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Rapid Functional Exhaustion and Deletion of CTL following Immunization with Recombinant Adenovirus¹

Philippe Krebs,*[†] Elke Scandella,* Bernhard Odermatt,[‡] and Burkhard Ludewig^{2*}

Replication-deficient adenoviruses (recombinant adenovirus (rec-AdV)) expressing different transgenes are widely used vectors for gene therapy and vaccination. In this study, we describe the tolerization of transgene-specific CTL following administration of β -galactosidase (β gal)-recombinant adenovirus (Ad-LacZ). Using MHC class I tetramers to track β gal-specific CTL, we found that a significant expansion of β gal-specific CTL was restricted to a very narrow dose range. Functional analysis revealed that adenovirus-induced β gal-specific CTL produced only very low amounts of effector cytokines and were unable to exhibit cytolytic activity in a ⁵¹Cr release assay. Furthermore, Ad-LacZ vaccination failed to efficiently clear established β gal-positive tumors. The impaired function of Ad-LacZ-induced CTL correlated with the presence of persisting β gal Ag in the liver. A further increase in the peripheral Ag load by injection of Ad-LacZ into SM-LacZ transgenic mice which express β gal as self-Ag exclusively in peripheral nonlymphoid organs, resulted in the physical deletion of β gal-specific CTL. Our results indicate first that CTL deletion in the course of adenoviral vaccination is preceded by their functional impairment and second, that the outcome of rec-AdV vaccination depends critically on the Ag load in peripheral tissues. *The Journal of Immunology*, 2005, 174: 4559–4566.

Recombinant AdV (rec-AdV)³ lacking both the E1 and E3 region (first generation adenoviruses) are mainly used as vaccination vectors, whereas the more attenuated second- and third-generation adenoviral vectors with additional deletions in the early gene region are better suited for gene transfer approaches (1). Adenoviruses attach to their target cells via the widely expressed Cocksackie-adenovirus receptor and subsequent delivery of the genetic cargo to the cell nucleus leads to efficient transduction. The route of administration mainly determines which cells are targeted by the vector. For example, after i.v. application of rec-AdV, >90% of the vector is transferred to the liver (2, 3), where mainly hepatocytes and, to some extent, Kupffer cells are transduced. Intramuscular administration of rec-AdV leads to transgene expression mainly in skeletal muscle cells close to the injection site (4). Furthermore, a substantial number of bone marrow-derived APC such as macrophages and dendritic cells (DCs) are transduced when the initial inoculum reaches secondary lymphoid organs (2). Importantly, rec-AdV infection of splenic APC is associated with an acute burst of inflammatory cytokines and activation/maturation of DC (2).

Due to their immunogenicity, first generation rec-AdV are now considered as promising vaccine vectors (5). Indeed, a comparative study has shown that rec-AdV elicited an effective immune response against a pathogenic HIV-SIV hybrid virus (6). Prime-boost vaccination with heterologous E1-deleted rec-AdV can fur-

ther augment the immunization efficacy (7). However, it is noteworthy that a number of studies achieved significant T cell activation or protective effects against various pathogens only if rec-AdV was used in prime-boost regimen with DNA (8, 9), protein in adjuvant (10), or in combination with other recombinant viruses such as attenuated vaccinia virus (11). These and other recent quantitative studies have shown that rec-AdV immunization usually elicits only rather low numbers of cytokine-producing effector CTL (<1% of CD8-positive T cells) (10, 12). Furthermore, adenoviral delivery of transgenes has been shown to be insufficient for the induction of tumor-specific CTL responses (13) and may even induce tolerance against self-Ags (14). Overall, these studies suggest that immunization with rec-AdV may elicit a spectrum of different T cell responses ranging from expansion and differentiation of effector T cells to complete abolishment of a functional response.

Several factors have been described that contribute to the fine tuning of antiviral immune responses including cellular tropism of the infectious agent, the overall level of costimulation (15), and potential viral escape strategies (16). However, it appears that the major factors determining duration and magnitude of an antiviral immune response are Ag localization, dose, and kinetics (17). For example, high doses of widely disseminating strains of the lymphocytic choriomeningitis virus (LCMV) exhaust antiviral CTL leading to establishment of a persistent infection (18). Physical deletion of anti-LCMV CTL is most likely preceded by their functional impairment with the inability to produce effector cytokines (19, 20). Likewise, persisting viral infections in humans, such as HIV or hepatitis C virus, are associated with dysfunctional CTL responses (21, 22). Moreover, a recent study by Wherry et al. (23) shows that not only the persistence of a viral Ag, but also the initial Ag level is an important factor determining the quality of the antiviral memory response.

Studies on the immunogenicity of antigenic peptides have shown that dose and route of application (24) together with their pharmacokinetic properties (25, 26) are critical for the efficacy of these vaccines. Rec-AdV represent delivery vehicles for antigenic peptides. It is thus important for the application of rec-AdV in

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³ Abbreviations used in this paper: rec-AdV, recombinant adenovirus; β gal, β -galactosidase; MCMV, mouse CMV; DC, dendritic cell; LCMV, lymphocytic choriomeningitis virus; BSS, balanced salt solution.

vaccination approaches, to correlate dose, route, and “pharmacokinetics” (initial distribution and persistence) of the genetically delivered Ag with a quantitative and functional analysis of the elicited immune response. In this study, we analyzed β -galactosidase (β gal)-specific CTL responses induced by a β gal-recombinant AdV (Ad-LacZ) using a combination of MHC class I tetramer analysis, intracellular cytokine staining, and cytotoxicity assays. Our results indicate that rec-AdV immunization is rather inefficient because 1) a significant expansion of CTL by rec-AdV is restricted to a narrow dose-range, 2) the majority of the elicited CTL are dysfunctional, and 3) vaccination with rec-AdV encoding self-Ags leads to physical deletion of the respective CTL population.

