Qualitative and Quantitative Analysis of Adenovirus Type 5 Vector-Induced Memory CD8 T Cells: Not as Bad as Their Reputation

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It has been reported that adenovirus (Ad)-primed CD8 T cells may display a distinct and partially exhausted phenotype. Given the practical implications of this claim, we decided to analyze in detail the quality of Ad-primed CD8 T cells by directly comparing these cells to CD8 T cells induced through infection with lymphocytic choriomeningitis virus (LCMV). We found that localized immunization with intermediate doses of Ad vector induces a moderate number of functional CD8 T cells which qualitatively match those found in LCMV-infected mice. The numbers of these cells may be sufficiently increased by additional adenoviral boosting, and, importantly, the generated secondary memory cells cannot be qualitatively differentiated from those induced by primary infection with replicating virus. Quantitatively, DNA priming prior to Ad vaccination led to even higher numbers of memory cells. In this case, the vaccination led to the generation of a population of memory cells characterized by relatively low CD27 expression and high CD127 and killer cell lectin-like receptor subfamily G member 1 (KLRG1) expression. These memory CD8 T cells were capable of proliferating in response to viral challenge and protecting against infection with live virus. Furthermore, viral challenge was followed by sustained expansion of the memory CD8 T-cell population, and the generated memory cells did not appear to have been driven toward exhaustive differentiation. Based on these findings, we suggest that adenovirus-based prime-boost regimens (including Ad serotype 5 [Ad5] and Ad5-like vectors) represent an effective means to induce a substantially expanded, long-lived population of high-quality transgene-specific memory CD8 T cells.

Most successful vaccine formulations in clinical use today induce potent humoral immune responses and often require multiple immunizations to sustain the immune response for long periods of time. However, development of preventive vaccines that effectively combat pathogens such as HIV, the malaria parasite, and hepatitis C virus has not yet been successful, in part probably due to the requirement for cellular immunity in disease control. An important task in modern vaccine development is therefore to develop a vaccine format capable of eliciting potent cellular immunity that can be sustained for life by repeated immunizations. Adenoviral (Ad) vectors have emerged as very promising candidates in this context on the basis of their documented immunogenicity and ability to induce host protection in multiple species, including humans (1–3). However, several reports have raised important concerns regarding the quality of the memory CD8 T cells induced through adenoviral vaccination. In particular, several groups have reported that adenovirus serotype 5 (Ad5) vectors induce dysfunctional CD8 T cells with a rather terminally differentiated phenotype and marked impairment in their capacity to undergo secondary expansion (4–7). However, we do not believe that the induction of dysfunctional CD8 T cells represents an irreversible outcome of immunization with Ad5 vectors, setting these qualitatively apart from other vaccine vectors with which they may be compared, e.g., other Ad serotypes or modified vaccinia virus (VV) Ankara. Rather, based on previous results (8, 9) indicating that while cell numbers are correlated with systemic dissemination of the adenoviral vector, effector quality decreases under the same conditions, we hypothesized that highly efficient memory CD8 T cells may be induced through Ad5 vector immunization, provided that extensive systemic dissemination is avoided. One problem under these conditions, however, may be that substantially lower numbers of memory CD8 T cells are generated, at least when the response is compared to that induced by infection with live virus. To resolve this problem, repeated immunization may be required, and this might then lead to impairment of cardinal memory cell features, such as the capacity to undergo secondary expansion (10–14). For this reason, it is very important that prime-boost regimens combining or using adenoviral vectors should be carefully evaluated regarding not only the magnitude but also the quality of the response, particularly as these parameters would appear to be independently regulated attributes of the induced memory response (8).

In the current study, we have addressed the issue of how to combine the generation of high-quality memory cells with the induction of a very substantially expanded CD8 T-cell memory population. For this purpose, we used an optimized adenoviral vaccine vector system in which the vector expresses the glycoprotein (GP) of lymphocytic choriomeningitis virus (LCMV) tethered to the major histocompatibility complex class II-associated invariant chain (II) (3). This enhances the transgene-specific CD8 T-cell response induced by the vector and is therefore likely to represent a modification of future clinical relevance. Here we show that memory CD8 T cells induced by local administration of adenoviral vectors modified in the described manner are phenotypically similar to and proliferate to the same extent as memory CD8 T cells induced by LCMV infection. Combining replication-deficient adenovirus vectors based on Ad5 and Ad35 in prime-boost regimens results in the induction of robust CD8 T-cell immunity.
memory that settles at high frequencies of transgene-specific memory cells. Notably, these high memory cell numbers are reached without an associated loss in cell quality. Thus, secondary memory CD8+ T cells induced by prime-boost vaccinations with Ad5 and Ad35 express essentially the same phenotypic markers as those induced by a single vaccination and also proliferate as well as memory CD8+ T cells induced by LCMV infection.

