

Dendritic Cell Migration Limits the Duration of CD8⁺ T-Cell Priming to Peripheral Viral Antigen[∇]

Amanda M. Schell,^{1†} Erica L. Granger,^{1†} Frank Koczot,²
Matthew A. Fischer,¹ and Christopher C. Norbury^{1*}

Department of Microbiology and Immunology, MC H107, Penn State M. S. Hershey College of Medicine, Hershey, Pennsylvania 17033,¹ and Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20892-0440²

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CD8⁺ T cells (T_{CD8+}) play a crucial role in immunity to viruses. Antiviral T_{CD8+} are initially activated by recognition of major histocompatibility complex (MHC) class I-peptide complexes on the surface of professional antigen-presenting cells (pAPC). Migration of pAPC from the site of infection to secondary lymphoid organs is likely required during a natural infection. Migrating pAPC can be directly infected with virus or may internalize antigen derived from virus-infected cells. The use of experimental virus infections to assess the requirement for pAPC migration in initiation of T_{CD8+} responses has proven difficult to interpret because injected virus can readily drain to secondary lymphoid organs without the need for cell-mediated transport. To overcome this ambiguity, we examined the generation of antigen-specific T_{CD8+} after immunization with recombinant adenoviruses that express antigen driven by skin-specific or ubiquitous promoters. We show that the induction of T_{CD8+} in response to tissue-targeted antigen is less efficient than the response to ubiquitously expressed antigen and that the resulting T_{CD8+} fail to clear all target cells pulsed with the antigenic peptide. This failure to prime a fully functional T_{CD8+} response results from a reduced period of priming to peripherally expressed antigen versus ubiquitously expressed antigen and correlated with a brief burst of pAPC migration from the skin, a requirement for induction of the response to peripheral antigen. These results indicate that a reduced duration of pAPC migration after virus infection likely reduces the amplitude of the T_{CD8+} response, allowing persistence of the peripheral virus.

The induction of effector CD8⁺ T cells (T_{CD8+}) is a vital step in the eradication or control of many viral infections. The induction of antiviral T_{CD8+} requires the presentation of virally derived peptides in complex with major histocompatibility complex (MHC) class I on the surface of specialized professional antigen-presenting cells (pAPC), most commonly a subset of dendritic cells (DC) that bear the CD8α chain (1, 29). The CD8α⁺ DC reside only in secondary lymphoid organs and not in the tissues, implying that cell-mediated transport or drainage of virus particles to a lymph node is required for initiation of a T_{CD8+} response. Partial inhibition of DC migration from the skin can impair the initiation of a T_{CD8+} response (2). After influenza infection in the lungs, there is a burst of DC migration, followed by a refractory period in which no DC migration occurs (19). The functional consequences of this refractory period of DC migration have not been explored.

A number of viruses, particularly human papillomaviruses, infect the skin and are ignored by the immune response for extended periods of time (31). We sought to explore the possibility that, after a low-level peripheral virus infection of the skin, changes in DC migration may limit the availability of antigen in the draining lymph node and thus the induction of a T_{CD8+} response. There are a number of confounding factors

that make the study of DC migration in the initiation of an antiviral T_{CD8+} response difficult. Virus particles may directly drain to the lymph node within seconds (11, 13, 25). In addition, many viruses will alter DC functions, including migration, after infection of the DC itself. This may occur via specific viral modulation of DC function (16) or via nonspecific shut down of host protein synthesis (26), both of which will affect migration. Thus, it is often not possible to distinguish between the effects of virus infection upon DC migration, drainage of virus directly to the lymph node, and the natural response that follows migration of DC responding to a peripheral virus infection.

There is currently no mouse model of a peripheral virus infection that is confined to the skin, as no natural mouse papillomavirus has ever been isolated. Therefore, to address these issues, we have made use of another small DNA virus, namely, an adenovirus vector that is replication deficient (rAd). These vectors express influenza virus nucleoprotein (NP) under the control of a ubiquitous (cytomegalovirus [CMV] immediate-early) or tissue-targeted promoter (K14, targeted to keratinocytes, the site of papillomavirus replication). Antigen driven by the K14 promoter is expressed only in skin cells, so only uninfected DC can present antigen in this system, removing the need to account for modulation of the function of virus-infected DC.

We demonstrate that when antigen is expressed in only keratinocytes in the skin, the efficiency of T_{CD8+} induction is reduced and the time period for which antigen is available to prime effector cells is reduced dramatically. DC-mediated transport is required for antigen to reach the lymph node where a T_{CD8+} response is initiated. The reduced time period

* Corresponding author. Mailing address: Room C6764E, Department of Microbiology and Immunology H107, Penn State M. S. Hershey College of Medicine, Hershey, PA 17033. Phone: (717) 531-7204. Fax: (717) 531-6522. E-mail: ccn1@psu.edu.

† A.M.S. and E.L.G. contributed equally to this study.

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of antigen presentation is the result of a transient blockade in DC migration from the site of infection. The blockade in DC migration reduced the delivery of viral antigen to the lymph node needed to induce a T_{CD8^+} response. The resulting T_{CD8^+} response to peripheral viral antigen is not capable of clearing all target cells presenting a viral peptide, thus allowing the persistence of peripheral virus-infected cells. These results provide a potential mechanism for the long-term evasion of the immune response by papillomaviruses following natural infection and also have important implications for tissue targeted gene therapy vectors.

MATERIALS AND METHODS

Mice. All mice were housed in specific-pathogen-free conditions in the animal facility at the Pennsylvania State M. S. Hershey College of Medicine. Experiments and breeding were conducted under the guidelines of the institutional animal care and use committee. F5 mice (21) were originally obtained from the NIAID contract facility at Taconic Farms (Germantown, NY) and, where indicated, were bred to B6.SJL congenic mice (Taconic). C57BL/6 mice were obtained from Charles River Laboratories (Wilmington, MA).

Recombinant viruses. The manufacture of replication-deficient (E1, E3 deleted) rAd vectors encoding influenza virus nucleoprotein derived from influenza virus A/PR8 and driven by CMV, K14, or surfactant protein C (SPC) promoters has been previously described (28). Viruses used here were made similarly, but the amino acid sequence of the nucleoprotein was altered at the D^p-restricted determinant (NP₃₆₆₋₃₇₄) from ASNENMETM to ASNENMDAM as previously described (25) in order to facilitate recognition by the F5 T-cell receptor (TCR) transgenic T_{CD8^+} . Recombinant vaccinia virus (rVACV) expressing influenza virus nucleoprotein from A/NT60/68 was kindly provided by Tim Elliott (Cancer Research UK Wessex Regional Medical Oncology Unit, University of Southampton, Southampton, United Kingdom).

