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*J Immunol* 2010; 184:4431-4439; Prepublished online 8 March 2010; doi: 10.4049/jimmunol.0900537

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Quality of the Transgene-Specific CD8+ T Cell Response Induced by Adenoviral Vector Immunization Is Critically Influenced by Virus Dose and Route of Vaccination

Peter Johannes Holst,* Cathrine Ørskov,† Allan Randrup Thomsen,* and Jan Pravsgaard Christensen*

Adenoviral vectors have been widely used for experimental gene therapy and vaccination, yet there is a surprising lack of knowledge connecting the route and dose of adenovirus administration to the induced transgene-specific immune response. We have recently demonstrated polyfunctional CD8+ T cells and protective memory responses using adenoviral vectors, which seem to contrast with recent reports suggesting that an exhausted CD8+ T cell phenotype is induced by inoculation with adenoviral vectors. Accordingly, we investigated the route and dose interrelationship for transgene-specific CD8+ T cells using adenoviral vectors encoding β-galactosidase applied either s.c. or i.v. Irrespective of the route of inoculation, most of the adenoviral inoculum was found to disseminate systemically as the dose was raised beyond 10^9 particles. The number of transgene-specific CD8+ T cells correlated positively with dissemination, whereas the functional capacity of the generated T cells correlated inversely with vector dissemination. A comparison of the immune response to s.c. or i.v. administration at moderate doses revealed that inoculation by both routes induced a transient peak of IFN-γ production. As the liver is a major site of adenoviral infection and a major site of adenoviral infection and dissection, the footpad (f.p.) was chosen because both transgene Ags vary [for example, see Holst et al. (11, 12)]. Elimination of adenoviral vectors expressing this Ag is well characterized and known to depend on CD4+ and CD8+ T cells and cytotoxicity. As the liver is a major site of adenoviral infection following, in particular, systemic inoculation, we also measured reports of induction of dysfunctional T cells contrast with our own data and clinical experience, we asked the question if differences in administration route and/or dosage might account for the observed discrepancies.

In this regard, the scientific literature is remarkably sparse, and most of the available information regarding the mechanisms of adenoviral elimination and induction of immune responses via different means of administration relies on the pioneering work in the laboratory of James Wilson in the 1990s, before the introduction of tools to accurately enumerate and functionally characterize Ag specific T cells (13–18). There have been some efforts in determining requirements for induction of mucosal immunity (19–22), but only one recent study has compared induction of systemic immunity by different relevant routes, yet this study did not address the role of dosage of the adenovirus used or investigate the functional capabilities of the primed cells, nor did it follow the cells over time to allow assessment of the quality of immunological memory (23).

In this study, we compare the CD8+ T cell response directed against the histochemical marker Ag β-galactosidase expressed from an adenoviral vector and injected via the i.v. or s.c. route in the footpad (f.p.). This Ag was chosen because both transgene product and vector-derived viral proteins contribute to the elimination of the viral vector (18), and the immune responses toward different transgene Ags vary [for examples, see Holst et al. (11, 12)]. Elimination of adenoviral vectors expressing this Ag is well characterized and known to depend on CD4+ and CD8+ T cells from them expressing CD40L and IFN-γ (13, 15, 16, 24). We measured the generation of transgene-specific CD8+ T cells in the spleen together with their capacity for ex vivo cytokine production and cytotoxicity. As the liver is a major site of adenoviral infection following, in particular, systemic inoculation, we also measured
the accumulation of intrahepatic transgene-specific CD8+ T cells and their ex vivo cytokine production. Finally, adenoviral transduction of different target organs and the ability of the vaccine-induced T cells to be mobilized and cause peripheral inflammation were also evaluated, and, importantly, the immunized mice were followed for several months. These analyses revealed profound differences in the quantity and quality of the transgene-specific effector and memory CD8+ T cells as function of virus dose and route of administration. We believe that these findings provide important new information regarding parameters of critical importance for the induction of efficient T cell responses following adenoviral vaccination.

Materials and Methods

Mice

C57BL/6 mice were obtained from Taconic M&B (Ry, Denmark). B6.SJL mice were bred in-house. All experiments were performed in accordance with national guidelines and the criteria outlined in the Guide for the Care and Use of Laboratory Animals (National Institutes of Health publication 86-23, revised 1985).