Finally, we find that a DNA-primed immune response can be effectively boosted by the Ad35 vector and that this response can be further boosted by the Ad5 vector. We show that this series of vaccinations leads to a memory CD8+ T-cell population characterized by relatively low CD27 expression and high CD127 and killer cell lectin-like receptor subfamily G member 1 (KLRG1) expression. Nevertheless, these memory CD8+ T cells are capable of proliferating in response to viral challenge and protecting against infection, as measured in terms of the amount of infectious virus in the organs of challenged mice. Moreover, the acute response stimulated by viral challenge of these mice is followed by very limited contraction and a sustained increase in the numbers of memory CD8+ T cells. Notably, despite the repetitive antigenic stimulation, the induced memory T cells do not appear to have been pushed toward exhaustive differentiation, as evidenced by low or absent expression of inhibitory receptors.

**MATERIALS AND METHODS**

**Mice.** Female C57BL/6 (CD45.2) mice were obtained from Taconic Farms (Ry, Denmark), and B6.SJL (CD45.1) mice were bred locally from breeder pairs originally obtained from The Jackson Laboratory (Bar Harbor, ME). All mice used in this study were 6 to 8 weeks old at initiation of the experiments and housed in a pathogen-free facility. All experiments were approved by the local animal ethics council and performed in accordance with national guidelines on animal experiments.

**Adenoviral vectors and vaccination.** Replication-defective Ad5 expressing LCMV GP tethered to II (Ad5-IiGP) was produced as described previously (15).

For production of the Ad35-IiGP vector, the IiGP insert was excised from the pacCMV plasmid via restriction enzymes and cloned into the pHMCMV5 plasmid (kindly provided by Hiroyuki Mizuguchi, Osaka University, Osaka, Japan), the shuttle vector for the Ad35 vector cloning system that was originally developed by Mizuguchi and Kay (16, 17). From the shuttle vector, the insert was cloned directly into the Ad35 backbone with an E1 deletion (also kindly provided by Hiroyuki Mizuguchi) via homing endonucleases PI-SceI and I-CeuI. The Ad35-IiGP DNA was transfected into Hek-E1b cells (kindly provided by Hiroyuki Mizuguchi) to support production of virus particles. Adenoviral particles were purified using standard methods, aliquoted, and frozen at −80°C in 10% glycerol. The IiGP insert sequence was verified by sequencing and restriction enzyme digestion. The infectivity of the adenovirus stocks was determined with a QuickTiter adenovirus titer immunosassay kit (Cell Biolabs).

Mice to be vaccinated were anesthetized and unless otherwise stated subcutaneously (s.c.) injected with 2 × 10^7 (Ad5) or 5 × 10^7 (Ad35) infectious units (IFU) in 30 μl phosphate-buffered saline (PBS) in the hind footpad. Mice to be vaccinated a second time always received the second injection in the contralateral hind footpad.

**Gene gun immunization.** DNA was coated onto 1.6-nm gold particles at a concentration of 2 μg DNA/mg gold, and the DNA-gold complex was coated onto plastic tubes such that 0.5 mg gold was delivered to the mouse per shot (1 μg DNA per shot). These procedures were performed according to the manufacturer’s instructions (Bio-Rad, CA). Mice were immunized on the abdominal skin using a hand-held gene gun device employing compressed helium (400 lb/in^2) as the particle motive force (18). Mice were inoculated twice with an interval of 3 weeks and then allowed to rest for 60 days before vaccination with adenoviral vectors.

**Viruses.** In addition to Ad, VV and LCMV were utilized in this study. Recombinant VV expressing GP of LCMV (VV-GP) was originally obtained from D. H. L. Bishop (Oxford University, Oxford, United Kingdom) via Annette Otenius (SFFIT, Zürich, Switzerland) and grown on CV-1 cells at a low multiplicity of infection; quantification was performed as described previously (19, 20). Mice to be infected with VV were injected intraperitoneally (i.p.) with 2 × 10^6 PFU in 300 μl PBS. LCMV Traub was produced, stored, and quantified as previously described, and mice were infected intravenously (i.v.) using 200 PFU (21). LCMV Armstrong (Arm) and clone 13 were originally provided by M. B. A. Oldstone (The Scripps Research Institute, La Jolla, CA) and further propagated in-house using BHK cells. For infection of mice, an i.v. dose of 2 × 10^6 PFU LCMV clone 13 or 1 × 10^6 PFU LCMV Arm was used, unless otherwise specified.

**Organ virus titers.** To determine virus titers in organs, the organs were first homogenized in PBS to yield 10% organ suspensions, and viral titers were subsequently determined as previously described (20, 22).

**Splenocyte preparation.** Splenocytes from mice were removed aseptically and transferred to Hanks balanced salt solution (HBSS). Single-cell suspensions were obtained by pressing the spleens through a fine steel mesh (mesh size, 70 μm), followed by centrifugation and two washes in HBSS, before resuspension in RPMI 1640 cell culture medium containing 10% fetal calf serum supplemented with NaHCO3, 2-mercaptoethanol, l-glutamine, and penicillin-streptomycin.

