The Journal of Immunology

A Ceramide Analogue Stimulates Dendritic Cells To Promote T Cell Responses upon Virus Infections

Curtis J. Pritzl, Young-Jin Seo, Chuan Xia, Madhuvanthi Vijayan, Zachary D. Stokes and Bumsuk Hahm

J Immunol 2015; 194:4339-4349; Prepublished online 25 March 2015;
doi: 10.4049/jimmunol.1402672
http://www.jimmunol.org/content/194/9/4339

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

A Ceramide Analogue Stimulates Dendritic Cells To Promote T Cell Responses upon Virus Infections

Curtis J. Pritzl, Young-Jin Seo, Chuan Xia, Madhuvanthi Vijayan, Zachary D. Stokes, and Bumsuk Hahm

The ceramide family of lipids plays important roles in both cell structure and signaling in a diverse array of cell types, including immune cells. However, very little is known regarding how ceramide affects the activation of dendritic cells (DCs) in response to viral infection. In this study, we demonstrate that a synthetic ceramide analog (C8) stimulates DCs to increase the expansion of virus-specific T cells upon virus infection. Exogenously supplied C8 ceramide elevated the expression of DC maturation markers such as MHC class I and costimulatory molecules following infection with the clone 13 strain of lymphocytic choriomeningitis virus (LCMV) or influenza virus. Importantly, ceramide-conditioned, LCMV-infected DCs displayed an increased ability to promote expansion of virus-specific $CD8^+$ T cells when compared with virus-infected DCs. Furthermore, a locally instilled ceramide analog significantly increased virus-reactive T cell responses in vivo to both LCMV and influenza virus infections. Collectively, these findings provide new insights into ceramide-mediated regulation of DC responses against virus infection and help us establish a foundation for novel immune-stimulatory therapeutics. ***The Journal of Immunology*****, 2015, 194: 4339–4349.**

Ceramide describes a family of sphingolipids that are composed of a sphingosine molecule linked to a fatty-acyl chain by an amide bond (1). These bioactive lipids can have both structural and signaling roles. Ceramide and small-chain analogs of ceramide have been shown to have a multitude of effects on a wide array of different cell types. Synthetic, short-chain ceramide molecules have proven to be much more soluble than endogenously produced long-chain ceramides and therefore have been used frequently in many different experimental systems (2–4). Primarily, small-chain ceramide analogs have been described as cell cycle arrest agents (5–7) or proapoptotic molecules (8–11). However, ceramide molecules have also been designated as a trigger of cellular differentiation (2, 12, 13), and shown to be involved in inflammatory processes (14).

The functions of ceramide and ceramide-metabolizing enzymes in immune responses are only beginning to be understood. The ceramide-metabolizing enzyme acid sphingomyelinase has been shown to play a key role in the degranulation of T cells, a mechanism critical to their effector function (15). Moreover, it was shown that the cross-linking of CD28 activates acid sphingomyelinase, which enhances the transmission of the signal to NF-κB in Jurkat T cells (16). In a recent lipidomic study, an increase in the production of 24-carbon ceramide has been demonstrated to occur during LPS-induced dendritic cell (DC) maturation, which again suggests roles for ceramide or ceramide-metabolizing enzymes during immune responses (17). However, it has also been shown that ceramide can inhibit the production of inflammatory cytokines from LPS-stimulated mast cells (18). These data suggest that ceramide may have as of yet unknown functions in the initiation or maintenance of pathogen-induced immune responses.

DCs are key regulators of immune responses (19). These cells efficiently transmit the pathogens' danger signal to pathogen-specific T lymphocytes (20). DCs sense pathogen-associated molecular patterns through highly conserved pattern-recognizing receptors such as TLRs (21, 22). Following pathogen-associated molecular pattern recognition, DCs undergo maturation, which involves the upregulation of molecules for Ag presentation and the costimulation of T lymphocytes.

Because of this key role in the initiation of immune responses, many viruses, such as HIV and the clone 13 strain of lymphocytic choriomeningitis virus (LCMV Cl 13), have evolved to subvert DC maturation or evade detection by these DCs (23–26). Unlike its parental strain, LCMV Cl 13 has been shown to persist in mice (27) by nullifying the function of host immune system including the suppression of DC maturation (23, 28) and antiviral T cell immunity (29–31). Currently, the most successful treatments have involved the blockade of inhibitory receptor interactions (29, 32) or interference with pro- and anti-inflammatory cytokine production (33–36). Although ceramides have been shown to have regulatory functions in many cell types, their roles in DC maturation or the suppression of DC responses by viruses have not been investigated. In this study, we provide evidence that conditioning DCs with an exogenous, short-chain ceramide analog results in more potent DC function in vitro and in vivo. In addition, local administration of the ceramide analog to mice induces more robust $CD8^+$ and $CD4^+$ T cell responses to viral infections.

Materials and Methods

Mice

C57BL/6 (The Jackson Laboratory) and C57BL/6-Thy1.1$^+$D^{b}GP$_{33–41}$ (GP33-specific) TCR transgenic (tg), mice, which are also known as P14

Department of Surgery, University of Missouri, Columbia, MO 65212; and Department of Molecular Microbiology and Immunology, University of Missouri, Columbia, MO 65212

Received for publication October 20, 2014. Accepted for publication February 24, 2015.

This work was supported by National Institutes of Health/National Institute of Allergy and Infectious Diseases Grant R01AI091797 (to B.H.).

Address correspondence and reprint requests to Prof. Bumsuk Hahm, One Hospital Drive, NW301C, University of Missouri, Columbia, MO 65212. E-mail address: hahmb@health.missouri.edu

The online version of this article contains supplemental material.

Abbreviations used in this article: DC, dendritic cell; i.n., intranasal; LCMV, lymphocytic choriomeningitis virus; MFI, mean fluorescence intensity; MHC-I, MHC class I; MOI, multiplicity of infection; NP, nucleoprotein; PD-L1, programmed death ligand 1; PI, propidium iodide; p.i., postinfection; tg, transgenic.

mice, were used (37). Mice were bred and maintained in a closed breeding facility according to institutional guidelines and with protocols approved by the Animal Care and Use Committee of University of Missouri (Columbia, MO).

Virus and infections

The LCMV Cl 13 was propagated on baby hamster kidney cells (37, 38). LCMV titers were determined by plaque assay on Vero cells (37). Influenza virus A/WSN/33 (H1N1) (39) and the influenza virus containing LCMV epitopes (FLU-LCMV) (40, 41) were provided by Y. Kawaoka (University of Wisconsin, Madison, WI) and used in this study. Mice were infected by intranasal (i.n.) or i.v. administration of 2×10^6 PFU LCMV Cl 13 or infected with influenza virus (1×10^5 PFU) or FLU-LCMV i.n. (1×10^5 PFU). Uninfected mice were used in all in vivo experiments as background controls (data not shown).

Bone marrow–derived DCs

DCs were derived from bone marrow cells by culture with RPMI 1640 medium containing 20 ng/ml recombinant mouse GM-CSF (PeproTech) for 10 d (37, 38, 42). DCs were infected with LCMV Cl 13 (multiplicity of infection [MOI] = 10) or influenza virus (MOI = 1 or 5) and treated with either 10 μM C8 ceramide (Cayman Chemical) or an equivalent volume of solvent (DMSO) unless otherwise indicated. DC maturation was assessed at 1 or 2 d postinfection (p.i.). DC viability was assessed 1 or 2 d p.i. by annexin V and propidium iodide (PI) staining.

In vitro T cell proliferation assay

LCMV epitope GP33-specific $CD8^+$ T cells (GP33/$CD8^+$ T cells) were purified from LCMV GP33-specific TCR tg mice using EasySep $CD8^+$ T cell enrichment reagents (Stemcell Technologies) and stained with CFSE (Invitrogen). DCs were infected overnight with LCMV Cl 13 (MOI = 10) or treated with TLR7 agonist loxoribine (0.5 mM) in the presence of GP33 peptide (1 μg/ml) for 1 h. These DCs or GP33/$CD8^+$ T cells were treated with a solvent control or C8 ceramide (10 μM) for 1 h. After washing, GP33/$CD8^+$ T cells were mixed with DCs at a DC:T cells ratio of 1:50. After 3 d, the proliferation of Ag-specific T cells was evaluated by the decrease in CFSE fluorescence observed in flow cytometric analyses.

In vivo DC-mediated T cell proliferation assay

$Thy1.1^+$,LCMV GP33/$CD8^+$ T cells were enriched from LCMV GP33–specific TCR tg mice using EasySep $CD8^+$ T cell enrichment reagents (Stemcell Technologies). A total of 1×10^5 enriched cells were adoptively transferred into C57BL/6 $Thy1.2^+$ recipient mice. The following day, LCMV Cl 13–infected, C8 ceramide, or solvent-treated DCs (5×10^5 DCs/mouse) were adoptively transferred into the recipient mice. At day 7 post-DC transfer, splenocytes were obtained from recipient mice and $CD8^+$ $Thy1.1^+$ cells were enumerated by flow cytometric staining.

Lymphocyte isolation

Although mice were under anesthesia, lungs were perfused with 20 ml PBS. Lung tissue was excised and minced into ~1-mm pieces and then incubated with 1 U/ml collagenase D (Roche) and 1 U/ml DNase I (Fermentas) for 45 min at 37°C with agitation (40, 41). Single-cell suspensions were obtained by forcing lung tissue through stainless steel mesh. Splenocyte suspensions were obtained by forcing spleens through nylon mesh. Cells were collected, and RBCs were lysed. Cells were washed and used for the flow cytometric analysis.