Adenovirus production and purification

An El and E3 deleted adenovirus expressing a nuclear-targeted β-galactosidase (Ad-β-gal) under control of the human CMV promoter was provided by Christoffer B. Newgard (Duke University Medical Center, Durham, NC). The viral stock was resuspended in a plaque assay, amplified in HEK293 cells, precipitated in polyethylene glycol, and purified by CsCl gradient centrifugation as described (25). The concentration of viral particles was determined by 260 nm absorption and infectious unit titers with Adenov-X Rapid Titer purification systems (Clontech, Mountain View, CA). The Ad-β-gal viral stock used in this study had a particle/infectious unit ratio of 62:1.

Vaccinia virus production and organ titration

A seed stock of vaccinia virus expressing β-galactosidase and EBV EBNA3C was provided by Julia Hurwitz at the St. Jude Children’s Research Hospital, Memphis, TN (26). Following receipt, the viral lysate was amplified in CV-1 cells, released by freeze-thaw cycles, purified by centrifugation, and titered using a plaque assay as previously described (27). For challenge studies, mice were infected i.p. with 2 × 10⁵ PFU of β-galactosidase expressing vaccinia virus. Five days later, ovaries from infected mice were surgically removed and stored at 80˚C. For organ virus titers, the ovaries were grinded with sterile sand to release cell-associated virus and 10% w/v organ suspensions were serially diluted for plaque titration as described above.

Single-cell suspensions

Whole spleens were homogenized and rinsed in HBSS. Livers were perfused with PBS through the inferior caval vein after creation of an outlet in the portal vein. Livers were then homogenized, the suspension pelleted and resuspended in a heparin-containing Percoll gradient buffer, and pelleted again. Remaining erythrocytes were lysed using Gey’s solution, and lymphocytes were counted after two washes in HBSS. This protocol contains minor modifications from that of Watanabe et al. (28).

Flow cytometry

For analysis of functionality of transgene-specific intracellular cytokine production, spleen cells were incubated in vitro for 5 h with or without the β-galactosidase–derived peptide DAPIYTNV (β-gal96–103) (0.1 mg/ml) (29) in the presence of monensin (3 μM) and murine IL-2 (10 U/well). Cells were then surface stained for CD8 and CD44 and in selected experiments also for CD19, CD27, CD127, and/or CD45.2. For staining of intracellular cytokines, cells were surface stained, permeabilized, and resuspended in a heparin-containing Percoll gradient buffer, and pelleted again. Remaining erythrocytes were lysed using Gey’s solution, and lymphocytes were counted after two washes in HBSS. This protocol contains minor modifications from that of Watanabe et al. (28).

Cytotoxicity

The cytotoxicity of activated CD8+ T cells was assessed in [51Cr]-release assay. Targets for evaluation of β-galactosidase–specific cytotoxicity were EL-4 cells pulsed with β-gal96–103 or unpulsed cells served as control targets. Assay time was 5 h, and the percentage of specific lysis was calculated as: (sample – spontaneous release)/(total – spontaneous release) × 100 (30).

In vivo cytotoxicity

The cytotoxicity of activated CD8+ T cells was assessed by transferring a mixture of CFSE-labeled cells from either C57BL/6 (CD45.2+) or B6. SJL (CD45.1+) mice pulsed with β-gal96–103 and an irrelevant peptide, respectively. The ratios of the two populations in immunized recipients’ spleens were determined 6 h later by surface staining for CD45.1, and the baseline ratios were determined in unvaccinated mice.

Delayed-type hypersensitivity

Mice were vaccinated in the left f.p. At the indicated time points, f.p. thickness was measured, and the mice received a single injection of 150 μg β-gal96–103 in the previously unvaccinated right hind f.p., and f.p. thickness of both hind feet was recorded 16, 24, 48, and 72 h later.

Serum amino transferase activity

Mice were anesthetized and exsanguinated through the periorbital venous plexus. Serum samples were assayed on a Modular analysis machine (Roche, Indianapolis, IN). Results are depicted in units per liter.

Adenoviral clearance and histochemistry

Frozen sections of mouse liver, 5–10 μm thick, were fixed with 4% paraformaldehyde and stained for β-galactosidase using a β-galactosidase staining kit (Roche). In each section, the total number of β-galactosidase–stained cells was counted, the size of the section was measured using Image Pro Plus 5.0 (Media Cybernetics, Bethesda, MD), and the number of β-galactosidase–stained cells per mm² was then calculated.