**Flow cytometry.** The frequencies of epitope-specific CD8+ T cells were determined by intracellular cytokine staining or surface staining using GP-specific tetramers. For surface staining, cells were stained with antibodies (Abs) for CD8 and CD44 in combination with GP33-, GP34-, and GP276-specific phycocyanin (PE) or allophycocyanin (APC)-conjugated tetramers (23). Note that in some experiments, staining with GP33 and GP34 tetramers was combined in order to enable a direct comparison of tetramer-defined cells to GP-specific cells, as defined by intracellular cytokine staining, which cannot clearly separate GP33- and GP34-specific cells. Intracellular cytokine staining was performed after 5 h of incubation with relevant peptides (0.1 μg/ml GP33 or GP276) at 37°C in 5% CO2. After incubation, the cells were stained with Abs for cell surface markers (peridinin chlorophyll protein-Cy5.5-CD8, APC-Cy7-CD44, fluorescein isothiocyanate-Cy7-CD27, PE-KLRG1, PE-Cy7-CD127) and Ab for intracellular cytokine (APC-gamma interferon [IFN-γ]). Samples were run on an LSRII flow cytometer (BD biosciences) and analyzed using FlowJo software (TreeStar).

**In vivo proliferation assay.** Spleen cells from vaccinated C57BL/6 (CD45.2) mice were negatively selected for CD4- and Ig-positive cells using relevant antibodies and magnetic beads and transferred to naive B6.SJL (CD45.1) recipients so that each recipient received 10^7 GP-specific CD8+ T cells, as measured by staining with tetramers targeting GP33-, GP34-, and GP276-specific T-cell receptors. One day after adoptive cell transfer, recipient mice were challenged i.p. with 2 × 10^6 PFU VV-VG, and 6 days later, spleen cells were stained with GP-specific tetramers in combination with anti-CD8 and anti-CD45.2 to evaluate donor cell proliferation.

**Statistical evaluation.** A nonparametric Mann-Whitney U test was used to compare quantitative data. GraphPad Prism (version 4) software was used for statistical analysis.

**RESULTS**

Phenotypic changes during effector to memory cell differentiation following Ad5 and LCMV infection. Due to prior reports stating that adenoviral vectors induce CD8+ T cells of suboptimal quality (4, 5), we found it pertinent to investigate the phenotype and function of CD8+ T cells induced by our optimized adenoviral vectors expressing GP of LCMV tethered to the invariant chain. As we had reasons to assume that an inferior quality of adenovirus-induced CD8+ T cells is related to the degree of systemic dissemination of the vector construct (8), we initially used a slightly suboptimal dose (2 × 10^6 IFU) of Ad5 to address the following
question: could adenoviral antigen presentation be brought to
induce the generation of high-quality CD8 T cells? As the “gold
standard” in this respect, we used GP-specific CD8 T cells gener-
at in mice infected in parallel with LCMV Arm i.v. To charac-
terize the cytokine-producing CD8 T cells generated in either case,
we used the cell surface markers CD27, CD127, and KLGR1. In the
LCMV system, the differentiation of virus-specific CD8 T cells has
previously been extensively studied, and during the contraction
phase, the initial reduction in the expression of CD127 and CD27
is followed by increased expression, while the reverse is seen for
KLGR1 (24,25). Particularly when it comes to the upregulation of
interleukin-7 (IL-7) receptor/CD127, this has been found to be
regulated in mice given adenovirus. Finally, with regard to the costimulatory
molecule CD27, we observed a delayed and prolonged decrease in
expression on adenovirus-stimulated CD8 T cells, unlike the sit-
uation in LCMV-infected mice, where expression began to in-
crease again at between 2 and 4 weeks postinfection (p.i.), as pre-
viously reported (25). Nevertheless, by the end of the observation
period (4 weeks p.i.), cytokine-producing CD8 T cells from
LCMV-infected and adenovirus-immunized mice presented with
a very similar phenotype. Thus, from a phenotypic point of view,
while the precise kinetics of changes in marker expression might
differ somewhat depending on the virus used for immunization,
the CD8 phenotypes at 4 weeks p.i. seemed quite similar, and from
this we deduce that the differences previously reported in the
literature would appear to be more associated with the exact condi-
tions of Ad5 immunization than with intrinsic qualities of this
vector system.

Phenotypic and functional comparison of GP-specific mem-
ory CD8 T cells generated through immunization with adenovi-
rus versus live LCMV. To further explore potentially important
differences between CD8 memory T cells induced by adenoviral
vectors compared to live virus, mice were immunized using opti-
mal doses (see Materials and Methods) of 2 commonly employed
serotypes of adenovirus, Ad5 and Ad35, and the memory CD8 T
cells present 2 months after immunization were compared to
those present in mice infected with either of 2 strains of LCMV,
LCMV Arm and LCMV Traub, with the latter causing a more
prolonged yet transient infection in i.v. infected mice (21). As can
be seen in Fig. 2A, both adenoviral vectors induced CD8 T-cell
populations which were much inferior in size compared to those
observed in LCMV-infected mice. However, regarding the phen-
typic profile of cytokine-producing memory CD8 T cells, Ad5-
primed cells did not stand out compared to memory cells induced
by live virus. Thus, despite small, statistically significant differ-
ences, the expression of all phenotypic markers fell within the
ranges set by the populations generated in the context of infection
with live viruses, and, moreover, we observed only slight differ-
ences in the phenotypic profile of Ad5 versus that of Ad35 (Fig. 2B
and C).

