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Prolonged Antigen Presentation following an Acute Virus Infection Requires Direct and Then Cross-Presentation

Erica L. Heipertz, Michael L. Davies, Eugene Lin, and Christopher C. Norbury

Antiviral CD8+ T cell recognition of MHC class I–peptide complexes on the surface of professional APCs is a requisite step in an effective immune response following many potentially lethal infections. Although MHC class I–peptide production is thought to be closely linked to the continued presence of virus, several studies have shown that the persistence of Ag presentation occurs for an extended period of time following the clearance of RNA viruses. However, the mechanism responsible for Ag presentation persistence following viral clearance was unknown until now. In this study, we used a recombinant DNA virus expressing different forms of model Ag to study the mechanism of prolonged Ag presentation in mice. We determined that the persistence of Ag presentation consists of three distinct mechanistic phases, as follows: ongoing viral replication, persistence of virally infected cells, and cross-presentation of Ag. These data will allow manipulation of the form of Ag contained within viral vectors to produce the most effective and protective CD8+ T cell response to be generated following vaccination.  

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D8+ T cells (T CD8+) play a crucial role in immunity to viruses. Antiviral T CD8+ are initially activated by recognition of MHC class I–peptide (pMHC-I) complexes on the surface of professional APCs (pAPC), but recognition of pMHC-I complexes on pAPC is also most likely required for efficient activation of memory T CD8+ (1, 2). Ag presentation of pMHC-I by pAPC is generally held to be downregulated before the clearance of Ag or bacterial pathogen (3–5). However, several studies have shown that the persistence of Ag presentation occurs for an extended period of time following clearance of RNA viruses that cause acute, but not persistent, infection (6–8). The mechanisms responsible for continued Ag presentation following clearance of detectable levels of virus remain unknown.

Generation of pMHC-I by pAPC can occur via at least two physically and mechanistically distinct presentation pathways: direct or cross-presentation. In the case of a virus infection, direct presentation occurs from any cells that are infected with virus, and peptides conjugated to MHC class I are generated efficiently from short-lived protein substrates that may be incorrectly folded or translated (9, 10). In contrast, cross-presentation is the internalization of proteinaceous material from virus-infected cells by uninfected pAPC, and generally involves the transfer of longer-lived antigenic substrates (11–13). Exogenous Ag was retained in dendritic cells (DC) for days, potentially implicating cross-presentation of Ag in the prolonging of Ag presentation (14). In this study, we used a recombinant Ag, OVA, expressed in a form that can be presented by both the cross- and direct presentation pathways (OVA full length [FL]). We compared OVA FL with an antigenic form (OVA minigene [MG]) that multiple independent laboratories (12, 13, 15, 16) have demonstrated is restricted exclusively to the direct presentation pathway, most likely because the t 1/2 of this form of Ag is too short to facilitate transfer to another cell without additional stabilization (17). Although a small number of minimal antigenic peptides can be cross presented, the OVA peptide studied in this work is completely restricted to the direct presentation pathway in vivo (18). By comparing the activation of naive Ag-specific T cells following infection with recombinant viruses, we were able to examine the contribution of direct and cross-presentation to the persistence of Ag presentation.

We examined persistence of Ag following infection with recombinant vaccinia virus (rVACV), a DNA virus that is unlikely to integrate its nucleic acids into infected cells as it is highly cytotoxic and replicates wholly in the cytosol of infected cells. Replicating vaccinia virus (VACV) can only be detected for 2 wk postinfection, but activation of adoptively transferred naive T CD8+ can be detected for 40 days postinfection. After detectable levels of virus are cleared, direct presentation persists, implying the existence of virus-infected cells for this period. A final phase of Ag presentation involves cross-presentation of Ag. The data yielded in this study will allow manipulation of the form of Ag contained within viral vectors or other vaccine preparations to allow the presentation of Ag for different periods of time, allowing the most effective and protective T CD8+ response to be generated following vaccination.

Materials and Methods

Mice

C57BL/6 mice were purchased from Charles River Laboratories (Wilmington, MA). OT-I TCR RAG1−/− (19, 20) transgenic mice were obtained from the National Institute of Allergy and Infectious Diseases Exchange Program (line 4175). Where indicated, OT-I mice were bred to B6.SJL mice (Taconic). MAPA mice (21) were purchased from The Jackson Laboratory. CD11cDTR/GFP mice (22) were purchased from Jackson Laboratory and subsequently backcrossed to C57BL/6 mice to achieve N10. All mice were maintained under specific pathogen-free conditions in the animal facility at the Pennsylvania State M.S. Hershey...
College of Medicine. All experiments and breeding were approved by the Penn State College of Medicine Institutional Animal Care and Use Committee.