Materials and Methods

Mice

C57BL/6 and SM-LacZ (27) mice were obtained from Charles River Laboratories or from the Institut für Labortierkunde (University of Zurich), respectively, and were used in sex-matched groups in the age of 6–8 wk. Animals were kept under specific pathogen-free conditions. All animal experiments were performed in accordance with the Swiss Federal legislation on animal protection.

Viruses and peptides

Recombinant adenovirus expressing the β gal protein under the control of the human CMV promoter (Ad-LacZ) and lacking E1 and E3 genes was kindly provided by Dr. S. Rusconi (University of Fribourg, Fribourg, Switzerland). Ad-LacZ was propagated on permissive HER-911 cells obtained from Dr. R. C. Hoeben (Leiden University Medical Center, Leiden, The Netherlands) and was purified by double cesium chloride gradient. Virus titer was determined in a cytopathic effect assay. In brief, serial dilutions of the adenovirus were used to infect HER-911 cells on a microtiter plate and cytopathic effect was determined after 13 days by microscopy. Tissue culture infectious dose 50% was calculated by the Reed-Muench method. Ad-LacZ aliquots were stored at -80°C in a buffer containing 2 mM MgCl_2 , 5% sucrose, 10 mM Tris pH 8.0. The β gal peptide 497–504 (ICPMYARV) (28) was purchased from Neosystem.

Isolation of liver and lung lymphocytes

Perfused livers were smashed through a metal grid. Lymphocytes were purified by Ficoll (Biochrom) gradient centrifugation ($600 \times g$, 15 min). Lungs were minced with razor blades and incubated in balanced salt solution (BSS) containing 1 mg/ml DNase (Fluka) and 2 mg/ml collagenase I (Sigma-Aldrich) at 37°C for 30 min. Cell aggregates were dispersed by passing the digest through a 18-gauge syringe, and lymphocytes were isolated by Ficoll gradient centrifugation.

Antibodies

Anti-CD8-FITC, anti-CD8-PE, anti-CD8-PerCP, anti-IFN- γ -FITC, anti-CD62L-FITC, anti-CD44-FITC were obtained from BD Pharmingen. For PBL samples, erythrocytes were lysed with FACS lysing solution (BD Pharmingen). Cells were analyzed with a FACSCalibur flow cytometer using the CellQuest software (BD Biosciences).

Construction of tetrameric MHC class I-peptide complexes and flow cytometry

MHC class I (H-2K^b) monomers complexed with β gal peptide were produced as described (29) and tetramerized by addition of streptavidin-PE (Molecular Probes). At the indicated time points following immunization, animals were bled and single-cell suspensions were prepared from spleen and lymph nodes. Aliquots of 5×10^5 cells or three drops of blood were stained using $50 \mu\text{l}$ of a solution containing tetrameric MHC class I-peptide complexes at 37°C for 10 min followed by staining with anti-CD8-FITC (BD Pharmingen) at 4°C for 20 min. The cells were analyzed by flow cytometry gating on viable leukocytes. The specificity of β gal-tetramers was controlled using splenocytes from naive C57BL/6 mice (0.07% β gal tet⁺ of CD8⁺ T cells) or from C57BL/6 mice immunized with enhanced GFP-recombinant AdV (0.1% β gal tet⁺ of CD8⁺ T cells). Absolute cell counts were determined by counting leukocytes in an improved Neubauer chamber. Statistical data analysis was performed using Prism 3.03 (GraphPad).

Cytotoxic T cell response

Specific ex vivo cytotoxicity was determined in a standard ^{51}Cr release assay as described (30). The supernatants of the cytotoxicity assay cultures were counted in a Cobra II Gamma Counter (Canberra Packard). Percentage of specific lysis was calculated as (experimental release – spontaneous release)/(total release – spontaneous release) $\times 100$. Spontaneous release was always below 20%.

Preparation of DCs

Generation of DCs from bone marrow cultures of C57BL/6 mice has been described (30). DCs were pulsed with β gal peptide at a concentration of 10^{-6} M for 60 min at 37°C . Cells were washed three times with BSS and 3×10^5 cells were injected i.v. twice in a volume of 0.5 ml of BSS.

Intracellular cytokine staining and ELISPOT

Specific ex vivo production of cytokine was measured both with intracellular cytokine staining and with ELISPOT assay. Organs were removed at the indicated time points following immunization with Ad-LacZ or β gal-loaded DC. Single-cell suspensions of 1×10^6 splenocytes, lymph node cells, or liver or lung lymphocytes were incubated for 5 h at 37°C in 96-well round-bottom plates in $200 \mu\text{l}$ of culture medium containing 25 U/ml IL-2 and 5 $\mu\text{g/ml}$ brefeldin A (Sigma-Aldrich). Cells were stimulated with PMA (50 ng/ml) and ionomycin (500 ng/ml) as positive control or left untreated as a negative control. For analysis of peptide-specific responses, 10^6 cells were stimulated with 10^{-6} M β gal peptide and then stained as described elsewhere (31). The percentage of CD8⁺ T cells producing IFN- γ was determined using a FACSCalibur flow cytometer.

