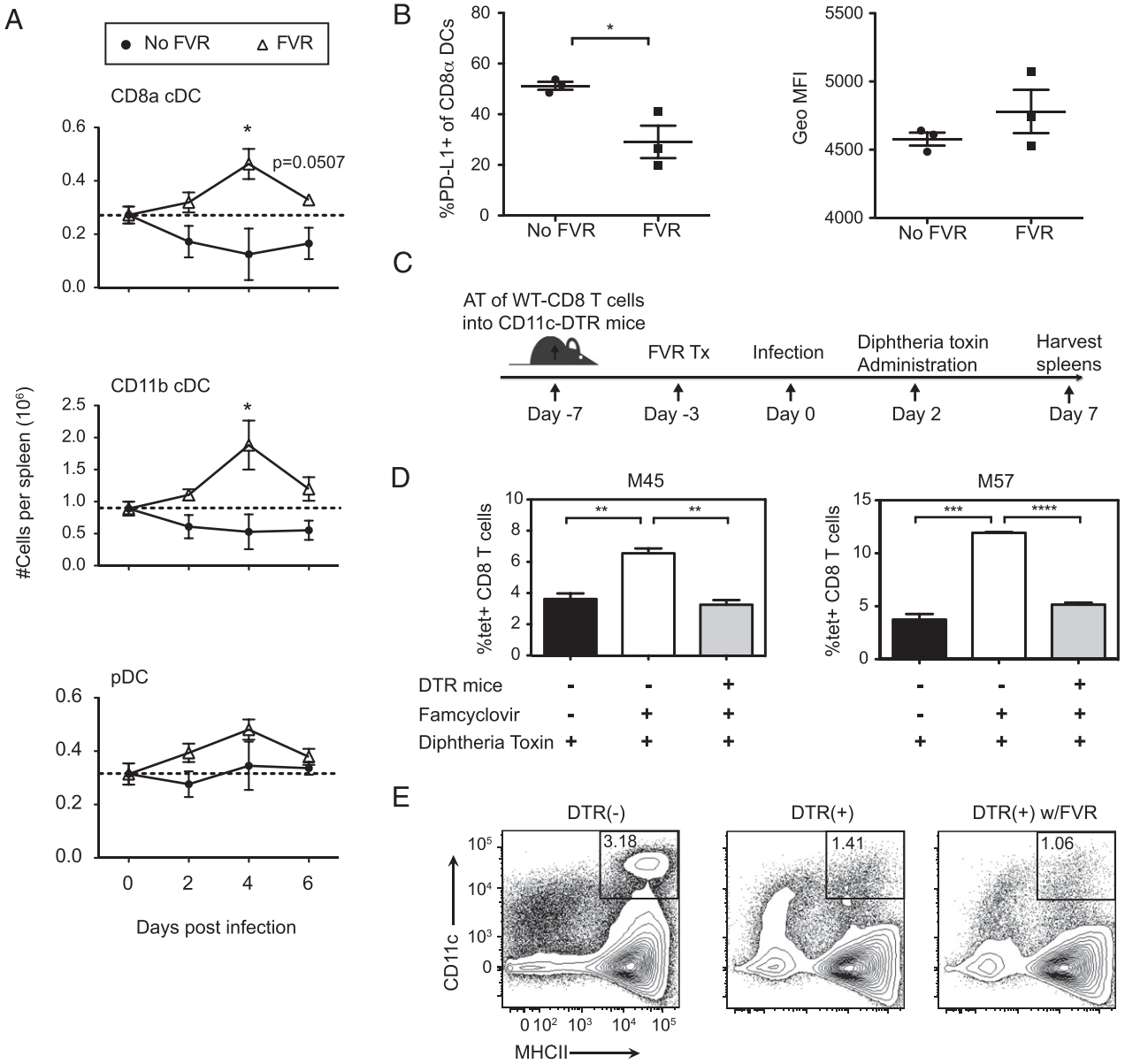


**FIGURE 5.** Depletion of CD11c DCs during acute MCMV-TK infection abrogates the M45 and M57 T cell response. (**A**) Line graphs show the absolute numbers of splenic cDCs ($CD11b^+$ and $CD8\alpha^+$) and pDCs on days 0–6 pi in MCMV-TK–infected mice, with or without FVR treatment. The dashed line represents absolute numbers from naive mice ($n = 4$). (**B**) Graphs show PD-L1 frequency and MFI on $CD8\alpha^+$ DCs at 4 d pi. (**C**) CD11c-DTR and littermate control mice were recipients of bead-purified WT (CD45.1/CD45.2) CD8 T cells at 1 wk prior to infection and were given one dose of DT at 2 d pi. (**D**) Bar graphs show the frequency of the M45- and M57-specific response in CD11c-DTR and littermate control animals at 7 d pi. (**E**) Plots show DC depletion from DTR+ and WT littermate mice at 2 d post-DT treatment, gated on the $CD3^-$ population. Graphs represent the average + SEM. Each time point or bar represents three mice per group. The experiments were done twice. $*p < 0.05$, $**p < 0.005$, $***p < 0.0005$, $****p < 0.0001$, Student $t$ test.

administered the TLR3 agonist poly(I:C) on days 1 and 2 pi. Fig. 6A shows that poly(I:C) markedly reduced the ability of famciclovir to enhance the $CD8^+$ T cell response. Although poly(I:C) induced multiple cytokines that were also induced by replicating CMV, we were particularly interested in the type I IFN response. Type I IFN was reported to increase DC turnover in vivo (28, 29). Specifically, in