Viruses

rVACV expressing OVA and MG were provided by J. Yewdell and J. Bennink (National Institutes of Health, Bethesda, MD). Female C57BL/6 mice (6–10 wk old) were injected i.p. with 10^6 PFU rVACV. Virus was titrated from tissue after three freeze-thaw cycles. For real-time RT-PCR analyses, tissue was homogenized and RNA was purified using the RNeasy Mini Kit (spleens) or RNeasy Plus Mini Kit (ovaries). After measuring RNA concentration and purity, we converted mRNA into cDNA and used a Bio-Rad iQ Powermix and an ABI 9700HT or Bio-Rad Opticon 2 to detect FL OVA cDNA. For OVA PCR, we used the protocol and primer concentrations from Klochkov et al. (23), and primers were as follows: OVA forward, 5'-GCA GCA CAT GCA GAA ATC-3'; OVA reverse, 5'-AGA AGA GAA CGG CGT TGG T-3'; and OVA probe, (FAM)-5'-CAT TCT TCT GTATCA AGC ACATGC-3'-BHQ. As positive controls and standard curves, pRB21-eGFP-OVA-FL plasmid (24) was used for OVA PCR. This assay could reliably detect 10 copies of the sequence in question. Similar results were obtained using a PCR assay to detect VACV E9L cDNA.

Cell lines and cultures

All media were purchased from Invitrogen (Grand Island, NY). IMDM supplemented with 10% FBS, antibiotics (penicillin and streptomycin), and 2 mM glutamine was used during effector assays and for adoptive transfers. Where shown, β2-microglobulin (β2M^-/-) cells (13) were maintained in DMEM containing 10% FBS supplemented with penicillin/streptomycin and 2 mM t-glutamine.

UV/Psoralen treatment

To treat virus stocks, 4.5'-8-trimethylpsoralen (Psoralen; Sigma-Aldrich, St. Louis, MO) was added to 1 ml rVACV stock (1 x 10^6 PFU/ml) to give a final concentration of 10 μg/ml. The stock was then exposed to UVA (295 nm) for times indicated by placing a portable UV lamp ∼1 cm above the liquid (25). Virus exposed to UVA for >10 min always failed to produce any plaques in a standard titration. To treat virus-infected cells, psoralen was added to β2M^-/- cells infected with virus for 5 h to give a final concentration of 10 μg/ml. Cells were exposed to UVC (254 nm), as described, with mechanical agitation every 5 min. Prior to exposure to UVC and psoralen, ∼5 x 10^6 PFU could be liberated from 5 x 10^6 infected cells previously exposed to VACV at a multiplicity of infection of 10, but, after exposure to UVC and psoralen, this was reduced to undetectable levels, indicating full ablation of VACV replication by this treatment.

In vivo cross-presentation

For in vivo infection, mice were injected i.p. with 5 x 10^6 β2M^-/- fibroblasts that were infected with VACV-OVA at a multiplicity of infection of 10 for 5 h, and then treated with psoralen and UV light (UVC) and γ irradiated at 20,000 rad, as previously described (13). Greater than 90% of β2M^-/- fibroblasts were infected and expressing detectable viral Ag following UVC and psoralen treatment. Injection of CpG oligonucleotides or LPS 12 h prior to injection of β2M^-/- fibroblasts completely ablated proliferation of adoptively transferred TcD8, indicating that cross-presentation was the pathway of Ag presentation used (26).

Adaptive transfer of TCR transgenic T cells

T cell–enriched populations were obtained from TCR transgenic (Tg) mice, as follows. Lymph nodes (popliteal, inguinal, brachial, axillary, and superficial cervical) and spleens were harvested, and a single-cell suspension was generated using a homogenizer. Live cells were isolated by centrifugation over lymphocyte separation medium (LSM). Cells were washed and resuspended, and 1–5 x 10^6 cells were injected into each recipient via the lateral tail vein using a 27-gauge needle.

Analysis of cell division in vivo

To analyze cell division in vivo, adoptively transferred TCR Tg T cells were labeled with the dye 5(and 6)-carboxyfluorescein diacetate, succinimidyl ester (CFDA-SE; Molecular Probes, Eugene, OR). Mononuclear cells isolated over a LSM gradient were washed and then labeled with 5 nM CFDA-SE for 10 min and then centric and washed, and injected i.v. into recipient mice. Primary lymphocytes were harvested at the times indicated following infection and analyzed for cell division, as indicated, by dilution of the CFDA-SE dye.