Flow cytometric analysis

Intracellular cytokine staining was performed as described previously (37, 38, 43). Abs used in this study were specific for murine CD11c, MHC class I (MHC-I) ($H\text{-}2K^b$), MHC-II ($I\text{-}A^b$), 4-1BBL, CD40, CD80 (B7-1), programmed death ligand 1 (PD-L1), CD86 (B7-2), CD8α, CD3, CD4, CD90.1 (Thy1.1), IFN-γ, and TNF-α (BD Pharmingen or eBioscience). LCMV-infected DCs were detected by using a rat anti-LCMV nucleoprotein (NP) Ab (VL-4 clone; BioXCell) (37, 44). For the intracellular cytokine staining, lymphocytes were cultured in the presence of 4 μg/ml brefeldin A (Sigma-Aldrich) and 1 μg/ml GP33 (KAVYNFATC), NP396 (FQPQNGQFI), GP276 (SGVENPGGYCL), Flu NP366 (ASNENMDAM), or 5 μg GP61 (GLNGPDIYKGVYQFKSVEFD) peptide for 6 h and then, fixed, permeabilized, and stained with indicated Abs. LCMV GP33–specific $CD8^+$ T cells were identified using fluorochrome-linked GP33 tetramers described previously (45), which were provided by the National Institutes of Health Tetramer Core Facility. Splenocytes were incubated with tetramer and CD8α-specific Abs for 30 min at 4°C and washed, and data were acquired by flow cytometry. Degranulation was assessed by CD107a internalization. Splenocytes were stimulated for 5.5 h with GP33 peptide in the presence of 5 μM brefeldin A (Sigma-Aldrich), 2 μM monensin (Fisher), and CD107a-specific Ab (BD Pharmingen). After stimulation, $CD8^+$ T cells were identified using CD8α-specific Abs. Apoptotic cell death was detected by using an Annexin V-FITC apoptosis detection kit (BD Pharmingen). Briefly, cells (1×10^5) were washed twice with annexin V–binding buffer and then incubated with Annexin V-FITC and PI for 15 min at room temperature in the dark (38). Data were collected on a CyAn ADP flow cytometer (Beckman Coulter) and analyzed with FlowJo (Tree Star) software.

Cytokine ELISAs

IL-12p70 and TNF-α cytokine levels were determined in the supernatants of DC cultures at 48 h p.i. using BD OptiEIA reagents (BD Biosciences), according to the manufacturer's instructions.

Determination of virus titers

The left lung lobe was harvested from infected and uninfected mice at the time points indicated. Lung tissues were homogenized using a BeadBeater with 1.0-mm-diameter Zirconia/Silica beads (BioSpec Products). LCMV titers were determined by plaque assay on Vero cells (37), and influenza viral titers were determined by plaque assay on MDCK cells (39).

Statistical analysis

All error bars represent mean ± SEM, and averages were compared using a bidirectional, unpaired Student *t* test (37, 38, 40). Data are representative of two to three independent experimental repetitions.

Results

Ceramide enhances DC maturation in the absence of cytotoxicity upon LCMV infection

To investigate whether ceramide regulates DC maturation, DCs were coincubated with C8 ceramide, and the expression levels of MHC-I, MHC-II, and multiple costimulatory markers, including B7-1 (CD80), B7-2 (CD86), 4-1BBL, and CD40 were evaluated. In addition, the expression of the inhibitory cell surface marker PD-L1 was determined. As shown in Fig. 1A, the ceramide analog had no effect on the expression of any of these markers by DCs in vitro, suggesting that short-chain ceramide alone is not immunoregulatory. However, when DCs were infected with LCMV (LCMV Cl 13) and exposed to C8 ceramide, the increased expressions of MHC-I (mean fluorescence intensity [MFI], 127→211), MHC-II (MFI, 97→162), B7-1 (MFI, 153→230), B7-2 (MFI, 107→210), $4\text{-}1BBL^{hi}$ (46→60%), and CD40 (MFI, 21→42) were observed compared with the infected cells treated with the corresponding solvent (Fig. 1B). These data suggest that exogenously provided short-chain ceramide increased phenotypic maturation of DCs upon LCMV Cl 13 infection. However, the surface expression of the inhibitory receptor PD-L1 was not affected by the addition of ceramide (MFI, 30→26). Moreover, ceramide increased the ability of DCs to secrete the soluble proinflammatory cytokines IL-12p70 (240→490 pg/ml) and TNF-α (460→840 pg/ml) upon LCMV Cl 13 infection (Fig. 1C).

It is plausible that the ceramide affected the susceptibility of the DCs to LCMV Cl 13, which may have led to an enhancement of DC maturation. However, exogenously supplied C8 ceramide (open histogram, 37%) did not alter the expression of LCMV NP in the DCs compared with solvent-treated DCs (filled histogram, 39%) (Fig. 1D). Similarly, the analog did not change the production of infectious LCMV from baby hamster kidney cells (Supplemental Fig. 1). Therefore, the enhanced DC maturation by C8 ceramide

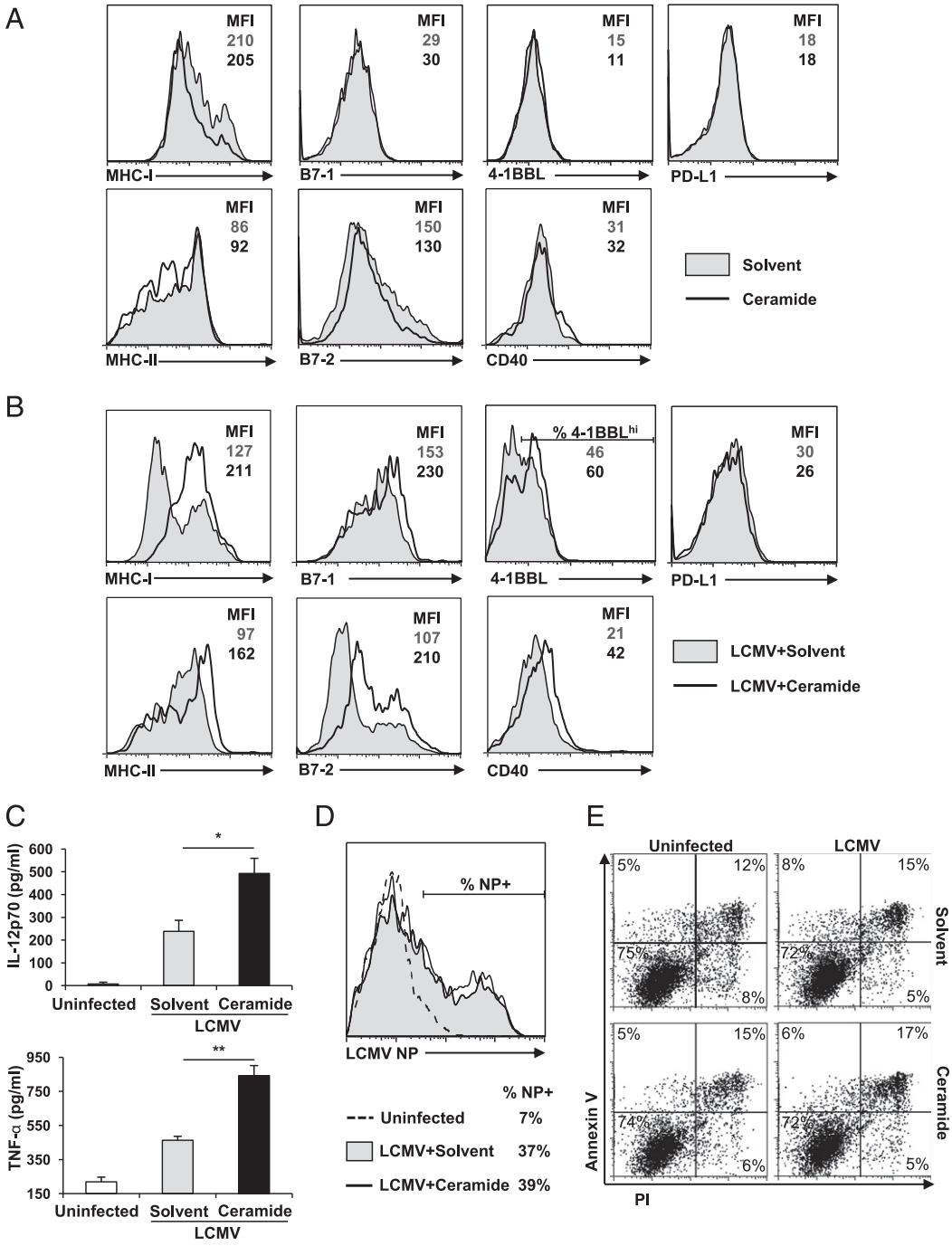

FIGURE 1. Exogenous ceramide increases the maturation of DCs upon LCMV Cl 13 infection without affecting viability. (**A**) DCs were incubated in the presence of 10 μM C8 ceramide (open histograms) or solvent (filled histograms) for 24 h. The cell surface expressions of MHC-I, MHC-II, B7-1, B7-2, 4-1BBL, CD40, and PD-L1 were assessed by flow cytometry. (**B**) DCs were infected with LCMV Cl 13 (MOI = 10) for 1 h, and then, C8 ceramide was added to a final concentration of 10 μM. The expression levels of MHC-I, MHC-II, B7-1, B7-2, 4-1BBL, CD40, and PD-L1 were assessed by flow cytometry 24 h postinfection. (**C**) IL-12p70 and TNF-α cytokines were detected in the supernatants from DCs that were uninfected or infected with LCMV Cl 13 (MOI = 10) and treated with 10 μM C8 ceramide or solvent for 48 h. (**D**) LCMV NP was detected by intracellular staining of uninfected or LCMV Cl 13–infected DCs that were treated with 10 μM C8 ceramide or its corresponding solvent at 48 h postinfection. (**E**) The apoptotic state of uninfected or LCMV Cl 13–infected (MOI = 10) $CD11c^+$ DCs that were incubated for 24 h with 10 μM C8 ceramide or solvent was identified by staining with FITC-labeled Annexin V and PI with flow cytometric analyses. Data shown are representative of two to three independently conducted experiments. Significance was determined by Student *t* test ($*p \leq 0.05$, $**p \leq 0.01$).

does not seem to be due to the altered permissiveness of the cells to LCMV infection.

Several reports have described a proapoptotic function of ceramide in a diverse array of cell types (4, 8–10, 46–51); however, the ability of C8 ceramide to induce apoptosis in DCs upon infection has not yet been described. Therefore, we determined the apoptotic state of uninfected and LCMV Cl 13–infected cells at 24 h p.i. in the presence of solvent or C8 ceramide. As shown in Fig. 1E, the addition of C8 ceramide did not increase the percentage of apoptotic or dead cells ($AnnexinV^+$ or PI^+ cells). This led us conclude that C8 ceramide displays DC-stimulatory activity without altering cell viability upon LCMV infection.