For ELISPOT analysis, spleens were harvested 2 wk after i.v. immunization with 2×10^9 PFU Ad-LacZ. As a positive control, mice were immunized i.v. with 2×10^9 PFU of a β gal recombinant mouse CMV (MCMV, kindly provided by E. Mokarsky, Stanford University). A total of 1.5×10^5 MACS-purified CD8⁺ T cells and 2×10^4 autologous DCs were incubated in presence or the absence of the β gal peptide in 96-well ELISPOT filter plates (Millipore) coated with an anti-IFN- γ capture Ab (R&D Systems). After 5 or 18 h, IFN- γ secretion was revealed using a biotin-conjugated anti-IFN- γ detection Ab (R&D Systems) and streptavidin-conjugated alkaline phosphatase (Jackson ImmunoResearch Laboratories). Reactive spots were visualized with 5-bromo-4-chloro-3-indolyl-phosphate-toluidin (Biomol) and nitroblue-tetrazoliumchlorid (Biomol).

Transplantation of tumor pieces and monitoring of tumor growth

EL4-LacZ tumor cells (28) in single-cell suspensions were injected s.c. into the flanks of T cell-immunodeficient mice (H-2^b RAG-1^{−/−}). Growing tumor pieces were dissected into small tumor pieces of $\sim 1 \times 1 \times 1$ mm and transplanted into the flanks of naive C57BL/6 recipient mice. Mice were immunized i.v. on day 2 after tumor transplantation with 2×10^9 PFU of Ad-LacZ or on days 2, 5, and 7 with 3×10^5 DC/ β gal. Tumor size was assessed at the indicated time points. Tumor volume was calculated by the formula, $V = \pi \times abc/6$, where a , b , and c are the orthogonal diameters.

Immunohistology

Freshly removed organs were immersed in HBSS and snap-frozen in liquid nitrogen. Frozen tissue sections were cut in a cryostat and fixed in acetone for 10 min. Sections were incubated with rabbit IgG fraction anti- β gal (ICN Pharmaceuticals) and anti-mouse mAb against CD8⁺ cells (YTS169), followed by goat anti-rat Ig (Caltag Laboratories) and alkaline phosphatase-labeled donkey anti-goat Ig (Jackson ImmunoResearch Laboratories). Nuclei were counterstained with hemalum.

Results

Determining the optimal dose-range for rec-AdV vaccination

In a first set of experiments, we determined the basic parameters for efficient induction of β gal-specific CTL responses by Ad-LacZ vaccination. Using MHC class I tetramers complexed with the H2-K^b-binding β gal_{497–504} epitope, we found that i.v. immunization with 2×10^7 PFU generally elicited only a weak expansion of β gal tet⁺ CD8⁺ T cells (Fig. 1a). In contrast, intermediate doses of virus (2×10^8 – 2×10^9 PFU) induced a significant proliferation of β gal-specific CD8⁺ T cells (Fig. 1, a–c). Examination of different organs revealed that the percentage of β gal-specific CTL was highest in nonlymphoid organs such as lung and liver (Fig. 1,

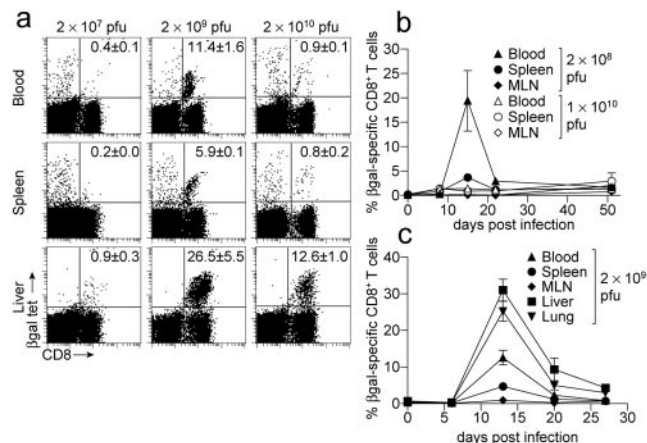


FIGURE 1. Expansion of β gal-specific CD8⁺ lymphocytes following Ad-LacZ immunization of C57BL/6 mice. *a*, Graded doses of Ad-LacZ were administered i.v. and β gal-specific CTL were analyzed in blood, spleen, and liver using MHC class I tetramers on day 12 postinfection. Mean percentages of β gal tet⁺CD8⁺ cells from CD8⁺ cells are indicated ($\pm$ SEM; $n = 3$). Representative data from one of four independent experiments are shown. *b*, Time course analysis of β gal-specific CD8⁺ cells following i.v. immunization of C57BL/6 mice with 2×10^8 (filled symbols) and 1×10^{10} PFU (open symbols) of Ad-LacZ. The percentage of β gal tet⁺ cells among CD8⁺ T lymphocytes was assessed in different organs at the indicated time points ($\pm$ SEM; $n = 3$). *c*, Mice were infected i.v. with 2×10^9 of Ad-LacZ, and the expansion of β gal-specific CD8⁺ cells (mean $\pm$ SEM; $n = 3$) was assayed in the indicated organs. MLN, mesenteric lymph nodes.

a and *c*), and close to the detection limit (0.1% tet⁺ cells in the CD8⁺ T cell compartment) in lymph nodes (Fig. 1, *b* and *c*). The β gal-specific CTL response peaked around day 12 after infection followed by the typical contraction phase and the establishment of a stable memory population with 1–2% of the CD8⁺ T cells being β gal-specific (Fig. 1, *b* and *c*). Importantly, a 5- to 10-fold increase of the optimal dose to $1\text{--}2 \times 10^{10}$ PFU led to a dramatic diminution of the elicited CTL response (Fig. 1, *a* and *b*). This pattern of reactivity in a remarkably narrow dose range of 3 log (low dose ($\leq 2 \times 10^7$), low response; intermediate dose ($10^8\text{--}10^9$ PFU), optimal response; high dose ($\geq 10^{10}$ PFU), reduced response) could also be observed after s.c. or i.p. infection with Ad-LacZ (data not shown).