MCMV infection, type I IFN depletes splenic DCs by preventing their replenishment from precursors (28), resulting in the previously reported impact of DC loss on the very early $CD8^+$ T cell response to MCMV in BALB/c mice (4). The type I IFN response to MCMV is biphasic: a small peak at 8 h is followed by a larger peak at 48 h (20, 30). Famciclovir pretreatment prevents the second type I IFN peak (Fig. 4, upper right panel). Therefore, we administered poly(I:C) on days 1 and 2 pi to famciclovir-treated mice in an attempt to recapitulate the second type I IFN peak. Fig. 6B shows that poly(I:C) treatment of famciclovir-treated MCMV-infected mice increased IFN-α levels at 48 h, although not quite to the level seen in mice not treated with famciclovir. Because we also know that cDCs are needed for the increased response, we next assessed the impact of poly(I:C) and type I IFN on splenic cDC numbers (Fig. 6C). Mice were infected with MCMV-TK in the presence of famciclovir and treated with poly(I:C) on days 1 and 2 pi, as before. In addition, they were treated with MAR1-5A3, an Ab against the IFN-α/β receptor subunit 1 that effectively neutralizes the activity of type I IFN in vivo, or a matched isotype control. DC numbers were analyzed in mice sacrificed on day 4 pi, the time point at which the greatest difference was seen in famciclovir-treated mice (Fig. 5A). The number of cDCs ($CD11c^+$) in the $CD11b^+$ and $CD8^+$ subsets was increased by famciclovir treatment, and poly(I:C) prevented

