Brief Report

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CpG pretreatment enhances antiviral T-cell immunity against cytomegalovirus

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Key Points

- Fully functional CD8 T-cell responses, control of infection, and protection from organ pathology are attained without cross-presentation.
- Direct presentation generates responses that limit disease and ensure host survival despite the presence of immunomodulatory viral proteins.

Major histocompatibility complex class I–restricted T-cell immunity is essential to control infection with cytomegalovirus (CMV), a clinically important virus that causes significant disease in immunocompromised individuals. Cross-presentation is considered the primary mode of antigen presentation to generate protective antiviral CD8$^+$ T-cell immunity. Herpesviruses, including CMV, encode numerous proteins that interfere with direct antigen presentation, leading to the paradigm that T-cell immunity to these pathogens necessitates cross-presentation. However, the antigen presentation requirements needed to generate a protective T-cell response to CMV remain unknown. Here, we show that a fully functional antiviral CD8$^+$ T-cell response can be generated in a system where cross-presentation is shut down by pretreatment with CpG. Notably, in this setting, CD8$^+$ T cells demonstrate accelerated control of infection, and organ pathology is limited. These data indicate that protective antiviral T-cell immunity to CMV is generated by direct presentation and can be enhanced by pretreatment with CpG. (*Blood*. 2013;122(1):55-60)

Introduction

The generation of antigen-specific CD8$^+$ T-cell responses is integral to effective cell-mediated antiviral immunity. Indeed, the control of infections with many viral pathogens is highly dependent on the timely and successful priming of naive T cells by professional antigen-presenting cells (APCs). It has been widely reported, and is now generally accepted, that cross-presentation, namely the acquisition of exogenous antigen for reprocessing and presentation on major histocompatibility complex class I (MHC-I) molecules, is crucial for antiviral CD8$^+$ T-cell immunity.[1] Cross-presentation allows dendritic cells (DCs) to initiate a response to pathogens that do not infect DCs, inactivate them, or eliminate them in the process of infection. Cytomegalovirus (CMV) has been studied in great detail with regard to the capacity to interfere with antigen presentation. Although CMV readily infects both DCs and macrophages,[2,3] the principal APC populations, cross-presentation has been postulated to be critical for the generation of antiviral CD8$^+$ T-cell responses because the virus encodes several proteins that impair antigen presentation.[4] However, the effects of these viral proteins may be limited, especially in vivo.[5]

Despite antiviral therapies, CMV still poses a significant clinical problem and remains the most predictable and problematic infection in bone marrow and solid organ transplantation, and a major health risk in immunocompromised patients generally.[6,7] Most pathology associated with CMV infection is due to an inability to generate appropriate CD8$^+$ T-cell immunity[8]; thus, a better understanding of the processes required to prime protective T-cell responses would be significant. Importantly, the critical question as to whether protective antiviral CD8$^+$ T-cell responses are elicited by cross-presentation has not been addressed.

Here, we assessed the importance of direct vs cross-presentation in the development of antiviral T-cell immunity in a model of CMV infection where antiviral CD8$^+$ T-cell responses are essential for host survival.[8-11] We used this system to analyze not only the development of antiviral CD8$^+$ T-cell immunity, but also whether the responses generated could afford protection. As such, this is the first study to define the role of direct vs cross-presentation in the generation of protective antiviral immunity to a clinically important viral pathogen.

Study design

Mice

BALB/c and congenic BALB.B6-CT6 (NK1.1$^+$Ly49H$^-$)[12] mice were used interchangeably. Other strains included: BALB/c.*IFNAR1*$^{-/-}$, BALB/c.*IFNAR2*$^{-/-}$, and CL4-TCR transgenic mice which express transgenic T-cell receptor (TCR) chains specific for the H2-K^d–restricted epitope IYSTVASSL from the influenza virus A/PR/8/34 hemagglutinin (HA)

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protein.[13] Experiments were performed with the approval of the Animal Ethics and Experimentation Committee of the University of Western Australia, in accordance with the guidelines of the National Health and Medical Research Council (NHMRC) Australia.