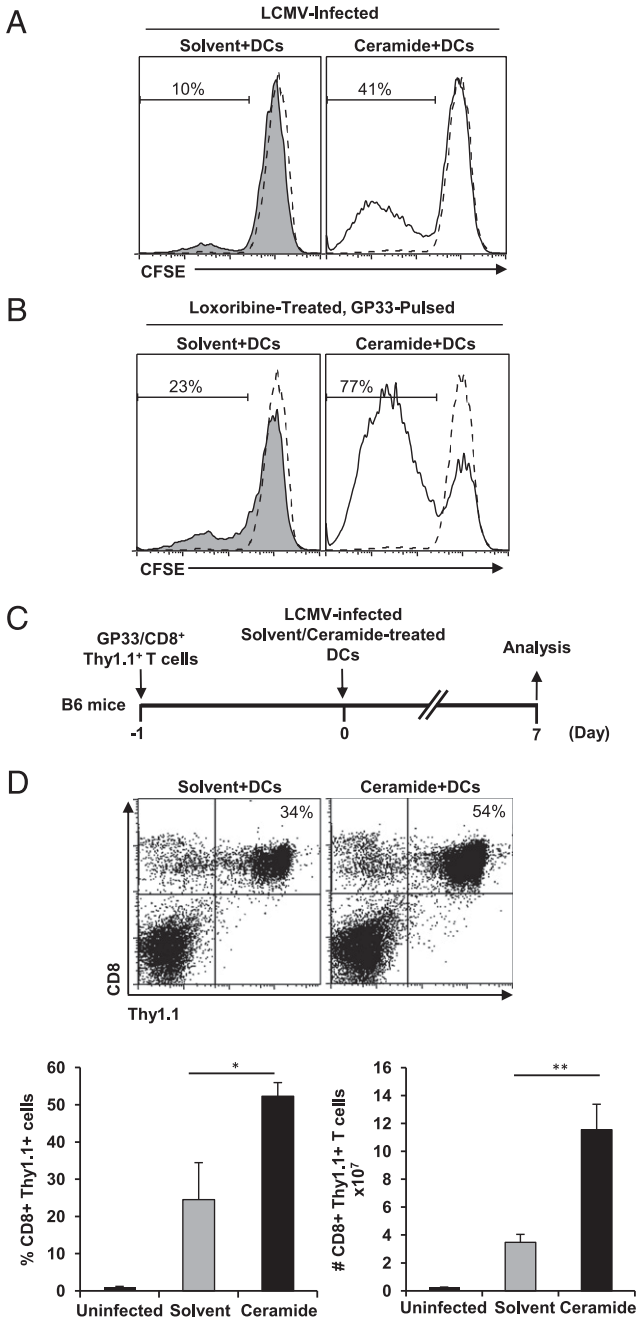

FIGURE 2. Exogenous ceramide increases the ability of DCs to stimulate Ag-specific $CD8^+$ T cell proliferation. (**A**) DCs were infected with LCMV Cl 13 (MOI = 1) for 1 h and then treated with either 10 μM C8 ceramide (open histogram, *right panel*) or solvent (filled histogram, *left panel*). LCMV-infected DCs were used to stimulate CFSE-labeled, naive $GP33/CD8^+Thy1.1^+$ T cells. Three days poststimulation, proliferation of $Thy1.1^+CD8^+$ T cells was determined by CFSE dilution and flow cytometry. Dotted histograms indicate T cells incubated with uninfected, unstimulated DCs. (**B**) DCs were incubated with loxoribine and pulsed with 1 μg/ml LCMV $GP_{33–41}$ peptide (GP33) for 1 h and then incubated in the presence of either 10 μM C8 ceramide (open histogram, *right panel*) or solvent (filled histogram, *left panel*) for 1 h, washed, and then used to stimulate CFSE-labeled, naive $GP33/CD8^+Thy1.1^+$ T cells for 3 d. Proliferation of $CD8^+Thy1.1^+$ T cells was determined by CFSE dilution and flow cytometry. Dotted histograms indicate T cells incubated with uninfected, unstimulated DCs. (**C** and **D**) GP33-specific $CD8^+Thy1.1^+$ T cells (1×10^5 cells/mouse) were adoptively transferred into wild-type C57BL/6 recipient mice (n = 3/group). These mice then received LCMV Cl 13–infected DCs that had been incubated with 10 μM C8 ceramide or solvent. At day 7 post-DC transfer, splenocytes were isolated and stained with Thy1.1 and CD8-specific Abs. The frequency and number of $Thy1.1^+CD8^+$ T cells in the live lymphocyte gate (determined by forward and side scatter profiles) were identified by flow cytometry. Data shown are representative of two experiments conducted independently (n = 3 recipient mice/group). Significance was determined by Student t test (*$p \leq 0.05$, **$p \leq 0.01$).

Ceramide-conditioned DCs elicit more robust $CD8^+$ T cell proliferation than untreated DCs

LCMV Cl 13–infected DCs that were incubated with ceramide exhibited higher expression levels of multiple phenotypic markers of DC maturation, which are involved in the priming of naive Ag-specific $CD8^+$ T cell responses. To determine whether ceramide enhances the ability of DCs to better prime naive $CD8^+$ T cells, DC-mediated T cell proliferation experiments were performed. Initially, DCs were infected with LCMV Cl 13 or left uninfected and then incubated with C8 ceramide or its solvent. The cells were used to stimulate CFSE-labeled, naive $GP33/CD8^+Thy1.1^+$ T cells, which are specific for the immunodominant GP33 epitope of LCMV. Importantly, LCMV Cl 13–infected DCs exposed to C8 ceramide induced more robust $CD8^+$ T cell proliferation than those that were not exposed to ceramide (10 versus 41%) (Fig. 2A). This indicates that ceramide-modulated DCs became more potent in inducing virus-specific T cell proliferation. To confirm that this effect was specific to DC maturation, solvent or C8 ceramide–treated DCs were stimulated with the TLR7 agonist loxoribine that mimics ssRNAs and pulsed with GP33 peptide. These DCs were then used to stimulate naive CFSE-labeled $GP33/CD8^+Thy1.1^+$ cells (Fig. 2B). As before, ceramide-treated DCs induced substantially more $CD8^+$ T cell proliferation than solvent-treated DCs (23 versus 77%). The data indicate that ceramide improves the ability of DCs to prime naive $CD8^+$ T cells upon LCMV infection or TLR7 stimulation in vitro.

Next, we determined whether ceramide-treated DCs increase the expansion of virus-specific T cells in vivo. To this end, LCMV Cl 13–infected, solvent/ceramide-conditioned DCs were adoptively transferred to C57BL/6 mice ($Thy1.2^+$) that had received 1×10^5 $GP33/CD8^+Thy1.1^+$ T cells (Fig. 2C). Seven days following DC transfer, amplified $GP33/CD8^+Thy1.1^+$ T cells were enumerated in the spleens of the recipient mice. Indeed, ceramide-conditioned DCs induced both a higher percentage and a higher total number of $GP33/CD8^+$ T cells when compared with mice that had received DCs preincubated in solvent alone (Fig. 2D), which suggests there was a significantly greater expansion of virus-specific T cells in vivo. Therefore, we could conclude that direct conditioning of DCs with C8 ceramide improves the capacity of DCs to induce virus-specific $CD8^+$ T cell expansion.

Systemically administered C8 ceramide fails to improve antiviral $CD8^+$ T cell responses

Because ceramide-conditioning strongly increased DC activation, we determined whether C8 ceramide is able to increase antiviral immune responses when administered systemically to virus-infected mice. For this purpose, mice were infected with LCMV Cl 13 and injected i.v. with C8 ceramide or its solvent. However, a single systemic administration with C8 ceramide did not enhance virus-specific T cell responses (Fig. 3A). We then increased the injection frequency of C8 ceramide by administering C8 ceramide into mice at days 1, 4, and 7 p.i. At day 8 p.i., the $CD8^+$ T cell responses in the spleen were analyzed to determine the effects of multiple ceramide treatments on virus-specific T cell responses. As shown in Fig. 3B, multiple administrations during the course of the infection did not increase the virus specific $CD8^+$ T cell responses.

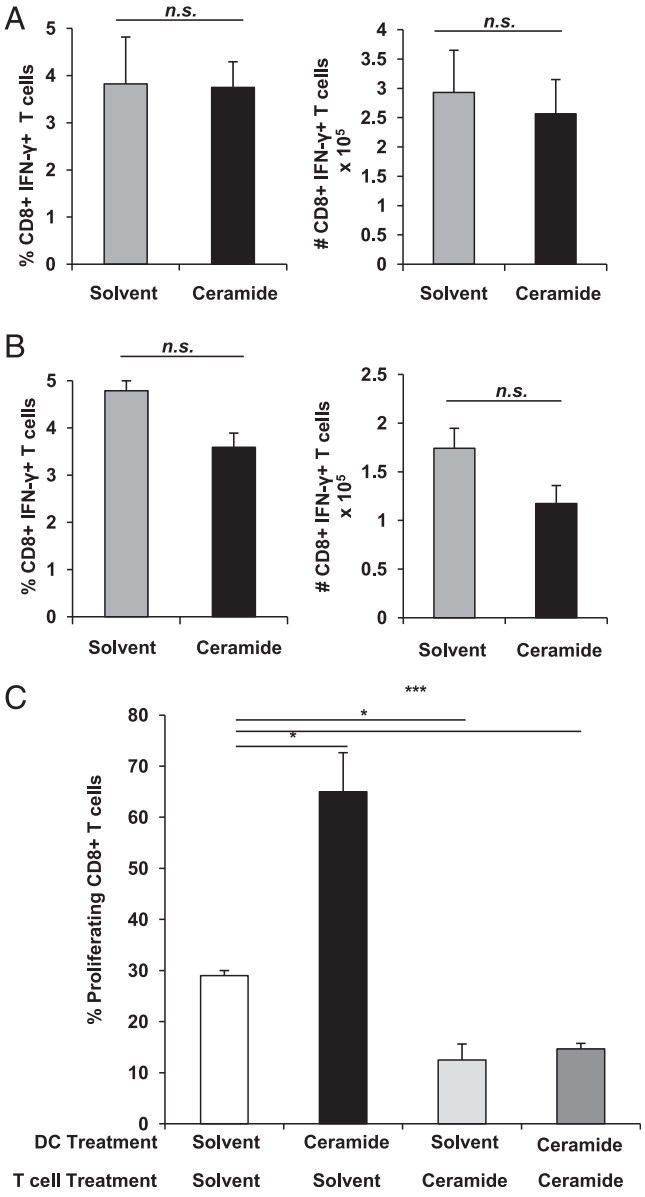

FIGURE 3. Systemic ceramide treatment fails to enhance antiviral $CD8^+$ T cell responses. (**A** and **B**) Groups of C57BL/6 mice (n = 5/group) were infected with LCMV Cl 13 via i.v. inoculation. (A) The day of infection mice received either 0.1 mg/kg C8 ceramide (black bar) or an equivalent volume of solvent (gray bar) by i.v. administration. (B) Mice received 0.1 mg/kg C8 ceramide (black bar) or solvent (gray bar) at days 1, 4, and 7 p.i. by i.v. administration. At day 8 p.i., the frequency and number of GP33-specific $CD8^+$ T cells were determined in the spleens of infected mice by intracellular cytokine staining (A and B). (**C**) GP33-specific $CD8^+$ T cells were purified from GP33-specific TCR tg mice, labeled with CFSE, and treated with solvent or 10 μM C8 ceramide as indicated for 1 h. GP33/$CD8^+$Thy1.1$^+$ T cells were then coincubated with LCMV Cl 13–infected DCs that were treated with solvent or 10 μM C8 ceramide as indicated for 3 d, and proliferation was determined by CFSE dilution and assessed by flow cytometry (n = 3/group). cytometry (n = 3/group). Data shown are representative of two to three independently conducted experiments. Significance was determined by Student t test ($*p \leq 0.05$, $***p \leq 0.001$).