Adenoviral clearance and real-time PCR

For determining the kinetics of adenovirus elimination, liver pieces and whole paw samples were digested with SDS and proteinase K, proteins were precipitated in high-salt solution, and DNA was precipitated from supernatant with ethanol and resuspended in TE buffer. PCR analysis was performed with the following primers and probes: GAPDHsenseB: caatgtgtccgtcgtgga; GAPDHreverseB: gatgcctgcttcaccacc; and GAPDHBp: cgctggaagaacgctcgct; 5’ HEX conjugated and 3’ BHQ-1 conjugated for genomic GAPDH and with LacZ246F: tactgtcgtcgtcccctcaaa; LacZ263R: taacacggtctggatc; and LacZprobe313T: tatccagacctgctcgcc 5’ conjugated to 6-FAM and 3’ conjugated to BHQ-1 to detect β-galactosidase DNA (31). Samples were run on a Stratagene MX3000 real-time PCR machine and analyzed using MX3000P software (Stratagene, La Jolla, CA). β-galactosidase amplification was then normalized to genomic GAPDH according to the following formula: viral DNA = 2^(ΔCqAdenovirus-Cq-galactosidase).

Results

I.v. vaccination versus f.p. vaccination: role of virus dose for systemic virus dissemination

To study the distribution of adenovirus delivered via s.c. or i.v. injection at various dosages, groups of mice were injected with 10⁹, 10¹⁰, and 10¹¹ particles either i.v. or f.p., and real-time PCR analyses for viral DNA were performed on total DNA isolated from popliteal LNs (only draining LNs in the f.p. group), the spleen, liver, and whole paw (site of injection in the f.p. group) 5 d after virus inoculation (Fig. 1). f.p. infection was preferred over i.m. inoculation as it provides an easier accessible and more defined data were collected on an LSRII instrument (BD Biosciences) and analyzed using FlowJo software (Tree Star, Ashland, OR).

Ex vivo cytotoxicity

The cytotoxicity of activated CD8+ T cells was assessed in [51Cr]-release assay. Targets for evaluation of β-galactosidase–specific cytotoxicity were EL-4 cells pulsed with β-gal96–103 or unpulsed cells served as control targets. Assay time was 5 h, and the percentage of specific lysis was calculated as: (sample – spontaneous release)/(total – spontaneous release) × 100 (30).
i.v. caused a paw transduction equivalent to 10^9 particles injected and 77-fold following an increase from 10^10 to 10^11 particles in the f.p. inoculation. Remarkably, the converse is also true. Thus, 10^11 particles injected efficiently as i.v. inoculation in reaching the systemic circulation.

Similarly, transduction of the spleen increased a remarkable 215-fold when the inoculum was increased from 10^9 to 10^10 particles in the f.p. injection, however, dose-dependent increases in transduction of the target organs were much smaller than would have been expected (70% and 73% increases in paw transduction and 52% and 57% increase in the draining LNs for each 10-fold increase in dose in liver and 5.8- and 11-fold in the spleen). In contrast, i.v. inoculation resulted in limited transduction of the paw except at the highest virus dose, and the popliteal LN was inefficiently transduced with no clear dose correlation. Following f.p. injection, however, dose-dependent increases in transduction of the target organs were much smaller than would have been expected (70% and 73% increases in paw transduction and 52% and 57% increase in the draining LNs for each 10-fold increase in dose injected into the f.p.). In contrast, liver transduction increased 9.6-fold when the inoculum was increased from 10^9 to 10^10 particles and 77-fold following an increase from 10^10 to 10^11 particles injected. Similarly, transduction of the spleen increased a remarkable 215-fold when the inoculum was increased from 10^10 to 10^11 particles. Thus, it is evident that systemic virus spread increases substantially more than target organ transduction as the dose of virus injected into the f.p. is increased. Also, at very high dose, so little virus is retained in the target organs that f.p. inoculation becomes as efficient as i.v. inoculation in reaching the systemic circulation. Remarkably, the converse is also true. Thus, 10^11 particles injected i.v. caused a paw transduction equivalent to 10^10 particles injected directly into this site. However, in this case, it should be stressed that the localization of the transduced cells within the paw may differ considerably depending on the route of inoculation.