Cross-priming assay

BALB/c mice were injected IV with 5×10^6 to 1×10^7 per mouse carboxyfluorescein diacetate succinimidyl ester (CFSE)–labeled leukocytes from CL4-TCR transgenic mice. Mice were then injected intraperitoneally (IP) with saline or 20 nmol CpG-1668 and 18 hours later were infected IP with 5×10^3 plaque-forming units (PFU) per mouse of MCMV-K181-Perth. Cell-associated HA (8×10^5 cells per mouse) was delivered IV. Non-HA–containing lysate was used to confirm that in vivo proliferation of transgenic CL4-$CD8^+$ T cells was specific to the HA antigen. Proliferation of CL4-$CD8^+$ T cells was measured by CFSE dilution.

Details of additional methods are available as supplemental Methods (see the supplemental Methods link at the top of the online article).

Results and discussion

Preexposure to Toll-like receptor (TLR) ligands, like CpG, has been shown to impair cross-presentation of exogenous antigens and thereby interfere with the development of $CD8^+$ T-cell responses to a number of viruses including influenza and herpes simplex virus 1.[14,15] We confirmed these findings in BALB/c mice using a novel H2-K^d–restricted HA system. Mice received CpG and CFSE-labeled $CD8^+$ T cells from CL4 TCR transgenic mice prior to challenge with cell-associated HA. Cell-associated HA was prepared by infecting NIH 3T3 fibroblasts with a recombinant murine CMV (MCMV) expressing the CL4 epitope from the HA protein of the mouse influenza virus (see supplemental Methods). In this system, the virus is used as a source of the CL4 HA epitope. Because 3T3 fibroblasts do not express H2-K^d, proliferation of the transferred T cells, as measured by CFSE dilution, will only occur if the CL4 epitope is cross-presented by host DCs. Pretreatment with CpG shut-down cross-presentation as demonstrated by the lack of CL4 proliferation after challenge with cell-associated HA (Figure 1A). Given the intention to use CpG pretreatment to shut-down cross-presentation prior to MCMV infection, we next examined whether the impairment in cross-presentation observed after CpG pretreatment is maintained when the host is also exposed to a viral infection that may recruit new DC progenitors. Newly recruited DC progenitors may provide an additional source of APCs, thereby bypassing the CpG-induced impairment in cross-presentation. Cross-presentation, however, remained impaired when CpG-treated mice were also infected with MCMV at the time of challenge with cell-associated HA (Figure 1B). These results demonstrate that viral infection is unable to bypass the impairment in cross-presentation induced by TLR ligand pretreatment.

Systemic treatment of mice with CpG can severely impair pathogen-specific $CD8^+$ T-cell immunity.[14,15] In BALB/c mice, $CD8^+$ T cells specific for an immunodominant epitope derived from the MCMV IE1 gene product are critical to control acute MCMV infection.[9] To determine whether impaired cross-presentation could affect MCMV-specific $CD8^+$ T-cell responses, we examined the numbers and functions of $CD8^+$ T cells both ex vivo and in vivo. Total numbers of $CD8^+$ T cells remained constant over the course of MCMV infection and IE1-specific $CD8^+$ T cells became detectable at days 6 and 10 postinfection (PI) (Figure 1C). CpG alone did not affect $CD8^+$ T-cell numbers, yet treatment of mice with CpG prior to infection with MCMV resulted in significant $CD8^+$ T-cell expansion, such that total $CD8^+$ T cells and IE1-specific $CD8^+$ T-cell numbers were 2-fold higher in the spleen at days 6 and 10 PI compared with mice infected with MCMV alone (Figure 1C).

Surface and intracellular expression of CD107a were used to enumerate degranulating $CD8^+$ T cells. Compared with untreated mice, CpG-treated mice had significantly ($P = .004$) higher numbers of $CD107a^+CD8^+$ T cells during infection (Figure 1C). Next, we measured the cytotoxic capacity of $CD8^+$ T cells both ex vivo and in vivo. For the ex vivo assay, splenocytes from untreated and CpG-treated mice were harvested at days 6 and 10 after MCMV infection and cultured ex vivo with Cr^{51}-labeled target cells expressing the viral antigen IE1 (IE1-pulsed P815 target cells). The ex vivo cytotoxic activity of splenocytes from MCMV-infected mice was maximal at day 6 PI and was not affected by CpG treatment (Figure 1D). Similarly, the lytic activity of IE1-specific $CD8^+$ T cells in vivo, measured using an in vivo CTL assay, demonstrated that robust and efficient antiviral $CD8^+$ T-cell responses were generated in a system where cross-presentation was absent because of prior exposure to CpG (Figure 1E).