Three days after injection, spleens were harvested and homogenized. Cells were harvested from the spleen as infections were performed i.v., and so the spleen is the most relevant site at which to examine T cell proliferation. In addition, by examining proliferation 3 d after injection, there is sufficient time for proliferating cells to circulate systemically from sites of activation to the spleen. Mononuclear splenocytes were isolated by centrifugation over LSM. Cells were incubated in 2.4G2 (27) supernatant/20% normal mouse serum for 20 min on ice to block FcR-mediated binding of Ab, and then stained with anti-CD8-PE Cy5 (clone 53-6.7) and anti-CD45.1-PE (clone A20) for 40 min on ice. Only cells expressing CD8 and CD45.1 were analyzed for CFDA-SE staining.

Depletion of cells in vivo

For systemic DC depletion, CD11c-DTR/GFP transgenic mice were injected i.p. with 10^6 PFU rVACV-OVA FL. On day 21 postinfection, single-cell suspensions were prepared from lymph nodes and spleens of OT-1 TCR β^-/- mice, following isolation of live cells, as described above. Frequency of CD8^+ Vβ5.1 TCR^+ cells was determined by flow cytometry. A total of 10^6 Vβ5.1^+ OT-1 TCR Tg T cells was then transferred i.v. into the infected mice.

Seven days posttransplant, spleens were harvested, and single-cell suspensions were prepared. A total of 2 x 10^6 cells per sample was stimulated for 5 h with 1 μg/ml SIINFEKL peptide in the presence of 10 μg/ml brefeldin A (Sigma-Aldrich) at 37°C, 5% CO2. After stimulation, Fc receptors were blocked with 2.4G2 supernatant/10% normal mouse serum prior to staining with PerCP-Cy5.5-conjugated anti-CD45.1 and V450-conjugated anti-CD8a. Cells were then fixed for 20 min at room temperature in 2% paraformaldehyde and permeabilized in 2.4G2 supernatant/10% mouse serum containing 0.5% saponin for 15 min on ice. Cells were then stained with allophycocyanin-conjugated anti-mouse IFN-γ (clone XM1G1.2) in permeabilization buffer for 30 min on ice. Finally, cells were washed three times in 1 x PBS/2% FCS/0.5% saponin and twice in HBSS/0.1% BSA.

Flow cytometric analysis

Cells were washed thoroughly prior to analysis (typically a minimum of three washes), and then data were acquired using either a FACScan, FACSCalibur, LSR II, or FACSCan flow cytometer (BD Biosciences, San Jose, CA) and analyzed using FlowJo software (Tree Star).

Results

To study the mechanism by which Ag is able to persist following an acute virus infection, it was essential to fully document the presence of virus in infected mice. We examined the presence of rVACV, a large cytotoxic DNA virus that replicates cytosolically and is not known to incorporate nucleic acids into the genome of infected cells. We used rVACV encoding the recombinant Ag OVA (rVACV-OVA FL). We infected mice with rVACV and titered virus from the primary site of replication, the ovaries. Titration is more sensitive in our hands than examination of the presence of individual VACV proteins or reporter genes such as GFP or β-galactosidase. Replicating rVACV was detectable for 12–14 d postinfection, but was never detected after day 15 postinfection (Fig. 1A). We have previously published that VACV is cleared rapidly from secondary lymphoid organs following peripheral or systemic infection (28), but, to ensure that this was the case in this study, we titered virus from spleen following i.v. injection. Even when we removed RBCs to increase the sensitivity of the assay by removing potentially cytotoxic effects on the monolayer used during titration, we could never find replicating VACV at day 14 postinfection (Supplemental Fig. 1). A plaque assay measures the...
presence of replicating virus. However, to extend this observation, we assayed the presence of viral RNA from the ovaries or spleen of infected mice by real-time quantitative PCR. The probes we used were specific either for the E9L DNA polymerase, a clinically validated assay used to measure the presence of multiple poxviruses, or for OV A, the Ag used in our presentation assays. Our results using quantitative PCR were similar to those found with the plaque assay, as no detectable signal was present later than day 14 postinfection (Supplemental Fig. 2).