Divergent correlations between the inoculum dose and transgene-specific CD8^+ T cell numbers and quality

To address the consequences of virus dose and route of inoculation on the transduced specific CD8^+ T cell response, groups of mice were injected with 10^9, 10^10, and 10^11 particles either i.v. or f.p., and 80 d later, all the mice were sacrificed for analysis of β-gal96–103-specific CD8^+ T cell numbers and quality (MFI; Fig. 2). A similar dose dependence was found for the accumulation of intrahepatic β-gal96–103-specific CD8^+ T cells following f.p. immunization, yet no clear dose correlation was found following i.v. immunization. In contrast, when T cell quality was assessed using mean fluorescence intensity (MFI) of IFN-γ-producing CD8^+ T cells as a parameter, an inverse correlation was found between the dose of peripherally administered adenovirus and MFI (Fig. 2B). Thus, at the highest dose of 10^11 particles f.p., the MFI dropped to the uniformly low levels seen following i.v. administration. Although IFN-γ MFI is gaining popularity as a marker of CD8^+ T functionality, considerably more experience has been obtained assessing T cell quality by measuring the ability of Ag-specific cells to produce multiple cytokines (35–39). In this respect, a hierarchy has been observed in which decreasingly protective cells produce an increasingly less diverse array of cytokines (40, 41). As the transition from IFN-γ/TNF-α double positive to IFN-γ single positive is often considered the last step before anergy (41), we also determined the fraction of IFN-γ/TNF-α double-positive CD8^+ T cells out of all IFN-γ-positive cells (Fig. 2C). This fraction was found to follow the MFI of the IFN-γ-producing cells with an inverse dose correlation following f.p. immunization and a uniformly low fraction obtained after i.v. immunization in both spleen and liver. Thus, the degree of viral dissemination as determined by liver DNA load on day 5 postinfection (p.i.) correlated positively with magnitude (number of IFN-γ-positive cells in spleen; Fig. 2D, left panel) and negatively with quality (MFI; Fig. 2D, right panel) of the induced CD8^+ T cell response, irrespective of the route of immunization.

Kinetics of the transgene CD8^+ T cell response in the spleen following i.v. or f.p. infection

As viral dissemination obviously impacts the quality of the induced CD8^+ T cell memory, we next undertook a longitudinal evaluation of the response following either peripheral or systemic activation. The purpose of this approach was to determine how the two types of responses develop over time. We chose a dose of 10^9 particles for inoculation via the f.p. and 10^10 particles for the i.v. route in all subsequent analyses. These doses were chosen because 10^9 particles in the f.p. induced polyfunctional CD8^+ T cell memory without the dissemination seen at higher doses; moreover, an inoculum of at least 10^10 virus particles was required to elicit transgene-specific CD8^+ T cell memory via the i.v. route.

Accordingly, cohorts of mice were vaccinated either i.v. or f.p., and the ensuing T cell response was measured at different time points p.i. using either H-2K^b/β-gal96–103 dextramers or β-gal96–103 peptide-induced IFN-γ production (Fig. 3). Following f.p. infection, a population of IFN-γ-producing β-gal96–103-specific CD8^+ T cells emerged 14 d.p.i. in the spleen, rising in numbers to a sharp peak at day 21 p.i. Following this, the transgene-specific CD8^+ T cell population contracted, and cell numbers declined to 25–40% of peak values between days 28 and 120 p.i. As observed by Tatsis et al. (10), the transgene-specific CD8^+ T cell response in
the spleen was even slower following i.v. infection. In this case, only low numbers of β-gal96–103–specific CD8+ T cells were present in the spleen at days 14 and 21 p.i., but numbers continued to rise throughout the observation period. Another difference between f.p.- and i.v.-infected mice was that virtually none of the T cells initially present in the spleen were IFN-γ producers upon ex vivo stimulation. Prompted by the delayed systemic accumulation of β-gal96–103–specific CD8+ T cells in the spleen following i.v. infection, we hypothesized that β-gal96–103–specific CD8+ T cells might be sequestered in the predominantly transduced organ, the liver, and therefore decided to analyze how the systemic immune response resulting from i.v. infection with adenovirus correlated with the intrahepatic immune response, virus elimination, and pathology. Accordingly, we performed analysis of virus elimination using real-time PCR of viral DNA and X-gal staining of β-galactosidase–producing hepatocytes. These findings were compared with enumerations of intrahepatic β-gal96–103–specific CD8+ T cells and serum transaminase levels. As expected, in f.p.-infected mice, viral DNA was rapidly eliminated from the vaccinated paw between days 14 and 21 p.i., mirroring the peak in β-gal96–103–specific CD8+ T cells in the spleen, yet some animals still had small but detectable amounts of viral DNA present at day 28 p.i. (data not shown). Analysis of the clearance of intrahepatic viral DNA in i.v.-infected mice revealed an initial rapid decline between days 7 and 14 p.i. followed by a slow decline until day 28 p.i. and a reduction to barely detectable levels thereafter (Fig. 4A). Analysis of the relative density of transduced cells visualized by staining for β-galactosidase activity gave similar results (Fig. 4A). In accordance with the virus-elimination data, serum transaminase levels were normal at day 7 p.i., high at day 14, and then declined to baseline levels at day 28 p.i. (Fig. 4B). Serum transaminase levels were initially parallelly expression of mRNA for proinflammatory cytokines and chemokines (TNF-α, IFN-γ, CCL5, and CXCL10) within the liver with values rising to a sharp peak at day 14 p.i. However, cytokine and CCL5 mRNA expression were maintained at values above baseline even at day 60 p.i. (Fig. 4C). The accumulation of β-gal96–103–specific CD8+ T cells in the liver mirrored virus clearance, cytokine induction, and serum transaminase levels. Thus, β-gal96–103–specific T cells were present at low numbers at day 7 p.i., reached a peak at day 14 p.i., and stabilized at days 21–60 p.i. (Fig. 4D). In contrast, intrahepatic CD8+ T cells only expressed ability to produce IFN-γ at a significant frequency on day 21 p.i., at which time most β-gal96–103–specific CD8+ T cells were cytokine producers. Numbers of IFN-γ–producing cells in the liver then declined and remained low at days 28 and 60 p.i. (Fig. 4D). Thus, our data suggest that near elimination of virus is associated with a sudden and transient rise in intrahepatic IFN-γ–producing T cells that precedes and is temporarily separated from the detection of these cells in the spleen. Notably, the kinetics of the appearance of IFN-γ–producing T cells in the liver after i.v.
vaccination actually follows that seen in the spleen of f.p.-infected mice, consistent with the sequestration of these T cells in the major adenovirus-transduced target organ.