In BALB/c mice, control of acute MCMV infection is highly dependent on virus-specific $CD8^+$ T cells, with IE1-specific $CD8^+$ T cells essential to reduce viral replication and protect from viral-induced disease.[9] Pretreatment with CpG resulted in a significant reduction in viral loads in visceral organs of MCMV-infected mice, but had less impact on viral replication in the salivary glands (Figure 2A). Reduced viral titers were observed from day 2 PI, suggesting that CpG pretreatment improves early innate immune responses. Further decreases in viral titers were observed at days 4 and 6 PI, and MCMV infection resolved earlier in mice pretreated with CpG (Figure 2A). Because control of late acute MCMV infection is dependent on $CD8^+$ T cells, these results indicate that the $CD8^+$ T-cell responses generated in the context of CpG pretreatment and impaired cross-presentation can confer protection.

Infection of BALB/c mice with a virulent MCMV strain results in spleen necrosis and atrophy (Figure 2B), a phenotype characterized by a general loss of architecture with regions of severe necrosis and scaring (Figure 2C). Remarkably, the spleens of CpG-treated mice showed no macroscopic signs of necrosis, nor a loss of splenic architecture (Figure 2B-C). Thus, while CpG administration did not alter the course of disease in terms of spread of virus to target organs, it significantly reduced the severity of MCMV infection and disease.

Given the early reduction in viral titers observed in the visceral organs of MCMV-infected mice that were pretreated with CpG, we investigated the role of innate cytokines. Interferon-α (IFN-α) was detected as early as 6 hours after CpG treatment (Figure 2D). To determine whether type I IFNs promote the early decreases in viral titers observed in CpG-treated mice, we examined viral loads in BALB/c.*IFNAR1*$^{-/-}$ and BALB/c.*IFNAR2*$^{-/-}$ mice. In mice unable to respond to type I IFNs, viral titers were equivalent with or without CpG pretreatment (Figure 2E). Thus, the induction of type I IFNs accounts for the early reduction in viral loads observed in mice pretreated with CpG. Treatment of fibroblasts with type I IFNs directly impairs the replication of human CMV and MCMV by interfering with the transcription of *IE* genes.[16,17] Our current results confirm the role of these cytokines in limiting early MCMV replication.

Because antiviral $CD8^+$ T-cell responses were generated effectively in a system where cross-presentation is disabled, we depleted $CD8^+$ T cells to determine whether the improved control of MCMV infection observed after CpG pretreatment was indeed dependent on $CD8^+$ T-cell–mediated responses. On days 2 and 4 PI, viral titers were equivalent in CD8-depleted and undepleted mice