Assessing functional impairment of Ad-LacZ-induced CTL

The CTL reactivity pattern after Ad-LacZ immunization closely resembles that observed after peptide immunization with induction or tolerization of specific T cells being dependent on the dose and timing of application (24, 25). To assess the effector function of β gal-specific CD8⁺ T cells after Ad-LacZ infection, direct ex vivo cytolytic activity was determined at the peak of the response (Fig. 2*a*) or following secondary in vitro restimulation for 5 days (Fig. 2*b*). Direct comparison of the cytolytic activity of the two CTL preparations by adjusting the E:T values for the numbers of tetramer-positive cells, revealed that Ad-LacZ-induced CTL are functionally impaired and require in vitro restimulation for several days to gain cytolytic activity (Fig. 2).

Ad-LacZ-induced CTL showed not only an impairment in their cytolytic activity but also in the production of the effector cytokine IFN- γ (Fig. 2*b*). The absolute numbers of β gal-specific CD8⁺ splenocytes secreting IFN- γ in a 5- or 18-h ELISPOT assay was more than one logarithmic unit lower than the absolute amount of β gal tet⁺CD8⁺ splenocytes (Fig. 2*b*). As a positive control for the ELISPOT assay, splenocytes from β gal recombinant MCMV-

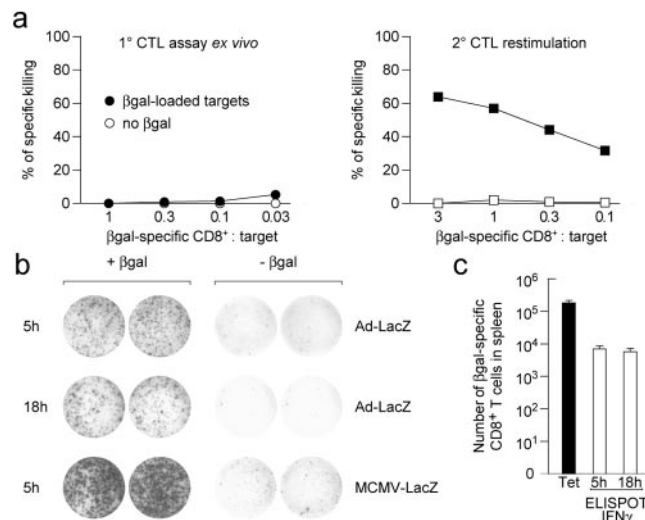


FIGURE 2. Impaired immediate effector function of Ad-LacZ-induced β gal tet⁺CD8⁺ cells. *a*, C57BL/6 mice were immunized with 2×10^9 PFU Ad-LacZ, and direct ex vivo cytolytic activity of splenocytes was assayed on day 12 postinfection (left panel). In addition, splenocytes were cultured in vitro for 5 days in the presence or absence of IL-2. For both assays, the E:T ratio was adjusted for the number of β gal tet⁺CD8⁺ cells. Values indicate specific lysis (mean $\pm$ SEM, $n = 3$) of β gal-peptide-loaded (closed symbols) or unpulsed (open symbols) ^{51}Cr -labeled EL4 cells. One representative of three experiments is shown. *b*, Impaired IFN- γ secretion of Ad-LacZ-elicited β gal-specific CD8⁺ T cells as determined by ELISPOT analysis. C57BL/6 mice were immunized i.v. either with 2×10^9 PFU Ad-LacZ or 2×10^6 PFU MCMV-LacZ (positive control) and spleens were harvested at days 13 and 6 postimmunization, respectively. IFN- γ -ELISPOT data from Ad-LacZ and MCMV-LacZ immunized mice are shown. Representative data from one of four mice are shown. *c*, The total of β gal tet⁺CD8⁺ spleen cells (closed bars) and the absolute numbers of CD8⁺ spleen cells secreting IFN- γ after β gal peptide restimulation for 5 or 18 h are shown (open bars) ($n = 4$). Percentages of β gal tet⁺ cells in the CD8⁺ compartment were 4.6% for Ad-LacZ and 1.4% for MCMV-LacZ.

LacZ-infected mice were used. Although Ad-LacZ elicited an ~ 3 -fold higher expansion of β gal-specific CD8⁺ T cells compared with MCMV-LacZ (4.6 vs 1.4% β gal tet⁺ of CD8⁺ T cells, respectively), MCMV-induced β gal-specific CD8⁺ T cells secreted significantly more IFN- γ (Fig. 2*c*).

To further analyze the functional status of rec-AdV-induced CTL, we compared the response induced by 2×10^9 PFU Ad-LacZ with that elicited by immunization with β gal peptide-loaded DCs (DC/ β gal). DC priming leads to a maximal expansion of CTL after 6–8 days (32) and β gal peptide-pulsed DC have been shown to elicit strong CTL responses against tumor- and self-Ags (33). Ad-LacZ and DC/ β gal vaccination generated comparable amounts of β gal-specific CTL in spleens and livers of naive C57BL/6 mice (Fig. 3*a*). Furthermore, the phenotype of Ad-LacZ- and DC/ β gal-induced tet⁺CD8⁺ cells was similar and characteristic for activated CTL with up-regulation of CD44 and down-regulation of CD62L molecules (Fig. 3*b*). However, Ad-LacZ-induced CTL showed an impaired ability to secrete IFN- γ and TNF- α after short-term peptide stimulation, whereas 100% of DC/ β gal-induced CTL produced IFN- γ and TNF- α (Fig. 3*c*). The inability of β gal-specific CTL to secrete effector cytokines was even more pronounced in peripheral organs such as liver and lung (Fig. 3*d*). However, in vitro restimulation for 5 days restored the functional activity of Ad-LacZ-induced CTL with 100% of tetramer-binding CTL being able to secrete both IFN- γ and TNF- α (data not

