Rapid and sustained CD4+ T-cell-independent immunity from adenovirus-encoded vaccine antigens

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Many novel vaccine strategies rely on recombinant viral vectors for antigen delivery, and adenovirus vectors have emerged among the most potent of these. In this report, we have compared the immune response induced through priming with adenovirus vector-encoded full-length viral protein to that elicited with an adenovirus-encoded minimal epitope covalently linked to β2-microglobulin. We demonstrate that the β2-microglobulin-linked epitope induced an accelerated and augmented CD8+ T-cell response. Furthermore, the immunity conferred by vaccination with β2-microglobulin-linked lymphocytic choriomeningitis virus (LCMV)-derived epitopes was long-lived and protective. Notably, in contrast to full-length protein, the response elicited with the β2-microglobulin-linked LCMV-derived epitope was CD4+ T-cell independent. Furthermore, virus-specific CD8+ T cells primed in the absence of CD4+ T-cell help were sustained in the long term and able to expand and control a secondary challenge with LCMV. Our results demonstrate that modifications to the antigen used in adenovirus vaccines may be used to improve the induced T-cell response. Such a strategy for CD4+ T-cell-independent immunity from adenovirus vectors offers prospects for vaccination against opportunistic pathogens in AIDS patients and possibly immunotherapy in chronic virus infections.

INTRODUCTION

During an acute viral infection, several branches of the innate and adaptive immune system work together to control the intruder. In most cases, the culmination is the generation of large numbers of effector CD4+ and CD8+ T cells that control the acute infection. Whereas there is little doubt that CD8+ T cells are crucial in controlling many primary virus infections, their role in protection against reinfection remains an issue of critical importance and some uncertainty (Wodarz et al., 2000; Zinkernagel & Hengartner, 2004). Thus, during some of the most important chronic infections, notably human immunodeficiency virus (HIV), hepatitis C virus (HCV) and human T-cell leukaemia virus (HTLV), antibodies are unable to control viral spread and replication, and sustained virus control or elimination depends critically on the generation and sustained functional integrity of virus-specific CD8+ effector T cells (Grakoui et al., 2003; Vanniasinkam & Ertl, 2005; Cohen, 2005). CD8+ T-cell integrity in turn seems to be dependent on the generation of virus-specific CD4+ T cells, and a poor CD4+ T-cell response is associated with poor prognosis in humans chronically infected with HIV or HCV (Gandhi & Walker, 2002; Bowen & Walker, 2005). As CD8+ T-cell responses are both long-lived and capable of controlling viral infections, considerable effort has been invested in the development of vaccine formulations capable of inducing robust and stable CD8+ T-cell responses (Lau et al., 1994; Gomez-Roman & Robert-Guroff, 2003; Gourley et al., 2004). To date, the most notable of such formulations are live attenuated vaccines, plasmid DNA vaccines and recombinant viral vaccines (Shiver et al., 2002). While issues regarding their safety remain, recombinant virus vaccines appear to be both potent and versatile (Wu et al., 2001, 2005; Shiver et al., 2002; Boyer et al., 2005; Vanniasinkam & Ertl, 2005). Important unsolved questions regarding the use of such vaccine vectors are their ability to prime CD4+ T cells and the dependence on CD4+ T cells for CD8+ T-cell priming. Thus, CD8+ T-cell memory independent of CD4+ T cells could protect AIDS patients from many opportunistic viral infections, and the potential use of immunotherapy in the hundreds of millions of people presently infected with viruses such as HIV, HCV and HTLV may require the vaccines to work without virus-specific CD4+ T-cell help. In the present study, we used recombinant replication-deficient adenovirus to express either full-length protein or minimal MHC class I-restricted epitopes from lymphocytic choriomeningitis virus (LCMV). Linkage of epitopes to human β2-microglobulin results in presentation of the inserted epitope covalently fused to the MHC complex and
the epitope therefore has a high chance of docking into the MHC peptide-binding groove (Uger & Barber, 1998). Previously, it has been found that this methodology acts by increasing the stability and subsequently the number of peptide/MHC complexes on cell surfaces, as evidenced by enhanced stability and antigenicity of epitopes mutuated in MHC-anchoring amino acids (Uger et al., 1999), and the minimal epitopes presented here have previously been tested as plasmid DNA vaccines (Bartholdy et al., 2003, 2004b). We hoped that they would also perform better than the full-length LCMV protein when expressed from a highly immunogenic adenovirus vector. Recombinant adenovirus has emerged in several settings as a superior vector for induction of antiviral CD8+ T-cell responses and is today incorporated in many experimental anti-HIV vaccine formulations (Shiver et al., 2002; Shiver & Emini, 2004; Vanniasinkam & Ertl, 2005).

