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Direct Priming and Cross-Priming Contribute Differentially to the Induction of CD8\(^+\) CTL Following Exposure to Vaccinia Virus Via Different Routes\(^1\)

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To explore the relative importance of direct presentation vs cross-priming in the induction of CTL responses to viruses and viral vectors, we generated a recombinant vaccinia vector, vUS11, expressing the human CMV (HCMV) protein US11. US11 dislocates most allelic forms of human and murine MHC class I heavy chains from the lumen of the endoplasmic reticulum into the cytosol, where they are degraded by proteasomes. Expression of US11 dramatically decreased the presentation of viral Ag and CTL recognition of infected cells in vitro without significantly reducing total cell surface MHC class I levels. However, because US11 is an endoplasmic reticulum resident membrane protein, it cannot block presentation by non-infected cells that take up Ag through the cross-priming pathway. We show that the expression of US11 strongly inhibits the induction of primary CD8\(^+\) CTLs when the infection occurs via the i.p. or i.v. route, demonstrating that direct priming is critical for the induction of CTL responses to viral infections introduced via these routes. This effect is less dramatic following i.m. infection and is minimal after s.c. or intradermal infection. Thus, classic MHC class I Ag presentation and cross-priming contribute differentially to the induction of CD8\(^+\) CTLs following exposure to vaccinia virus via different routes. The Journal of Immunology, 2002, 169: 4222–4229.

A n important arm of the immune response to intracellular pathogens, especially viruses, is the CD8\(^+\) CTL response (1–6). CTL recognize virally infected cells based on their presentation of unique viral peptides bound to MHC class I molecules on the cell surface. Ags can be presented to CD8\(^+\) CTL through the classic MHC class I-restricted, Ag processing pathway (direct priming) in which endogenous Ags localized to the cytoplasm of infected cells are broken down into peptides that are presented complexed with MHC class I molecules on the cell surface (7). In addition, there is substantial evidence for an exogenous or cross-priming pathway in which exogenous Ags that are not expected to gain access to the cytoplasm of an APC are in some manner presented on MHC class I molecules (8–12). In special situations, CTLs can be induced in vivo by soluble or particulate Ags (13–16). The relative contribution of each of these pathways to the induction of CD8\(^+\) CTLs is an important issue. A detailed accounting of their contributions is essential for the rational design of vaccines.

Recently, Sigal et al. (17, 18) showed that virally infected non-hemopoietic cells are unable to stimulate primary CTL responses directly. The Ag produced by these non-hemopoietic cells has to be cross-presented by bone marrow-derived APCs to induce anti-viral CTL responses. Another group (19) also found that Ag under the control of tissue-specific promoters can elicit CTL responses without the expression of Ag in APCs. Cross-priming has been shown to be important in the induction of CTL responses to certain tumors and in the development of peripheral tolerance to adoptively transferred T cells specific for a transgenic Ag expressed in pancreatic \(\beta\) cells (20–22). Therefore, presentation of endocytosed material may be far more prevalent than originally recognized (23).

A major challenge in separating the contributions of the two class I-restricted Ag processing pathways under physiological conditions is that it is difficult to inhibit one pathway without affecting the other. In our study we took advantage of a human CMV (HCMV)\(^3\) gene product, US11, to achieve this purpose and dissect the relative contributions of direct priming and cross-priming to the induction of vaccinia-specific CTL in a mouse model under conditions in which the cell types infected (bone marrow-derived APCs or non-hemopoietic cells) are influenced by the route of exposure to the virus.

HCMV is a ubiquitous herpes virus that can establish life-long infection. After periodic reactivation from latency, it uses a variety of immune evasion proteins to survive and replicate in the face of a robust, fully primed host immune response (24–26). The HCMV gene product US11, an endoplasmic reticulum (ER) resident, type I transmembrane glycoprotein, has been shown in transfection experiments to cause the selective degradation of endogenous class I molecules (27). In the presence of US11, class I heavy chains are dislocated from the lumen of the ER into the cytoplasm, where they are deglycosylated by host N-glycanase and then degraded by the proteasome (28). A second HCMV gene product, US2, has also been observed to carry out a similar function (27, 29). In human astrocytoma cells transfected with the HCMV US11 gene, the K\(^b\), D\(^\alpha\), D\(^\beta\), and L\(^\alpha\) molecules expressed via recombinant vaccinia virus vectors are rapidly degraded, while in US2-transfected cells, only D\(^\beta\) and D\(^\alpha\) are significantly destabilized (30). Recently it was

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\(^{3}\) Abbreviations used in this paper: HCMV, human CMV; ER, endoplasmic reticulum; \(\beta\)-gal, \(\beta\)-galactosidase; i.d., intradermal.
shown that unlike US11, US2 causes the degradation of two essential proteins in the MHC class II Ag processing pathway: HLA-
DR-α and DM-α. The expression of US2 in cells reduces or abolishes their ability to present Ag to CD4+ T lymphocytes (31).