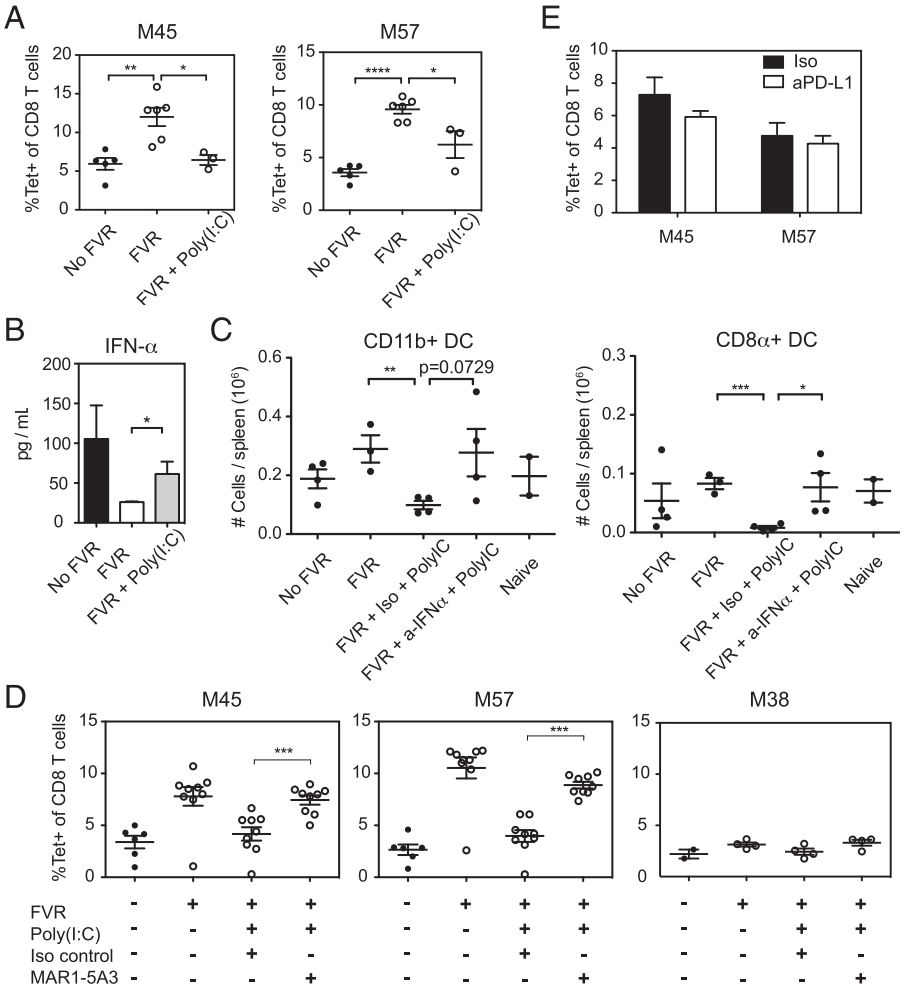


**FIGURE 6.** Sterile inflammation reduces CD8 T cell responses in famciclovir-treated mice. MCMV-TK–infected mice were given poly(I:C) at day 1 and 2 pi. (**A**) Representative graphs showing the frequency of M45- and M57-tetramer+ splenocytes at day 7 pi. (**B**) Plasma was collected from infected mice given poly(I:C), and peripheral IFN-α was quantified by an ELISA at 48 h pi. At day 4 pi, cDCs were shown to be preserved with FVR treatment (see Fig. 5). (**C**) Decrease in number of cDCs in MCMV-TK infection with the administration of poly(I:C) treatment and the recovery when these mice are treated with neutralizing IFN-αR1 mAb (MAR1-5A3). (**D**) Frequency of M45-, M57-, and M38-tetramer specific CD8 T cell populations in response to poly(I:C), along with the combination of MAR1-5A3 mAb or isotype control. (**E**) Ag-specific T cells were measured at day 7 pi after MCMV infection with the addition of blocking PD-L1 mAb. Graphs represent the average + SEM. Individual plot points and bars represent two to nine mice per group. Experiments were done twice. $*p < 0.05$, $**p < 0.005$, $***p < 0.0005$, $****p < 0.0001$, Student $t$ test.

this increase, consistent with the interpretation that inflammatory cytokines suppress cDC numbers during MCMV infection. However, when type I IFN activity was neutralized, cDC numbers were restored to the levels seen in the absence of poly(I:C).

To test whether this increase in type I IFN could account for the impact of poly(I:C) on the CD8$^+$ T cell response in famciclovir-treated MCMV-infected mice, mice were infected with MCMV-TK and administered poly(I:C) and MAR1-5A3 as before, and the CD8$^+$ T cell response was analyzed on day 7 (Fig. 6D). Whereas poly(I:C) reduced the ability of famciclovir to enhance the response to M45 and M57, the neutralization of type I IFN reversed this effect. Thus, neutralization of type I IFN in poly(I:C)-treated mice restored the elevated CD8$^+$ T cell responses seen in mice treated with famciclovir alone (Fig. 6A). We also measured the response to M38, an inflationary epitope that we previously observed to be minimally impacted by famciclovir treatment (Fig. 1F). Consistent with previous results, famciclovir caused only a slight nonsignificant increase in the response to M38. Similarly, poly(I:C) treatment and IFN-α/βR blockade had little impact on the response to this inflationary epitope. These results suggest that the impact of famciclovir on the T cell response to conventional epitopes is attributable to the fact that a large type I IFN response does not develop in famciclovir-treated mice. Together, these results indicate that type I IFN, whether induced by replicating virus or by poly(I:C), depletes cDCs, and this negatively impacts the acute CD8$^+$ T cell response to MCMV infection. During chronic lymphocytic choriomeningitis (LCMV; clone 13) infection, DCs that express PD-L1 and IL-10 can suppress the CD8$^+$ T cell response. These suppressive DCs are dependent on type I IFN (31, 32). It was possible that type I IFN could be inducing similarly suppressive DCs during acute MCMV infection. It was shown that PD-L1 blockade reverses exhaustion during chronic LCMV infection (31, 32). To test whether PD-L1 on DCs induces a suppressive mechanism on M45- and M57-specific CD8 T cells, we used anti–PD-L1 Ab blockade during acute MCMV infection. Fig. 6E shows that PD-L1 blockade did not increase the CD8$^+$ T cell response in MCMV-infected mice and suggests that M45- and M57-specific CD8 T cells are not suppressed by a PD-1/PD-L1 mechanism.