To determine the persistence of Ag presentation following infection with rVACV, we infected mice and adoptively transferred OT-I TCD8+, which are specific for the OV A 257–264 peptide presented on MHC class I, at various times postinfection. We used this methodology, as the only alternative, ex vivo culture of pAPC populations with naive TCD8+, is much less sensitive (29). We labeled the OT-I TCD8+, with CFDA-SE, transferred them into the previously infected mice, and measured TCD8+ proliferation 3 d later. We used proliferation as the most sensitive measure of Ag presentation, as a functional assay of T cell activation may be biased by the phenotype and activation status of the APC, with some APC inducing T cell proliferation, but functional unresponsiveness, following Ag presentation. We were able to detect TCD8+ proliferation of cells in the spleen for >40 d postinfection (Fig. 1B, 1C), although the proliferation observed did decline significantly between 16 and 21 d postinfection in multiple experiments. As expected after an i.v. infection, the proliferation observed in the spleen was always greater than that observed at any other site, including lymph nodes or bone marrow (data not shown). To explore whether the proliferation of TCD8+ was accompanied by an acquisition of effector function, we adoptively transferred OTI TCD8+ (CD45.2+) into B6.SJL (CD45.1+) mice infected with rVACV-OVA FL 21 d previously. On day 7 posttransfer, spleens were harvested and cells were stimulated ex vivo for 5 h with SIINFEKL in the presence of brefeldin A. IFN-γ production by donor (CD45.1+) and recipient (CD45.1+) TCD8+ cells was assessed by intracellular cytokine staining assay. Flow plots are gated on CD8α+ cells and are representative of two independent experiments with four mice each.

Although we used multiple assays to characterize the clearance of VACV from infected mice, it is not formally possible to exclude the presence of low levels of virus that are present below the level of detection of our assays, or in obscure tissues that we did not examine for the presence of virus. Therefore, to assess the role of virus replication in the prolonged Ag presentation, we treated stocks of rVACV with UVA and the DNA intercalating agent psoralen to render the virus replication deficient. This is a method

FIGURE 1. The persistence of virus and Ag presentation following infection with rVACV. (A) Mice were infected with rVACV, and ovaries were removed at times indicated. A plaque assay was used to determine rVACV titer. Error bars represent the SE. (B) OT-I.SJL TCD8+, cells were labeled with CFDA-SE and transferred i.v. into mice previously infected with rVACV-OVA FL. Three days after transfer, spleens were removed and analyzed for CFDA-SE dilution. Representative histograms display gated CD45.1+ TCD8+, from individual mice of five replicate experiments with similar results. Numbers represent the mean percentage of CD45.1+ TCD8+, that have undergone proliferation. (C) Graphical representation of the percentage of replicating OT-I.SJL TCD8+, cells at each time point after VACV infection. Error bars represent the SE. (D) OT-I TCD8+ cells (CD45.2+) were transferred i.v. into B6.SJL (CD45.1+) mice infected with rVACV-OVA FL 21 d previously. On day 7 posttransfer, spleens were harvested and cells were stimulated ex vivo for 5 h with SIINFEKL in the presence of brefeldin A. IFN-γ production by donor (CD45.1+) and recipient (CD45.1+) TCD8+, cells was assessed by intracellular cytokine staining assay. Flow plots are gated on CD8α+ cells and are representative of two independent experiments with four mice each.
we have previously used extensively (13, 25), and we ensured that each batch of virus produced following treatment is not replication competent. Treatment with UVA and psoraleen for 20 min allowed production of early proteins while preventing production of late proteins and thus virus replication (30) (Supplemental Fig. 3). We infected mice i.v. with UVA/psoraleen-treated rVACV-OVA FL at the times indicated. TCD8+ proliferation was detectable for ~30 d postinfection with replication-deficient rVACV (Fig. 2), as compared with ~45 d following infection with replicating rVACV (Fig. 1B). Interestingly, this reduction in the duration of Ag presentation (~15 d) closely corresponded to the time period that we were able to detect replicating virus by viral titers (Fig. 1A). The reduced duration of Ag presentation was not due to reduced Ag load upon infection, as equivalent levels of Ag driven by the early promoter driving OVA were produced upon UVA/psoraleen exposure (Supplemental Fig. 3) and equivalent numbers of cells were infected by treated or untreated VACV stocks in previous studies (25). Therefore, we can accurately account for the requirement for replication in continued Ag presentation, and, surprisingly, VACV replication is essential for only about one-third of the time during which Ag presentation occurs.