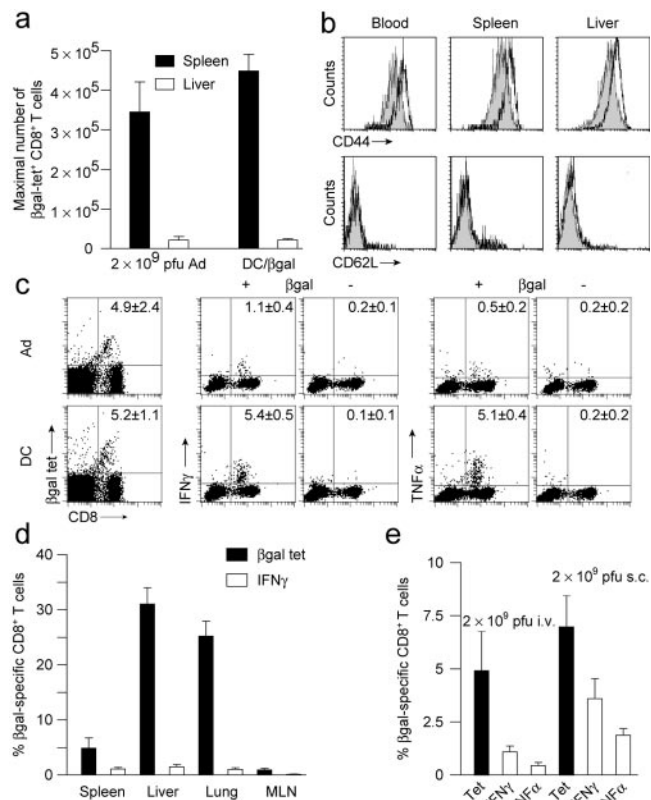


FIGURE 3. Phenotypic and functional characterization of Ad-LacZ-induced CTL. Immunization with β gal peptide-pulsed DC (3×10^5 on days 0 and 2) served as positive control. *a*, Absolute numbers of tet⁺CD8⁺ lymphocytes in spleens and livers after immunization with 2×10^9 PFU Ad-LacZ (day 12) or 3×10^5 DC/ β gal (day 8) ($n = 3$). *b*, Expression of CD44 (upper row) and CD62L (lower row) on tet⁺ CD8⁺ cells from Ad-LacZ (gray plots)- or DC/ β gal-immunized C57BL/6 mice (open plots) at the respective maximum of expansion. Representative data of one mouse from a group of four animals are shown for blood and spleen. Liver-infiltrating lymphocytes were pooled from four animals. *c*, Production of effector cytokines after immunization of C57BL/6 mice with 2×10^9 PFU Ad-LacZ (upper row) or β gal peptide-pulsed DC (lower row). Tetramer analysis and the corresponding intracellular cytokine production assay for IFN- γ and TNF- α in the presence or absence of β gal peptide are shown. Mean percentages of β gal-specific CD8⁺ cells are indicated ($\pm$ SEM, $n = 4$). Representative data from one of three separate experiments are shown. *d*, Percentage of β gal-specific CD8⁺ T cells measured by tetramer analysis (closed bars) vs intracellular IFN- γ production (open bars) in different organs on day 13 after Ad-LacZ infection ($\pm$ SEM, $n = 8$) (MLN, mesenteric lymph nodes). Pooled data of three distinct experiments are represented. *e*, Influence of different infection routes. C57BL/6 mice were infected with 2×10^9 PFU Ad-LacZ either i.v. or s.c. Percentage of β gal tet-positive, IFN- γ - or TNF- α -secreting CD8⁺ splenocytes on day 12 postinfection are shown (mean $\pm$ SEM, $n = 3$).

shown). Furthermore, the defective cytokine secretion in Ad-LacZ-induced CTL was not restricted to i.v. immunization; β gal-specific CD8⁺ T cells showed a substantial reduction in effector cytokine production after s.c. injection (Fig. 3*e*). Importantly, therapeutic vaccination with an optimal dose of the Ad-LacZ vector did not provide efficient antitumor protection compared with the treatment with peptide-pulsed DCs (Fig. 4). Taken together, these results indicate that even in a dose range where optimal expansion of CTL can be achieved, rec-AdV immunization elicited functionally impaired CTL responses.

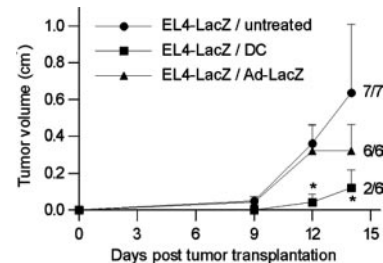


FIGURE 4. Therapeutic antitumor treatment. Solid EL4-LacZ tumor pieces ($1 \times 1 \times 1$ mm) were transplanted s.c. into the flanks of naive C57BL/6 mice. Tumor-transplanted mice were either left untreated, or immunized i.v. on day 2 after tumor transplantation with 2×10^9 PFU of Ad-LacZ ($\blacktriangle$) or on days 2, 5, and 7 with 3×10^5 β gal peptide-pulsed DCs ($\blacksquare$). Tumor volume $\pm$ SEM was determined at the indicated time points. Numbers indicate the proportion of mice displaying tumor growth. Significant differences ($p < 0.05$, Mann-Whitney U test) between DC/ β gal-immunized and untreated tumor-transplanted mice are indicated by an asterisk (*). No significant differences were found between Ad-LacZ-immunized and untreated tumor-transplanted mice.