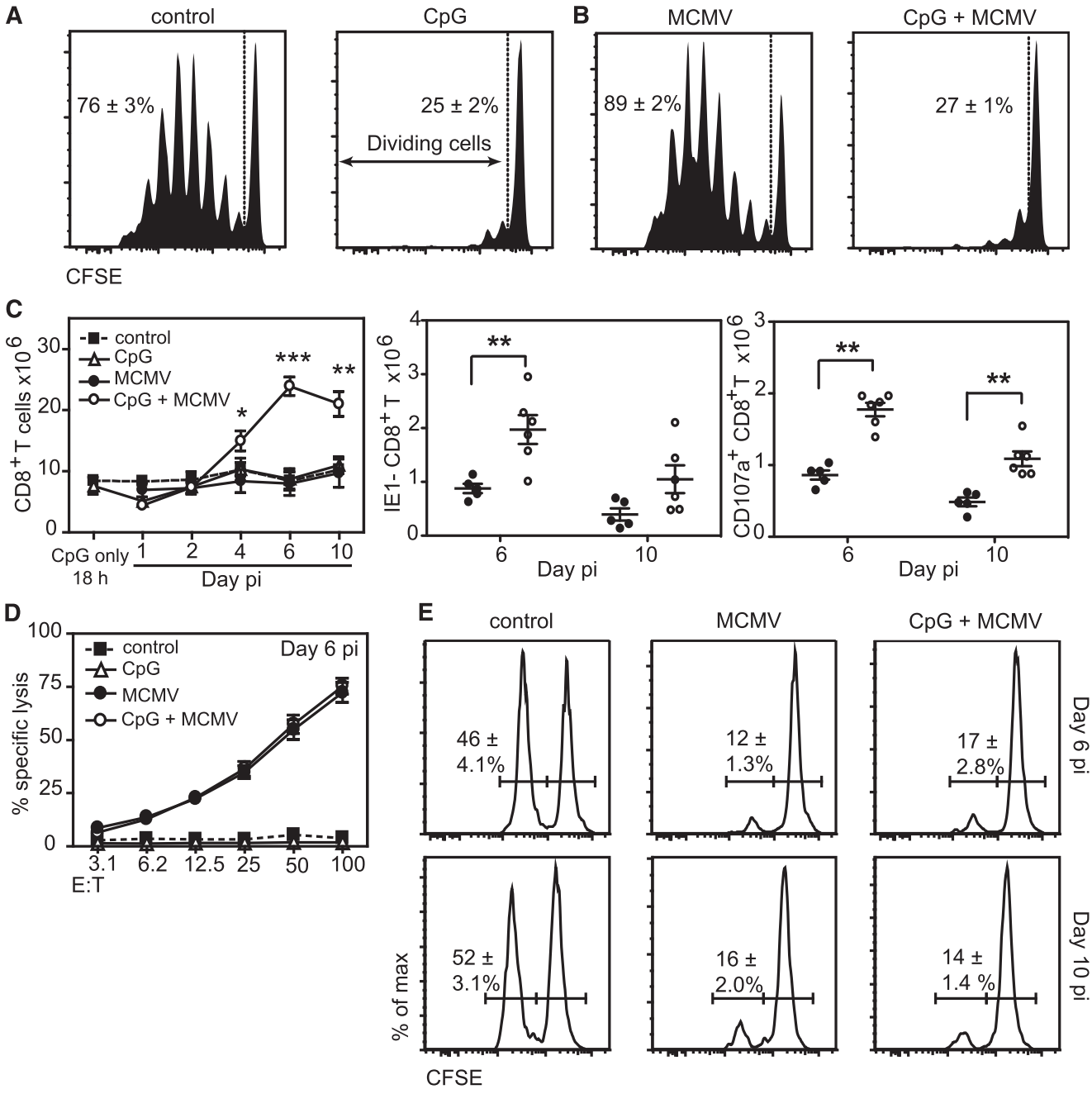

Figure 1. Effective MCMV-specific CD8$^+$ T-cell responses are elicited after inhibition of cross-presentation. (A) The cross-presenting capacity of DCs after CpG administration was assessed by measuring the proliferation of CL4 TCR transgenic HA-specific CD8$^+$ T cells. BALB/c mice treated with saline (control) or 20 nmol CpG (CpG) received CFSE-labeled leukocytes from CL4 transgenic mice. Cell-associated HA was administered 18 hours later. Proliferation of CL4 CD8$^+$ T cells was measured in the spleen 4 days after transfer. Results are representative of 3 experiments with at least 2 mice per group. (B) The cross-presenting capacity of DCs after CpG activation and MCMV infection was assessed as in panel A, except that mice were infected IP with MCMV at the time of injection of cell-associated HA. Results are representative of 3 experiments with at least 2 mice per group. (C) BALB/c mice received either saline or CpG 18 hours prior to infection with MCMV (5×10^3 PFU per mouse) and T-cell responses were measured at various times PI. CD8$^+$TCRβ^+ (left), IE1-specific CD8$^+$TCRβ^+ (middle), and CD107a$^+$CD8$^+$TCRβ^+ (right) cells were enumerated in the spleens of mice that were infected with MCMV with or without CpG pretreatment. Data are pooled from 2 to 4 independent experiments (where n = 5-12 mice per time point), except at day 1 PI (where n = 3) (*$P < .05$, **$P < .01$, ***$P < .001$). (D) The lytic activity of antiviral, IE1-specific CD8$^+$ T cells was measured ex vivo by harvesting splenocytes on day 6 PI and culturing them with Cr^{51}-labeled, IE1-pulsed P815 target cells for 4 hours at various E:T ratios. Specific lysis was determined as noted in supplemental Methods. Data are from 2 independent experiments (where n = 6 infected mice per group). (E) The lytic activity of antiviral, IE1-specific CD8$^+$ T cells was measured using an in vivo CTL assay. Untreated or CpG pretreated mice were infected with MCMV (5×10^3 PFU per mouse IP); at days 5 and 9 PI, the mice received a 1:1 mixture of CFSE^{lo} IE1 peptide-pulsed and CFSE^{hi} unpulsed splenocytes IV. Spleens were harvested on days 6 and 10 PI and IE1-specific CTL killing measured by flow cytometry as the loss of CFSE^{lo} IE1-pulsed targets compared with CFSE^{hi} unpulsed targets. Histograms are representative of 2 independent experiments (n = 4-6 mice per group per time point). Percentages shown are mean ± SEM. E:T, effector to target ratio.