To date, we showed that virus replication was required for 12–14 of the 40+ days of Ag presentation persistence. However, ~30 d of Ag presentation was not explained by this mechanism. It is possible that persistently infected cells were directly presenting Ag, or that RNA or DNA had been transferred from infected cells to uninfected cells and could result in continued production of Ag for up to 30 d. To study the mechanism of persistent Ag presentation following cessation of virus replication, we used rVACV expressing a MG, the minimal antigenic determinant from OVA, residues 257–264 (SIINFEKL). rVACV-expressing OVA MG has a short t1/2 of 7 s and can only be directly presented by infected cells (17). We treated rVACV-OVA MG with UVA and psoraleen, a treatment that does not alter generation of the p-MHC I complex (25) and examined proliferation of OVA-specific TCD8+ at various times postinfection. Ag-specific proliferation of TCD8+ returned to background levels between 14 and 16 d postinfection (Fig. 3A, 3C), indicating that direct presentation by infected cells, or cells that have obtained nucleic acids encoding Ag, persists for ~2 wk following an initial infection. As virus replication can be detected for ~14 d, we would expect that the duration of Ag presentation following infection with replicating rVACV-OVA MG would be the period of replication (~14 d) plus the period of direct presentation following infection (~14 d) to give a total of 28 d. Upon infection with replicating rVACV-OVA MG, levels of proliferation of adoptively transferred OT-I returned to background levels following adoptive transfer between 26 and 30 d postinfection (Fig. 3B, 3D), as we had predicted.

To this point, we had been able to account for 26–30 d of the 45-d period for which Ag presentation occurs following infection with rVACV-OVA. FL OVA has a t1/2 of ~280 min (24) and is available for cross-presentation (12, 13) as well as direct presentation by VACV-infected cells. However, OVA MG can only be directly presented by infected cells (13), raising the intriguing possibility that the deficit of 18 d of Ag presentation we observed postinfection with rVACV-OVA MG versus OVA FL was due to a lack of cross-presentation (Fig. 3B, 3D). To examine the contribution of cross-presentation in the persistence of Ag presentation, we used cells that do not express MHC I and are unable to directly present Ag. We have been unable to directly detect Ag transfer from injected cells to host cells over many years of experimentation, so we again assayed TCD8+ proliferation, which in this case can only occur via cross-presentation of Ag following immunization with these cells. β2M−/− cells were infected for 5 h to produce levels of Ag greater than or equal to levels of Ag found in cells ex vivo following infection with replication-competent VACV. Mice were immunized with 5 × 106 β2M−/− cells of which >80% were detectably infected, to produce a similar number of infected cells, and similar Ag load, to immunization with 5 × 106 PFU of nonreplicating VACV. When mice were immunized with β2M−/− cells infected with rVACV-OVA FL, we were able to detect TCD8+ proliferation for ~18 d (Fig. 4). This was the exact difference in the number of days between mice infected with rVACV-OVA MG (direct presentation, 26 d) and OVA FL (direct and cross-presentation, 45 d). Thus, cross-presentation of Ag accounted for the remaining days of Ag presentation in our system.

To determine whether DC were specifically responsible for one or more phases of Ag persistence, we used CD11cDTR/GFP mice (22), in which DC express GFP and can be depleted by injecting the mice with DT (Fig. 5A–D). Although other studies have indicated that DT treatment of these mice can deplete other cell
types (31, 32), we found that CD11c+ DC, but not F4/80+ monocytes/macrophages, were depleted in VACV-infected mice treated with DT (Fig. 5D). DT was administered once, and Ag presentation was assayed within 4 d of depletion, a time point at which functional Ag presentation is not restored in our hands (data not shown). In CD11cDTR/GFP mice infected with rVACV-OVA FL, if DT was given during the phase of direct presentation by virally infected pAPCs, no difference in TcD8+ proliferation was seen between DT-injected and vehicle-injected mice (Fig. 5E). However, if DT was given during the later phase of cross-presentation by uninfected pAPCs (day 36 postinfection), TcD8+ proliferation was reduced to background levels in the DT-injected mice (Fig. 5F). We also used clodronate liposomes to deplete macrophages on day 20 postinfection, a procedure that leaves

**FIGURE 3.** The requirement for presentation by rVACV-infected cells in prolonged Ag presentation. C57BL/6 mice were infected with nonreplicating (A) or replicating (B) rVACV-OVA MG at times indicated. Virus replication was blocked with a 20-min treatment with UVA/psoralen. OT-I.SJL T cells were labeled with CFDA-SE and transferred i.v. into previously infected mice. Three days after transfer, spleens were removed and analyzed for CFDA-SE dilution. Representative histograms display gated CD45.1+ TcD8+ from individual mice of replicate experiments using six mice per condition. Gates represent the percentage of CD45.1+ TcD8+ cells that proliferated. (C and D) Graphical representation of the percentage of replicating OT-I.SJL TcD8+ cells at each time point after VACV infection in (A) and (B), respectively. Error bars represent the SE.