Although C8 ceramide had a stimulatory effect on DCs, it is possible that the lipid may have differential effects on $CD8^+$ T cells. To test this, an in vitro T cell proliferation assay was conducted. LCMV Cl 13–infected DCs were incubated with C8 ceramide as in Fig. 2A and used as a control for the immune-stimulatory activity of C8 ceramide (29→65%; Fig. 3C). In this experiment, CFSE-labeled enriched GP33/$CD8^+$Thy1.1$^+$ T cells were also preincubated with C8 ceramide or the solvent. C8 ceramide–treated or solvent-treated DCs and T cells were mixed in the combinations as indicated in Fig. 3C. Interestingly, when naive $CD8^+$ T cells were conditioned with C8 ceramide, fewer live cells with a diluted CFSE profile were observed in the culture (14.5%). Furthermore, C8 ceramide–treated T cells proliferated and/or accumulated less efficiently than solvent-treated T cells, regardless of DC conditioning with C8 ceramide (Fig. 3C). These data suggest that exposure to short-chain ceramides prevents naive $CD8^+$ T cells from proliferating/accumulating.

Locally delivered C8 ceramide improves T cell responses to a LCMV infection

It is possible that the systemic ceramide treatment failed to improve LCMV-specific T cell responses because of failure to encounter DCs in sufficient concentrations or at the appropriate time and/or location. In an attempt to account for this, mice were infected with LCMV Cl 13 via i.n. instillation and then given a single i.n. dose of C8 ceramide or solvent. The rationale was to provide ample exposure of C8 ceramide to the DCs in the infected site by local administration. Because LCMV Cl 13 was administered locally via i.n. administration, robust T cell responses were observed in the lungs of infected mice at 7 d p.i. Importantly, these responses were significantly increased in the mice that had received an i.n. dose of C8 ceramide. The data show that mice that were treated with C8 ceramide by local administration exhibited nearly a 2-fold increase in the frequency and a 4-fold increase in the number of LCMV GP33-tetramer$^+$$CD8^+$ T cells compared with LCMV-infected mice that were treated only with solvent (Fig. 4A). C8 ceramide treatment also significantly enhanced the frequency of $CD8^+$ T cells expressing IFN-γ, whereas the number of $CD8^+$ T cells producing IFN-γ following GP33 stimulation increased ∼4-fold (Fig. 4B). Moreover, both the frequency and number of $CD8^+$ T cells expressing both TNF-α and IFN-γ in response to GP33 significantly increased in the lungs of mice locally receiving C8 ceramide (Fig. 4C). Furthermore, the function of these GP33-specific $CD8^+$ T cells was demonstrated using a CD107a degranulation assay. As with cytokine production, the frequency of CD107a$^+$ cells following GP33 stimulation was increased ∼2-fold, whereas the number of CD107a$^+$ cells was increased >3-fold in the ceramide-treated mice compared with those treated with only solvent (Fig. 4D). In addition, the frequency of $CD4^+$ T cells responding to an immunodominant LCMV-specific GP61 peptide stimulation was increased from 9 to 17% (Fig. 4E). The numbers of LCMV GP61–specific $CD4^+$ T cells also significantly increased (Fig. 4E). Collectively, these data demonstrate the immune stimulatory ability of C8 ceramide when administered to the site of infection, rather than systemically.

To establish whether the amplification in T cell responses observed in the lungs of ceramide-treated mice is associated with increases in the activation state of DCs in the lung, groups of -mice were infected with LCMV Cl 13 by intranasal administration, and treated with solvent (filled histograms) or ceramide (open histograms). Groups of uninfected mice served as baseline controls (dashed histograms). At 2 d p.i., cells were isolated from the lungs and the expression levels of MHC-I, MHC-II, 4-1BBL, and CD40 were determined on the surface of CD11c$^+$ cells (Fig. 5). Following LCMV Cl 13 infection, the locally instilled ceramide analog elevated the expression levels of these molecules (MHC-I, MFI 24→37; 4-1BBL, MFI 24→44; MHC-II^{hi}, 38→51%; CD40^{hi}, 34→62%). This is consistent with the results from the experiments performed with DCs in vitro (Fig. 1B) and suggests a potential mechanism for the boosted antiviral T cell responses following ceramide treatment.

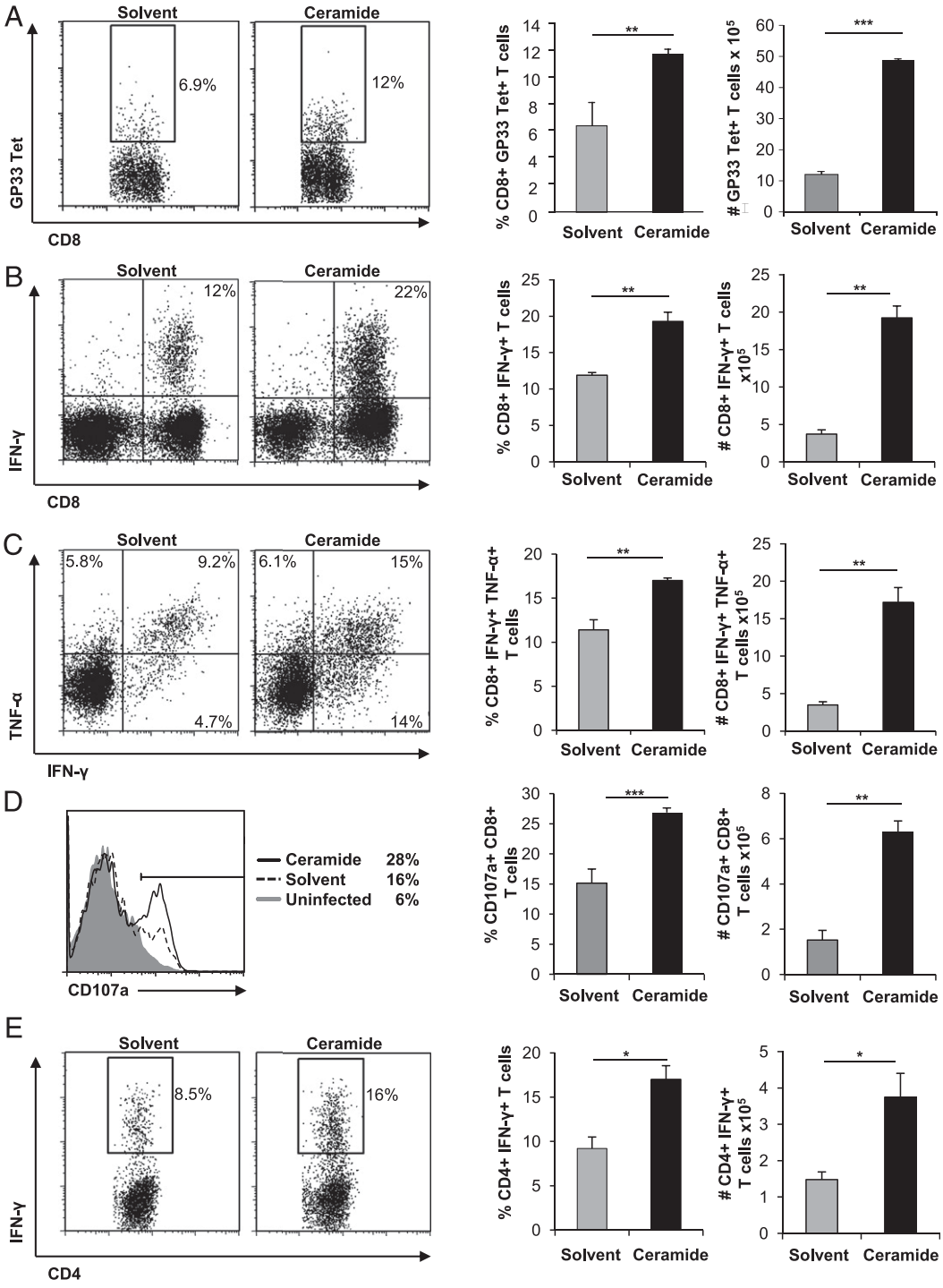

FIGURE 4. Locally administered ceramide enhances LCMV Cl 13–specific T cell responses. Groups of C57BL/6 mice (n = 4–5) were infected with LCMV Cl 13 by intranasal inoculation. The day of infection, mice were treated with either solvent (gray bars) or C8 ceramide (0.1 mg/kg, black bars) by intranasal administration. At day 7 p.i., LCMV-specific T cell responses were assessed in the lungs. (**A**) LCMV GP33–specific $CD8^+$ T cells were identified by tetramer staining. (**B** and **C**) $CD8^+$ T cell function was determined by stimulation with $GP_{33–41}$ (GP33) peptide and IFN-γ production (B) or TNF-α and IFN-γ production (C) by $CD8^+$ T cells was determined by intracellular cytokine staining and flow cytometry. (**D**) Degranulation in response to GP33 peptide stimulation was assessed by CD107a staining. (**E**) Functional LCMV-specific $CD4^+$ T cell responses were determined by stimulation with LCMV $GP_{61–80}$ (GP61) peptide, followed by intracellular cytokine staining for IFN-γ. Cell numbers were calculated from the total splenocytes counts. Data shown are representative of two to three independently conducted experiments. Significance was determined by Student t test (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$).