In this study we evaluated direct and cross-priming pathways using a recombinant vaccinia vector, vUS11, that encodes HCMV US11 under control of a vaccinia early/late promoter. The vector also encodes β-galactosidase (β-gal), which we used as a model Ag, under control of the vaccinia late promoter. By coexpressing HCMV US11 with test Ags using recombinant vaccinia virus, we were able to block the presentation of Ags synthesized endogenously within infected cells (direct priming). Since US11 is an ER resident membrane protein, it cannot be efficiently transferred in functional form from one cell to another. Thus, MHC class I re-
stricted presentation by non-infected cells that take up Ags released by infected cells (cross-priming) is not significantly af-fected. Therefore, this strategy allows selective inhibition of the classic MHC class I Ag presentation pathway. Using this system, we compared the induction of primary vaccinia-specific and β-gal-specific CD8+ CTLs in mice challenged with vUS11 or control vaccinia vSC8, a recombinant vaccinia expressing only β-gal un-der control of the vaccinia late promoter. Our results suggest that classic MHC class I Ag presentation and cross presentation contribute differentially to the induction of CD8+ CTL following exposure to vaccinia virus via different immunization routes.

Materials and Methods

Mice

Six- to 8-wk-old female BALB/c mice were purchased from National Can-cer Institute (Bethesda, MD). B10.A(5R) mice were purchased from The Jackson Laboratory (Bar Harbor, ME).

Antibodies

FITC-conjugated mouse anti-H-2Kb, FITC-conjugated mouse anti-H-2Dd, FITC-conjugated mouse IgG2a, κ, rat anti-mouse IFN-γ, and biotin-con-jugated rat anti-mouse IFN-γ were purchased from BD PharMingen (San Diego, CA). Mouse anti-β-gal was purchased from Life Technologies (Gaithersburg, MD). HRP-conjugated goat anti-mouse IgG (H+L) was purchased from Bio-Rad (Hercules, CA). FITC-conjugated goat anti-mouse IgG (H+L) was purchased from Caltag Laboratories (Palo Alto, CA). The N-terminal Kα peptide-specific for SINIFKEK (OVAα26–39/H-2Kb) complexes (32) was obtained from Dr. R. Germain (National Institutes of Health, Bethesda, MD).

Generation of recombinant vaccinia virus

The full-length US1 gene was amplified by PCR using DNA from purified HCMV strain AD169 and the following primer pair: 5′ primer, GCCCTCTC AGATGAAACTTGTAATGCTTATTC; and 3′ primer, CCTCTAGACTACCAGTCGGCAACCGAT. The 5′ primer contains an XhoI site, and the 3′ primer contains an XbaI site. Vector pSC11.MCS1-US11 was gen-
erated by ligating the US11 PCR product cut with XhoI and XbaI into the Sall and Nhel sites of pSC11.MCS1 (33). This placed US11 under control of vaccinia early/late promoter p7.5. The vector also encodes β-gal under control of vaccinia late promoter p11. The US11 insert of pSC11.MCS1-US11 was sequenced. Recombinant vaccinia virus pUS11 was generated according to standard methods (34, 35). In brief, CV-1 cells were infected with wild-type vaccinia virus (vWR-Lvar), followed by transfection (Lipofectamine 2000; Life Technologies) of the infected cells with pSC11.MCS1-US11. Recombinant virus was selected on the basis of bro-modeoxyuridine resistance and screened by the expression of β-gal. The recombinant virus was carried through three rounds of plaque purification under selective conditions. The control vaccinia vector vSC8 (34, 35) was obtained from the National Institutes of Health AIDS Research and Re-erence Reagent Program. It encodes β-gal under control of vaccinia late promoter p11. Vaccinia viruses were amplified and then purified by zonal sucrose gradient centrifugation. Viral stocks used in a single experiment were titrated simultaneously using CV-1 cells. The two viruses had similar plaque sizes and had the same efficiency of replication in both HeLa S3 and CV-1 cells. Construction of vaccinia vector pXSO is described in detail elsewhere (X. Shen, C. B. Buck, S. B. J. Wong, and R. F. Siliciano, manu-
script in preparation). Briefly, this vector encodes a rapidly degraded, epitope-shuffled form of the HIV-1 Gag protein consisting of an N-terminal methionine residue, followed in sequence by the following segments of the gag protein (HXB2R coordinates): 238–506, 505–567, 103–136, and 236–283. The peptide sequence LEQLESINIFKEK derived from OVA is ap-
pended at the C terminus. It includes the K1-restricted SINIFKEK epitope. Complexes of SINIFKEK and H-2Kb can be detected by 25-D1.16 mAb on MC57G cells infected with this virus.