Transgene-specific CD8\(^+\) T cells emerging after systemic adenovirus infection are dysfunctional, but retain cytotoxic capabilities ex vivo and in vivo

Prompted by the reduced capacity for cytokine production by transgene-specific CD8\(^+\) T cells from i.v. and high-dose primed mice, we next performed a more thorough evaluation of memory cell phenotypes and effector functions following i.v. and f.p. immunization. Following viral infection, specific cell surface markers such as CD27 and CD127 and the capacity for IL-2 production have been associated with the ability of memory CD8\(^+\) T cells to persist in an Ag-independent manner and to expand upon rechallenge (40, 42–46). In contrast, the loss of cytolytic effector functions is frequently a relatively late-appearing functional impairment immediately preceding physical deletion (47, 48). In our experimental model, we compared memory cells induced i.v. and f.p. immunization and found that f.p. immunization indeed was associated with higher levels of CD27 and CD127 expression on IFN-γ-producing cells. However, even in f.p.-immunized mice, a significant proportion of cells were CD127 negative consistent with an effector memory phenotype (Fig. 5A). Similarly, when assessing the coproduction of IL-2 and IFN-γ, the proportion of double-positive cells was higher in the f.p.-immunized mice (Fig. 5A). As expected from the normally late disappearance of cytotoxic capacity, we found that transgene-specific T cells generated through immunization by either route were able to exert substantial ex vivo as well as in vivo cytotoxicity (Fig. 5B, 5C) and that cytotoxic activity correlated with the expected number of splenic β-galactosidase–specific CD8\(^+\) T cells (cf. Fig. 3).

T cells remaining after peripheral and systemic challenge efficiently control an acute viral challenge

As the transgene-specific cells induced by systemic or peripheral immunization varied considerably in frequency and phenotype, we sought to further determine their antiviral efficacy and capacity for recall expansion during an acute challenge with β-galactosidase-expressing vaccinia virus. To assess the degree of protection following immunization by either route, we infected i.v.- or f.p.-immunized mice i.p. with \(2 \times 10^7\) PFU of β-galactosidase–expressing vaccinia virus and measured the virus load in the ovaries 5 d later. Mice vaccinated by any route ~100 d earlier completely cleared the virus within 5 d postchallenge (Fig. 6A).

To evaluate the capacity for recall expansion, we transferred 50 million spleen cells from mice immunized ~4 mo earlier into naive B6.SJL recipients via the i.v. route. B6.SJL mice express the CD45.1 allotype, and it is therefore possible to stain and identify donor cells in the capacity of their CD45.2 expression. The day after cell transfer, recipients were either left uninfected or infected with \(2 \times 10^7\) PFU of β-galactosidase–expressing vaccinia virus and measured the virus load in the ovaries 5 d later. Mice vaccinated by any route ~100 d earlier completely cleared the virus within 5 d postchallenge (Fig. 6A).