To further explore the responses in these locally treated mice, splenocytes were harvested and LCMV-specific T cell responses were assessed. As shown in Fig. 6A, the local administration of C8 ceramide increased the frequency of IFN-γ–producing $CD8^+$ T cells in spleens following stimulation with GP33 peptide from 10 to 20%, which corresponded to an increase in the number of GP33-specific IFN-γ–producing cells. In addition, the frequency as well as the number of GP33-specific functional $CD8^+$ T cells expressing both TNF-α and IFN-γ increased in spleens from mice that had received C8 ceramide (Fig. 6B). C8 ceramide appeared to have globally increased LCMV-specific $CD8^+$ T cell responses because it did not alter the dominance of the $CD8^+$ T cell epitopes

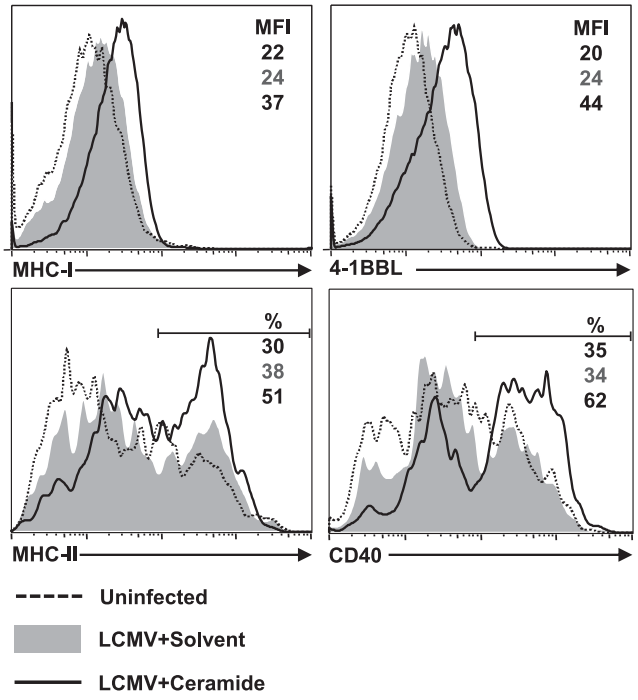

FIGURE 5. Intranasal ceramide administration increases the expression of activation markers on CD11c$^+$ cells in the lungs upon infection. Groups of C57BL/6 mice (n = 3–4) were left uninfected (dashed histograms) or infected with LCMV Cl 13 by intranasal inoculation. The day of infection, mice were treated with either solvent (filled histograms) or C8 ceramide (0.1 mg/kg, open histograms) by intranasal administration. At day 2 p.i., the surface expression of MHC-I, MHC-II, 4-1BBL, and CD40 by CD11c$^+$ cells isolated from the lungs was determined by flow cytometry. Data shown are representative of two independently conducted experiments.

GP33, NP396, and GP276 (Supplemental Fig. 2). Similarly, C8 ceramide treatments also significantly increased the frequency and number of LCMV GP61–specific CD4$^+$ T cells in the spleens of infected mice compared with control mice (Fig. 6C). Taken together, these data demonstrate that administration of C8 ceramide in proximity to the infection enabled the lipid analog to enhance systemic adaptive immune responses to a viral infection.

To our surprise, the enhanced antiviral T cell response was not able to improve the control of the LCMV Cl 13 infection in the lungs. As shown in Fig. 6D, virus titers in the lungs at 8 d p.i. were not different between the solvent-treated mice and the C8 ceramide–recipient mice. Intranasal infection did not appear to have developed into a systemic infection because no virus was detected in the serum at 8 d p.i. (data not shown). In addition, LCMV Cl 13 does not progress to a persistent infection in the lungs following intranasal infection because no virus was detected in the lungs at 14 d p.i. (Fig. 6D).

C8 ceramide displays immune-stimulatory activity in response to influenza virus infection

C8 ceramide has been shown to be immunostimulatory to DCs in response to an immunosuppressive LCMV Cl 13 infection. Furthermore, it has demonstrated these properties in vivo when administered locally to LCMV Cl 13–infected mice. To determine whether this effect was specific to an LCMV Cl 13 infection or whether the lipid analog had broader effects, DCs were infected with influenza virus A/WSN/33 (H1N1) and treated with C8 ceramide or an equivalent volume of solvent. The expression levels of both MHC-I (Fig. 7A) and B7-2 (Fig. 7B) have increased in the C8 ceramide–treated DCs when compared with the solvent-treated DCs. Therefore, C8 ceramide displays immune-stimulatory effects during influenza virus infection of DCs.

To determine whether the enhancement of DC responses to influenza would translate into better stimulation of CD8$^+$ T cell responses, LCMV GP33/CD8$^+$Thy1.1$^+$ T cells were transferred into C57BL/6 (Thy1.2$^+$) mice (Fig. 7C). The following day, the mice were infected with influenza virus expressing the LCMV epitope GP33 (FLU-LCMV) via i.n. inoculation. The mice received an intranasal instillation of C8 ceramide or solvent and the frequency of GP33/CD8$^+$Thy1.1$^+$ T cells was determined at 6 d p.i. The data shown in Fig. 7D demonstrate that C8 ceramide induced a 4-fold increase in the frequency of GP33/CD8$^+$Thy1.1$^+$ T cells in the lungs of the FLU-LCMV–infected mice and a much greater increase in the accumulation of these cells, suggesting that C8 ceramide is also immune stimulatory during influenza virus infections.

To investigate the effects of C8 ceramide during the endogenous immune response to influenza, C57BL/6 mice were infected with WSN strain of influenza virus. At day 8 postinfection, the influenza viral NP–specific CD8$^+$ T cell response in the lungs was assessed. Similar to the observation with LCMV, C8 ceramide increased the CD8$^+$ T cell response to influenza in the lungs following local administration (Fig. 7E). The frequency of NP-specific CD8$^+$ T cells in producing IFN-γ in the lungs of infected mice was significantly increased from 2.1 to 4.3%. This increased frequency also resulted in a significantly increased number of NP-specific CD8$^+$ T cells (Fig. 7E). Collectively, these data demonstrate the immune-stimulatory activity of locally administered C8 ceramide during an influenza virus infection. Similar to that observed with LCMV Cl 13, the enhanced, influenza-specific T cell response appeared unable to accelerate virus clearance because both solvent and ceramide-treated mice had similar virus titers in the lung at 8 d p.i. (Fig. 7F).

Discussion

The data presented in this study demonstrate a novel DC-stimulatory function of a synthetic ceramide analog upon virus infection. C8 ceramide enhanced phenotypic maturation and functional activation of DCs in response to both LCMV Cl 13 as well as influenza virus infections.

Previously, ceramide and its short-chain synthetic analogs were reported to have both structural and signaling functions and inhibit virus replication during HIV or hepatitis C virus infection (52, 53). However, C8 ceramide did not alter LCMV Cl 13 replication (Fig. 1D, Supplemental Fig. 1). Also, ceramide molecules have been predominantly shown to act as secondary lipid messengers to mediate apoptotic signals because of cell stress (3, 6, 11, 54–56). In contrast to its known roles in the induction of apoptosis in tumor cells, C8 ceramide did not induce cell death of DCs in vitro (Fig. 1E). Thus, it should be possible to safely modulate DCs with short-chain ceramides in vitro/ex vivo or administer ceramides as immune-therapeutic application. It is possible, however, that the lipid analog may affect the ability of DCs to withstand NK cell–mediated killing or suppression in vivo, which has been shown to regulate immune responses to viral infections (57, 58). Further studies into the exact effects of ceramide on DCs in vivo are required to fully use ceramide in immunotherapeutic applications.

Ceramide and its derivatives have been implicated in immune regulation. Lipidomic profiling has shown that DCs upregulate the production of C24 ceramide upon LPS stimulation (17). Furthermore, inflammatory cytokines such as TNF-α elevate the level of ceramide in DCs (59). However, a role for this increase in

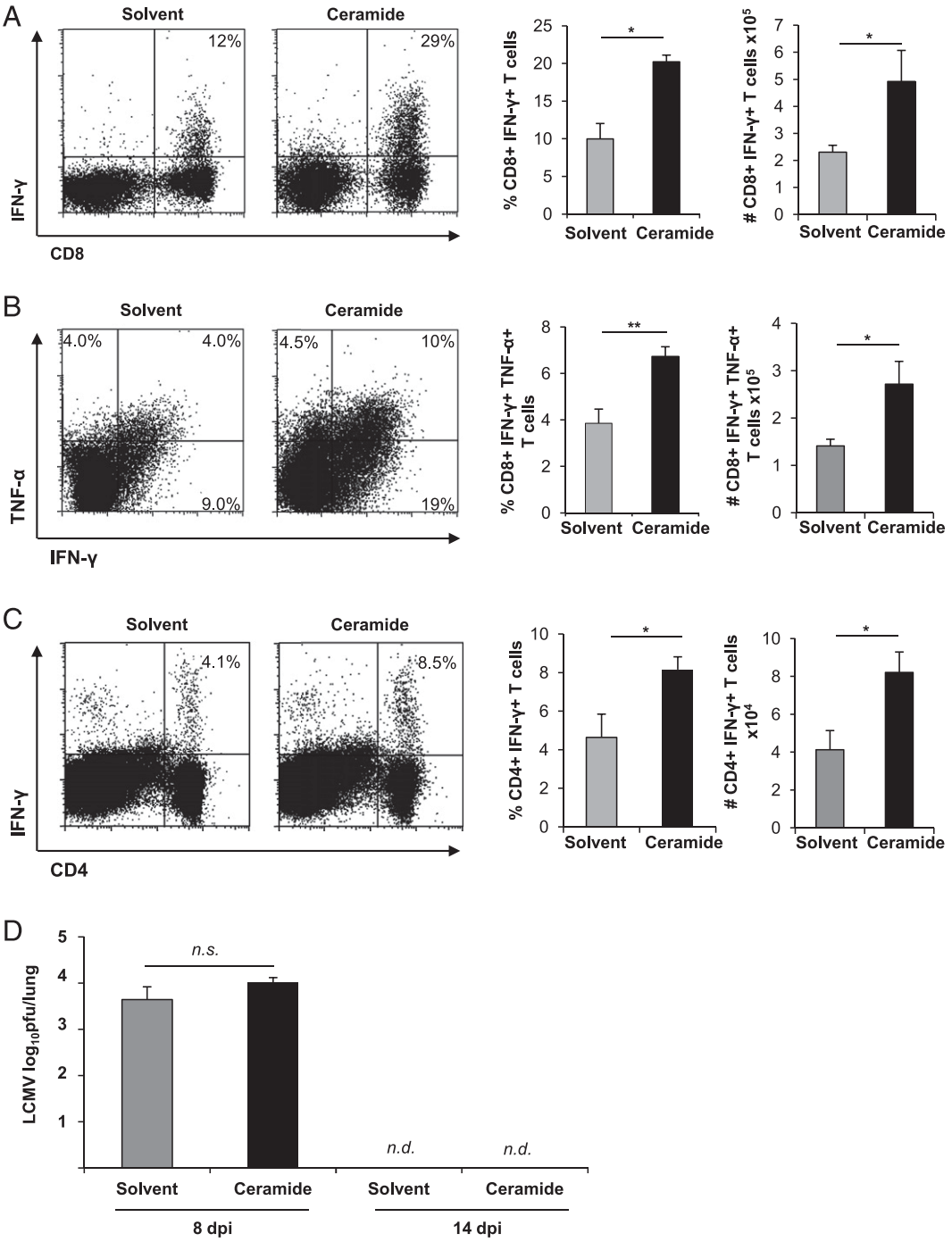

FIGURE 6. Local ceramide treatment amplifies the T cell responses in the spleen upon infection. Groups of C57BL/6 mice ($n = 4–5$) were infected with LCMV Cl 13 by intranasal inoculation. The day of infection, mice were treated with either solvent (gray bars) or C8 ceramide (0.1 mg/kg, black bars) by i.n. administration. At day 7 p.i., LCMV-specific T cell responses in the spleens were determined by intracellular cytokine staining. Splenocytes were stimulated with LCMV GP33 peptide and IFN-γ–producing $CD8^+$ T cells (**A**) and $CD8^+$ T cells producing IFN-γ and TNF-α (**B**) were identified by flow cytometry. (**C**) $CD4^+$ T cells producing IFN-γ were determined by stimulation with LCMV GP61 peptide, followed by intracellular cytokine staining. Cell numbers were calculated from the total splenocytes counts. (**D**) LCMV Cl 13 titers were measured in the lungs at 8 and 14 d p.i. by plaque assay on Vero cells.). Data shown are representative of two to three independently conducted experiments. Significance was determined by Student *t* test (*$p \leq 0.05$, **$p \leq 0.01$; n.d., not detected).