Immunizations. Female C57BL/6 mice (6 to 10 weeks old) were injected intravenously (i.v.) with 10^7 PFU of rVACV or rAd in the dosages indicated. To generate T_{CD8^+} after i.v. injection, splenocytes from mice immunized with viruses were stimulated *in vitro* for 6 days with splenocytes pulsed with antigenic peptides at a concentration of 1 ng/ml.

For intradermal (i.d.) ear injections, mice were anesthetized, and rAd was injected into each ear pinna (~20 μ l/ear). At the times indicated postinfection, the spleens and draining cervical lymph nodes were removed and homogenized, and T-cell expansion and/or effector activity was assessed as described below. For all T_{CD8^+} assays, whether directly *ex vivo* or after *in vitro* culture, live cells were recovered via centrifugation over a lymphocyte separation medium gradient (LSM; density = 1.077 g/ml; Mediatech, Herndon, VA), and viable cells were harvested from the LSM-medium interface. Cells were then tested for effector activity or proliferation.

Cell lines and cultures. All media were purchased from Invitrogen (Grand Island, NY). Cytotoxic T-cell culture was in Roswell Park Memorial Institute (RPMI) 1640 medium containing 10% fetal bovine serum (FBS), 5×10^{-5} M β -mercaptoethanol, antibiotics (penicillin and streptomycin), nonessential amino acids, sodium pyruvate (1 mM), and 2 mM glutamine (RP-10). Iscove modified Dulbecco medium supplemented with 10% FBS, antibiotics (penicillin and streptomycin), and 2 mM glutamine was used during effector assays and for adoptive transfers where shown.

Adoptive transfer of TCR Tg T cells. T-cell enriched populations were obtained from TCR transgenic (TCR Tg) mice as follows. Lymph nodes (popliteal, inguinal, brachial, axillary, and superficial cervical) and spleens were harvested, and a single-cell suspension was generated. Live cells were isolated by centrifugation over LSM. Cells were washed and resuspended, and 1×10^6 to 5×10^6 cells were injected into each recipient via the lateral tail vein. Unless the experimental protocol required otherwise, mice were immunized 24 h after the adoptive transfer of T cells.

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 an LSM gradient were washed and then labeled with 5 mM CFDA-SE for 10 min at 37°C, washed, and injected i.v. into recipient mice. Primary lymphocytes were harvested at the times indicated after immunization and analyzed for cell division as indicated by dilution of the CFDA-SE dye.

Spleens were harvested from two mice per group and homogenized separately.

Mononuclear cells were isolated by centrifugation over LSM. Cells were incubated in 2.4G2 (9) supernatant–20% normal mouse serum for 20 min on ice to block Fc receptor-mediated uptake of antibody and then stained with anti-CD8-phycoerythrin (PE) Cy5 (clone 53-6.7) and either anti-Vb11-PE (clone RR3-15) or anti-CD45.1-PE (clone A20) for 40 min on ice.

Flow cytometric analysis. Cells were washed thoroughly prior to analysis (typically a minimum of three washes), and then data were acquired by using either a FACScan, a FACSCalibur, or a FACSCanto flow cytometer (Becton Dickinson, San Jose, CA) and analyzed by using FlowJo software (Treestar, San Carlos, CA). Only cells expressing CD8 and either V β 11 or CD45.1 were analyzed for CFDA-SE staining. All antibodies were from BD Pharmingen (San Diego, CA).

Intracellular cytokine production analysis. Splenocytes were harvested, and mononuclear cells were isolated over an LSM gradient. The resulting cells were restimulated in the presence of APC bearing the NP₃₆₆₋₃₇₄ peptide. At 6 days after restimulation, live cells were harvested by centrifugation over LSM. The resulting cells were incubated with or without 1 μ M NP₃₆₆₋₃₇₄ peptide for 2 h, at which time 5 μ g/ml of brefeldin A was added, and the cells were incubated for an additional 4 h. Cells were then stained with anti-CD8-PE-Cy5 antibody and fixed with 1% paraformaldehyde for 20 min. After a washing step, cells were permeabilized with 0.5% saponin and stained with anti-gamma interferon (IFN- γ)-fluorescein isothiocyanate (FITC; clone XMG 1.2) for 40 min on ice and analyzed as indicated above. The results shown are the percentages of CD8⁺ cells that produced IFN- γ .

In vivo killing assay. Red blood cell-depleted splenocytes were purified from B6.SJL mice by suspension in ACK lysis buffer (Cambrex, Walkersville, MD). The resulting cells were washed once and incubated with either the concentrations of NP₃₆₆₋₃₇₄ shown or 1 μ M control peptide from chicken egg ovalbumin (OVA) (OVA₂₅₇₋₂₆₄; SIINFEKL) for 40 min at room temperature. All peptides were made in the Macromolecular Core Facility at Penn State College of Medicine. Cells were then washed twice to remove unbound peptide and labeled with 5 μ M (NP peptide-pulsed) or 0.5 μ M (OVA peptide-pulsed) CFDA-SE. Cells were counted and mixed in equal ratios, and 2×10^6 cells/peptide were injected into previously immunized mice. At 18 h after injection, the spleens were harvested from recipient mice and homogenized, and the red blood cell-depleted splenocyte population was analyzed for the presence or absence of CFDA-SE-labeled cells by flow cytometric analysis. Similar results were obtained when the spleens were removed from recipients 4 h after the injection of CFDA-SE-labeled cells.

DC migration assay. FITC (8 mg/ml, Sigma) was dissolved in equal amounts of dibutyl phthalate (Sigma) and acetone and then vortexed vigorously. C57BL/6 mice were immunized with approximately 4×10^6 PFU rAdLacZ i.d. in each ear. Immunized or nonimmunized mice received 25- μ l aliquots of FITC solution applied to each ear with a pipette tip. After 18 h, the draining lymph nodes were harvested, microdissected, and incubated in 2 mg/ml of collagenase D (Roche Diagnostics, Indianapolis, IN) at 37°C for 30 min. The resulting single cell suspensions were analyzed by flow cytometry.

Ex vivo proliferation. DC obtained from lymph nodes were incubated with CD11c microbeads (Miltenyi Biotec, Auburn, CA) and positively sorted by using an AutoMACS system. A total of 5×10^4 CD8-enriched CFDA-SE-labeled TCR-Tg cells were added to 10^5 sorted DCs in 200 μ l of RPMI 1640 containing 10% FBS, 1% nonessential amino acids, penicillin-streptomycin, sodium pyruvate, and 2 mM L-glutamine in 96-well U-bottom plates (Costar; Corning, Corning, NY). Each culture was performed in duplicate. Cultures were analyzed for proliferation after 60 h.