CD8\(^+\) T cells emerging after systemic adenovirus infection are unable to respond to peripheral peptide challenge

It is clear that the transgene-specific CD8\(^+\) T cells emerging late after systemic/high-dose infection have deficiencies in cytokine production and secondary expansion, yet are proficient in ex vivo and in vivo cytotoxicity and that the immunized mice can control

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**FIGURE 4.** Intrahepatic immune response following i.v. adenovirus infection. A cohort of mice was injected i.v. with \(10^{10}\) particles of Ad-β-gal on day 0, and mice were sacrificed on days 7, 14, 21, 28, 60, and 120 d.p.i. Unvaccinated mice were included for comparison and plotted as day 0. A, Relative levels of viral DNA and number of β-galactosidase–expressing cells per area in liver sections. Data are averages ± SEMs; \(n = 4\) in each group. B, Measurements of serum aspartate aminotransferase and alanine aminotransferase activity. Data are averages ± SEMs; \(n = 4\) in each group. C, Measurements of selected cytokine and chemokine mRNAs that were overexpressed in virus-transduced livers as determined by RNAse protection analysis. All values were normalized to L32 expression and shown as percent induction over unvaccinated controls. Data are averages ± SEMs; \(n = 4\) in each group. D, Total number of Ag-specific intrahepatic CD8\(^+\) T cells, as determined by intracellular IFN-γ staining or H-2Kb-β-gal96–103 dextramer binding. Data are averages ± SDs; \(n = 4\) in each group.
a vaccinia virus challenge. This could suggest that the late emerging cells might indeed be protective and provide the cellular basis for long-lasting and even expanding T cell-mediated immunity. However, in many ways, cytotoxicity assays only require limited functional capacity of effector cells, as target cells and effector cells are directly mixed together (this is the case even in vivo, in which the target cells remaining in the spleen are measured), and, regarding the control of vaccinia challenge, f.p.-immunized mice were as efficient despite lower numbers of Ag-specific cells. Combating an infection in most cases also requires the effector CD8+ T cells to be able to migrate into sites of infection and cause inflammatory responses and cell killing. A recent report has indicated that exhausted CD8+ T cells arising during chronic infections have altered expression of genes associated with migratory capacity (49), which could suggest that this nonredundant effector function might be more affected than the cytotoxicity. To assay for a functional contribution of the surplus of Ag-specific cells emerging late after systemic/high-dose infection, we evaluated the ability of the primed T cells to migrate into peripheral tissues and cause inflammation by performing a CD8+ T cell-mediated delayed-type hypersensitivity (DTH) test (50) (Fig. 6). As expected, f.p.-vaccinated mice responded to injection of the β-galactosidase–expressing vaccinia virus, and viral titers in the ovaries were determined 5 d later by plaque assays. Shown are titers in individual mice with the dashed line depicting the assay sensitivity. B. Fifty million splenocytes from groups of four mice vaccinated i.v. or f.p. 120 d previously were transferred i.v. into B6.SJL recipients. The following day, half the recipient mice were challenged with 2 × 10^7 PFU of β-galactosidase. Shown are total numbers of CD45.2^+CD8^+CD44^+ cells in individual recipient mice. *Statistically significant difference from uninfected mice.

![FIGURE 5](image-url)

![FIGURE 6](image-url)

**Discussion**

The presented analysis of viral dissemination and the expansion of transgene-specific CD8+ T cells after adenoviral immunization have important implications for our understanding of the immunobiology of systemic viral infections and for the use of adenoviral vectors as vaccine carriers. In particular, we demonstrate that viral dosage markedly influences viral dissemination, which in turn were high (Fig. 7, left panels). Notably, the responses at 60 and 120 d p.i. were not confidently discernible, probably reflecting the contraction of the transgene-specific CD8+ T cell population. Surprisingly, transgene-specific DTH in i.v.-vaccinated mice paralleled the pattern after f.p. infection, thus demonstrating that some of the functional T cells present in i.v.-vaccinated mice at day 21 p.i. can be recruited and cause inflammation. However, the large number of β-galactosidase–specific CD8+ T cells found in these mice at days 60 and 120 p.i. were completely inefficient in causing peripheral inflammation. Although only a short window of immediate peripheral responsiveness was found in the infected mice irrespective of the route of adenoviral priming, memory T cells persisted at the site of original adenovirus administration in the right f.p. of the f.p.-vaccinated mice, as evidenced by their ability to initiate inflammation triggered by the peptide injected into the left f.p. (Fig. 7, right panels) (three independent experiments have verified that the most pronounced swelling occurs at the original site of adenovirus administration and not in the peptide-injected f.p.). This response at the original injection site could be observed as late as 17 mo postimmunization providing strong yet indirect evidence that transgene-primed T cells persisted in the f.p. of these vaccinated mice at a level sufficient to maintain local immune surveillance.
Right paw