In the analysis presented above, GP-specific cells were consis-
tently defined on the basis of their cytokine production. During
LCMV infection, virtually all antigen-specific cells differentiate
into cytokine-producing cells (26, 27); however, this is not typi-
cally the case, since following most infections, the numbers of
tetramer-positive cells exceed those defined by intracellular cyto-
kine staining (28, 29). To see if defining GP-specific cells by
tetramer staining instead of cytokine production would make an
impact on our results with regard to phenotype, we compared
Ad5- and LCMV Arm-primed CD8 T cells, analyzing tetramer-
positive cells side by side with cytokine-producing cells. As can be
seen in Fig. 3A, confirming the literature, the ratio between
tetramer-positive and cytokine-producing cells was close to 1 for
LCMV-primed cells. In contrast, the ratio for Ad5-primed cells
was nearly 2, indicating that there are some Ad5-primed cells
which have not fully differentiated and reached a cytokine-pro-
ducing state. Interestingly, despite the inclusion of these addi-
tional CD8 T cells in the case of Ad5-primed cells, the phenotypic
characterization was not markedly affected, although overall there
was a tendency to observe slightly bigger phenotypic differences
between antigen-specific cells defined by the two criteria in the

FIG 1 Expression of CD27, CD127, and KLGR1 on GP-specific CD8 T cells as
a function of time after inoculation with Ad5 or LCMV. C57BL/6 mice were
vaccinated with 2 × 10^6 IFU of Ad5-IiGP foot pad or 10^5 PFU of LCMV Arm
i.v., and the expression of CD27, CD127, and KLGR1 on cytokine (IFN-γ)-
producing CD8 T cells was analyzed on the indicated days, identified by d. and
the specific day; results for GP33-specific cells are presented, but similar results
were obtained for GP276-specific cells. Each point represents one animal; dotted
lines represent marker expression on CD8 T cells from uninfected mice. Data are representative of two independent experiments.

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case of Ad5-primed cells than in the case of LCMV-primed cells (Fig. 3B). Thus, for Ad5-primed CD8 T cells, there tends to be fewer cells expressing KLRG1 and, correspondingly, more single CD127^-H11001^ cells (memory precursor effector cells [MPECs]), when analyzing tetramer-positive cells than cytokine-producing cells. Most importantly, the comparative analysis presented above does not provide any technical explanation as to why our results differ from those in recently published studies (6, 7). For the remainder of this report, we therefore continue to define GP-specific CD8 T cells through their capacity for cytokine production, as we believe these to represent the most functionally relevant subset, except when proliferative capacity is studied, in which case tetramer staining was used for enumeration of the relevant populations.

Despite the relatively low number of GP-specific CD8 T cells in adenovirus-immunized mice, we have previously found such vaccinated mice to be able to resist intracerebral challenge with LCMV for nearly a year, and even i.v. challenge with a high dose of rapidly invasive LCMV clone 13 is efficiently controlled (3). Regarding functional characterization, we have previously shown that Ad5-IiGP-induced memory cells match LCMV-driven memory cells in terms of polyfunctionality, and significant *in vivo* cytotoxicity may be observed for at least 2 month postvaccination (3, 19, 28, 30). However, one important quality not previously addressed is the capacity of adenovirus-primed memory CD8 T cells to undergo secondary expansion; it could be argued that neither of the challenge models mentioned above that has been studied would critically gauge this quality. Given that quite different num-

FIG 2 Head-to-head comparison of Ad- and LCMV-primed GP-specific memory CD8 T cells. Mice were vaccinated with Ad35-IiGP, Ad5-IiGP, LCMV Arm, or LCMV Traub, and splenocytes were analyzed 60 days later. The number (A) and phenotype (B) of the generated cells were determined using the same markers used for Fig. 1; results and representative plots (C) are depicted for GP33-specific cells. Each point represents one animal. Data regarding Ad35-IiGP, Ad5-IiGP, and LCMV Arm are representative of two experiments, while LCMV Traub infected mice were included in only one experiment. A comparison of Ad5-IiGP- and LCMV Arm-immunized mice at 120 days after vaccination gave similar results. *, P < 0.05; **, P < 0.01.
bers of memory cells are present in LCMV-infected versus adenovirus-primed mice, we needed to perform an analysis where the expansion of equal numbers of memory cells could be directly compared. Therefore, approximately $10^4$ GP-specific (as determined by tetramers detecting GP33-, GP34-, and GP276-specific cells) CD8 T cells from either Ad5- or LCMV Arm-immunized mice were transferred to naive recipients, which were then challenged on the next day with GP-expressing vaccinia virus. Six days later, the numbers of GP-specific donor cells recovered in the recipients’ spleens were enumerated. As can be seen in Fig. 3C, similar numbers of donor-derived GP-specific CD8 T cells were recovered from the recipients irrespective of the immunization regimen of the donors. Consequently, even though the CD8 T-cell memory response induced through adenoviral vaccination clearly does not match that following LCMV infection in quantitative terms, adenovirus-primed cells did not stand out as being qualitatively inferior to those in LCMV-infected mice.