Western blotting

The A20 cell line, a BALB/c B cell lymphoma, was infected with recom-binant vaccinia vectors at a multiplicity of infection of 3 for 2 h and then incubated overnight to allow protein expression. The cells were washed, pelleted, resuspended in SDS sample buffer, and boiled for 10 min. The cell lysates were pelleted at maximum speed in a microcentrifuge for 10 min, and an aliquant equivalent to 105 cells/lane was separated by SDS-PAGE on a 4–12% gradient Nupage minigel (Novex, San Diego, CA). The proteins were transferred onto nitrocellulose, and the nitrocellulose was blotted ac-
cording to ECL kit protocol (NEN, Boston, MA; primary Ab, mouse anti-β-gal at 1 μg/ml; secondary Ab, HRP-conjugated goat anti-mouse at 1/10,000 dilution).

Infection of SCID mice with vaccinia viruses

Two groups of two SCID mice each were injected i.p. with 3 × 106 PFU of either vSC8 or vUS11 diluted in HBSS. These mice were sacrificed 3 days later, and their ovaries were recovered. Ovarian homogenates were sonicated, serially diluted, and used to infect CV-1 cells preplated overnight in six-well plates at a concentration of 5 × 103 cells/well. Two days later these wells were overlaid with crystal violet solution, and the plaques were counted.

Stimulation of a primary CTL response

Group of three to five mice were immunized with 3 × 106 PFU of vSC8 or vUS11 in 0.1 ml sterile PBS via different routes. Seven or 8 days after immunization, mice were sacrificed, and their spleens were harvested. The spleens were homogenized, and the suspension was passed through a 70-μm pore size cell strainer. Pelleted spleenocytes were resuspended in ACK lysis buffer (BioSource International, Camarillo, CA); 150 mM NH4Cl, 1 M KHC2O3, and 10 mM EDTA, pH 7.2) and incubated at room temperature for 5 min to remove RBC. The spleenocytes were washed three times and then resuspended in RPMI containing 10% FCS supplemented with antibiotics, counted, and used as effectors directly in a 51Cr release assay or an ELISPOT assay.

Induction of β-gal-specific CTL

Two groups of three BALB/c mice (National Cancer Institute) each were immunized i.v. with either HBSS alone or 1 × 106 PFU of replication-incompetent adenovirus vector encoding β-gal (a gift from Dr. J. K. Donahue, The Johns Hopkins University School of Medicine, Baltimore, MD) diluted in HBSS. These mice were sacrificed 2 wk later, and their spleens were recovered. Single-cell spleenocyte suspensions were prepared as described above. After thorough washing, these effector cells were pos-
tively enriched for CD8+ cells by magnetic separation using Ab-labeled microbeads according to the manufacturer’s suggested protocol (Miltenyi Biotec, Auburn, CA). This resulted in a cell population that was >95% CD8+ by flow cytometric analysis. These effectors were resuspended at a concentration of 0.5 × 106 cells/ml in complete culture medium (RPMI 1640 medium supplemented with 10% FCS, 50 μM 2-ME, penicillin, streptomycin, IL-2, and Glutamax (Invitrogen, Carlsbad, CA)). Stimulator cells were isolated from the spleens of syngeneic BALB/c mice, gamma-
irradiated (3000 rad), and resuspended in complete culture medium at a concentration of 0.5 × 107 cells/ml. Effector cells (1 × 105) from each group of mice were incubated for 6 days with 5 × 105 stimulator cells in a total volume of 4 ml complete culture medium in the presence of 10 μg/ml of the L4-restricted β-gal peptide, TPHPARILG.

Cytolytic T cell assays

Cells of the mastocytoma cell line P815 (H-2b) were infected with the control vaccinia vector vSC8 or with vUS11 at a multiplicity of infection of 10 for 6 h. Infected cells were labeled with 51Cr for 2 h at 37°C, then washed three times to remove the excess 51Cr. Splenocyte effectors from immunized mice were mixed with target cells at varying E:T cell ratios in a 96-well plates. The plates were spun briefly and incubated for 8–10 h at 37°C. Cells were then spun again, and 40 μl medium from each well was transferred into corresponding wells in a LumaPlate (Packard Instrument, Meriden, CT). The radioactivity was counted with a TopCount
instrument. The percent specific lysis (%SL) is defined as: [(counts experimental lysis - counts medium lysis)/counts Nonidet P-40 lysis - counts medium lysis)] × 100%. Vaccinia-specific lysis is defined as: [%SL of uninfected targets] - [%SL of uninfected targets].