pretreated with CpG (Figure 2F), consistent with the role of type I IFNs described in the previous paragraph. However, after day 4, viral titers increased in mice depleted of CD8$^+$ T cells to levels equivalent to those observed in undepleted mice that had not received CpG prior to MCMV infection (Figure 2F). These results demonstrate that in a system where cross-presentation is shut down,

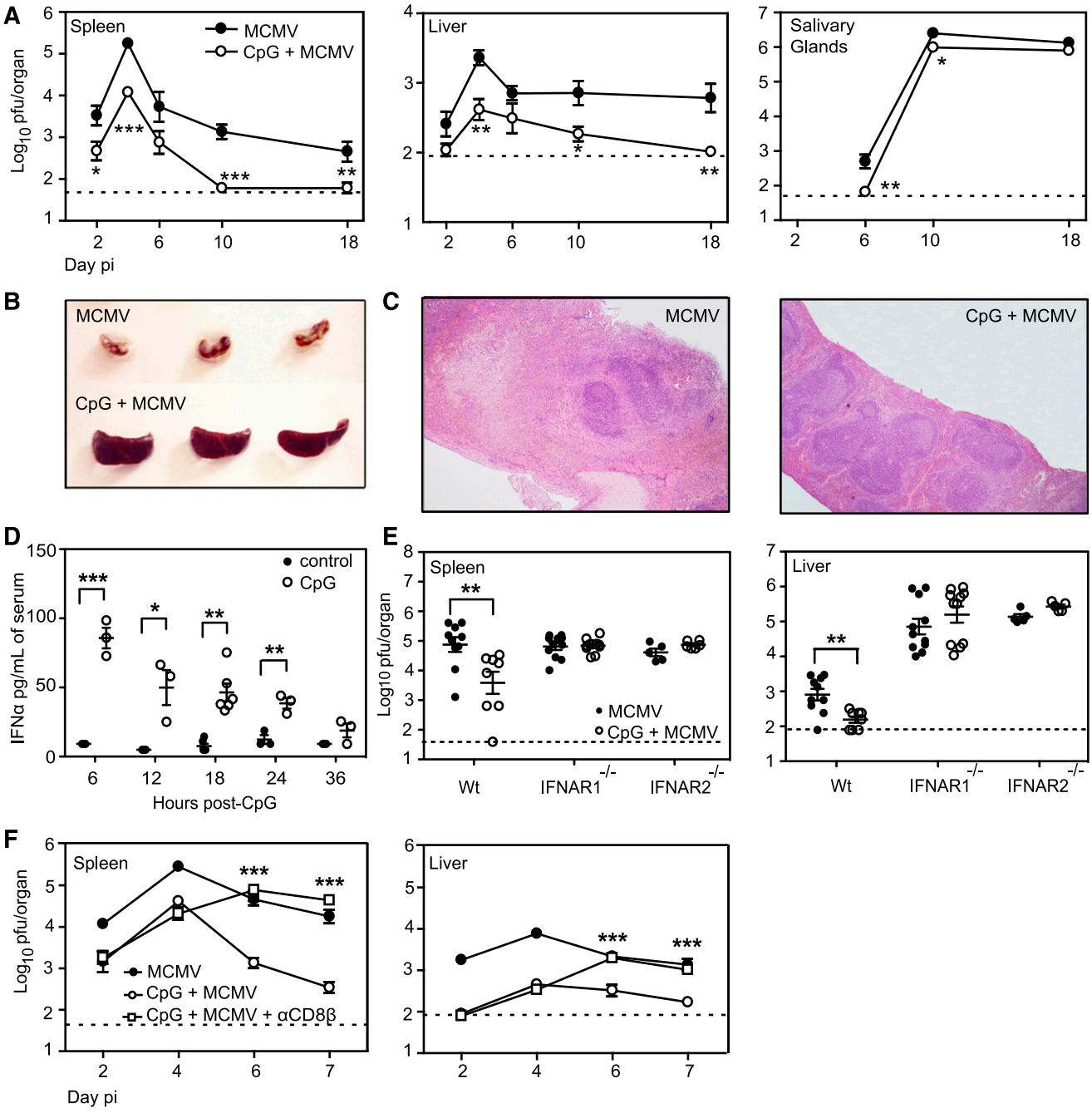

Figure 2. Improved control of MCMV infection and limited virus-induced pathology after CpG pretreatment are due to activities mediated by type I IFNs and improved CD8$^+$ T-cell responses elicited in the absence of cross-presentation. (A) Viral titers were measured in the organs of BALB/c mice treated with CpG 15 to 18 hours prior to MCMV infection. The indicated organs were harvested at various times PI and viral titers determined by plaque assay. Data are pooled from 4 independent experiments (n = 5-14 mice per time point). Mean ± SEM are plotted. The dashed line represents the limit of detection of the assay. Mice were injected with saline or CpG 18 hours prior to infection with MCMV and (B) gross spleen morphology and (C) spleen histology examined at day 42 PI. For histology, spleens were stained with H&E and images were taken at ×4 magnification. (D) Serum IFN-α levels were measured by ELISA at the indicated times after CpG administration (n = 3-6 mice per group at each time point). (E) Viral titers were measured in the organs of BALB/c (Wt), BALB.IFNAR1$^{-/-}$, and BALB.IFNAR2$^{-/-}$ mice pretreated with CpG 18 hours prior to MCMV infection. Viral titers in spleens and livers at day 4 PI are shown as mean ± SEM (where n = 5-11 mice per group), pooled from 3 independent experiments. The dashed