Removal of the infection site. At times indicated after i.d. ear infection, the mice were anesthetized, and the site of infection was surgically removed. At 3 days postimmunization, the draining lymph nodes were removed and analyzed for cell division *in vivo*.

Statistical analysis. Statistical analyses were determined where indicated using a two-tailed unpaired Student *t* test. In each case, tests were used to distinguish between values obtained after immunization with either AdK14NP versus AdCMVNP, AdK14NP versus AdSPCNP, or AdK14NP versus no immunization. Where analysis of *in vivo* killing assays was performed, percentages of cells remaining in the CFDA-SE high peak were compared. Where statistics are shown superimposed on representative histograms, they represent statistics from at least three repeat experiments. All error bars shown on plots represent the standard error of the mean, and the statistical significance is indicated (*, $P < 0.05$; **, $P < 0.01$).

RESULTS

Characterization of rAd. In order to study the initiation of a T_{CD8^+} response to a peripheral virus infection in the skin, we

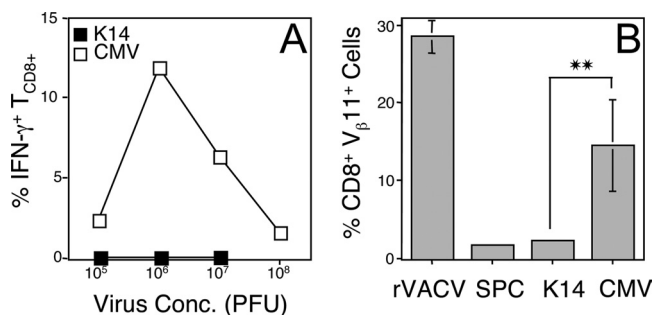


FIG. 1. T_{CD8}⁺ responses after i.v. immunization with AdCMVNP or AdK14NP. Mice were immunized i.v. with AdCMVNP (□) or AdK14NP (■) (A) or additionally with AdSPCNP or rVACV-NT60 NP (B). (A) Production of IFN- γ in response to NP₃₆₆₋₃₇₄ peptide by *in vitro* restimulated splenocytes removed 30 days after immunization. Error bars are too small to observe. There was no statistically significant difference between the response observed in mice infected with either AdK14NP or AdSPCNP compared to uninfected mice. (B) Expansion of adoptively transferred F5 T_{CD8}⁺ (CD8⁺ V β 11⁺) 4 days after immunization. **, $P < 0.01$.

designed a system in which a nonreplicating adenovirus vector expresses a model antigen, influenza virus nucleoprotein (NP) from the A/NT60 strain, driven by a tissue-targeted promoter. The use of NP as an antigen allows the use of F5 transgenic mice bearing a TCR specific for an antigenic peptide (NP₃₆₆₋₃₇₄) in complex with D^b, facilitating both the study of early events after infection and the study of the duration of antigen presentation. The production of NP was driven either by the ubiquitous CMV promoter (AdCMVNP), by a promoter that drives the expression primarily in keratinocytes (K14, AdK14NP) or, as a negative control, by a promoter that drives expression in type II pneumocytes in the lung (SPC, AdSPCNP).

Although many promoters have been described as “tissue specific,” these promoters may also drive expression in other tissues, which would be a concern in the interpretation of our results. Therefore, we infected mice i.v. with each of our rAd to allow access of the injected virus to the maximal number of tissues, including pAPC populations. We then examined the functional relevance of the T_{CD8}⁺ response to antigen expressed in tissues other than our target tissues. As shown (Fig. 1A), cells from mice primed with AdCMVNP generated a T_{CD8}⁺ response, as measured by production of IFN- γ by *in vitro* restimulated splenocytes after exposure to the NP₃₆₆₋₃₇₄ peptide. We used an assay where cells were restimulated with peptide, since it is the most sensitive assay that can detect a response when no response can be detected directly *ex vivo*. In contrast to the response observed with AdCMVNP, the response to AdK14NP was not significantly different ($P < 0.01$) from that observed with no immunization in five separate experiments. These results demonstrate that cells that are infected after i.v. immunization, including pAPC, do not express K14-driven antigen at a level sufficient to generate effector T_{CD8}⁺. Similar results were obtained when primary *ex vivo* activation of T_{CD8}⁺ was examined (not shown).

Throughout our studies we used activation and expansion of TCR transgenic T_{CD8}⁺ as a measure for T_{CD8}⁺ priming. To ensure that the higher precursor frequency of F5 T_{CD8}⁺ after adoptive transfer did not result in a differential ability of tissue-

targeted antigen to expand T_{CD8}⁺, we immunized mice i.v. with AdK14NP, AdCMVNP, or AdSPCNP. As shown in Fig. 1B, neither AdK14NP nor AdSPCNP expanded the numbers of adoptively transferred TCR transgenic T_{CD8}⁺ above background levels. In contrast, specific T_{CD8}⁺ were dramatically expanded in mice immunized with either AdCMVNP or rVACV encoding nucleoprotein from A/NT60.

Induction of T_{CD8}⁺ specific for viral antigen expressed in the skin. In order to allow expression of rAd-encoded antigen driven by the K14 promoter, we infected mice in the skin, where rAd can infect keratinocytes. To examine whether rAd infection could drive proliferation of specific naive T_{CD8}⁺ after infection of keratinocytes, we immunized mice i.d. in the ear pinnae with AdCMVNP, AdSPCNP, or AdK14NP. At 24 h prior to infection, we transferred CFDA-SE-labeled F5 splenocytes into mice to allow specific T_{CD8}⁺ proliferation to be measured. At 3 days postimmunization, the superficial cervical lymph nodes draining the ear were removed and a single-cell suspension analyzed for dilution of the CFDA-SE stain in adoptively transferred F5 T_{CD8}⁺. As expected, AdSPCNP failed to trigger proliferation of F5 T_{CD8}⁺, since the virus did not drain to areas containing cells that are able to produce protein driven by the SPC promoter (Fig. 2A). In contrast, significant proliferation was observed after i.d. immunization with either AdK14NP (Fig. 2B) or AdCMVNP (Fig. 2C). There was a significant decrease ($P < 0.01$) in the number of F5 T_{CD8}⁺ proliferating in response to AdK14NP (45.7%) compared to AdCMVNP (94%), which likely reflects both an enhanced expression of antigen driven by the CMV promoter within keratinocytes, as well as expression of antigen within other cell types, including pAPC.