For experiments with β-gal-specific CTL, P815 cells were infected with vaccinia virus that expressed (vSC8) or did not express β-gal (vWR) at a multiplicity of infection of 5 in a 100-μl volume. After 2 h the target cells were resuspended in 2 ml RPMI 1640 medium supplemented with 10% FCS for a total of 18 h. The target cells were labeled with 51Cr for 1.5 h, washed, counted, and resuspended at a concentration of 1.75 × 106 cells/ml in RPMI 1640 medium supplemented with 10% FCS, penicillin, streptomycin, and glutamine. Live effector cells, as determined by trypan blue exclusion, were mixed with labeled target cells at E:T cell ratios of 100, 33, 11, and 3.7 in V-bottom, 96-well plates. Five hours later 50 μl supernatant from each well was sampled and counted as described above.

**Peptides**

The Kb-restricted peptide (pβ-gal-Kb), β-gal457-505 (ICPMYARV), and the L4-restricted peptide (pβ-gal-L4), β-gal378-383 (TPHAPARIDL), were synthesized by The Johns Hopkins University School of Medicine Peptide Synthesis Facility and were purified to >95% before use.

**ELISPOT assays**

MultiScreen-HA plates (Millipore, Bedford, MA) were coated with rat anti-mouse IFN-γ mAb at a concentration of 10 μg/ml in PBS at 4°C overnight. The Ab was discarded, and the coated plates were then washed six times with PBS containing 0.05% Tween 20 (PBST). Each well was then blocked with 200 μl assay medium for at least 1 h at 37°C. The medium was discarded. Various dilutions of effector cells with peptide and stimulator cells were added to coated/blocked wells and incubated at 37°C for 10–12 h in 200 μl RPMI containing 10% FCS supplemented with IL-2 (20 U/ml; Biogen, Cambridge, MA) and antibiotics. The cells were then discarded, and the plates were washed six times with PBST. Biotin rat anti-mouse IFN-γ at a concentration of 5 μg/ml in PBST was added to each well, and the plate was incubated at 4°C overnight. The Ab solution was discarded, and the plates were then washed six times with PBST. Avidin-alkaline phosphatase (Sigma-Aldrich, St. Louis, MO) was added at a concentration of 1.25 μg/ml in PBS and incubated for 2 h at room temperature. The plate was then washed six times with PBST, and 50 μl 5-bromo-4-chloro-3-indolylphosphate/nitro blue tetrazolium solution (Sigma-Aldrich) was added to each well. After incubation at room temperature for 15 min, the reaction was stopped by adding water. The plate was air-dried, and the spots were counted using a dissecting microscope.

**Results**

**Generation and characterization of the recombinant vaccinia vector vUS11**

In this study we used two recombinant vaccinia viruses to study the role of direct presentation and cross-priming in CTL induction. The previously described vector vSC8, which expresses the well-characterized, H-2Kb-restricted chicken OVA epitope SIINFEKL (pOVA) fused to the C terminus of a rapidly degraded form of HIV-1 Gag. After the fusion protein is processed by the class I pathway, surface expression of SIINFEKL/H-2Kb complexes can be detected using the mAb 25-D1.16 (32). Nine hours after the coinfection, FACS analysis showed that the expression of US11 did not significantly affect the total H-2Kb level on the cell surface (Fig. 1B), consistent with results obtained in A20 and P815 cells. On the other hand, we observed a significantly lower level of SIINFEKL/Kb on cells coinfected with vUS11 when compared with cells coinfected with vSC8 (Fig. 1D). These results provide direct demonstration that US11 reduces cell surface presentation of a defined class I epitope. In this regard the expression of HCMV US11 produces the same result as treatment of cells with brefeldin A, a specific inhibitor of the transport of newly synthesized proteins from ER to Golgi apparatus. Brefeldin A inhibits the lysis of virally infected cells by CTL without producing a measurable drop in the cell surface concentration of class I MHC molecules (38, 39).

**vUS11 vector** can be used to distinguish between the direct and cross-priming pathways only if US11 expression in an infected cell could be affected by introducing the US11 gene with a recombinant vaccinia vector, A20 or P815 cells were infected with vSC8 or vUS11. Sixteen hours after infection, FACS analysis showed no significant difference in the levels of H-2Dd molecules on the cell surface between cells infected with the two vectors (data not shown). This result probably reflects the long half-life of cell surface MHC class I molecules (37).