## Discussion

Increased virus replication usually results in an increased peak CD8$^+$ T cell response by increasing the overall amount of Ag and inducing inflammatory cytokines that promote CD8$^+$ T cell proliferation (signal 3). The inflammatory cytokines also promote CD8$^+$ T cell differentiation to an SLEC phenotype. In this article we describe a situation in which increased virus replication causes the expected increase in virus-specific SLECs; however, its impact on virus-specific CD8$^+$ T cell numbers is opposite from what would be expected, with 2–3-fold lower peak responses to major epitopes in the presence of virus replication. We show that a large type I IFN response elicited by replicating virus depletes cDCs and that depletion of cDCs is responsible for the lower CD8$^+$ T cell response.

In most experimental and clinical situations the profound impact of type I IFNs on viral load is the principal determinant of any

immunological consequences; however, type I IFNs are pleiotropic cytokines with the potential to impact the immune response at multiple levels (33). They promote DC maturation for effective costimulation and cross-presentation (34, 35), and they act directly on TCR-activated $CD8^+$ T cells to provide signal 3 to promote proliferation (36–39). Consequently, they usually have a positive impact on CD8 T cell response to vaccination (40, 41). Conversely, for certain single-cycle vaccine vectors, inhibition of viral gene expression by type I IFN can reduce the $CD8^+$ T cell response (42, 43). Type I IFNs can also be directly immunosuppressive. At the point of T cell priming, if IFN signaling precedes TCR engagement, the impact of IFN is suppressive (44). Type I IFNs can promote IL-10–secreting DCs and $CD4^+$ T cells (31, 32, 45, 46). In fact, type I IFN blockade results in clearance of chronic LCMV infection (31, 32), which results from the induction of suppressive DCs that secrete IL-10 and express PD-L1.

Type I IFNs were described to impact DC numbers in several experimental models. In particular, type I IFN was reported to decrease cDC numbers in the spleen, most likely by increasing their turnover (28) and/or preventing their replenishment from the bone marrow (35). Type I IFNs particularly affect the $CD8\alpha^+$ subset (28), which is required for cross-presentation.

In the study most analogous to our work, Dalod and colleagues (4) studied the $CD8^+$ T cell response to acute MCMV infection in BALB/c mice, focusing on a single epitope. They found that higher virus titers, whether due to lack of NK cells or to a drug-sensitive recombinant MCMV, resulted in a delayed initial appearance of $CD8^+$ T cells without impacting their peak numbers. They showed that type I IFN reduced splenic $CD11c^+$ DCs during acute MCMV infection (4) and that this was responsible for the delayed appearance of $CD8^+$ T cells in BALB/c mice with higher virus titers. In C57BL/6 mice, we found a similar impact of virus-induced type I IFN on cDCs in the spleen; however, the consequent impact on $CD8^+$ T cells was different. We found no difference in the time of appearance of MCMV-specific T cells, and instead report a later increase in the $CD8^+$ T cell response that resulted in a substantially higher peak response. As discussed further below, high virus titers impacted responses to only a subset of epitopes. The difference between our results and those of Dalod and colleagues (4) is likely due to the different nature of the epitope-specific $CD8^+$ T cell response in the two mouse strains. The acute $CD8^+$ T cell response in C57BL/6 mice consists of two populations: inflationary and noninflationary. In contrast, in BALB/c mice, both of the $CD8^+$ T cell responses that are immunodominant during acute infection, including the IE1-specific T cells studied by Dalod and colleagues (4), also induce memory inflation. It is now known that, after the T cells have been primed, memory inflation is sustained by Ag presentation on nonhematopoietic cells (22, 23). Our results show that type I IFN–induced depletion of DCs results in reduced peak $CD8^+$ T cell responses specifically to noninflationary immunodominant epitopes while having little impact on inflationary epitopes.