Prime-boost regimens with Ad5 and Ad35 increase memory CD8 T-cell numbers without impacting CD8 T-cell phenotype or proliferative capacity. The requirements to make a successful vaccine are to induce a robust, efficient, and long-lasting memory response. In the above-described experiments, our focus had been on getting high-quality memory cells rather than high memory cell numbers, and even though adenovirus-vaccinated mice have previously been found to be effectively protected in the models that we have employed so far (3, 19, 20, 31), it is obvious that increases in memory cell numbers might be critical in other situations. Therefore, to address the issue of generating high memory cell numbers, we tested combinations of Ad5 and Ad35 in prime-boost regimens and measured the induced CD8 T-cell memory level 2 months later to evaluate the full potential of these two vectors in vaccine development. Mice were initially vaccinated with Ad5-IiGP or Ad35-IiGP, followed by vaccination with the homologous or heterologous vector 60 days later. The GP-specific CD8 T-cell response was measured on day 60 after the final vaccination.

We found that priming with the Ad5 vector and boosting with either the Ad5 or the Ad35 vector increased the number of CD8 memory cells equally well, although it seemed that the heterologous combination gave more consistent results (Fig. 4A). These results confirm our earlier observation that priming and boosting with the Ad5 vector are possible, in spite of preexisting immunity.

**FIG 3** Comparative phenotypic and proliferative analysis of GP-specific memory CD8 T cells using either tetramer staining (tet) or intracellular cytokine staining (intra) as the defining parameter. Mice were vaccinated with Ad5-IiGP or LCMV Arm, and splenocytes were analyzed 60 days later. The numbers (A) and phenotypes (B) of the generated cells were determined using the same markers used for Fig. 1 and 2. For tetramer staining of GP33-specific cells, tetramers GP33/H-2D^b^ and GP34/H-2K^b^ were combined in order to allow a direct comparison to results obtained by intracellular cytokine staining using GP33 peptide, which does not distinguish these two epitopes. The results of phenotypic analysis for GP33-specific cells are presented, but similar results were obtained for GP276-specific cells. (C) For evaluation of proliferative capacity, $10^4$ GP-specific CD8 T cells (determined by staining with tetramers for GP33-, GP34-, and GP276-specific T-cell receptors) from immunized CD45.2 donors were transferred into naive CD45.1 recipients. On the next day, recipients were infected i.p. with $2 \times 10^4$ PFU vaccinia virus expressing GP of LCMV; uninfected recipients served as controls. Six days later, GP-specific donor-derived CD8 T cells in the spleen were enumerated. Each point represents one animal. Data are representative of two experiments. *, $P < 0.05$; **, $P < 0.01$. 

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Primming with Ad35 and boosting with Ad5 also very efficiently increased the number of memory CD8 cells. In contrast, priming and boosting with Ad35-IiGP significantly increased only the GP276-specific response, and both the GP33 and the GP276 responses were found to be very low. This observation correlates well with previous results obtained in our group, which indicated that the Ad35 vector is more prone to inhibition by preexisting vector immunity (20). Overall, the heterologous prime-boost regimens seemed to have a greater vaccination potential than homologous combinations: priming with Ad35 and boosting with Ad5 resulted in the greatest increase in GP33-specific CD8 T-cell numbers in the spleen, while GP276 responses were not significantly different between the two combinations.

We also compared the expression of the phenotypic markers CD127, CD27, and KLRG1 on the various populations of vaccine-induced memory cells. It is of interest to note that the phenotype of second-generation memory cells (Fig. 4B) is quite similar to that of primary memory cells (Fig. 2B), indicating that the ex-
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Expanded population of memory cells generated by the second vaccination is qualitatively similar to primary memory cells.

Priming with the Ad35 vector seems to generate memory CD8 T cells, which are more CD127⁺ and less KLRG1 positive (KLRG1⁻) than cells generated through priming with the Ad5 vector, indicating that Ad35-primed cells may be less terminally differentiated than Ad5-primed cells. This may reflect the fact that the Ad35 vector is not as efficient in inducing primary T-cell expansion as the Ad5 vector. Thus, comparison of the heterologous prime-boost regimens reveals that priming with the Ad35 vector and boosting with the Ad5 vector generated not only the higher number of antigen-specific CD8 T cells but also cells which tend to be more CD127⁺ and less KLRG1⁻, and thus, regarding the absolute numbers of MPECs generated, this regimen seemed to be overall superior (data not shown). Based on this observation, we would therefore assume that this sequence of applying the two vectors would have the greatest potential for future vaccine use, and for this reason, memory CD8 T cells generated in this manner were compared head-to-head with LCMV-primed cells to establish whether the expansion of memory cell numbers came at the price of a reduced proliferative capacity. To study this, similar numbers of GP-specific cells from twice-primed mice and LCMV-infected mice were transferred to naive recipients, and vaccinia virus-induced donor cell expansion was evaluated. Based on the very similar expansion observed following adoptive transfer of antigen-specific CD8 T cells from twice-primed mice and LCMV-infected animals (Fig. 4C), we conclude that adenoviral boosting causes a significant increase in memory cell numbers without negatively impacting the proliferative capacity of the primed cells.

Multiple immunizations with DNA, Ad35, and Ad5 induce high numbers of antigen-specific memory CD8 T cells with a defined phenotype. In continuation of the findings presented above, we were interested in evaluating the potential of using adenoviral vectors in combination with DNA vaccination, in order to investigate whether initial priming with DNA could be used to further increase memory cell numbers and whether this would result in terminally differentiated cells impaired in their capacity to proliferate and protect against viral challenge.