Cells often proliferate prior to deletion or entry into an unresponsive state during the induction of peripheral tolerance (12), and expression of constitutively expressed antigen within keratinocytes may induce tolerance of T_{CD8}⁺ (6, 35). Antigen-specific proliferation of TCR transgenic T_{CD8}⁺, therefore, does not necessarily imply maturation to effector and memory status. To examine whether cells responding to tissue-targeted rAd-encoded antigen acquired lytic function, we adoptively transferred F5 splenocytes and immunized the recipient mice with rAd. We then examined the clearance of CFDA-SE-labeled splenocytes pulsed with either the NP₃₆₆₋₃₇₄ peptide or a control peptide (OVA₂₅₇₋₂₆₄). As shown in Fig. 2D to F, we found that the adenoviruses did not stimulate clearance of OVA peptide-pulsed cells. AdCMVNP-immunized mice completely cleared NP₃₆₆₋₃₇₄ pulsed cells (Fig. 2F), with the number of target cells remaining not being statistically significant from zero. AdSPCNP-immunized mice failed to clear any cells pulsed with the NP₃₆₆₋₃₇₄ peptide (Fig. 2D), but AdK14NP-immunized mice showed significant clearance of cells pulsed with the NP₃₆₆₋₃₇₄ peptide (Fig. 2E), demonstrating induction of specific cytolytic activity by the AdK14NP virus. However, in seven experiments examining cytolytic activity in a primary response, the number of residual NP peptide-pulsed cells in mice immunized with AdK14NP consistently remained statistically different ($P < 0.05$) from those in mice immunized with AdCMVNP (Fig. 2G). The reduced clearance of NP peptide-pulsed cells indicates a reduction in the efficiency of antigen-specific cytolytic activity compared to AdCMVNP, similar to that seen during initiation of the proliferative response. The

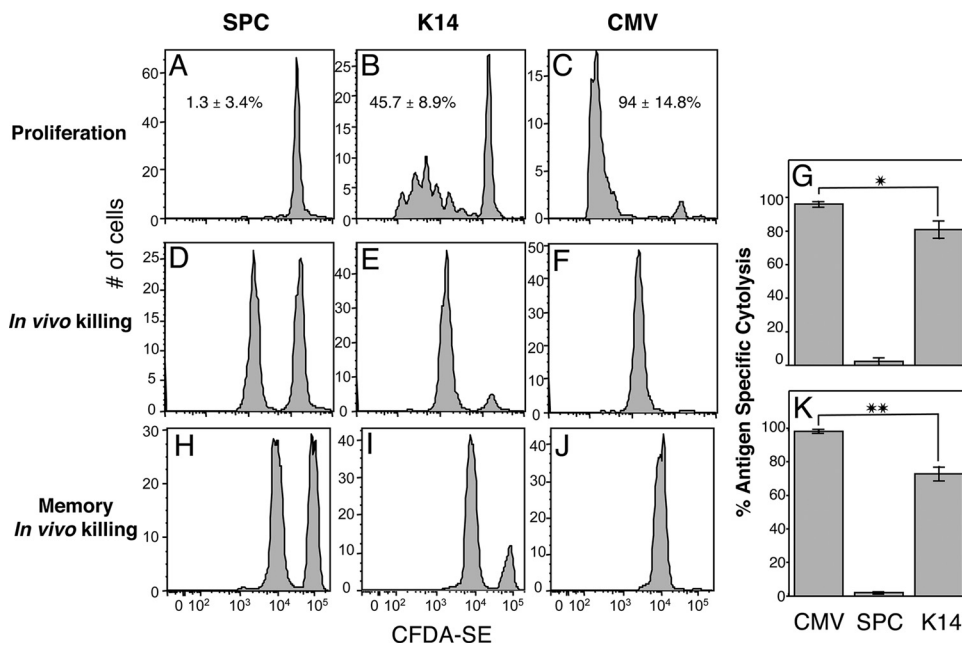


FIG. 2. Proliferation and effector activity of T_{CD8^+} after i.d. immunization with AdCMVNP or AdK14NP. Mice were immunized i.d. in the ear pinnae with AdSPCNP (A and D), AdK14NP (B and E), or AdCMVNP (C and F). Proliferation of adoptively transferred F5 T_{CD8^+} in the draining cervical lymph node was assessed 3 days postinfection by monitoring CFDA-SE dilution (A to C). The data shown are representative, but the statistics shown are derived from four repeat experiments. (D to K) Cytotoxic activity was assessed 3 days (D to G) or 30 days (H to K) postinfection by examining clearance of cells pulsed with either the OVA₂₅₇₋₂₆₄ peptide (labeled with 0.5 μ M CFDA-SE, lower peak) or with the NP₃₆₆₋₃₇₄ peptide (labeled with 5 μ M CFDA-SE, upper peak) from the spleen 18 h after injecting target cells. Similar results were obtained when splenocytes were taken 4 h after the injection of labeled cells. *, $P < 0.05$; **, $P < 0.01$. The data in panels D to F and H to J are representative, and cumulative data from three independent experiments are shown with statistical analyses in panels G and K.

reduced efficacy of cytolytic activity of T_{CD8^+} primed with AdK14NP is preserved in the memory response (Fig. 2K, $P < 0.01$), since 30 days postinfection the clearance of NP₃₆₆₋₃₇₄ pulsed cells was incomplete after immunization with AdK14NP (Fig. 2I) but not after immunization with AdCMVNP (Fig. 2J).

Localization of effector responses. After immunization with AdK14NP, we detected proliferation of F5 T_{CD8^+} in the lymph node draining the site of infection but only minimal proliferation in the spleen (Fig. 3A). In contrast, proliferation was readily detectable in both the draining node and spleen after immunization with AdCMVNP (Fig. 3A). Lower numbers of F5 T_{CD8^+} were present in the spleen 5 days after immunization with AdK14NP (Fig. 3B, $P < 0.01$), even when the amount of AdK14NP used to immunize was 100-fold higher than that of AdCMVNP (data not shown). This deficit in localization of responding T_{CD8^+} to the spleen appeared more pronounced in the memory response where, 30 days after immunization, very few adoptively transferred F5 T_{CD8^+} could be detected after immunization with AdK14NP (Fig. 3C, $P < 0.01$). The decrease in detectable T_{CD8^+} was not due to clearance of the adoptively transferred F5 T_{CD8^+} since these cells were easily detectable after immunization with AdCMVNP (Fig. 3C).