horizontal line represents the limit of detection of the assay. (F) BALB/c mice were injected with saline or CpG 18 hours prior to MCMV infection. Mice were depleted of CD8$^+$ T cells at days −2, 0, 2, and 6 with respect to MCMV infection, using an anti-CD8β antibody (53.5.8). Spleens and livers were harvested at the indicated time points and viral titers measured. Data are pooled from 2 independent experiments (where n = 5-10 mice per time point). *P* values were determined between CpG + MCMV and CpG + MCMV + anti-CD8β (*$P < .05$, **$P < .01$, ***$P < .001$). ELISA, enzyme-linked immunosorbent assay; H&E, hematoxylin and eosin; Wt, wild type.

antiviral CD8$^+$ T-cell responses elicited by direct presentation effectively limit MCMV infection.

Pretreatment with TLR ligands has been associated with both improved[18-20] and impaired[14,15] immunity to subsequent pathogen challenge. Such paradoxical effects are likely to have a variety of explanations, but where CpG is concerned, the importance of cross-presentation for generating protective immunity may be a key consideration. Here, we show that systemic treatment with CpG improves

the control of MCMV infection despite disabling cross-presentation. CpG pretreatment accelerated the control of replicating virus and protective antiviral CD8$^+$ T-cell–mediated immunity developed in the absence of cross-presentation. The implications of these findings are twofold: first, direct presentation of viral antigen is sufficient to generate an effective CD8$^+$ T-cell response to CMV, and second, the influence of CpG on improved T-cell immunity may be attributable, at least in part, to the fact that direct presentation may be a more efficient means to elicit a protective antiviral T-cell response.