LCMV is a natural mouse pathogen and a prototypic arenavirus. Infection with this virus is a long-exploited model system for both acute and chronic viral infections in mice. Local administration of LCMV in the brain results in acute and lethal meningitis, whereas intravenous (i.v.) inoculation with high doses of rapidly replicating virus variants often results in sustained virus replication and CD8+ T-cell dysfunction, which mimics that observed during chronic viral infection in humans (Zajac et al., 1998; Kristensen et al., 2002; Barber et al., 2006; Day et al., 2006). Most vaccine strategies using recombinant viruses express full-length native or modified proteins from the relevant infectious organism. This favours the possible generation of CD8+ T-cell responses against multiple epitopes, relative independence of the MHC haplotype of the recipient and, if relevant given the chosen protein (i.e. expression on the surface of the organism in question), the generation of a protective antibody response. One downside of this is that it is harder to draw conclusions regarding the contribution and relative importance of CD8+ T cells. Another possible downside could be suboptimal antigen presentation. Thus, we have recently observed a stronger recall response (i.e. upon secondary virus challenge) in mice vaccinated with DNA plasmids expressing minimal epitopes covalently linked to β2-microglobulin than in mice primed using a conventional minigene construct (Bartholdy et al., 2003). The results presented here indicate that a minimal immunodominant epitope covalently linked to β2-microglobulin induces a CD8+ T-cell response that is faster and more consistent than the CD8+ T-cell responses induced by the full-length viral glycoprotein. Furthermore, the generation of functional CD8+ T cells directed against epitopes in the full-length glycoprotein is dependent on the presence of CD4+ T cells, whereas the generation of virus-specific CD8+ T cells using an adenovirus-expressed β2-microglobulin-linked epitope is not. The generated CD8+ T cells provide long-term protection from virus infection, thus adding support to the role of CD8+ T-cell memory in protection from lethal infection. Accordingly, the recombinant viral expression of a minimal CD8+ T-cell epitope has allowed us to explore the properties of immunological memory based solely on CD8+ T cells, induced by a potent vaccine vector candidate.

**METHODS**

**Mice.** C57BL/6, BALB/c and MHC H2−/− mice (B6.129-H2-Ab1m(C57N12)) were obtained from Taconic M&B (Ry, Denmark). All mice used were between 7 and 10 weeks old and were housed in a specific-germ-free facility. All experimental procedures were performed according to local experimental guidelines.

**Adenovirus vectors.** For construction of E1- and E3-deleted adenovirus expressing LCMV-derived epitopes, PCR products containing the minimal epitope [H-2Dβ/GP33–41 (AdGP33), H-2Dβ/ NP396–404 (AdNP396) or H-2Lb/NP118–126 (AdNP118)] constructs described by Bartholdy et al. (2003) or the full-length LCMV glycoprotein (AdGP) were cloned into the pACCMV shuttle vector under the control of the immediate-early cytomegalovirus promoter and enhancer as well as the simian virus 40 polycl signal (a plasmid encoding the full-length LCMV glycoprotein was used as a template for construction of the AdGP shuttle vector; this plasmid was a kind gift from Michael B. Oldstone). The obtained plasmid was co-transfected with pJM17 into HEK293 cells and viral lysates were obtained. These were cloned by plaque purification before sequencing, large-scale production and purification by CsCl gradient centrifugation as described previously (Becker et al., 1994). After purification, adenovirus stocks were aliquotted immediately and frozen at –80 °C in 10% glycerol. Virus particles were then determined by spectrophotometry at 260 nm and the infectivity of adenovirus stocks was determined with the Adeno-X Rapid Titre kit (Clontech), which determines infectious replication in HEK293 cells. The virus particle/infectious focus-forming units (vp/i.f.u.) ratio was then calculated. The virus stocks used in the paper were AdGP33 (vp/i.f.u. ratio 45), AdNP396 (vp/i.f.u. ratio 31), AdNP118 (vp/i.f.u. ratio 48.1) and AdGP (vp/i.f.u. ratio 46.1). In control experiments where AdGP33, AdNP396, AdNP118 and AdGP stocks were administered in the footpad, viral loads were determined to be similar by real-time PCR 3 days after vaccination (not shown). Before vaccination experiments, dilution factors were calculated and virus stocks were thawed and diluted in PBS and then transported to the animal facility on ice for immediate administration.

**Vaccinations.** In all studies, mice to be vaccinated were anaesthetized and injected with 2×107 i.f.u. in the right hind footpad in a volume of 0.03 ml.

**Virus infection.** Mice were infected intra-cerebrally (i.c.) with 20 p.f.u. LCMV Armstrong clone 53b in a volume of 0.03 ml or i.v. with 106 p.f.u. LCMV clone 13 in 0.3 ml. Intra-cerebral infection induces a fatal CD8+ T-cell-mediated meningitis to which immunocompetent mice succumb on days 7–10 post-infection (Christensen et al., 1994).