ceramide content has not yet been elucidated (17). Exogenous C2 ceramide was reported to inhibit the ability of human DCs to take up soluble Ags to impair their ability to stimulate the Ag-specific T cell clones in vitro (60). However, the experiment was conducted in the absence of any inflammatory stimulus such as pathogenic infection. Furthermore, C2 ceramide did not inhibit conventional MHC-I–mediated peptide presentation pathways to stimulate T cells (60). In contrast to the immune-suppressive activity of C2 ceramide, our paper reports a newly characterized role of a ceramide analog in the stimulation of DCs upon virus infection by enhancing virus-specific T cell responses both in vitro and in vivo. It is possible that the presence of inflammatory signals, the DC source, and the model used may have affected the differential outcome.

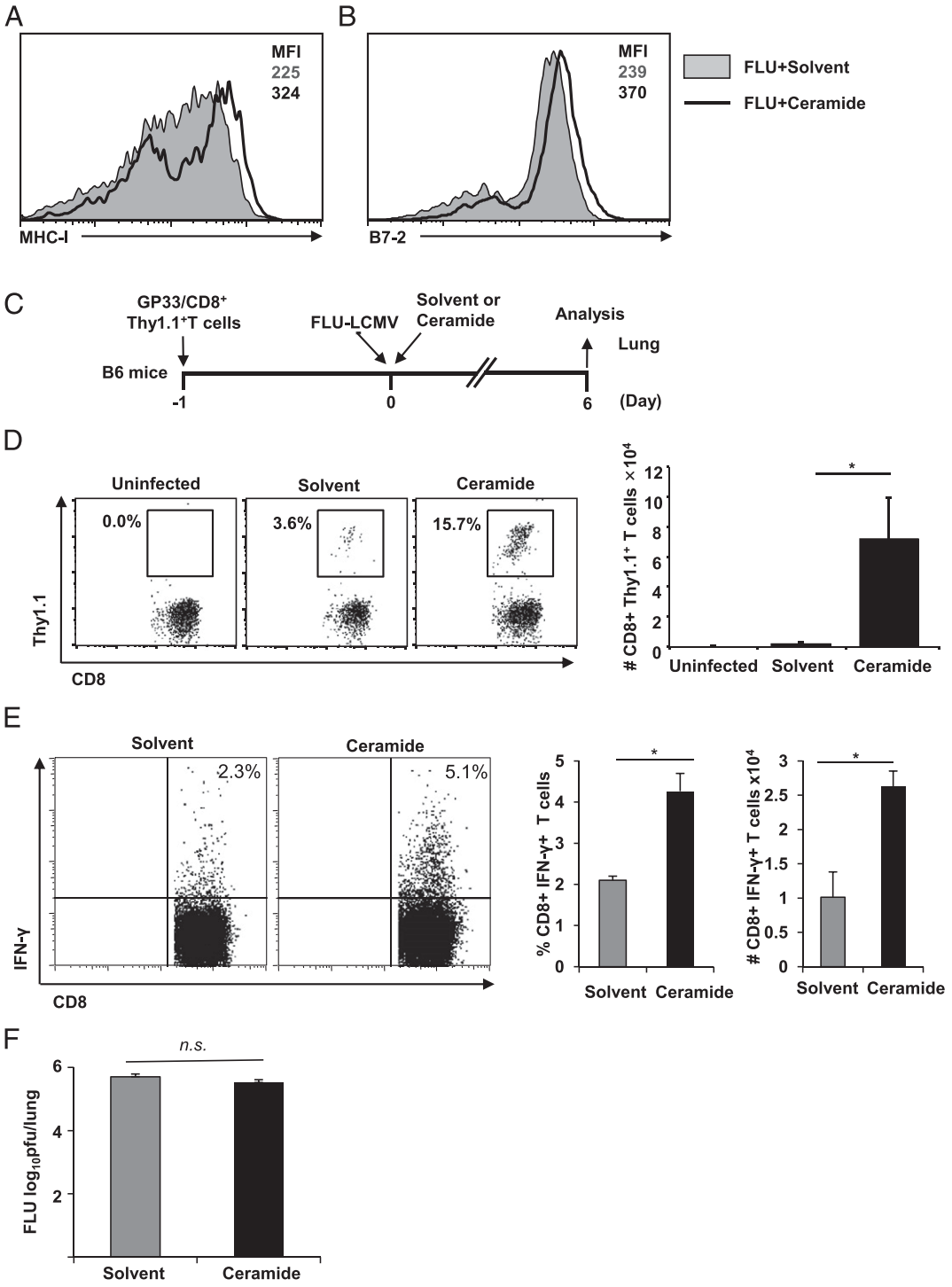

FIGURE 7. Exogenous ceramide improves influenza-specific immune responses. (**A** and **B**) DCs were infected with influenza virus and then coincubated with 10 μM C8 ceramide (open histograms) or solvent (filled histograms) for 24 h. Surface expression of MHC-I (A) and B7-2 (B) was determined by flow cytometry. (**C** and **D**) A total of 5×10^4 GP33/CD8$^+$Thy1.1$^+$ T cells were adoptively transferred into groups ($n = 4$) of C57BL/6 recipients. The following day, recipient mice were infected with 1×10^5 PFU LCMV GP33–expressing influenza virus (Flu-LCMV) via intranasal inoculation and treated with 0.1 mg/kg C8 ceramide or solvent by intranasal administration. Uninfected, untreated control mice were used as negative controls. At day 6 p.i., Thy1.1$^+$,CD8$^+$ cells were identified in the lungs by flow cytometry. (**E** and **F**) Groups of C57BL/6 mice ($n = 4$) were infected with the WSN strain of influenza (1×10^5 PFU) by intranasal inoculation. At 1 h p.i., mice were treated with 0.1 mg/kg C8 ceramide by intranasal administration. At day 8 p.i., IFN-γ expression by Flu NP366–specific CD8$^+$ T cells was determined by intracellular cytokine staining (E). Influenza virus titers were determined in the lungs at 8 d p.i. by plaque assay on MDCK cells (F). All data shown are representative of at least two independent experiments. Significance was determined by Student t test (*$p \leq 0.05$).

Our data demonstrate that C8 ceramide increases the surface expression of MHC-I and MHC-II along with costimulatory markers in response to virus infection (Figs. 1B, 5, 7A, 7B) and that these DCs stimulate T cell responses when directly infected or pulsed with peptides (Fig. 2). Importantly, costimulatory molecules 4-1BBL and CD40 that were reported to be important for generation of LCMV-specific T cell responses (61) were increased by ceramide in vitro and in vivo. However, it is unclear whether the heightened T cell expansion was due to ceramide-mediated increased proliferation signal and/or enhanced T cell accumulation signal triggered

in DCs. Thus, ceramide-induced signaling pathways in DCs as well as the detailed effect of ceramide-conditioned DCs on T cell activation, proliferation, and accumulation remain to be further investigated. In addition, it is possible that the lipid analog may affect Ag uptake by DCs or their ability to cross-present Ag, which have been shown to be important mechanisms in LCMV and influenza-specific immune responses (60, 62, 63) and thus warrants further exploration.

When ceramide was administered systemically, no increase was observed in LCMV-specific T cell responses (Fig. 3A, 3B). However, when it was administered locally via intranasal administration, significant increases of anti-LCMV and anti-influenza T cell responses were observed (Figs. 4, 6, 7). There are several possible explanations for this observation. First, systemic administration of C8 ceramide may dilute or degrade too rapidly to reach an effective concentration near populations of DCs. If this is the case, higher doses with more consecutive administrations would be required. Second, ceramide appeared to have an inhibitory effect on naive T cells during their activation in vitro (Fig. 3C), which may have occurred in the systemically administered experimental regimen. Either of these conditions may have been overcome by directly administering C8 ceramide to the respiratory tract at early time points postinfection (Figs. 4–7), which led to more virus-specific T cells.

Surprisingly, the increased virus-specific T cell responses observed in the lungs following C8 ceramide treatment (Figs. 4, 6, 7) did not appear to enhance the clearance of LCMV Cl 13 or influenza (Figs. 6D, 7F). Respiratory infections of these viruses seem to be efficiently cleared by strong host immune responses in vivo because no viruses were detected in the serum at 8 d p.i. (data not shown), and similar level of viruses were found in the lungs of mice treated with or without ceramide (Figs. 6D, 7F). C8 ceramide treatment did not decrease the functionality of the activated $CD8^+$ T cells (Fig. 4) because more $CD8^+$ T cells expressed functional antiviral cytokines (IFN-γ/TNF-α) and exhibited CD107a positivity, an indication of degranulation. Also, the increased T cell response was observed both in the secondary lymphoid organ (spleen) as well as in the peripheral tissue (lungs). In addition, C8 ceramide did not appear to affect the dominance of $CD8^+$ T cell epitopes during LCMV Cl 13 infections (Supplemental Fig. 2), indicating that higher numbers of virus-specific $CD8^+$ T cells should be circulating and able to lyse infected cells. However, high levels of ceramide accumulation can lead to severe pathological conditions in the lung (64, 65). It is also conceivable that locally administered C8 ceramide may affect the nonimmune cells in an unforeseen harmful manner including enhanced cell death (65). This may have compromised the increased T cell responses to clear viruses from the lungs of infected, C8 ceramide–treated mice.