Replicating virus caused a loss of DCs that was most pronounced 2–4 d pi, and this could be recapitulated by treatment of CD11c-DTR mice with DT on day 2. In either case, the loss of cDCs after the initial priming of $CD8^+$ T cells resulted in reduced numbers of $CD8^+$ T cells at the peak of infection. These results suggest that a maximal $CD8^+$ T cell response required continued Ag presentation and/or costimulation beyond day 3. Although 24 h of Ag exposure is sufficient to program the responding T cell population to proliferate for an initial clonal burst (47), prolonged Ag exposure was shown to increase the size of the clonal burst in several models (38, 48–52). Thus, the increased response to M45 and M57 that we observed with nonreplicating virus is likely caused by prolonged Ag presentation because of preserved cDC populations. Our results reinforce the concept that, although initial priming programs a substantial clonal expansion, the continued presence of Ag can give T cells a "second wind" and increase the ultimate size of the response.

In chronic LCMV infection, type I IFN induces a DC population that suppresses $CD8^+$ T cell responses by expressing PD-L1 and secreting IL-10 (31, 32), In our experiments, it is possible that, in addition to impacting $CD8^+$ T cell numbers, type I IFN modulates the phenotype and, perhaps trafficking, of cDCs. These factors could be involved in the overall suppressive role for type I IFN in this model. However, blockade of PD-L1 did not alter the immunosuppressive effect of type I IFN in our experiments (Fig. 6E). Furthermore, the fact that cDC depletion on day 2 pi decreased the $CD8^+$ T cell response is evidence for a predominant postpriming immune-stimulatory role for cDCs in drug-treated mice, rather than a predominant immunosuppressive role in untreated mice.

It is interesting that famciclovir primarily impacted the responses to two immunodominant epitopes, M45 and M57, whereas it did not increase the frequency of $CD8^+$ T cell responses to subdominant epitopes or to two other dominant epitopes, m139 and M38. The subdominant epitopes may not be able to compete with the dominant responses when Ag becomes limited, which probably explains their subdominance and their failure to respond to famciclovir's preservation of cDCs. However, within the dominant responses, the selective boosting of only M45- and M57-specific populations is quite striking. Unlike M45 and M57, dominant responses to m139 and M38 are subject to memory inflation. Memory inflation is the term used to describe the sustained or increased numbers of responses to a subset of MCMV epitopes that occurs during chronic infection. As in other infections, DCs are likely responsible for the very initial priming of inflationary and noninflationary responses. Remarkably, however, two recent studies (22, 23) used elegant bone marrow chimera models to demonstrate that the Ag presentation that sustains memory inflation takes place entirely on cells of nonhematopoietic origin. Thus, we propose that, after initial priming by DCs, Ag presented on nonhematopoietic cells is likely sufficient to drive optimal $CD8^+$ T cell responses to M38 and m139, even when DCs were depleted. In contrast, the responses to M45 and M57 do not inflate and, therefore, are likely to be dependent on Ag presented by DCs. Several lines of evidence suggest that the acute response to MCMV is driven predominantly or exclusively by cross-presented Ag (25, 26). Indeed, the antigenic peptide from M45 ($M45_{985–993}$) is dependent on the immunoproteasome for production (53). Hence, depletion of DCs would remove Ag for those responses. Our results are somewhat analogous to a recent study (52) of the primary $CD8^+$ T cell response to influenza virus. In that study, depletion of $CD11c^+$ DCs at day 6 pi reduced the peak (day 8) $CD8^+$ T cell response to the nucleoprotein Ag, which is strongly cross-presented by DCs at day 6 pi. In accordance with that study, we conclude that Ag cross-presented by DCs beyond day 3 of infection can increase the size of the primary $CD8^+$ T cell response and further bias the response to immunodominant epitopes.

Overall, our data show that modulation of Ag-presenting cDCs after T cell priming can have dramatic effects on the size of the acute $CD8^+$ T cell populations and the numbers of memory cells that are formed. Our work points to type I IFN as a critical mediator of this process and shows that inflammation can have diverse effects on a developing T cell population.

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## Disclosures

A.B.H. is a witness for a civil case regarding an autoimmune disease. A.B.H. has equity in a startup company; patents are pending regarding the use of CMV as a tumor vaccine vector. The other authors have no financial conflicts of interest.

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