Although infection with vUS11 did not significantly reduce surface class I levels over the course of 16 h, it did have a dramatic effect on the presentation of viral Ags expressed in infected cells (Fig. 1). In these experiments cells of the murine fibrosarcoma line MC57G (H-2b) were coinfected with a recombinant vaccinia vector, vXS0, and with either vUS11 or the control vaccinia vector, vSC8. The vXSO vector expresses the well-characterized, H-2Kb-restricted chicken OVA epitope SIINFEKL (pOVA) fused to the C terminus of a rapidly degraded form of HIV-1 Gag. After the fusion protein is processed by the class I pathway, surface expression of SIINFEKL/H-2Kb complexes can be detected using the mAb 25-D1.16 (32). Nine hours after the coinfection, FACS analysis showed that the expression of US11 did not significantly affect the total H-2Kb level on the cell surface (Fig. 1B), consistent with results obtained in A20 and P815 cells. On the other hand, we observed a significantly lower level of SIINFEKL/Kb on cells coinfected with vUS11 compared with cells coinfected with vSC8 (Fig. 1D). These results provide direct demonstration that US11 reduces cell surface presentation of a defined class I epitope. In this regard the expression of HCMV US11 produces the same result as treatment of cells with brefeldin A, a specific inhibitor of the transport of newly synthesized proteins from ER to Golgi apparatus. Brefeldin A inhibits the lysis of virally infected cells by CTL without producing a measurable drop in the cell surface concentration of class I MHC molecules (38, 39).

**FIGURE 1.** The vUS11 vector reduces presentation of viral Ag on the cell surface. Uninfected MC57G cells, MC57G coinfected with vXS0 and vSC8, and MC57G cells coinfected with vXS0 and vUS11 were analyzed for surface expression of H-2Kb or SIINFEKL/Kb complexes by FACS 9 h after infection. A and C, Background staining with isotope controls for 25-D1.16 (irrelevant mouse IgG1 mAb), respectively. B and D, Staining with anti-H-2Kb and mAb 25-D1.16, respectively. Multiplicities of infection were 1 for vXS0 and 10 for vUS11 and vSC8.
cell inhibits presentation of viral Ags by the infected cell but not by other adjacent cells that do not express US11. US11 is an integral membrane protein localized to the ER, making it unlikely that such a transfer could take place. In coculture experiments using two different APC lines, we were unable to show that US11 expressed by one cell could be transferred in a functional form to other cells in the culture as judged by FACS analysis of K\(^{b}\)/SIINFEKTL on the cell surface (not shown). Therefore, US11 should only affect direct presentation mediated by infected APCs that express US11.

Expression of US11 reduces presentation of vaccinia Ags to vaccinia-specific CTLs in vitro and decreases priming of vaccinia-specific CTL responses in B10.A(5R) mice

Because the expression of US11 inhibits the presentation of the H-2K\(^{b}\)-restricted SIINFEKTL model peptide on the surface of infected cells, we reasoned that CTL recognition of viral Ags might be impaired in the case of vUS11-infected cells. To investigate this question, we generated vaccinia-specific effector cells by i.p. infection of B10.A(5R) mice with 3 \(\times 10^6\) PFU of vSC8 or vUS11. B10.A(5R) mice were used because the class I alleles expressed by this strain (H-2K\(^{b}\), H-2D\(^{d}\), H-2L\(^{d}\)) are all susceptible to US11-mediated rapid degradation (30). Seven days after infection, spleens were harvested from the immunized mice, and the splenocytes were used directly as effectors in a \(^{51}\)Cr release assay with mediated rapid degradation (30). Seven days after infection, spleens were harvested from the immunized mice, and the splenocytes were used directly as effectors in a \(^{51}\)Cr release assay with vSC8- or vUS11-infected P815 cells as targets (H-2D\(^{d}\), H-2L\(^{d}\)-restricted lysis). By using relatively high E:T cell ratios and long (8-h) incubation periods, the induction of CTL can be directly measured without an in vitro stimulation (40). The vaccinia-specific effectors generated by i.p. priming with the control vaccinia virus vSC8 lysed vSC8-infected P815 targets, but were unable to lyse vUS11-infected P815 targets (Fig. 2A). These results demonstrate that the expression of US11 dramatically reduces the presentation of vaccinia Ags to vaccinia-specific CTLs in vitro. Compared with the US11-mediated inhibition of Ag presentation as measured by K\(^{b}\)/SIINFEKTL staining (Fig. 1), the inhibition of lysis of vaccinia-infected targets by US11 was more complete. This may reflect the fact that in the setting of coinfection with vUS11 and vXSO, some of the SIINFEKTL peptide was processed for MHC class I presentation before US11 was fully active. Because the SIINFEKTL peptide expressed by vXSO is linked to a rapidly degraded Ag (see Materials and Methods), the peptide may have been processed for MHC class I presentation more rapidly than peptides derived from vaccinia early Ags. If this were so, then some H-2K\(^{b}\)/SIINFEKTL complexes may have formed and exited the ER before US11 became fully active. In any event, the results presented in Fig. 2A show that under appropriate conditions US11 can very effectively block the presentation of viral Ags to CTL.