Timing of the T_{CD8^+} response. In our system it is possible that AdCMVNP virus particles drain to the lymph node and infect pAPC that can directly present NP peptide to T_{CD8^+} , a scenario we have previously observed after infection with VACV (25). In contrast, antigen expressed from AdK14NP must be translated within keratinocytes, transferred to pAPC (likely DC), and transported via afferent lymphatics to the

draining lymph node. To examine the differences in the requirement for pAPC-mediated transport from the site of infection, we removed the site of infection at various times postinfection. By removing the site of infection, we could remove the contribution of cell-mediated transport, which requires infection of cells at the site, antigen expression, transfer of antigen to pAPC, and then migration of these pAPC to the lymph node. However, we would not remove the contribution of drainage of particles directly to the lymph node and infection of cells *in situ*. We found that immediate removal of the site of infection reduced the proliferation of adoptively transferred F5 T_{CD8^+} in response to AdK14NP infection to almost background levels but did not impact the response after infection with AdCMVNP (Fig. 4A). In contrast, removal of the site of infection 24 h postinfection did not alter the response to AdK14NP. These results are consistent with a differential requirement for cell-mediated trafficking of antigen from the site of infection, with AdK14NP requiring cell-mediated trafficking and AdCMVNP generating a response in the absence of this process.

Drainage of solute, and presumably of virus particles, to the lymph node can occur within seconds of injection (11, 14), but the migration of DC from the skin to draining lymph nodes is thought to take at least 10 to 16 h (14, 20). Thus, it might be expected that a response to a peripheral viral antigen may be delayed compared to the response to a comparable antigen that can be expressed in secondary lymphoid organs. To address whether T_{CD8^+} respond to peripheral viral antigen with different kinetics of proliferation and acquisition of effector

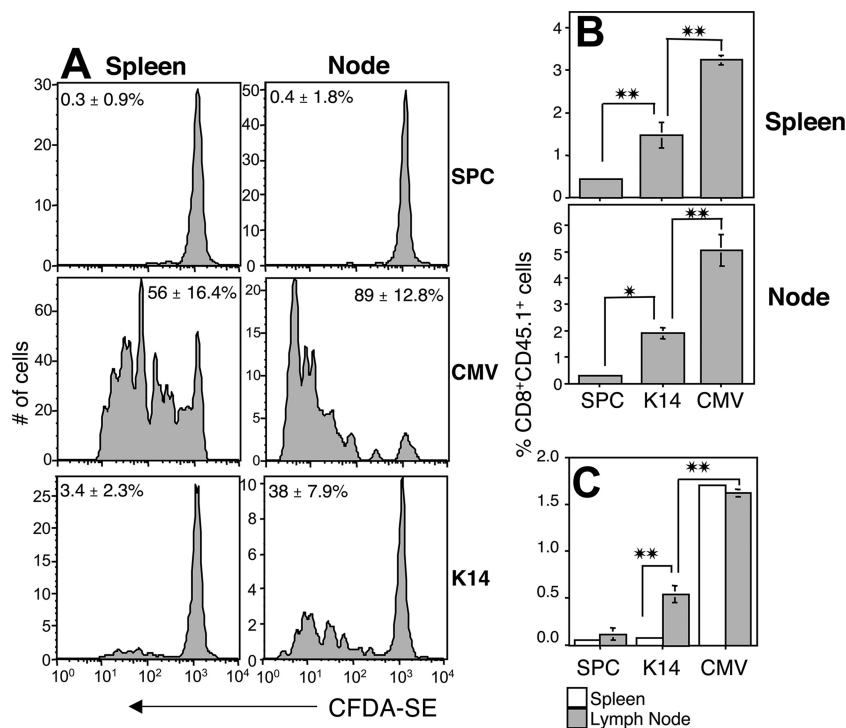


FIG. 3. Localization of T_{CD8^+} after i.d. immunization with AdCMVNP or AdK14NP. CFDA-SE-labeled (A) or unlabeled (B and C) F5 T_{CD8^+} were adoptively transferred into mice that were immunized 24 h later with rAd as shown. Spleens and lymph nodes were removed 3 days (A), 4 days (B) or 30 days (C) after immunization, and F5 T_{CD8^+} ($CD8^+$, $CD45.1^+$) proliferation (A), quantified by dilution of CFDA-SE, or the numbers of cells (B and C) were measured by flow cytometry. (A) Representative plots are shown, but the statistics from three independent experiments are included. The numbers of proliferating cells in both spleen and node were different in AdCMVNP- and AdK14NP-infected mice ($P < 0.01$). Panels B and C: **, $P < 0.01$; *, $P < 0.05$.

function, we examined the proliferation of adoptively transferred F5 T_{CD8^+} at various times after i.d. immunization with AdK14NP or AdCMVNP. As shown in Fig. 4B, we observed that the number of proliferating cells after immunization with AdK14NP was lower than after immunization of AdCMVNP ($P < 0.01$). However, there were no gross differences in the kinetics of proliferation of F5 T_{CD8^+} after immunization with AdCMVNP or AdK14NP, with proliferation beginning at 3 days postinfection for both. Similarly, mice immunized with either virus acquired the ability to clear injected splenocytes pulsed with the NP₃₆₆₋₃₇₄ peptide at 3 days postinfection (Fig. 4C). These results demonstrate a clear correlation between the initiation of proliferation and the acquisition of effector activity that has previously been demonstrated after immunization with herpes simplex virus type 1 (HSV-1) (24). Our results show, somewhat surprisingly, that there is no major difference in the time course of priming of T_{CD8^+} to peripheral antigen or antigen that can be expressed within secondary lymphoid organs.

We had observed that the ability of T_{CD8^+} primed by peripheral viral antigen to clear peptide-pulsed cells was incomplete (Fig. 2E, G, I, and K). This observation could be due to the lower number of cells primed by peripheral antigen (Fig. 3) or to a difference in the ability of individual T_{CD8^+} to lyse target cells. To examine whether there was a deficiency in the ability of T_{CD8^+} primed by peripheral viral antigen to lyse target cells, we adoptively transferred F5 splenocytes and immunized recipient mice with AdSPCNP, AdK14NP, or

AdCMVNP. CFDA-SE-labeled splenocytes loaded with either the OVA peptide as a control or with titrated amounts of the NP₃₆₆₋₃₇₄ peptide were injected, and clearance of these cells was measured 18 h after injection. We found that in mice immunized with AdCMVNP, complete clearance of cells pulsed with NP peptide concentrations down to 10^{-7} M was observed (Fig. 4D). Progressively less clearance was observed at peptide concentrations down to 10^{-10} M. Clearance of cells pulsed with 10^{-11} M peptide was almost indistinguishable from clearance of cells pulsed with the control peptide or from mice immunized i.d. with the AdSPCNP virus. As previously observed, AdK14NP did not clear all of the NP peptide-pulsed cells at any concentration used (Fig. 2E, I, G, and K). However, we found that despite the inability to clear all of the peptide-pulsed cells, AdK14NP-primed cells cleared NP peptide-pulsed cells over a similar range of peptide concentrations as AdCMVNP, although there was a minor difference in the lysis of cells at a concentration of 10^{-9} M peptide. Thus, the failure of AdK14NP-primed cells to exert the complete cytolytic effects seen after priming with AdCMVNP is likely related to limited expansion of cells (Fig. 2B and 3) rather than a lowered affinity of these cells for NP peptide-D^b complexes.