A method to evade the possibly harmful effect of C8 ceramide on various cell types and to achieve the critical ceramide concentration near DCs would be to modulate the form of C8 ceramide and specifically target C8 ceramide to DCs. This DC-specific targeting could be achieved using DC-specific Abs, such as DEC-205 (66–68) linked to ceramide molecules. Alternatively, ceramide-containing liposomes, which may be efficiently taken up by DCs (69, 70), could be used or efficiently targeted to DCs using DC-specific Abs in vivo (71, 72). These approaches remain to be investigated.

Collectively, our findings describe a role for a short-chain synthetic ceramide analog to enhance the activation of DCs upon virus infection to induce T cell immunity. These newly characterized immune-stimulatory effects of synthetic ceramide analogs may provide a basis to develop a novel immunotherapeutic against viral infections.

Acknowledgments

We thank Michael Oldstone (The Scripps Research Institute) and Yoshihiro Kawaoka (University of Wisconsin, Madison, WI) for provision of C57BL/6-Thy1.1$^+$D^{b}GP$_{33–41}$-TCR tg mice and influenza viruses, respectively. We thank the National Institutes of Health Tetramer Core Facility for graciously supplying GP33 tetramer. Also, we thank the Cell and Immunology Core and Animal Care facilities at University of Missouri.

Disclosures

The authors have no financial conflicts of interest.

References

1. Park, J. W., W. J. Park, and A. H. Futerman. 2014. Ceramide synthases as potential targets for therapeutic intervention in human diseases. *Biochim. Biophys. Acta* 1841: 671–681.
2. Okazaki, T., A. Bielawska, R. M. Bell, and Y. A. Hannun. 1990. Role of ceramide as a lipid mediator of 1 α,25-dihydroxyvitamin D3-induced HL-60 cell differentiation. *J. Biol. Chem.* 265: 15823–15831.
3. Li, H., P. Junk, A. Huwiler, C. Burkhardt, T. Wallerath, J. Pfeilschifter, and U. Förstermann. 2002. Dual effect of ceramide on human endothelial cells: induction of oxidative stress and transcriptional upregulation of endothelial nitric oxide synthase. *Circulation* 106: 2250–2256.
4. Hartfield, P. J., G. C. Mayne, and A. W. Murray. 1997. Ceramide induces apoptosis in PC12 cells. *FEBS Lett.* 401: 148–152.
5. Jayadev, S., B. Liu, A. E. Bielawska, J. Y. Lee, F. Nazaire, L. M. Pushkareva MYu, Obeid, and Y. A. Hannun. 1995. Role for ceramide in cell cycle arrest. *J. Biol. Chem.* 270: 2047–2052.
6. Bourbon, N. A., J. Yun, and M. Kester. 2000. Ceramide directly activates protein kinase Cζ to regulate a stress-activated protein kinase signaling complex. *J. Biol. Chem.* 275: 35617–35623.
7. Zhu, X. F., Z. C. Liu, B. F. Xie, G. K. Feng, and Y. X. Zeng. 2003. Ceramide induces cell cycle arrest and upregulates p27kip in nasopharyngeal carcinoma cells. *Cancer Lett.* 193: 149–154.
8. Mizushima, N., R. Koike, H. Kohsaka, Y. Kushi, S. Handa, H. Yagita, and N. Miyasaka. 1996. Ceramide induces apoptosis via CPP32 activation. *FEBS Lett.* 395: 267–271.
9. Gill, J. S., and A. J. Windebank. 2000. Ceramide initiates NFκB-mediated caspase activation in neuronal apoptosis. *Neurobiol. Dis.* 7: 448–461.
10. Park, J. Y., M. J. Kim, Y. K. Kim, and J. S. Woo. 2011. Ceramide induces apoptosis via caspase-dependent and caspase-independent pathways in mesenchymal stem cells derived from human adipose tissue. *Arch. Toxicol.* 85: 1057–1065.
11. Yabu, T., H. Shiba, Y. Shibasaki, T. Nakanishi, S. Imamura, K. Touhata, and M. Yamashita. 2015. Stress-induced ceramide generation and apoptosis via the phosphorylation and activation of nSMase1 by JNK signaling. *Cell Death Differ.* 22: 258–273.
12. Bielawska, A., C. M. Linardic, and Y. A. Hannun. 1992. Modulation of cell growth and differentiation by ceramide. *FEBS Lett.* 307: 211–214.
13. Riboni, L., A. Prinetti, R. Bassi, A. Caminiti, and G. Tettamanti. 1995. A mediator role of ceramide in the regulation of neuroblastoma Neuro2a cell differentiation. *J. Biol. Chem.* 270: 26868–26875.
14. Józefowski, S., M. Czerkies, A. Łukasik, A. Bielawska, J. Bielawski, K. Kwiatkowska, and A. Sobota. 2010. Ceramide and ceramide 1-phosphate are negative regulators of TNF-α production induced by lipopolysaccharide. *J. Immunol.* 185: 6960–6973.
15. Herz, J., J. Pardo, H. Kashkar, M. Schramm, E. Kuzmenkina, E. Bos, K. Wiegmann, R. Wallich, P. J. Peters, S. Herzig, et al. 2009. Acid sphingomyelinase is a key regulator of cytotoxic granule secretion by primary T lymphocytes. *Nat. Immunol.* 10: 761–768.
16. Boucher, L. M., K. Wiegmann, A. Fütterer, K. Pfeffer, T. Machleidt, S. Schütze, T. W. Mak, and M. Krönke. 1995. CD28 signals through acidic sphingomyelinase. *J. Exp. Med.* 181: 2059–2068.
17. Santinha, D. R., D. R. Marques, E. A. Maciel, C. S. Simões, S. Rosa, B. M. Neves, B. Macedo, P. Domingues, M. T. Cruz, and M. R. Domingues. 2012. Profiling changes triggered during maturation of dendritic cells: a lipidomic approach. *Anal. Bioanal. Chem.* 403: 457–471.
18. Chiba, N., A. Masuda, Y. Yoshikai, and T. Matsuguchi. 2007. Ceramide inhibits LPS-induced production of IL-5, IL-10, and IL-13 from mast cells. *J. Cell. Physiol.* 213: 126–136.
19. Steinman, R. M. 2012. Decisions about dendritic cells: past, present, and future. *Annu. Rev. Immunol.* 30: 1–22.
20. Austyn, J. M., R. M. Steinman, D. E. Weinstein, A. Granelli-Piperno, and M. A. Palladino. 1983. Dendritic cells initiate a two-stage mechanism for T lymphocyte proliferation. *J. Exp. Med.* 157: 1101–1115.
21. Medzhitov, R., P. Preston-Hurlburt, and C. A. Janeway, Jr. 1997. A human homologue of the *Drosophila* Toll protein signals activation of adaptive immunity. *Nature* 388: 394–397.
22. Pasare, C., and R. Medzhitov. 2005. Toll-like receptors: linking innate and adaptive immunity. *Adv. Exp. Med. Biol.* 560: 11–18.
23. Ng, C. T., B. M. Sullivan, and M. B. Oldstone. 2011. The role of dendritic cells in viral persistence. *Curr. Opin. Virol.* 1: 160–166.
24. Colonna, M. 2004. Viral immunosuppression: disabling the guards. *J. Clin. Invest.* 113: 660–662.