Our results also indicated that when introduced by i.p. injection, vUS11 induced significantly lower levels of vaccinia-specific CTL than did the control vaccinia vector vSC8, as shown by a \(^{51}\)Cr release assay with vSC8-infected P815 cells as targets (Fig. 2A). Similar results were obtained when the induction of CTL responses to defined peptide epitopes expressed by these viruses were evaluated. The K\(^{b}\)-restricted CTL response to \(\beta\)-gal peptide 497–504 (p\(\beta\)-gal-K\(^{b}\)) and the L\(^{d}\)-restricted response to \(\beta\)-gal peptide 876–884 (p\(\beta\)-gal-L\(^{d}\)) were measured by ELISPOT assay (Fig. 2B). The response was readily measurable using effector cells from mice infected with vSC8, but was extremely low with effectors from mice immunized with vUS11 despite equivalent expression of \(\beta\)-gal by vSC8 and vUS11 (Fig. 2C). This difference does not reflect any intrinsic difference in the replication of the two viruses. Both viruses replicate equivalently in vitro. Equivalent in vivo replication was also demonstrated following i.p. inoculation of 3 \(\times 10^6\) PFU of each virus. Recovery of virus from the ovaries demonstrated 2.4 \(\times 10^7\) PFU/ovary for vSC8 and 3.3 \(\times 10^7\) PFU/ovary for vUS11. In the vSC8 and vUS11 vectors, \(\beta\)-gal expression is driven off a late vaccinia promoter. To demonstrate that the Ag can be directly presented by infected cells, we generated \(\beta\)-gal-specific CTL by priming BALB/c mice with an adenovirus vector expressing \(\beta\)-gal and then restimulated splenocytes from immunized mice with the L\(^{d}\)-restricted \(\beta\)-gal peptide, TPHPARGL. The resulting effectors lysed target cells infected with vSC8, but not target cells infected with a control vaccinia vector that does not express \(\beta\)-gal (vWR; Table I). Effector cells from unprimed mice did not lyse either target. This result shows that \(\beta\)-gal expressed from the late vaccinia promoter can be directly presented in our system. Taken together, these results demonstrate that the expression of US11 interfered with the in vivo priming of Ag-specific CTL responses in mice. The inhibitory effect was particularly dramatic in the case of the L\(^{d}\)-restricted response to \(\beta\)-gal peptide. Strong, but less complete, inhibition was seen in the case of the K\(^{b}\)-restricted \(\beta\)-gal peptide. This may reflect different rates of US11-mediated degradation of K\(^{b}\) and L\(^{d}\) (30). Therefore, in subsequent experiments we
focused on the Ld-restricted CTL responses to β-gal (using pβ-gal-Ld for the ELISPOT assay). Taken altogether, the in vitro and in vivo results suggest that direct presentation of β-gal and vaccinia Ags can be blocked by the expression of US11 and that direct presentation plays an important role in CTL priming after i.p. infection of vaccinia virus as it can be significantly reduced by the expression of US11.

Expression of US11 affects the priming of CTL responses differentially depending on the route of exposure

The data collected in mice infected i.p. were consistent with direct priming as a principal mode of presentation of viral Ags for the induction of CTL responses. Because direct priming is thought to be dependent on the infection of professional APCs, which may be abundant in the peritoneal compartment, we reasoned that other vaccination sites, where professional APCs might be less prevalent, might be more reliant on cross-priming for CTL induction. To test this hypothesis, groups of three to five mice were inoculated with vSC8 and vUS11 by various routes, and CTL responses were determined as described above. As observed previously, i.p. inoculation of vUS11 resulted in greatly diminished CTL responses as measured by either ELSPOT or 51 Cr release (Fig. 3, A and B). In the case of i.v. inoculation of vaccinia virus, the expression of US11 also inhibited CTL induction, while for i.m. inoculation the inhibitory effects of US11 were much less pronounced (Fig. 3, A and B). Interestingly, in the case of infection by s.c. or intradermal (i.d.) routes, we did not observe significantly lower levels of β-gal- or vaccinia-specific CTLs induced by vUS11 compared with vSC8. The pattern was observed in response to a defined peptide Ag (β-gal; Fig. 3A) and was also observed in the overall response to vaccinia virus (Fig. 3B). These experiments were repeated three or more times with all routes of inoculation using different preparations of virus stocks with similar results. In summary, our results indicate that the effect of inhibition of direct priming on CTL induction is dramatic in the case of i.p. and i.v. infection, less significant for i.m. infection, and minimal in the case of s.c. or i.d. infection. Based on these results, we suggest that classic MHC class I Ag presentation and cross-priming contribute differentially during the induction of CD8+ CTLs following infection by vaccinia virus via different routes.