Duration of antigen presentation. We observed no difference in the kinetics with which a T_{CD8^+} response is initiated to peripheral viral antigen versus antigen that can be expressed within pAPC (Fig. 4B to D). To assess the duration of antigen presentation following immunization with each rAd, we immunized mice i.d. and isolated an enriched population of lymph

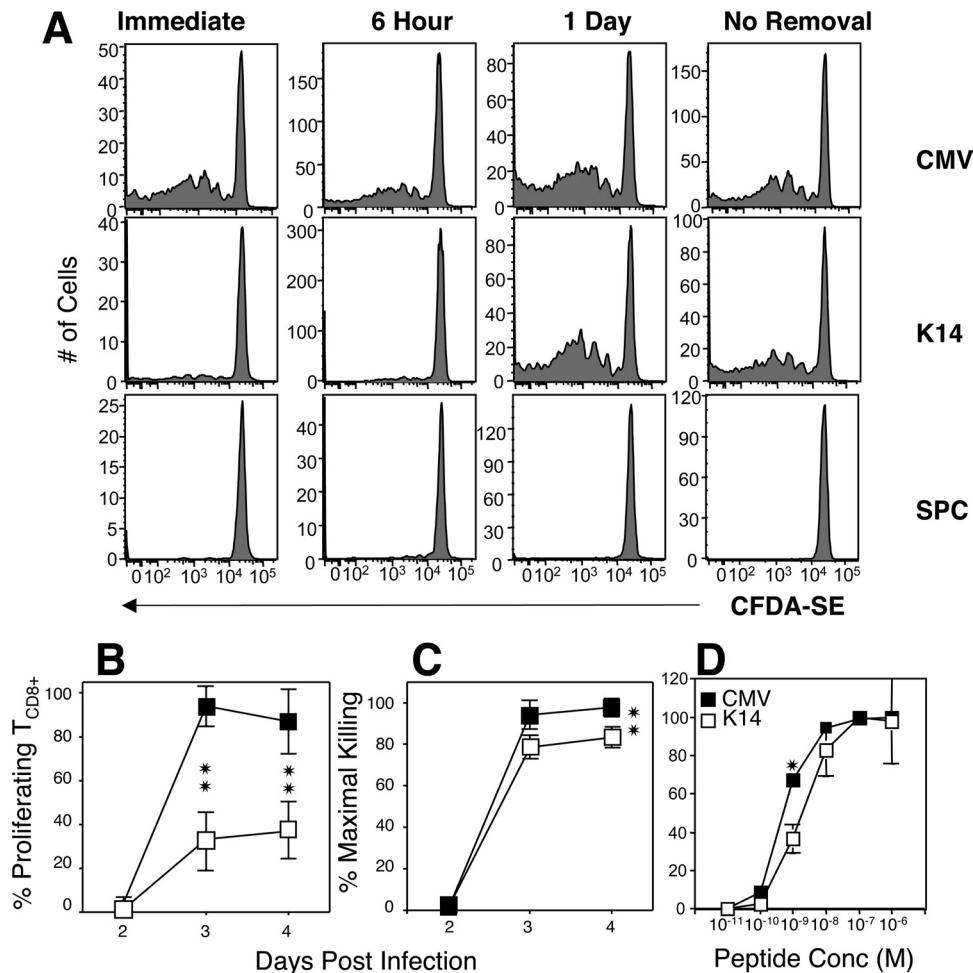


FIG. 4. Time course and efficacy of T_{CD8+} activation after i.d. infection with AdCMVNP or AdK14NP. Mice were immunized i.d. and tested for either the ability to stimulate proliferation of adoptively transferred F5 T_{CD8+} via CFDA-SE dilution (A and B) or the effector activity via clearance of specific peptide-pulsed CFDA-SE^{HI} labeled targets (C) on day 3 (A) or days 2, 3, and 4 (B and C) postinfection. (A) The site of infection was removed at the times shown postinfection. (D) The sensitivity of effector F5 T_{CD8+} was assessed by measuring the clearance of cells pulsed with various amounts of NP₃₆₆₋₃₇₄ peptide 6 days after i.d. immunization. The data in panel C are expressed as a percentage of the maximal killing of NP₃₆₆₋₃₇₄ peptide-pulsed CFDA-SE^{HI} cells compared to levels after immunization with AdSPCNP. *, $P < 0.05$; **, $P < 0.01$.

node CD11c⁺ cells at 48 h postinfection. We incubated these pAPC with CFDA-SE-labeled F5 T_{CD8+} for 60 h and assessed the ability of the pAPC populations to trigger T_{CD8+} proliferation. As a positive control, we purified DC from mice immunized 24 h before with rVACV expressing influenza virus NP, a virus to which *ex vivo* proliferation has previously been demonstrated. DC from mice immunized with rVACV-NP triggered antigen-specific proliferation of F5 T_{CD8+}, but DC from mice immunized with AdCMVNP failed to reproducibly stimulate antigen-specific T_{CD8+} proliferation in four similar experiments (Fig. 5A).

As an alternative approach to measure the duration of antigen presentation, we adoptively transferred CFDA-SE-labeled F5 splenocytes at various times ranging from the day of immunization to 7 days postimmunization and assayed for dye dilution at 3 days after transfer. We previously established that 3 days posttransfer is the optimal day to observe proliferation of F5 T_{CD8+} after immunization (Fig. 4B). Proliferation of naive F5 T_{CD8+} requires both costimulation and a “danger”

signal (data not shown), so this assay measures antigen presentation by pAPC but not by somatic cells. Despite the fact that the rAd used are replication deficient, antigen presentation was sufficient to initiate proliferation of F5 T_{CD8+} at least 7 days postimmunization (Fig. 5B). In contrast to the long-lived ability of AdCMVNP-immunized mice to trigger F5 T_{CD8+} proliferation, pAPC within AdK14NP-immunized mice presented antigen for less than 72 h (Fig. 5B), and at time points after this there was no significant difference between immunization with AdK14NP and AdSPCNP. Thus, although the T_{CD8+} response is initiated with similar kinetics, the duration of presentation of peripheral viral antigen is markedly reduced compared to systemically expressed antigen.