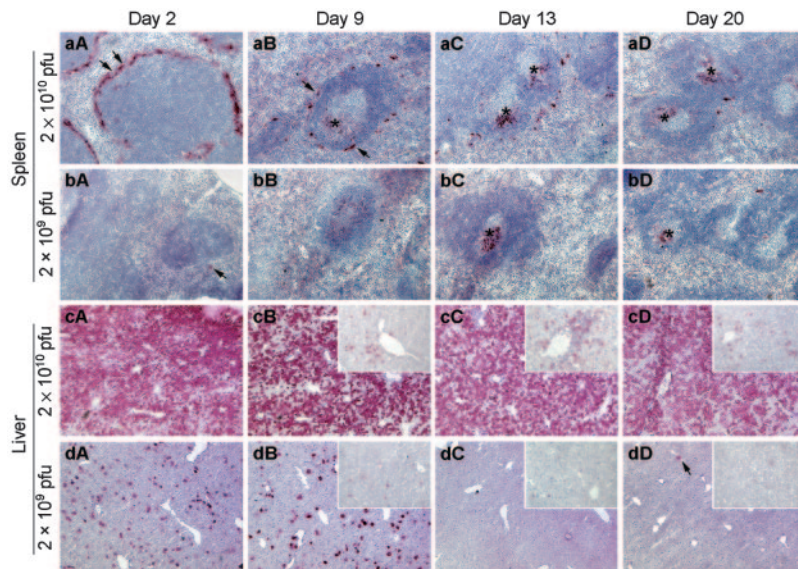
Ag distribution and persistence after Ad-LacZ infection

To determine the Ag expression pattern after Ad-LacZ infection, we analyzed the tissue distribution of the β gal Ag in different organs by immunohistochemistry after administration of 2×10^9 or 2×10^{10} PFU. We found that the Ag was rapidly trapped in the marginal zone of the spleen (Fig. 5, *aA* and *bA*). However, the vast majority of the Ag was found in the liver (Fig. 5, *cA* and *dA*) corroborating earlier studies showing that rec-AdV immunization via the i.v. route mainly leads to the transduction of nonprofessional APC such as hepatocytes (2, 3). The β gal Ag was present in the spleen for at least 20 days after infection with 2×10^{10} (Fig. 5*a*) and 2×10^9 PFU (Fig. 5*b*). It appears that Ag persistence was associated with ongoing germinal center formation (Fig. 5, *a* and *b*). Transgene expression in the liver could be found for >20 days after infection with 2×10^{10} PFU (Fig. 5*c*), whereas the Ag was cleared from the liver around day 13 postinfection with 2×10^9 PFU (Fig. 5*dC*). It is important to note that the loss of Ag expression in the liver after immunization with 2×10^9 PFU coincided with the maximum of the β gal-specific CTL response, whereas Ag persistence in a peripheral organ after infection with 2×10^{10} PFU was associated with strongly diminished CTL responses (compare Figs. 1 and 5). It is likely that the high amounts of Ag in the liver contributed to the functional exhaustion of β gal-specific CTL which were recruited to this organ throughout the observation period (Fig. 5, *cB*–*cD*, insets).

Deletion of Ad-LacZ induced CTL in the presence of peripherally expressed self-Ag

The above findings suggested that the persistence of rec-AdV-delivered Ag in the periphery critically determines the activation of transgene-specific CTL. In principle, other mechanisms such as immune evasion which has been shown to play a role in natural AdV infection (16, 34) might influence the dose-dependent down-regulation of Ad-LacZ-induced CTL responses. To exclude the latter possibility, we injected Ad-LacZ into transgenic SM-LacZ mice which constitutively express the bacterial β gal protein under the control of the smooth muscle protein 22 α promoter. In SM-LacZ mice, β gal is exclusively expressed in cardiomyocytes and arterial smooth muscle cells (27). This restricted peripheral expression pattern results in immunological ignorance such that high-avidity CTL can still be induced, for example, by immunization with β gal-peptide-pulsed DC (33). Here, SM-LacZ mice

FIGURE 5. Ag persistence following immunization with Ad-LacZ. C57BL/6 mice were injected i.v. with either 2×10^{10} (rows *a* and *c*) or 2×10^9 PFU (rows *b* and *d*) of Ad-LacZ. At the indicated time points, spleens (*a* and *b*) and livers (*c* and *d*) were analyzed by immunohistochemistry using anti- β gal and anti-CD8 Abs. Trapping of β gal Ag in the splenic marginal zone is indicated by arrows in (*aA*, *aB*, and *bA*). Progressive translocation toward and accumulation within germinal centers is indicated by asterisks in (*aB*–*aD* and *bC*–*bD*). The insets in (*cB*–*cD* and *dB*–*dD*) represent consecutive sections stained with anti-CD8. Magnification: *a*, *b* and insets in *cB*–*cD* and *dB*–*dD*, $\times 100$; *c* and *d*, $\times 50$.



were immunized with Ad-LacZ and β gal-specific expansion of CD8⁺ cells was assayed by tetramer analysis. Transgene-specific CTL responses remained very low following infection with 2×10^9 PFU Ad-LacZ with an average of 0.7 and 0.8% of the CD8⁺ T cells from blood and spleen, respectively; whereas DC pulsed with the β gal-peptide induced strong CTL expansion in SM-LacZ mice (Fig. 6*a*). Importantly, the frequency of tetramer-positive CTL in SM-LacZ following infection with 2×10^9 PFU Ad-LacZ was below the detection limit in $\sim 50\%$ of the mice (Fig. 6*b*). Effector cytokine production of CD8⁺ T cells from Ad-LacZ-infected SM-LacZ was not detectable after short-term in vitro re-

stimulation with β gal peptide (data not shown). CTL expansion in SM-LacZ mice remained low following i.v. infection with 2×10^7 PFU or 2×10^8 PFU Ad-LacZ (Fig. 6*c*). Moreover, specific CTL activity was absent in $>50\%$ of the mice even after restimulation for 5 days in culture (Fig. 6*d*), suggesting that Ad-LacZ infection of SM-LacZ mice may lead to physical deletion of β gal-specific CTL.