**FIGURE 4.** Prolonged presentation of cell-derived Ag (cross-presentation). (A) Mice were immunized with β2M knockout (β2M<sup>−/−</sup>) cells infected with rVACV-OVA FL for 5 h at times indicated. To prevent viral replication, cells were treated with UVC/psoralen for 20 min. OT-I.SJL T cells were labeled with CFDA-SE and transferred i.v. into previously immunized mice. Three days after transfer, spleens were removed and analyzed for CFDA-SE dilution. Representative histograms display gated CD45.1+ TcD8+ from individual mice of replicate experiments using eight mice per condition. Gates represent the percentage of CD45.1+ TcD8+ cells that proliferated. Numbers represent the SE of percentage of CD45.1+ TcD8+ proliferation. (B) Graphical representation of the percentage of replicating OT-I.SJL TcD8+ cells at each time point after VACV infection. Error bars represent the SE.
mature DC intact (Fig. 6B). We did not detect any reduction in CD11c$^+$ cells in CD11cDTR/GFP mice prevent Ag presentation persistence, only during the later stage that depends on cross-presentation. CD11cDTR/GFP mice were treated with 4 ng/g DT (A and D) or PBS (B and D). C57BL/6 mice were treated with 4 ng/g DT (C). Spleens were removed, and CD11c$^+$, GFP$^+$ cells were analyzed by flow cytometry 1 d later (A–C). (D) CD11cDTR/GFP mice were infected with $10^6$ PFU rVACV. Fifteen days postinfection, VACV-infected mice were injected with DT (as above) or PBS (i.p.). Spleens were isolated 24 h later, and single-cell suspensions of splenocytes were stained for monocytes/macrophages (F4/80$^+$, CD11c$^+$) and DC (CD11c$^+$ F4/80$^+$). Data represent the mean and SEM of at least four individual mice. (E and F) CD11cDTR/GFP mice were infected with rVACV expressing OVA FL (E and F). Sixteen days (E) or 38 d (F) postinfection, mice were treated with 4 ng/g DT. One day after DT treatment, OT-I SJL T cells were labeled with CFDA-SE and transferred i.v. into previously infected mice. Three days after transfer, spleens were removed and analyzed for CFDA-SE dilution. Representative histograms display gated CD45.1$^+$ TCD8$^+$ from individual mice of replicate experiments using four mice per condition. Gates represent the percentage of CD45.1$^+$ TCD8$^+$, cells that proliferated. Numbers represent the SE of percentage of CD45.1$^+$ TCD8$^+$, proliferation.

In agreement with data following acute infection with RNA viruses, we observed prolonged Ag presentation following an acute VACV infection. When mice were infected with rVACV-OVA FL, Ag presentation was observed for $>40$ d (Fig. 1B). The prolonged Ag presentation was weeks longer than we were able to detect replicating virus, viral DNA, or transcription of antigenic genes in mice (12–14 d; Fig. 1A). During this first phase of Ag presentation persistence, virus replication extended the amount of time Ag presentation was detected by 12–14 d. However, virus replication was not required for the persistence of Ag presentation, as mice infected with replication-deficient viruses also displayed prolonged Ag presentation (Figs. 2, 3A).

During the second phase of Ag presentation persistence, both direct and cross-presentation can occur. To address the role of only direct presentation in the persistence of Ag presentation, we used a nonreplicating rVACV-OVA MG with a $t_{1/2}$ in the order of seconds (13). Due to the short $t_{1/2}$, only the originally infected cells are able to directly present Ag because OVA MG is most likely degraded before it can be cross presented (13). $T_{CD8a}$, proliferation was observed for $\sim2$ wk in mice infected with rVACV-expressing OVA MG (Fig. 3A). The number of peptide complexes produced from the OVA MG construct, even from a UV-treated virus that does not replicate, is significantly greater than from a VACV-expressing FL OVA (25), indicating that reduced production of peptide does not account for a reduced persistence of presentation. In addition, there is no evidence that peptide produced from a MG or FL construct has a different $t_{1/2}$. Therefore, this result indicated that an originally infected cell was able to persist and directly present Ag for up to 2 wk. The ability of a persistently infected cell to resist virus-induced lysis and present Ag is intriguing because VACV is described to cause death of infected APC, both in vitro and in vivo (28, 35).
DC were originally thought to be nondividing cells with a reported life span of 2–3 d (36–38). If VACV is not causing DC death, the ability of DC to survive for 2 wk is particularly interesting due to their reported short life span. However, more recent data suggest that DC, particularly conventional DC precursors, persist for up to 10 d (39, 40). In addition, daughter DC may present Ags captured from their progenitors, possibly prolonging the duration of Ag presentation (39, 41). It is possible that replicating DC extend the duration of Ag presentation observed in our system. OVA MG has a short $t_{1/2}$, requiring continuous peptide generation or stabilization of the peptide. It is possible that DC retain low levels of viral RNA that we cannot detect ex vivo, allowing continued low-level synthesis of the peptide by infected cells. Genomic RNA is detectable for 2 mo following infection with vesicular stomatitis virus (VSV) (42) and may contribute to prolonged Ag presentation following acute infection with VSV (8). However, VACV is a DNA virus that can replicate in the cytosol with little or no contribution from the host cell nucleus, whereas VSV is a RNA virus that requires viral RNA to be transcribed in the nucleus during replication. Therefore, the difference in viral life cycle between VSV and VACV makes the contribution of integrated nucleic acids to prolonged Ag presentation by VACV unlikely. Another possibility is the stabilization of OVA MG peptide by chaperones or MHC class I molecules. However, this stabilization would have to occur for days and perhaps even weeks to account for the prolonged Ag presentation that we observe by infected cells. The $t_{1/2}$ of OVA257–264–Kb complexes is in the order of 360 min (43), making persistence for days unlikely. Even if peptide–MHC complexes are transferred to uninfected cells, a process known as cross-dressing (44) that is important in the