DC migration. Previous studies in the lung have described a period after virus infection during which tissue-resident DC are refractory to stimulation of migration to secondary lymphoid organs (19). We hypothesized that the reduced duration of antigen presentation after immunization with AdK14NP, an event that is dependent upon DC migration from the skin,

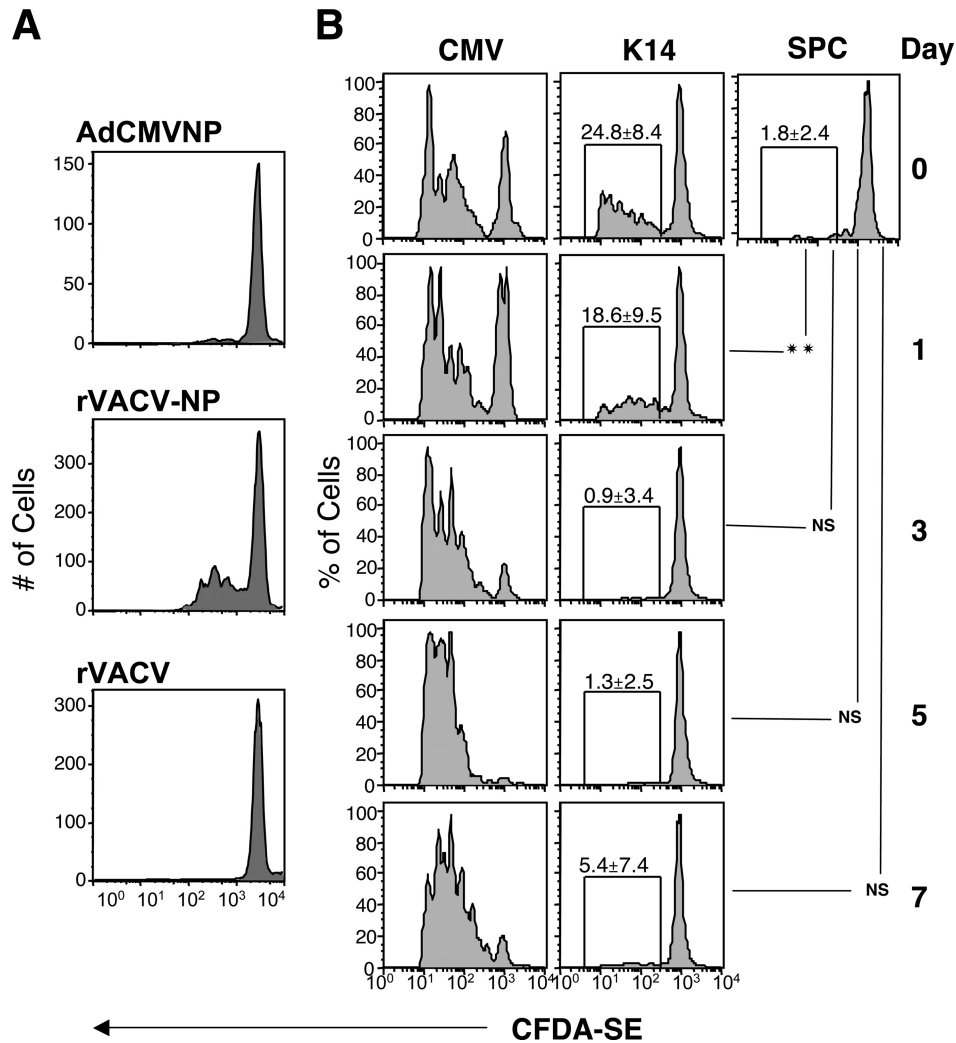


FIG. 5. Duration of antigen presentation. (A) Mice were immunized with viruses as shown, and enriched populations of DC were isolated from the draining lymph node at 24 h (VACV) or 48 h (rAd) postinfection. DC were incubated with CFDA-SE-labeled F5 T_{CD8}⁺ for 60 h, and proliferation of the T_{CD8}⁺ was measured. In four separate experiments, we observed no proliferation after immunization with AdCMVNP. (B) CFDA-SE-labeled F5 T_{CD8}⁺ were adoptively transferred into recipients at the days shown after i.d. infection. The proliferation of F5.SJL cells was assessed 3 days after adoptive transfer. The data shown are representative of three independent experiments, but the statistics of pooled data for AdK14NP and AdSPCNP stimulated proliferation are shown. Proliferation in response to AdK14NP was significantly different to that in response to AdSPCNP on 0 and 1 day postinfection ($P < 0.01$) but was not statistically different on days 3 through 7. Proliferation in response to AdCMVNP infection was statistically different ($P < 0.01$) from that observed after infection with either Adk14NP or AdSPCNP at all time points examined.

might be a consequence of such a blockade in DC migration. To examine this hypothesis, we used the classic technique of painting the skin with FITC at various time points after rAd infection and then measuring the number of FITC-positive DC in the draining lymph node 18 h later (Fig. 6). Reproducibly, we observed 10 to 15% of CD11c⁺ cells in the draining lymph node at this point were FITC⁺ (Fig. 6A), an observation that agrees with previous measurements that non-lymphoid organ-derived DC are typically rare in lymph nodes (15). Immunization with rAd increased the proportion of FITC⁺ DC in the node substantially, but this increase was transient and lasted for 1 to 2 days before falling back to, or below, steady-state levels (Fig. 6A).

Antigen presentation after infection with AdK14NP was undetectable as soon as 3 days postinfection (Fig. 5B), suggesting

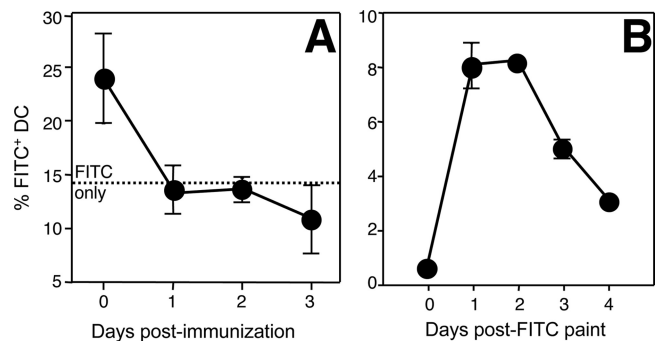


FIG. 6. DC Migration following rAd infection. (A) The ears of mice were immunized and then painted with FITC at the time periods shown postinfection with rAd. The percentage of FITC⁺ CD11c⁺ DC in the draining lymph node was quantified 18 h later. (B) Similar to panel A, but unimmunized mice were painted with FITC, and the numbers of FITC⁺ CD11c⁺ DC were quantified at the times shown postpainting.

that the migrating DC may have died or exhausted the supplies of antigen that they had taken up by that point. To address the life span of migrating DC, we painted the skin with FITC and examined the persistence of FITC⁺ DC in the draining lymph node over the following days. Equal numbers of FITC⁺ DC were observed in the node 24 and 48 h after painting, but at subsequent time points the number of FITC⁺ DC decreased significantly, in parallel with the decrease in antigen presentation.