Discussion

Induction of T cell immunity with rec-AdV is considered to be an option for vaccination approaches against infectious diseases and cancer (1). Tumor immunity requires the generation of T cells

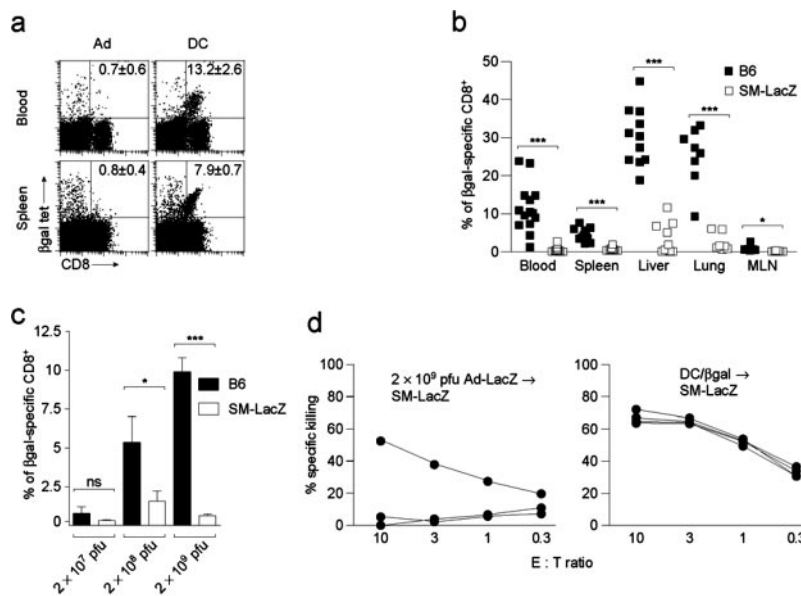


FIGURE 6. Deletion of β gal-specific CTL in SM-LacZ mice following Ad-LacZ immunization. *a*, SM-LacZ mice were infected with 2×10^9 PFU Ad-LacZ or immunized with DC/ β gal and tetramer analysis was performed at the peak of the response. Mean percentages of β gal tet⁺ cells in the CD8⁺ T cell compartment are indicated ($\pm$ SEM, $n = 3$). Data are representative for one of three different experiments. *b*, C57BL/6 or SM-LacZ mice were vaccinated i.v. with 2×10^9 PFU Ad-LacZ. β gal-specific CD8⁺ T cell responses were assessed in the indicated organs with β gal tetramers between days 11 and 13 postinfection. Pooled data from three separate experiments are shown. *c*, Peak expansion of β gal-specific CD8⁺ T cells in blood following i.v. infection with graded doses of Ad-LacZ. Mean percentages of β gal tet⁺ cells in the CD8⁺ T cell compartment are indicated ($\pm$ SEM). Pooled data from three separate experiments are shown. Results of the statistical analysis (Student's *t* test) are indicated in *b* and *c* (*, $p < 0.05$; ***, $p < 0.0001$; ns = not significant). *d*, CTL activity in individual SM-LacZ mice after immunization with 2×10^9 PFU Ad-LacZ or DC/ β gal. Splenocytes were restimulated for 5 days in vitro and tested in a CTL assay. E:T values were adjusted for the number of tet⁺CD8⁺ cells. Values indicate specific lysis of β gal-peptide-loaded ⁵¹Cr-labeled EL4 cells. Representative data from one of three separate experiments are shown.

directed against—in some cases abundantly expressed—self-Ags, whereas in the case of vaccination against infectious agents, the vaccinee has usually not previously encountered the Ag. The major goal of this study was to assess the efficacy of rec-AdV vaccination both in the context of adenoviral delivery of self-Ag and as a means to deliver foreign (microbial) Ag. We used a first generation rec-AdV expressing the *E. coli*-derived β gal protein to provide a detailed analysis of the kinetics and the functional properties of CTL elicited by this vaccination approach. Our study reveals that optimal Ad-LacZ-driven expansion of CTL in the absence of endogenous transgene expression is restricted to a rather narrow dose range of only 1–2 log. Expansion of specific CTL in C57BL/6 mice to levels of 5–15% in the CD8 T cell compartment by a single immunization, as seen in this study, is, per se, a good indicative for the potential of the vaccine.