Our results differ from those reported in a study that used Batf3-deficient mice, which exhibit defects in the development of CD8α$^+$ and CD103$^+$CD11b$^-$ DCs,[21] the classical cross-presenting DC populations,[1] to examine the relevance of cross-presentation during MCMV infection in C57BL/6 mice.[22] It is worth noting that in this study viral loads were equivalent in C57BL/6 wild-type and Batf3-deficient mice despite differences in CD8$^+$ T-cell responses.[22] The inability to generate MCMV-specific CD8$^+$ T-cell responses in C57BL/6 Batf3-deficient mice has been reported by a separate study which attributed the defect to a dependence on CD8α$^+$ DCs for priming of MCMV-specific T cells, but independent of cross-presentation, because normal antiviral CD8 T-cell responses were generated in CD11c-Rac mice which display an inability to uptake exogenous antigen resulting in a marked defect in cross-presentation.[23] The data in Batf3-deficient mice are further complicated by recent evidence showing that Batf3 is not required for the generation of CD8α$^+$ DCs in inflammatory settings.[24] Most importantly, however, it should be noted that, in C57BL/6 mice, CD8$^+$ T-cell responses are irrelevant for protection from MCMV infection (which is natural killer cell dependent) and indeed, depleting CD8$^+$ T cells does not affect viral replication nor host survival.[10,11] In contrast, we took advantage of the fact that systemic administration of CpG inhibits cross-presentation[14] to examine how this affects control of MCMV infection in BALB/c mice, a model where CD8$^+$ T-cell–mediated immunity is essential to protect the host from disease. We found that optimal protective antiviral CD8$^+$ T-cell responses were generated in the absence of cross-presentation. CD8$^+$ T-cell responses to vaccinia virus[25] are also unaffected in the absence of cross-presentation, but whether these responses are protective remains to be examined.

In addition to effectively blocking cross-presentation (Figure 1A-B), CpG induced a burst of type I IFNs (Figure 2D). Analysis of DC populations at 18 hours after CpG administration revealed a phenotypic loss of CD8α$^+$ DCs, an increase in CD11b$^+$ DCs, and concomitant DC activation, measured by the increased cell-surface expression of CD80 and CD86 (supplemental Figure 1A-C), effects which may be attributed to type I IFNs. As shown (Figure 2E), the CpG-induced type I IFNs also led to early decreases in viral loads, consistent with the fact that CMV does not effectively replicate in cells preexposed to these cytokines.[16,17] The enhanced T-cell response measured after CpG pretreatment, characterized by increased numbers of virus-specific IE1$^+$ CD8$^+$ T cells (Figure 1C), may reflect preservation of splenic viability (Figure 2B-C) as a consequence of type I IFN-dependent reductions in early viral burden. The possibility, however, remains that CpG-induced type I IFNs may also improve direct presentation through enhanced DC activation.

Despite the prediction that CMV gene products that interfere with MHC-I presentation function to evade recognition and killing of infected cells by cytotoxic CD8$^+$ T cells, previous studies have shown that MCMV genes that impede MHC-I presentation have no impact on the size, kinetics, and immunodominance hierarchy of antiviral CD8$^+$ T-cell responses,[5] suggesting that they may play a role in situations other than priming of antiviral T-cell immunity. Indeed, studies analyzing the role of rhesus CMV genes that encode inhibitors of MHC-I antigen presentation, elegantly demonstrated that these proteins are dispensable for the establishment and maintenance of primary and persistent infection, but are essential for superinfection of immunocompetent hosts with distinct CMV strains.[26] The results of our study prove that generation of protective antiviral CD8$^+$ T-cell immunity occurs independently of cross-presentation, and thus provide independent evidence that subversion of MHC-I presentation is unlikely to improve viral fitness during primary infection. Our results also provide the first evidence that CD8$^+$ T-cell responses generated in the absence of cross-presentation are sufficient to confer protection against CMV disease.

Our studies challenge the current dogma that cross-presentation is crucial for the generation of antiviral immunity and show that this is not the case, at least for some viruses. These results have the potential to change the current paradigm guiding the design of improved therapeutic strategies to combat infection with CMV, a pathogen able to cause severe clinical disease, especially in immune-compromised hosts.

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Authorship

Contribution: M.L.O. and M.E.W. generated and analyzed data and wrote the manuscript; P.F. generated and analyzed data; M.J.E. assisted with data analysis; P.J.H. provided critical reagents; G.R.H. assisted with experimental design and data analysis; and C.E.A. and M.A.D.-E. devised the project, designed the experiments, analyzed data, and wrote the manuscript.

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