The induction of primary CTL responses by vUS11 in BALB/c mice

Finally, we wanted to determine whether the phenomenon observed in B10A(5R) mice could be generalized. We inoculated BALB/c mice with $3 \times 10^9$ PFU of vSC8 or vUS11 via i.p., i.v., i.m., s.c., or i.d. routes. The induction of β-gal-specific CTLs was studied by measuring IFN-γ secretion using the ELISPOT assay (Fig. 4A), and the induction of vaccinia-specific CTLs was measured by $^{51}$Cr release assay (Fig. 4B). Significantly lower Ld-restricted β-gal-Ld-specific IFN-γ secretion and vaccinia-specific responses were induced by vUS11 than by vSC8 via the i.p. route, consistent with previous findings in B10.A(5R) mice. The effect of US11 on the induction of β-gal-Ld-specific and vaccinia-specific responses was not significant if the infections were conducted via other routes. This may reflect the fact that not all the
Methods are indicated. Seven days after infection, splenocytes were used to measure the same populations of splenocytes were measured by a 51Cr release assay pressing US11. This, in turn, dramatically reduces the capacity of restricted presentation of newly synthesized viral Ags in cells expressing US11 does significantly reduce the MHC class I-restricted presentation of previously synthesized viral Ags in cells expressing US11. This, in turn, dramatically reduces the capacity of infected cells to be recognized by virus-specific CD8+ T cells in either the induction or effector phase of the immune response. As a result, US11-expressing viruses can be used to determine whether direct priming contributes to the induction of CTL responses in vivo.

Using this system we have demonstrated that direct priming can contribute to the induction of primary CD8+ CTLs, that the magnitude of the contribution depends on the route of infection. In the case of i.p. or i.v. inoculation, US11-mediated inhibition of the classic MHC class I pathway in infected cells strongly inhibits the induction of primary CD8+ CTLs. This finding indicates that direct priming plays an important role in the induction of virus-specific CTL responses in this situation. It is not clear which cell types are infected through these routes. A likely explanation for our results is that i.p. or i.v. inoculation results in the direct infection of large numbers of professional APCs. Such infected APCs could express Ags endogenously, process them through the classic MHC class I processing pathway, and present them to naive CD8+ T cells. In this scenario, the expression of US11 would inhibit the induction of CD8+ CTLs. An alternative explanation is that vUS11 simply replicates poorly in the i.p. site and thus induces a poor CTL response. We consider this less likely, because the viruses replicate equally well in vitro and in vivo in immunodeficient mice.

We also showed that inhibition of the classic MHC class I pathway by US11 has less of an effect on the induction of primary CD8+ CTLs following i.m. infection and has a minimal effect following s.c. or i.d. infection. This observation may reflect the fact that cell types other than professional APCs are initially infected at these sites. For example, following i.m. injection, a large fraction of the viral inoculum may go into myocytes, which are not efficient at priming a CTL response (42–44). In fact, Sigal and colleagues (17, 18) have shown that chimeras constructed by lethally irradiating C57BL/6 mice and reconstituting them with bone marrow from TAP0/0 mice (also H-2b) do not generate CTL responses following infection with a large i.p. dose of vaccinia. This result suggests that virally infected non-hemopoietic cells are unable to stimulate primary CTL-mediated immunity, and that bone marrow-derived cells are required as APC. In this setting, one possibility is that virus-induced cytokines such as IFN-γ can reverse the US11 effect. This would allow direct presentation by infected APC, which would have been undetected by us. However, in this case it would be necessary to propose that this IFN-γ-mediated reversal operates only at some sites of infection.

Evidence that cross-priming can be an obligatory Ag presentation pathway for priming CTL responses to viruses that only infect non-hemopoietic cells comes from elegant studies of poliovirus receptor transgenic mice. Normal mice cannot be infected by poliovirus because they do not express the receptor for this virus. In mouse chimeras constructed by lethally irradiating poliovirus receptor transgenic mice and reconstituting them with bone marrow from normal mice, the priming of polio-specific CTL is similar to the priming in polio receptor transgenic mice (17). Strong evidence also came from the study by Prasad et al. (19) showing that Ag under the control of tissue-specific promoters can elicit CTL responses without the expression of Ag in APCs. Yewdell and colleagues (62) have recently used vaccinia vectors expressing US2 or US11 to demonstrate that both direct priming and cross-priming contribute to the induction of CTL responses in vivo, consistent with the findings presented here.