DISCUSSION

Many viruses penetrate the body at peripheral sites and are either poorly cleared or establish latency, allowing long-lived infection that can create many complications for the host. In the present study we designed a model system in which viral antigen is expressed at physiologically relevant levels in the periphery in somatic cells but not within pAPC. We observed that the T_{CD8}⁺ response to peripheral antigen was insufficient to clear cells pulsed with an identical peptide, even when these cells were introduced systemically. The inability to clear all cells presenting a viral peptide could account for the ability of viruses, such as papillomaviruses that infect peripherally, to avoid clearance by the adaptive immune response. This observation was revealed only following a carefully designed system in which antigen is expressed at low levels in a peripheral site. Thus, the conclusions drawn from studies in which large doses of virus are administered systemically may not be relevant for some peripheral human infections.

The lower numbers of T_{CD8}⁺ responding to peripheral antigen are likely the result of both lower levels of antigen and lower levels of antigen presentation in the draining lymph node. However, there are currently no direct methods available to quantify antigen presentation in a lymph node, and the culture of *ex vivo* DC from the draining node of mice infected with AdCMVNP with F5 T_{CD8}⁺ failed to reproducibly induce T_{CD8}⁺ proliferation. Despite the inability of this widely used assay to detect *ex vivo* antigen presentation, we found that almost all F5 T_{CD8}⁺ proliferated *in vivo* after adoptive transfer. The detection of *ex vivo* antigen presentation by F5 T_{CD8}⁺ may result from a difference in sensitivity versus other TCR transgenic lines, such as OT-1 (10). The affinity of activated F5 and OT-1 to peptide-pulsed targets is similar, but naive F5 T_{CD8}⁺ are not activated by peptide alone *in vitro* and require some form of inflammatory stimulus, such as virus infection (data not shown). However, it is clear that the relative insensitivity of the *ex vivo* presentation assay compared to the *in vivo* assay could lead to misinterpretation of results. A failure to observe *ex vivo* proliferation triggered by some populations of pAPC could be misinterpreted as an inability of these cells to present antigen *in vivo*.

Our inability to measure *ex vivo* antigen presentation from DC prevented us from examining the phenotype and maturation status of the pAPC presenting peripheral or systemic antigen to T_{CD8}⁺. The T_{CD8}⁺ response to antigen expressed constitutively in the skin appears to be defined both by the pAPC population that is responsible for antigen presentation and by the form of antigen that is expressed. Initially, it was reported that Langerhans cells presenting a peptide derived from a highly expressed secreted peptide induced T_{CD8}⁺-mediated autoimmunity (22, 23). However, subsequent studies

using full-length antigen indicated that T_{CD8}⁺ tolerance, rather than autoimmunity, was induced (3, 35). The induction of tolerance was mediated by antigen presentation by a Langerin⁺ non-Langerhans cell (6, 7, 36). Here we have demonstrated that full-length antigen expressed by virus-infected keratinocytes induces proliferation and functional activation of antigen-specific T_{CD8}⁺. The discrepancy between our results and those that were previously published likely results from the virus infection, which will activate the innate immune system via the ligation of pattern recognition receptors (17, 32).

One consequence of the activation of the innate immune system is initiation of a burst of DC, likely dermal DC (4) migration, from the skin to the draining lymph node. We confirmed that cell-mediated transport of antigen is required for presentation of peptide derived from NP driven by the K14 promoter, as removal of the infection site within 6 h of infection ablates the T_{CD8}⁺ response. However, our inability to ablate T_{CD8}⁺ priming after infection with AdCMVNP is consistent with AdCMVNP particles draining directly to the node, a process that may take seconds (11, 13, 25), wherein these particles could infect lymph node-resident pAPC and trigger naive T_{CD8}⁺. DC migration to the draining lymph node may occur 10 to 16 h after infection (14, 20), so one might expect the initiation of the T_{CD8}⁺ response to AdCMVNP to occur more rapidly than that to AdK14NP. However, when we examined the time course of T_{CD8}⁺ activation after infection with each virus we found similar kinetics. One possible explanation for this observation is that proliferation of F5 T_{CD8}⁺ could not be detected until 3 days postinfection with any rAd. This is 24 h slower than the time period required for proliferation after infection with either VACV (25) or HSV-1 (24). This delay might be accounted for by the relatively slow expression of antigen by rAd compared to VACV and HSV-1. However, the promiscuous expression of the receptor for rAd particles on parenchymal cells raises the possibility that all available rAd is bound and internalized by these cells and that none are able to drain to the lymph node. Thus, it appears likely that all rAd-encoded antigen must be transported to the node, whether within infected DC (5, 17, 30) or after pAPC-mediated internalization of antigen derived from infected cells.

We did observe a significantly pronounced difference in the duration of antigen presentation after infection with AdK14NP versus AdCMVNP, with presentation after peripheral expression of viral antigen lasting less than 3 days. Antigen presentation after infection with *Listeria monocytogenes* is transient, likely as a result of T_{CD8}⁺-mediated killing of pAPC (37). However, the presentation of virus-encoded antigens to T_{CD8}⁺ can occur for an extended period of time (33, 38) and may potentiate the further expansion of T_{CD8}⁺ after the initial exposure to antigen that triggers six to seven divisions of these cells (18, 27, 34). Antigen presentation after infection with AdK14NP correlated with a burst of DC migration from the skin that was followed by a refractory period in which migration was reduced significantly. The continued presentation of antigen remains a focus of our laboratory, but these data indicate that this phenomenon may either require the expression of virus genes within pAPC (33) or may require the deposition of large quantities of antigen that can be transferred between DC populations in the draining node (8) for an extended period of time.

Our observation that enhanced DC migration is transient is consistent with similar observations in the lung after influenza virus infection (19) but extend upon the previous observations by providing a functional consequence of the reduced DC migration at later times postinfection. Viruses that infect peripherally and express little antigen initially could utilize the refractory period of DC migration to establish a low-level infection, preventing adequate priming of T_{CD8^+} that can clear all infected cells. This may be the mechanism by which viruses establish latent peripheral infection. Further investigation of the mechanisms responsible for the refractory period of DC migration may yield an intervention that stimulates DC migration and so allows the early phase of peripheral infection to generate a more effective T_{CD8^+} response.

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