Consequently, mice were immunized twice 3 weeks apart with plasmid DNA containing the IIGP sequence and vaccinated with 2 × 10⁷ IFU Ad35-IIGP 60 days after the second DNA immunization. Finally, the mice were vaccinated with Ad5-IIGP after an additional resting period of 60 days. The GP-specific CD8 T-cell response was measured on day 60 after each viral vaccination. Initial priming with DNA increased the response from Ad35-IIGP vaccination significantly for both epitopes tested, and both memory populations were further expanded by the Ad5-IIGP booster vaccination (Fig. 5A). Phenotypically, the transgene-specific memory CD8 T cells from DNA-primed, Ad35-boosted mice looked very similar to those found in Ad5-primed mice (cf. Fig. 2B), whereas in multiply vaccinated mice, we noted an increase in both CD127 and KLRG1 expression as well as a decrease in CD27 expression (Fig. 5B); this could indicate that we were inducing an increasing number of memory CD8 T cells which might be getting more terminally differentiated and therefore loosing proliferative potential, while still being maintained predominantly through the action of IL-7. Nevertheless, multiply immunized mice were still effectively protected against challenge with a high dose (2 × 10⁸ PFU) of LCMV Arm i.p. (Fig. 4C).

Functional evaluation of memory CD8 T cells induced by multiple immunizations with DNA, Ad35, and Ad5. Due to the possible functional implications of the observed trend in memory phenotype, we found it critical to directly explore how efficiently the induced memory CD8 T-cell pool in multiply immunized mice could further expand in response to viral challenge. Mice that had previously been vaccinated with DNA, Ad35, and Ad5 were therefore challenged with vaccinia virus expressing LCMV GP, and the GP-specific CD8 T-cell response was measured 6 days later. The GP33-specific memory CD8 T-cell population expanded significantly in response to the viral challenge, causing a 4-fold increase in the numbers of antigen-specific CD8 T cells. On the other hand, the GP276-specific CD8 T-cell population did not expand much, and in this case, cell numbers increased only by a factor of 1.4 (Fig. 6A). However, the reduced expansion of transgene-specific CD8 T cells in multiply vaccinated mice compares to what we have previously observed in adenovirus-vaccinated, vaccinia virus-challenged mice (20) and could reflect very early efficient virus control limiting antigen stimulation. Consistent with this possibility, virus titers in the ovaries were significantly lower in previously vaccinated mice than naive mice, with 2 of the former mice having titers below 10⁵/g organ (Fig. 6B).

Thus, the memory CD8 T-cell population formed through DNA priming and adenovirus boosting 2 times clearly retained the capacity to protect against acute viral infection. However, as challenge of intact mice could not distinguish between effects obtained through increases in cell numbers and those obtained by distribution, perhaps compensating for a reduction in per cell functionality, we decided to perform additional adoptive transfer studies.

To evaluate the proliferative capacity on a per cell basis, similar numbers (10⁴ cells) of GP-specific CD8 T cells from singly and multiply immunized mice were transferred to naive recipients, which were subsequently challenged with vaccinia virus. As previously, the numbers of antigen-specific donor CD8 T cells recovered in the recipient spleens 6 days later were enumerated using tetramer staining. As seen in Fig. 6C, we observed a tendency toward a reduced expansion of CD8 T cells from multiply immunized mice; however, the difference was not statistically significant, and overlapping numbers of transgene-specific CD8 T cells were recovered from recipients receiving cells from singly and multiply immunized mice (Fig. 6C).

To further gauge the functionality of the CD8 T-cell responses present in multiply immunized mice, we transferred a high number of antigen-specific memory CD8 T cells from either singly or multiply vaccinated mice to recipients, which were subsequently challenged i.v. with a high dose of LCMV clone 13, causing exhaustive T-cell differentiation and chronic infection in naive animals. The dose of antigen-specific donor CD8 T cells selected for this cell transfer (10⁵ cells) was the lowest number of primed cells previously found to be capable of impacting the course of LCMV infection under these conditions (12). As can be seen in Fig. 6D, cells from singly and multiply immunized mice caused a similar reduction in the median spleen viral titer 10 days later, although only the effect of cells from multiply immunized mice was statistically significant compared to that for mice without adoptive transfer of primed cells, which were all heavily infected at 10 days postchallenge. As virus control under these conditions is believed to be quite demanding in terms of the proliferative capacity of the transferred donor cells (11, 12), we conclude that neither of our exper-
Experimental approaches points to a substantial reduction in the proliferative capacity of antigen-specific CD8 T cells found in mice being subjected to DNA priming and Ad35/Ad5 boosting. The acute CD8 T-cell response induced by vaccinia virus challenge stabilizes at a high memory level. Finally, we investigated whether vaccinia virus challenge of multiply vaccinated mice resulted in stable expansion of the memory cell pool in the challenged mice. In addition, we studied the expression of phenotypic markers on the surviving functional memory CD8 T cells, but this time we also included an analysis of the expression of known inhibitory receptors (PD-1, LAG-3, and TIM-3 [12, 32]) to further evaluate the quality of the memory CD8 T cells resulting from four immunizations. The number of GP-specific CD8 T cells decreased only slightly from day 6 to day 60 after vaccinia virus challenge and settled at a high level with cells phenotypically resembling those found in otherwise matched, but unchallenged mice (Fig. 7A; cf. Fig. 6A). Notably, it seemed that vaccinia virus challenge resulted in memory CD8 T cells with a slightly higher