Many viruses can infect both non-hemopoietic cells and bone marrow-derived cells. Our system has the advantage that both bone marrow-derived cells and non-hemopoietic cells are susceptible to infection by vaccinia, and the cell types that are infected are determined naturally by the route of exposure. From our studies of
i.p. or i.v. priming with vaccinia virus, we conclude that direct priming plays an important role in the induction of CTL responses when virus is introduced via these routes. Evidence supporting this conclusion also comes from the work of Bronte et al. (45). They studied the ability of a panel of recombinant vaccinia viruses expressing β-gal under the control of a number of early and late promoters to prolong the survival of mice bearing a lethal β-gal-expressing tumor. They found that via the i.v. route only those recombinant vaccinia viruses employing early promoters were effective in prolonging survival. Late promoters were ineffective regardless of their determined promoter strength. When a variety of cell types were infected with the panel of viruses in vitro, dendritic cells were found to express β-gal only under the control of the early promoters even though late promoters were intrinsically more active in other cell types (45). These results are consistent with the idea that direct infection of professional APCs by vaccinia viruses is important for the induction of a CTL response.

Our results have also shown that among all the immunization routes tested, i.p. and i.v. infection resulted in the highest levels of CD8+ CTL. This is consistent with the observation that i.v. immunization using recombinant modified vaccinia virus Ankara expressing multiple CTL epitopes induced stronger CTL than did i.m. immunization (46). The direct infection of APCs and efficient presentation of Ag by direct priming could contribute to this phenomenon. Unlike peptide-class II complexes, which have extremely long half-lives (exceeding 100 h), peptide-class I complexes have shorter half-lives (~10 h) (47). MHC class I presentation must therefore be sustained by continuous synthesis of class I molecules and loading from internal sources of Ag, which could be accomplished more efficiently by direct priming.

In our vaccinia virus system the expression of US11 significantly reduced the MHC class I-restricted presentation of newly synthesized viral Ags in cells expressing US11 without affecting the overall cell surface expression of previously synthesized MHC class I molecules. In cell lines stably expressing US11, where US11 can function for longer period of time, surface MHC class I levels were found to decrease (36). However, our results clearly demonstrate that HCMV US11 can prevent the presentation of viral Ags before it has any significant effect on the cell surface MHC class I level. These phenomena resemble the inhibition of Ag presentation by brefeldin A (38, 39), although US11 and brefeldin A operate through different biochemical mechanisms. Brefeldin A rapidly and reversibly blocks the transport of newly synthesized proteins out of the ER and has no apparent effect on endosome, endosome acidification, lysosomal function, or the trans-Golgi system (48).

For viruses that evade CD8+ CTLs by interfering with MHC class I expression, it is necessary to explain how at the same time they can evade NK cells. By ~72 h after infection, HCMV expresses an NK decoy, UL18, which mimics MHC class I (49). Recently, it has been proposed that another HCMV product, UL16, serves to mask NK recognition of UL16-binding proteins or MHC class I chain-related protein B, which are ligands for the activating receptor, NKG2D/DAP10 (50). In the case of HIV-1, the Nef protein down-regulates cell surface MHC class I by accelerating the endocytosis of class I complexes (51–54). The specific targeting of HLA-A and -B locus products, but not HLA-C or -E locus products, may be relevant for NK cell evasion (55). We proposed here that inhibition of MHC class I Ag presentation without significant reduction of cell surface class I levels, as observed here, could be another important mechanism. Especially for viruses with short replication cycles, this mechanism may be enough to allow viral replication in the presence of efficient CD8+ T cell and NK responses. Recently, the expression of HCMV US11 has been shown to be sufficient to trigger the cytotoxicity of NK cell clones expressing an inhibitory killer cell Ig-like receptor for HLA-C (56). Based on the long half-life of cell surface MHC class I, as shown here and by others, together with the fact that vaccinia is rapidly cytotoxic, we believe that triggering the cytotoxicity of NK cells is not a significant factor contributing to the phenomena we observed here, because total class I levels are not reduced during the time course of our experiments.

In terms of vaccine development, our results suggest that immunization routes should be taken into consideration when vaccines are designed or evaluated. Certain vaccine strategies rely on the classic MHC class I Ag presentation pathway; for instance, strategies involving the targeting of Ags for rapid degradation in cytosol (57, 58) are likely to be most effective in the setting of direct presentation. Strategies that involve targeting Ag-expressing cells for apoptosis (59–61) rely on cross-priming. The results presented here provide a rational basis for the choice of routes of immunization that are likely to be the best way for the particular virus vaccine strategies.

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References