25. Pritzl, C. J., Y.-J. Seo, and B. Hahm. 2012. Viruses strive to suppress host immune responses and prolong persistence. In *Recent Advances in Immunology to Target Cancer, Inflammation and Infections.* J. R. Kanwar, ed. InTech, Rijeka, Croatia, p. 23–42.
26. Hahm, B. 2009. Hostile communication of measles virus with host innate immunity and dendritic cells. *Curr. Top. Microbiol. Immunol.* 330: 271–287.
27. Matloubian, M., T. Somasundaram, S. R. Kolhekar, R. Selvakumar, and R. Ahmed. 1990. Genetic basis of viral persistence: single amino acid change in the viral glycoprotein affects ability of lymphocytic choriomeningitis virus to persist in adult mice. *J. Exp. Med.* 172: 1043–1048.
28. Sevilla, N., D. B. McGavern, C. Teng, S. Kunz, and M. B. Oldstone. 2004. Viral targeting of hematopoietic progenitors and inhibition of DC maturation as a dual strategy for immune subversion. *J. Clin. Invest.* 113: 737–745.
29. Barber, D. L., E. J. Wherry, D. Masopust, B. Zhu, J. P. Allison, A. H. Sharpe, G. J. Freeman, and R. Ahmed. 2006. Restoring function in exhausted CD8 T cells during chronic viral infection. *Nature* 439: 682–687.
30. Blackburn, S. D., H. Shin, W. N. Haining, T. Zou, C. J. Workman, A. Polley, M. R. Betts, G. J. Freeman, D. A. Vignali, and E. J. Wherry. 2009. Coregulation of CD8$^+$ T cell exhaustion by multiple inhibitory receptors during chronic viral infection. *Nat. Immunol.* 10: 29–37.
31. Wherry, E. J., S. J. Ha, S. M. Kaech, W. N. Haining, S. Sarkar, V. Kalia, S. Subramaniam, J. N. Blattman, D. L. Barber, and R. Ahmed. 2007. Molecular signature of CD8$^+$ T cell exhaustion during chronic viral infection. *Immunity* 27: 670–684.
32. Jin, H. T., A. C. Anderson, W. G. Tan, E. E. West, S. J. Ha, K. Araki, G. J. Freeman, V. K. Kuchroo, and R. Ahmed. 2010. Cooperation of Tim-3 and PD-1 in CD8 T-cell exhaustion during chronic viral infection. *Proc. Natl. Acad. Sci. USA* 107: 14733–14738.
33. Brooks, D. G., M. J. Trifilo, K. H. Edelmann, L. Teyton, D. B. McGavern, and M. B. Oldstone. 2006. Interleukin-10 determines viral clearance or persistence in vivo. *Nat. Med.* 12: 1301–1309.
34. Ejrnaes, M., C. M. Filippi, M. M. Martinic, E. M. Ling, L. M. Togher, S. Crotty, and M. G. von Herrath. 2006. Resolution of a chronic viral infection after interleukin-10 receptor blockade. *J. Exp. Med.* 203: 2461–2472.
35. Teijaro, J. R., C. Ng, A. M. Lee, B. M. Sullivan, K. C. Sheehan, M. Welch, R. D. Schreiber, J. C. de la Torre, and M. B. Oldstone. 2013. Persistent LCMV infection is controlled by blockade of type I interferon signaling. *Science* 340: 207–211.
36. Wilson, E. B., D. H. Yamada, H. Elsaesser, J. Herskovitz, J. Deng, G. Cheng, B. J. Aronow, C. L. Karp, and D. G. Brooks. 2013. Blockade of chronic type I interferon signaling to control persistent LCMV infection. *Science* 340: 202–207.
37. Seo, Y. J., and B. Hahm. 2014. Sphingosine analog AAL-R promotes activation of LCMV-infected dendritic cells. *Viral Immunol.* 27: 82–86.
38. Hahm, B., M. J. Trifilo, E. I. Zuniga, and M. B. Oldstone. 2005. Viruses evade the immune system through type I interferon-mediated STAT2-dependent, but STAT1-independent, signaling. *Immunity* 22: 247–257.
39. Seo, Y. J., C. J. Pritzl, M. Vijayan, K. Bomb, M. E. McClain, S. Alexander, and B. Hahm. 2013. Sphingosine kinase 1 serves as a pro-viral factor by regulating viral RNA synthesis and nuclear export of viral ribonucleoprotein complex upon influenza virus infection. *PLoS One* 8: e75005.
40. Marsolais, D., B. Hahm, K. H. Edelmann, K. B. Walsh, M. Guerrero, Y. Hatta, Y. Kawaoka, E. Roberts, M. B. Oldstone, and H. Rosen. 2008. Local not systemic modulation of dendritic cell S1P receptors in lung blunts virus-specific immune responses to influenza. *Mol. Pharmacol.* 74: 896–903.
41. Marsolais, D., B. Hahm, K. B. Walsh, K. H. Edelmann, D. McGavern, Y. Hatta, Y. Kawaoka, H. Rosen, and M. B. Oldstone. 2009. A critical role for the sphingosine analog AAL-R in dampening the cytokine response during influenza virus infection. *Proc. Natl. Acad. Sci. USA* 106: 1560–1565.
42. Seo, Y. J., C. J. Pritzl, M. Vijayan, C. R. Blake, M. E. McClain, and B. Hahm. 2012. Sphingosine analogue AAL-R increases TLR7-mediated dendritic cell responses via p38 and type I IFN signaling pathways. *J. Immunol.* 188: 4759–4768.
43. Hassett, D. E., J. Zhang, M. Slifka, and J. L. Whitton. 2000. Immune responses following neonatal DNA vaccination are long-lived, abundant, and qualitatively similar to those induced by conventional immunization. *J. Virol.* 74: 2620–2627.
44. Nayak, D., K. R. Johnson, S. Heydari, T. L. Roth, B. H. Zinselmeyer, and D. B. McGavern. 2013. Type I interferon programs innate myeloid dynamics and gene expression in the virally infected nervous system. *PLoS Pathog.* 9: e1003395.
45. Jeannet, G., C. Boudousquié, N. Gardiol, J. Kang, J. Huelsken, and W. Held. 2010. Essential role of the Wnt pathway effector Tcf-1 for the establishment of functional CD8 T cell memory. *Proc. Natl. Acad. Sci. USA* 107: 9777–9782.
46. Gulbins, E., R. Bissonnette, A. Mahboubi, S. Martin, W. Nishioka, T. Brunner, G. Baier, G. Baier-Bitterlich, C. Byrd, F. Lang, et al. 1995. FAS-induced apoptosis is mediated via a ceramide-initiated RAS signaling pathway. *Immunity* 2: 341–351.
47. Brugg, B., P. P. Michel, Y. Agid, and M. Ruberg. 1996. Ceramide induces apoptosis in cultured mesencephalic neurons. *J. Neurochem.* 66: 733–739.
48. Larocca, J. N., M. Farooq, and W. T. Norton. 1997. Induction of oligodendrocyte apoptosis by C2-ceramide. *Neurochem. Res.* 22: 529–534.
49. Siskind, L. J., and M. Colombini. 2000. The lipids C2- and C16-ceramide form large stable channels. Implications for apoptosis. *J. Biol. Chem.* 275: 38640–38644.
50. Lavrentiadou, S. N., C. Chan, T. Kawcak, T. Ravid, A. Tsaba, A. van der Vliet, R. Rasooly, and T. Goldkorn. 2001. Ceramide-mediated apoptosis in lung epithelial cells is regulated by glutathione. *Am. J. Respir. Cell Mol. Biol.* 25: 676–684.
51. Kurinna, S. M., C. C. Tsao, A. F. Nica, T. Jiffar, and P. P. Ruvolo. 2004. Ceramide promotes apoptosis in lung cancer-derived A549 cells by a mechanism involving c-Jun NH2-terminal kinase. *Cancer Res.* 64: 7852–7856.
52. Finnegan, C. M., S. S. Rawat, A. Puri, J. M. Wang, F. W. Ruscetti, and R. Blumenthal. 2004. Ceramide, a target for antiretroviral therapy. *Proc. Natl. Acad. Sci. USA* 101: 15452–15457.
53. Voisset, C., M. Lavie, F. Helle, A. Op De Beeck, A. Bilheu, J. Bertrand-Michel, F. Tercé, L. Cocquerel, C. Wychowski, N. Vu-Dac, and J. Dubuisson. 2008. Ceramide enrichment of the plasma membrane induces CD81 internalization and inhibits hepatitis C virus entry. *Cell. Microbiol.* 10: 606–617.
54. Basu, S., and R. Kolesnick. 1998. Stress signals for apoptosis: ceramide and c-Jun kinase. *Oncogene* 17: 3277–3285.
55. Mazière, C., M. A. Conte, L. Leborgne, T. Levade, W. Hornebeck, R. Santus, and J. C. Mazière. 2001. UVA radiation stimulates ceramide production: relationship to oxidative stress and potential role in ERK, JNK, and p38 activation. *Biochem. Biophys. Res. Commun.* 281: 289–294.
56. Sanvicens, N., and T. G. Cotter. 2006. Ceramide is the key mediator of oxidative stress-induced apoptosis in retinal photoreceptor cells. *J. Neurochem.* 98: 1432–1444.
57. Raulet, D. H. 2004. Interplay of natural killer cells and their receptors with the adaptive immune response. *Nat. Immunol.* 5: 996–1002.
58. Spaggiari, G. M., R. Carosio, D. Pende, S. Marcenaro, P. Rivera, M. R. Zocchi, L. Moretta, and A. Poggi. 2001. NK cell-mediated lysis of autologous antigen-presenting cells is triggered by the engagement of the phosphatidylinositol 3-kinase upon ligation of the natural cytotoxicity receptors NKp30 and NKp46. *Eur. J. Immunol.* 31: 1656–1665.
59. Wolff, R. A., R. T. Dobrowsky, A. Bielawska, L. M. Obeid, and Y. A. Hannun. 1994. Role of ceramide-activated protein phosphatase in ceramide-mediated signal transduction. *J. Biol. Chem.* 269: 19605–19609.
60. Sallusto, F., C. Nicolò, R. De Maria, S. Corinti, and R. Testi. 1996. Ceramide inhibits antigen uptake and presentation by dendritic cells. *J. Exp. Med.* 184: 2411–2416.
61. Tan, J. T., J. K. Whitmire, R. Ahmed, T. C. Pearson, and C. P. Larsen. 1999. 4-1BB ligand, a member of the TNF family, is important for the generation of antiviral CD8 T cell responses. *J. Immunol.* 163: 4859–4868.
62. Helft, J., B. Manicassamy, P. Guermonprez, D. Hashimoto, A. Silvin, J. Agudo, B. D. Brown, M. Schmolke, J. C. Miller, M. Leboeuf, et al. 2012. Cross-presenting CD103$^+$ dendritic cells are protected from influenza virus infection. *J. Clin. Invest.* 122: 4037–4047.
63. Wakim, L. M., and M. J. Bevan. 2011. Cross-dressed dendritic cells drive memory CD8+ T-cell activation after viral infection. *Nature* 471: 629–632.
64. Alayoubi, A. M., J. C. Wang, B. C. Au, S. Carpentier, V. Garcia, S. Dworski, S. El-Ghamrasni, K. N. Kirouac, M. J. Exertier, Z. J. Xiong, et al. 2013. Systemic ceramide accumulation leads to severe and varied pathological consequences. *EMBO Mol. Med.* 5: 827–842.
65. Petrache, I., V. Natarajan, L. Zhen, T. R. Medler, A. T. Richter, C. Cho, W. C. Hubbard, E. V. Berdyshev, and R. M. Tuder. 2005. Ceramide upregulation causes pulmonary cell apoptosis and emphysema-like disease in mice. *Nat. Med.* 11: 491–498.
66. Bonifaz, L., D. Bonnyay, K. Mahnke, M. Rivera, M. C. Nussenzweig, and R. M. Steinman. 2002. Efficient targeting of protein antigen to the dendritic cell receptor DEC-205 in the steady state leads to antigen presentation on major histocompatibility complex class I products and peripheral CD8$^+$ T cell tolerance. *J. Exp. Med.* 196: 1627–1638.
67. Guo, M., S. Gong, S. Maric, Z. Misulovin, M. Pack, K. Mahnke, M. C. Nussenzweig, and R. M. Steinman. 2000. A monoclonal antibody to the DEC-205 endocytosis receptor on human dendritic cells. *Hum. Immunol.* 61: 729–738.
68. Mahnke, K., M. Guo, S. Lee, H. Sepulveda, S. L. Swain, M. Nussenzweig, and R. M. Steinman. 2000. The dendritic cell receptor for endocytosis, DEC-205, can recycle and enhance antigen presentation via major histocompatibility complex class II-positive lysosomal compartments. *J. Cell Biol.* 151: 673–684.
69. Copland, M. J., M. A. Baird, T. Rades, J. L. McKenzie, B. Becker, F. Reck, P. C. Tyler, and N. M. Davies. 2003. Liposomal delivery of antigen to human dendritic cells. *Vaccine* 21: 883–890.
70. Pietersz, G. A., V. Apostolopoulos, and I. F. McKenzie. 2000. Generation of cellular immune responses to antigenic tumor peptides. *Cell. Mol. Life Sci.* 57: 290–310.
71. Badiee, A., N. Davies, K. McDonald, K. Radford, H. Michiue, D. Hart, and M. Kato. 2007. Enhanced delivery of immunoliposomes to human dendritic cells by targeting the multilectin receptor DEC-205. *Vaccine* 25: 4757–4766.
72. van Broekhoven, C. L., C. R. Parish, C. Demangel, W. J. Britton, and J. G. Altin. 2004. Targeting dendritic cells with antigen-containing liposomes: a highly effective procedure for induction of antitumor immunity and for tumor immunotherapy. *Cancer Res.* 64: 4357–4365.