FIG 5 Sequential immunizations with DNA, Ad35, and Ad5 induce high numbers of antigen-specific memory CD8 T cells with a characteristic phenotype. Mice were immunized twice 3 weeks apart with plasmid DNA containing the IiGP sequence and vaccinated with Ad35-IiGP 60 days after the second DNA immunization, followed by vaccination with Ad5-IiGP 60 days later; for each boosting step, previously naive mice receiving only the last immunization were included for comparison. (A) Numbers of vaccine-induced memory CD8 T cells measured on day 60 after the last viral vaccination. (B) Phenotype of multiply primed CD8 T cells; results for GP33-specific cells are depicted. (C) Spleen virus titers on day 3 after i.p. challenge with $2 \times 10^5$ PFU of LCMV Arm at least 60 days after the last vaccination. Each point represents one animal. *, $P < 0.05$; **, $P < 0.01$. 

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CD27 expression, perhaps reflecting a difference in the stimulatory pathways activated in the context of vaccinia virus versus adenovirus infection (Fig. 7B).

Notably, the number of antigen-specific CD8 T cells in the DNA-primed, adenovirus-boosted group decreased slightly from day 60 to day 120, while CD27 expression increased, perhaps reflecting the fact that the numbers of memory cells were slowly decreasing in animals that were not reinfected, with cells expressing CD27 having a selective advantage over CD27-negative cells. Importantly, the expression of PD-1, LAG-3, and TIM-3 was low and similar in challenged and unchallenged mice (Fig. 7C), indicating that viral challenge did not induce detectable exhaustion of the functional antigen-specific CD8 T cells.

**DISCUSSION**

Experimental analysis of memory CD8 T-cell differentiation is classically performed in singly immunized mice. However, in real life, repeated antigenic stimulation is not unusual and—at least when it comes to vaccines—is often required to induce and maintain a solidly protective immune response. There are two reasons for this: the first reason is that one immunization often does not drive the generation of a sufficient number of relevant T cells; the second reason is that for certain infections, it is critical that the first wave of T cells that meets the invading pathogen already be positioned near the sites of pathogen entry and/or possess immediate effector activity. To reach both of the last two goals, repeated vaccination may be essential (10). Replication-defective adenovirus vectors in many ways would seem to be the ideal vaccines in this context, were it not for their reported ability to induce partially exhausted T cells (4,5). In this study, we therefore focused on the potential of adenoviral vectors to generate high-quality memory CD8 T cells and how these cells may be further expanded without a substantial loss of functional capacity, particularly in terms of replicative capacity.
As primary tools in this analysis, we used the two adenoviral vectors Ad5 and Ad35 to induce transgene-specific CD8 T-cell responses. These vectors represent serotypes selected from opposite ends of the spectrum with regard to the immunogenicity of adenoviral vectors, with Ad5 being a very efficient inducer of cell-mediated immunity and with Ad35 being relatively poor in inducing a transgene-specific CD8 T-cell response (33). In accordance with this, we found that the number of CD8 T cells generated toward the dominant epitope during the acute-phase response was significantly higher after Ad5 vaccination than Ad35 vaccination (data not shown). However, comparable numbers of GP33-specific memory CD8 cells could be generated. On the other hand, the Ad35 vector proved to be an extremely weak inducer of the acute-phase CD8 T-cell response toward the subdominant GP276 epitope, and memory cell generation was in this case equally poor. Based on these findings, we agree that the Ad35 vector on its own is a weaker vaccine vector than the Ad5 vector; however, this would not exclude its use as a primer in the context of adenovirus-based prime-boost regimens, where priming with a weak vector might set the ideal stage for subsequent boosting with a stronger vector. Notably, in theory, the reduced ability of the Ad35 vector to induce an immune response in wild-type mice could be caused by the lack of expression of the known Ad35 receptor CD46 in female C57BL/6 mice (34). To address this issue, we compared Ad35-induced responses in CD46 transgenic mice and wild-type C57BL/6 mice and observed no statistically significant differences, although a trend toward a slightly higher GP276 response was noted in CD46 transgenic mice than wild-type mice (data not shown).

By comparing the CD8 T cells induced by localized immunization with these adenoviral vectors to those in LCMV-infected mice, we conclude that under the tested conditions, high-quality memory cells can be induced by immunization using adenoviral vectors, including Ad5. However, their numbers are low compared to those generated through infection with replicating virus, and even though the induced memory CD8 T cell level has been found to be sufficient for significant protection in all experimental setups tested so far, an increase in the frequency of memory CD8 T cells is clearly desirable. This can be obtained through boosting with homologous or heterologous adenoviral vectors, as previously demonstrated (13, 20, 35–38). The important point of this study is that we document that the secondary memory cells generated in this manner do not appear to be more terminally differentiated or detectably impaired in their per cell proliferative capacity. This might have been expected on the basis of recent findings showing that repeated immunization with most (live) vectors causes a reduction in the proliferative capacity of the resulting memory cells (10–14). As to the reason why adenoviral boosting in our hands does not have this effect, we would suggest...
that the T-cell expansion induced by localized administration of these replication-defective vectors is not nearly as substantial as that induced by any live agent, and the cumulative division history is therefore markedly different. The more gradual increase in expression of KLRG1 on GP-specific CD8 T cells in Ad5-immunized mice than LCMV-infected mice is consistent with this interpretation.