However, simple enumeration of CTL by tetramer analysis may lead to a misinterpretation of the nature of an ongoing T cell response (35). Our functional analysis of rec-AdV-induced CTL revealed that the majority of these cells had been anergized in vivo because prolonged in vitro restimulation was required for the restoration of the full array of effector functions. Several previous studies have reported that rec-AdV immunization may elicit only low numbers of cytokine producing CTL (10, 12). Furthermore, a rather strong disparity between the frequencies of tetramer-binding and IFN- γ -producing CTL has been observed after immunization of OVA peptide-expressing rec-AdV (36). This effect is most likely associated with a more pronounced reduction in the ability to produce other effector cytokines (TNF- α or IL-2) as described for the LCMV-induced functional exhaustion of CTL (20). It is particularly noteworthy that rec-AdV-induced peripheral “effector” CTL, i.e., CTL homing to nonlymphoid organs such as liver or lung, were most severely compromised in their ability to produce cytokines (Fig. 3d). Another stringent criterion for CTL effector function is the ability to lyse target cells in a short-term ^{51}Cr -release assay. This killing mechanism of CTL is contact-dependent and requires the synthesis and intracellular storage of perforin (37). The absence of the perforin-dependent ex vivo CTL activity after Ad-LacZ immunization and the restoration of this function following in vitro restimulation (Fig. 2) is a further indication for the functional impairment of CTL after rec-AdV infection. Transgene- and/or AdV-specific CTL may, nevertheless, contribute to the elimination of rec-AdV-transduced cells by perforin-independent mechanisms such as Fas/FasL (38). It is possible that the observed gradual loss of β gal-staining in the liver after infection with “optimal” doses of Ad-LacZ was mediated by the maximally expanded, but functionally impaired β gal-specific CTL population. Similarly, the elimination of peptide-pulsed lymphocytes from the circulation does not require fully differentiated CTL (39). It is thus likely that partially differentiated rec-AdV-induced CTL lacking perforin-dependent ex vivo CTL activity, may be able to perform effector functions in less-demanding readout systems such as the in vivo CTL assay based on the elimination of peptide-pulsed lymphocyte from the circulation as described by Yang and colleagues (36). Indeed, in vivo CTL assays revealed that Ad-LacZ-induced CTL were still capable of eliminating their targets from the circulation (data not shown).

The presented analysis, together with a previous study (7), shows that a major characteristic of rec-AdV vaccination is the lack of a linear correlation between rec-AdV dose and the magnitude of the CTL response. Furthermore, the efficient tolerization with physical deletion of Ag-specific CTL by rec-AdV immunization is a completely novel aspect of this vaccination approach. Induction of tolerance with anergization (functional exhaustion) or even deletion of T cells is thought to be determined by the micro-

environment in secondary lymphoid organs. The activation status of professional APCs such as DC is seen as the critical factor determining the quality (induction vs tolerance) of an immune response (40). Rec-AdV administration has been shown to efficiently transduce DC in the splenic marginal zone and to mediate their activation (2). Furthermore, Ad-LacZ injection is associated with a pronounced elevation in the production of inflammatory cytokines such as IL-6, TNF, or IL-12 (2). Thus, the observed tolerizing effects of rec-AdV appear, at a first glance, not to be mediated by a poorly stimulating microenvironment in secondary lymphoid organs and a lack of activating signals for DC.

Peripheral tolerization of self-reactive CD8-positive T cells involves to some extent transport of Ags to secondary lymphoid organs and their presentation by bone marrow-derived APC (most likely DC) (41–43). It is noteworthy that CTL are only tolerized by this mechanism if the Ag is highly expressed in nonlymphoid organs (44), whereas peripheral Ags expressed below a certain threshold remain immunologically ignored (44, 45). Importantly, not only naive, but also previously activated memory CD8⁺ T cells can be tolerized via these mechanisms (46). Taking the aforementioned studies into account, we consider the following as most likely scenario to explain the observed rec-AdV-mediated anergization/tolerization of CTL: rec-AdV can directly deliver their transgenic cargo to professional APC in secondary lymphoid organs which leads to DC maturation through local inflammation and elicits—once a certain threshold-dose of Ag has been reached—a significant response of fully differentiated effector CTL. However, high amounts of peripherally expressed Ag lead to rapid functional exhaustion and deletion of those early CTL in the periphery, i.e., the liver. Because the rec-AdV-mediated inflammation in secondary lymphoid organs is only transient (2), the inducing capacity of the APC in lymphoid organs gradually wanes. We consider it thus likely that, particularly in the high dose setting ($\geq 10^{10}$ PFU Ad-LacZ in C57BL/6 or 10^9 PFU AdLacZ in SM-LacZ), tolerance of CTL may be maintained through presentation of the exogenous β gal Ag by professional APC lacking appropriate costimulation. Whether and how other potential mechanisms of peripheral tolerance, e.g., the induction of regulatory T cells, contribute to the observed effects, remains to be determined.

Taken together, this study identifies an inherent problem of rec-AdV-based vaccination. Although DC and other professional APC are transduced to some extent, the promiscuity of this viral vector leads to systemic delivery and transduction of different cell types in multiple organs. Early Rec-AdV-mediated activation of innate immune responses in the liver with significant cytokine and chemokine release (47, 48) may lead to the preferential attraction of β gal-specific CTL to this peripheral organ. Particularly the liver may function as a lymphocyte “sink” where significant numbers of activated lymphocytes undergo apoptotic death (49, 50). Routing of the viral vectors to the relevant target cells can be achieved by transductional targeting. For example, rec-AdV type 5 vectors can be modified to target DC more specifically by replacing the fiber protein with that from the subgroup B human AdV (51) or by genetic modification of the AdV capsid protein with a protein bridge (52). Likewise, molecular adaptors (usually bi-specific Abs) can be used to alter the receptor-binding properties of the rec-AdV type fiber proteins (53). In addition, long-lasting activation of DC in secondary lymphoid organs through specific transduction with stimulatory molecules such as GM-CSF (54) or flt3L (55) may help to circumvent potential tolerizing effects. It is likely that such improvements are necessary for the efficient employment of rec-AdV vectors as vaccines against infectious agents or to overcome tolerance to tumor/self-Ags in the context of cancer immunotherapy.

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Disclosures

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