FIGURE 6. Depletion of phagocytes failed to prevent Ag presentation persistence during the phase of persistently infected cells. (A) C57BL/6 mice were infected with rVACV-expressing OVA FL. Twenty days postinfection, mice were treated with 200 μl clodronate liposomes, empty liposomes, or PBS i.v. One day after treatment, OT-I SJL T cells were labeled with CFDA-SE and transferred i.v. into previously infected mice. Three days after transfer, spleens were removed and analyzed for CFDA-SE dilution. Representative histograms display gated CD45.1+ TCD8+ from individual mice of replicate experiments using four mice per condition. Gates represent the percentage of CD45.1+ TCD8+ cells that proliferated. Numbers represent the SE of percentage of CD45.1+ TCD8+ proliferation. (B) Mice were infected with 10⁷ PFU rVACV, and, 15 d postinfection, VACV-infected mice were injected with clodronate liposomes (250 μl) or PBS (i.v.). Spleens were isolated 24 h later, and single-cell suspensions of splenocytes were stained for monocytes/macrophages (F4/80+, CD11c−) and DC (CD11c+ F4/80−). Data represent the mean and SEM of at least four individual mice.

FIGURE 7. Schematic of mechanisms responsible for prolonged Ag presentation following acute VACV infection.
expansion of memory TCD8+ following lymphocytic choriomeningitis virus or VSV infection (45), the short 11/2 of these complexes may preclude a meaningful contribution to prolonged Ag presentation. Some peptides may bind to cytosolic chaperones (18), allowing them to survive for periods of time sufficient for them to be transferred to other cells for cross-presentation. However, peptides that bind to chaperones under physiological conditions are the exception, not the rule, and this binding has not been demonstrated with the OVA 257–264 peptide used in this study (46, 47).

When we depleted CD11c+ DC or macrophages, both of which are infected by VACV in vivo (28) using multiple methodologies, we did not observe a repeatable effect upon Ag presentation if depletion occurred prior to day 20 postinfection (Figs. 5, 6). However, our attempts to deplete both DC and macrophages simultaneously led to death of the treated mice, so it is possible that both DC and macrophages can present persisting Ag, or that other infected immune cell types or somatic cells may contribute to the persistence of Ag presentation. We showed that the final 18 d of Ag presentation produced much lower levels of proliferation of Ag-specific TCD8+ made possible by cross-presentation of Ag. Immunization of mice with infected presentation-incompetent β2M−/− cells produced the same 18-d period of Ag presentation that was lost when short-lived Ag that was not a substrate for cross-presentation was expressed by rVACV. Depletion of CD11c+ cells during the final 18 d of presentation ablated the ability to trigger proliferation of OT1, most likely indicating a requirement for cross-presentation by specialized subsets of DC (Fig. 5E).