![Swelling x 10^2 mm](Left challenged paw)

**FIGURE 7.** DTH following i.v. or f.p. immunization. Six mice in each group were vaccinated either i.v. or f.p. with Ad-β-gal and injected in the left hind f.p. with β-gal\(_{96–103}\) peptide 21, 60, 120, and 520 d later (520 d was only performed on two f.p.-immunized mice); f.p. thickness of both hind paws was measured before peptide injection and 16, 24, 48, and 72 h later. Swelling in the injected paw is shown in the left panels and in the un.injected right paw in the right panels (the paw used for the original immunization in the f.p.-immunized group). Data are averages + (f.p. vaccinated) or — (i.v. vaccinated) SEMs.

positively impacts on memory T cell numbers, but negatively impacts on memory cell quality.

It is evident that i.v. and high-dose induced dissemination of a nonreplicative adenoviral vector causes a quantitatively robust transgene-specific systemic CD8\(^+\) T cell response that is tarnished by profound qualitative deficiencies. However, at moderate virus doses, these deficits are significantly influenced by the route of infection (Figs. 2, 3, 7).

When moderate doses of virus are given i.v., the CD8\(^+\) T cell response against the adenovirus-encoded Ag at first involves relatively few but cytokine-producing and migration-competent cells, with at least some preferential recruitment to the dominant infection site, the liver. It is unclear whether the CD8\(^+\) T cells detected within the liver during the first weeks of infection are deleted [as suggested by some reports (51–53)] or whether some survive. Whatever the explanation for the loss of the initial wave of Ag-specific CD8\(^+\) T cells in the liver, it is evident that a systemic expansion of Ag-specific CD8\(^+\) T cells is resumed following a reduction in the hepatic viral load. The quality of these primed cells is initially poor, with none of the Ag-specific cells expressing IFN-γ, and although the fraction of cytokine-producing cells gradually increases, these cells do not become fully functional with respect to cytokine production at least within 120 d after virus inoculation. A gradual recovery of exhausted cells may be similar to what has been reported in lymphocytic choriomeningitis virus (LCMV)-infected mice following gradual control of a chronic infection and in HIV-infected patients during immunological recovery initiated by highly active antiretroviral therapy (54–58). Importantly, that our data show a primary accumulation of antiviral CD8\(^+\) T cells within the liver in i.v.-immunized mice followed by a second systemic expansion does not contradict previous reports showing initial expansion and then deletion of Ag-specific T cells in related systems (51–53), as none of these previous studies evaluated CD8\(^+\) T cell responses at sufficiently late time points to make the observation of a partial T cell recovery with time as we have seen. Careful lineage analysis will be required to determine whether this secondary population originates from the first wave of activated T cells or is induced de novo by persistent Ag (59).

Even with the first phase of the virus-induced CD8\(^+\) T cell response, the numbers of Ag-specific cells in the liver continue to rise after the clearance of the vast majority of the viral DNA from the target organ, the peak in intrahepatic cytokine and chemokine production, and the rise in serum transaminases (Figs. 3, 4). The appearance of Ag-primed CD8\(^+\) T cells in the spleen after hepatic infection is further delayed, and the numbers eventually appearing are much higher than what is seen during the peak response in the liver. Despite the remarkable differences in virus biology (i.e., among others, recombinant adenovirus being replication deficient), these observed associations of T cell infiltration, virus elimination, and parenchymal damage roughly reproduces the dichotomy of hepatic and systemic immunity seen in hepatitis B- and C-infected human patients and monitored in experimentally infected chimpanzees. If the course of infection is synchronized based on the time of peak levels of intrahepatic virus, then the immune responses toward hepatitis B, hepatitis C, and replication-deficient adenovirus-encoded transgene become superimposable (60, 61). In future studies, it will be interesting to determine whether the immune response patterns we have observed are related to the ability of adenovirus to target the liver or whether it is a consequence of more general interrelations between Ag dissemination and persistence on the one hand and the immune system on the other. Recent discovery of adenovirus variants with reduced liver targeting suggest the possibility to address this issue (32, 33).