In this study, we also observed that DNA priming followed by two adenviral booster doses resulted in the generation of an expanded memory cell population dominated by CD127/KLRG1 double-positive and CD27low cells. Notably, this multiply primed memory cell population retained protective capacity and was able to expand and remain stably expanded in response to in vivo challenge with live virus. According to conventional theory, high KLRG1 expression and low CD27 expression are the hallmarks of senescent cells with a low proliferative capacity (24, 39–41); however, in line with other recent studies, our results may question this simplistic interpretation. Thus, in a study on the phenotype of antigen-specific cells expanded through multiple infections, Masopust et al. (10) reported that KLRG1 expression increased for each round of antigenic stimulation, while CD27 expression decreased (10). Nevertheless, as evaluated by adoptive transfer, secondary memory cells with a high KLRG1 expression exhibited the same proliferative capacity as primary memory cells with a much lower KLRG1 expression (10). Similarly, in a recent study from the group of Lefrançois, where secondary memory cells were sorted into KLRG1-negative (KLRG1−) CD127+ and KLRG1− CD127+ populations and adoptively transferred to naive recipients, the proliferative capacity of the latter cells in response to infectious challenge was only marginally reduced (~2-fold) compared to that of the former (29). These results point to the same conclusion as our own observations, namely, that KLRG1 may be expressed by cells that have experienced repeated antigen stimulation but its expression is not necessarily a marker of replicative senescence; instead, it may simply be an indicator of (recent?) antigenic experience. In line with this interpretation, neither we nor Lefrançois and colleagues (29) found the KLRG1-expressing CD8 T cells to coexpress inhibitory receptors such as PD-1, which further supports the conclusion that these cells are not functionally exhausted.

Current evidence suggests that adenviral vectors provide the immune system with a prolonged, low-grade antigenic stimulus (4, 5, 42–44), and it would therefore make sense that these vectors maintain a high level of KLRG1 expression on the memory CD8 T cells, whereas acute and quickly resolved infections would gradually lead to primed CD8 T-cell populations mostly characterized by lower levels of KLRG1 expression. At first glance, the expression of CD127 on these cells may seem paradoxical, as these markers are normally found to be expressed on separate, nonoverlapping cell subsets, except during the contraction phase (24). However, in a study of chronic human herpesviral infections, Ibe- gbu et al. also noted that coexpression of KLRG1 together with markers such as CD27 and CD127 may define a memory T-cell subset, whereas coexpression of KLRG1 and CD57, a marker of replicative senescence, may characterize truly terminally differentiated cells (45). Together these observations support the idea that the appearance of memory cells with the former phenotype is associated with chronic infection and prolonged antigen stimulation (24). This impression is reinforced by the results of a recent study on mice persistently infected with murine gammaherpesvi-

rus 68. Here it was found that persistent infection induced a population of KLRG1+ cells which coexpressed CD127, retained characteristics of polyfunctional effector cells (such as IFN-γ and tumor necrosis factor alpha production), and upon adoptive transfer were highly efficient in protecting against homologous challenge (46).

In conclusion, in the present study, we show that adenvirus-based prime-boost regimens, even when involving Ad5 similar vectors, can induce robust secondary T-cell expansion resulting in the generation of high numbers of transgene-specific memory CD8 T cells which appear to match other virus-induced CD8 T cells in terms of expression of critical cell markers, effector function, and replicative capacity. As to why other groups tend to find, in particular, Ad5-based vectors to induce functional exhaustion and decreased anamnestic potential, we would focus on the two critical factors setting the design of previous studies apart from that of our own: first, the use in published studies of very high doses of Ad5 for murine immunization and, second, a difference in the route of immunization, intramuscular versus s.c., which may also make a contribution. It has previously been demonstrated that systemic dissemination of the viral inoculum tends to increase CD8 T-cell numbers but decrease their functionality, and both dose and route of inoculation were found to be important in this respect (8, 9). Finally, a combination of DNA priming and repeated adenviral boosting resulted in the generation of even higher numbers of memory CD8 T cells which, despite certain effector memory features (low expression of CD27, high expression of KLRG1) still expressed CD127 (IL-7 receptor), could proliferate substantially in response to challenge with live virus and protect against chronic infection. Based on these findings, we argue that appropriately designed adenvirus-based prime-boost regimens can induce a long-lived population of predominantly effector memory-like cells which retain substantial proliferative capacity; such cells would have a strong potential for use in vaccine development within the fields of HIV infection, hepatitis C, malaria, and cancer, where robust and persistent CD8 T-cell responses seem to be essential (47, 48).

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