It has been proposed that DC have a unique ability to control the pH of the phagosome, with a pH of 7 and above that preserves Ag from rapid degradation and is optimal for cross-presentation (48). The gp91phox−/− mice that lack the gp91 subunit of NOX2, a NADPH oxidase that controls phagosomal pH, are deficient in cross-presentation of Ag targeted to the CD205 receptor (48). However, we did not detect a significant difference in cross-presentation of Ag between gp91phox−/− mice and C57BL/6 mice when virus or virus-infected cells were present (Supplemental Fig. 4). This may be because the presence of virus induces inflammation that overcomes the requirement for modulation of phagosomal pH in DC (49) or because the requirement for gp91 in cross-presentation is dependent upon targeting to the CD205 receptor. Ag may be retained long-term in recently discovered Ag storage compartments found in mature DC (14). These Ag compartments are separate from early endosomal loading compartments and allow for a continuous supply of MHC class I ligands for up to 2 wk (14). It is possible that these same storage compartments allow for the retention of Ag in DC following viral clearance, providing an Ag source for cross-presentation. Alternatively, Ag may be retained long-term in the form of immune complexes, which are stored long-term for the production of Ab on the surface of follicular DC (50). Immune complexes are a substrate for cross-presentation (51), and DC have been shown to cross-present long-lived Ab–Ag complexes during persistent Ag presentation during an influenza virus infection (52, 53). It is possible that intact Ag within these complexes can be processed to maintain MHC class I–restricted presentation during the cross-presentation phase following VACV infection.

Many memory TCD8+ responses are studied 30 d postinfection; however, we show in this study that original Ag presentation is still occurring at this time point following infection with rVACV. Ag presentation after pathogen clearance may be capable of generating memory TCD8+ cells (8). Therefore, the memory response observed 30 d postinfection may not be an accurate representation of the memory T cell population. It has previously been proposed that prolonged Ag presentation can trigger proliferation TCD8+ that are subsequently deleted or enter an unresponsive state during the induction of peripheral tolerance (54). However, we found that VACV-specific TCD8+ triggered by persisting Ag presentation did become functionally active, albeit at a lower level than those triggered during the initial infection. Recently, persistent Ag presentation following an acute RNA virus infection has been implicated in programming of memory TCD8+ (52). The study of the phenotype of effector and memory TCD8+ produced following persistent Ag presentation postinfection with the DNA virus VACV is beyond the scope of this study. However, the data yielded in this study will allow manipulation of the form of Ag contained within viral vectors or other vaccine preparations to allow the presentation of Ag for different periods of time, allowing the most effective and protective TCD8+ response to be generated following vaccination.

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Disclosures

The authors have no financial conflicts of interest.

References


Supplemental Figures

Figure S1. Female C57BL/6 mice were infected i.v. with $10^6$ pfu rVV-FL-OVA. At 3 or 14 days post-infection, mice were sacrificed and spleens were taken for analysis. For half the samples, the whole spleen was freeze-thawed 3x, then Dounce homogenized, filtered through a 40um strainer, and sonicated. For half the samples, the whole spleen was incubated in 1 mg/ml collagenase D for 60 minutes, then ground through a 40um filter and incubated in ACK lysis buffer for 10 minutes to lyse red blood cells. The remaining cells were spun down and resuspended in 5ml HBSS, then freeze-thawed 3x, then homogenized, filtered and sonicated. All homogenized samples were subjected to a series of 10-fold dilutions and titered in triplicate on TK-minus cell monolayers. Dotted lines indicate the limit of detection.
Figure S2.

Female C57BL/6 mice (n=4) were infected i.v. with $10^6$ pfu of rVACV-OVA. At each day post-infection, ovaries were extracted and frozen in $-80^\circ$C. Tissue was ground in a mortar and pestle over liquid nitrogen, and RNA was extracted and transcribed into cDNA. OVA primers were used to detect the presence of actively replicating virus. Limit of detection was 10 copies of cDNA in a volume representing 10% of total ovary tissue.
Figure S3

WT3 cells were infected with rVACV that had been treated with psoralen and UVA for the times indicated. β-gal was either driven by the early/late promoter p7.5 (red) or by the late promoter p11 with (white) or without AraC (blue). β-gal production was measured by incubating with a β-gal substrate that contained 0.125% Igepal and chloropheno red-β-D-galactopyranoside. A$_{570}$ was determined with an MRX plate reader.
**Figure S4:** gp91phox<sup>−/−</sup> mice do not show a defect in cross presentation of antigen following immunization with β2M<sup>−/−</sup> cells infected with rVACV expressing OVA FL.

gp91phox<sup>−/−</sup> or C57BL/6 mice were immunized with β2M<sup>−/−</sup> cells infected with rVACV expressing OVA FL at times indicated. OT-1.SJL T cells were labeled with CFDA-SE and transferred i.v. into previously immunized mice. 3 days after transfer, spleens were removed and analyzed for CFDA-SE dilution. Histograms were gated on CD45.1<sup>+</sup> T<sub>CD8+</sub> and are representative of two individual mice. Gates represent the percentage of CD45.1<sup>+</sup> T<sub>CD8+</sub> cells that proliferated. Numbers represent the standard error of percent CD45.1+ T<sub>CD8+</sub> proliferation.