Regarding the use of adenovirus as vaccine vectors, we have noted the remarkable similarity in experimental findings among a number of studies using leishmanias, HIV (Gag), and LCMV Ags and the response induced by i.v. administration or peripheral over dosing shown in this study (9, 10, 39). The late i.v.-induced CD8\(^+\) T cells we observed bear all the markings of a dysfunctional T cell population: lower fraction of cytokine-producing cells out of total specific cells, lower IFN-γ MFI, lower fraction of cells coproducing TNF-α or IL-2 and IFN-γ, lower expression of IL-7R (CD127), absence of a contraction phase normally suggestive of a memory response, and an impaired ability to migrate into tissues and exert effector functions. This is in contrast to the CD8\(^+\) T cell response in f.p.-primed mice, which has a more normal contraction phase (25–40% as opposed to no contraction (9) or expansion (10)] (Fig. 3); most cells are competent polyfunctional cytokine producers and can initiate peripheral inflammation and immunological surveillance of the target organ. Of note, similar results for low-dose peripheral vaccination have previously been reported for the f.p.-induced response using LCMV gp (11, 12) and the studies using leishmanias Ags by Darrah et al. (39), which also reported an inverse relationship between vector dose and T cell cytokine-producing competence. We decided to look further for a common denominator between high-dose peripheral immunization and systemic (i.v.) vaccination by quantitating viral vector dissemination during our dose-escalation study. This study demonstrated that f.p.-administered virus is only retained in the target organ when injected in a low dose, and the virus disseminates systemically following an increase in the inoculum, and it is the degree of virus
dissemination that negatively impacts on the capacity for cytokine production by the ensuing CD8+ T cells rather than the level of local transduction or the route. At the doses used by Tatsis et al. (10), f.p. administration was as efficient as i.v. administration in causing systemic dissemination, and thus it is no surprise that these authors found no differences in immune response relating to the route of immunization. Just as f.p. injection gave a nonlinear correlation of dose and systemic virus transduction, i.v. immunization induced a remarkably efficient transduction of the f.p. at the highest dose only. This could reflect the prolonged presence of virus in the circulation at very high inocula; thus, it has previously been demonstrated that increasing the stability of adenovirus in plasma can enhance transduction of peripheral target tissues such as cancers (62). If this is the explanation, this observation may shed light on reports that i.m. administration is capable of inducing mucosal immune responses, as a high-dose i.m. administration might be distributed to peripheral mucosal sites or lymphoid organs draining such sites (21). Conversely, our reports of highly persistent immunological surveillance in the f.p.-injected target organ may help explain why intranasal virus administration may induce persistent T cell responses at this site only (63).

We have demonstrated that even CD8+ T cells primed by i.v. infection can mediate cytotoxicity ex vivo and in vivo and protect against a vaccinia virus challenge i.p.; thus, even though these cells cannot easily be mobilized to a site of infection, it remains a possibility that mucosal immune responses induced by high-dose vaccination will prove effective against a localized mucosal challenge (which has not yet been addressed in published studies) (21). However, it must be stressed that previous studies using adenoviral vectors in humans have found a dose ceiling of ~10^11 particles (7). Adjusting for size, this corresponds to <10^6 particles for a mouse, and, thus, even the 10^6 particles given in the f.p. immunization regimen remain on the high side of what is relevant for human vaccine studies. Our data further indicate that an attempt to use a high-dose regimen in humans would lead to systemic dissemination of virus at levels that are greater than what has previously been associated with idiosyncratic case fatalities in humans and that attempts to induce systemic responses with low doses of virus may fail to induce a quantifiable transgene-specific CD8+ T cell response (64).

In conclusion, the use of adenoviral vectors at relevant low-to-moderate doses induces a systemic CD8+ T cell response that retracts into a stable splenic memory population of functional cytotoxic-producing cells with persistent surveillance of the originally transduced target organ. These are desirable traits for the use of virus vectors for vaccination. In contrast, systemic immunization with adenoviral vectors induces a protracted and dysfunctional CD8+ T cell response with evidence of exhaustion and lack of ability to migrate into a peripheral site and cause Ag-specific inflammation. These are clearly undesirable traits for a vaccine-induced immune response and would likely be difficult to induce safely in humans. Accordingly, systemic or high-dose administration of adenoviral vectors may represent an interesting tool to study the immunobiology of chronic infections, but is inappropriate for vaccine and vaccine study purposes.

Acknowledgments
We thank Grethe Thørner Andersen and Lone Malte for expert laboratory assistance and Grazyna Hahn for technical assistance. MHC dextramers were kindly donated by Jørgen Schtaller, DakoCytomation, Glostrup, Denmark. Vaccinia virus-expressing β-galactosidase was a kind gift from Julia Hurwitz, St. Jude Children’s Research Hospital, Memphis, TN.

Disclosures
The authors have no financial conflicts of interest.

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