**Survival study.** Mortality was used to evaluate the clinical severity of acute LCMV-induced meningitis. Mice were checked twice daily for a minimum of 2 weeks after i.c. inoculation; deaths occurring less than 5 days after infection were excluded from analysis.

**Organ virus titres.** To determine virus titres in organs, these were first homogenized in PBS to yield 10% (w/v) organ suspensions, and serial 10-fold dilutions were prepared. Each dilution was then plated in duplicate on MC57G cells. Forty-eight hours after infection, infected cell clusters were detected using monoclonal rat anti-LCMV (VL-4) antibody, peroxidase-labelled goat anti-rat antibody and...
o-phenylenediamine (substrate) (Battegay et al., 1991). The numbers of p.f.u. were counted, and results are expressed as p.f.u. (g tissue)^{-1}.

**Cell preparations.** Single-cell suspensions of spleen cells were obtained by pressing the organs through a fine steel mesh and, when required, erythrocytes were lysed by 0.83 % NH_4Cl treatment. Livers were perfused with PBS through the caval vein and then homogenized and lymphocytes were purified on a Percoll gradient as described by Liu et al. (2001).

**Antibodies for flow cytometry.** The following monoclonal antibodies were purchased from BD PharMingen as rat anti-mouse antibody: Cy-chrome-conjugated anti-CD8, FITC-conjugated anti-CD44, phycoerythrin (PE)-conjugated anti-IFN-γ and PE-conjugated IgG1 isotype standard.

**Detection of antigen-specific CD8\(^+\) T cells by MHC class I dextramer.** LCMV-specific CD8\(^+\) T cells were enumerated by binding of PE-conjugated H-2D\(^b\)/GP33–41, H-2D\(^b\)/NP396–404 or H-2L\(^d\)/NP118–126 dextramers obtained from DakoCytomation.

**Flow cytometry analysis.** Staining of cells for flow cytometry was performed according to standard laboratory procedure. For enumeration of LCMV-specific CD8\(^+\) T cells, splenocytes were incubated in vitro for 5 h at 37 °C in 5 % CO\(_2\) with relevant peptide (0.1 μg ml\(^{-1}\)) in the presence of monensin (3 μM; Sigma) and murine recombinant IL-2 (10 U per well; R&D Systems). After incubation, cells were surface-stained, washed, fixed and permeabilized using 0.5 % saponin. Cells were then stained with anti-IFN-γ or IgG1 isotype control for 20 min at 4 °C. Samples were analysed using a Becton Dickinson FACSCalibur, and at least 10^6 mononuclear cells were gated using a combination of low angle and side scatter to exclude dead cells and debris. Data analysis was conducted using Cell Quest Pro (B&D Biosciences).

**Statistical analyses.** Quantitative results were compared using the Mann–Whitney U test. Survival rates were compared using Fisher’s exact test. A value of P<0.05 was considered statistically significant.

**RESULTS**

**T-cell responses and protective efficacy after vaccination with adenovirus-encoded full-length LCMV glycoprotein and a minimal β2-microglobulin-linked epitope**

We chose the footpad route for immunization, since it is at least as efficient as the intramuscular and subcutaneous routes (not shown) and this site is easier to infect consistently in each experiment. Thus, following footpad vaccination with 2×10^7 i.f.u. of either AdGP (expressing full-length glycoprotein from LCMV; Fig. 1a) or AdGP33 (expressing the immunodominant epitope GP33–41 of the LCMV glycoprotein covalently linked to β2-microglobulin; Fig. 1a), animals were analysed for the presence of antigen-specific, IFN-γ-producing CD8\(^+\) T cells (Fig. 1b). The results consistently showed earlier and numerically superior expansion of antigen-specific CD8\(^+\) T cells in mice receiving the AdGP33 vector and, importantly, a good response was consistently induced. Further observations at 80–90 days after vaccination indicated that AdGP33-induced responses were also better maintained. Further observations demonstrated that AdGP-induced responses to subdominant CD8\(^+\) T-cell epitopes (GP92-101, GP118-125 and GP276-284) and the CD4\(^+\) T-cell epitope GP61-80 were minimal (mean below 0.11, 0.09 and 0.28 % of CD8\(^+\) T cells and 0.06 % of CD4\(^+\) T cells, respectively) when measured at the peak of the response, 21 days after vaccination, indicating that the AdGP33-vaccinated mice would not lack significant responses to other epitopes. In subsequent analyses, 21 days after immunization was used as an early time point, as our own observations (Fig. 1b and not shown) and evaluations of other adenovirus-expressed epitopes have indicated that CD8\(^+\) T-cell responses have peaked at this time (Bruna-Romero et al., 2001; Fitzgerald et al., 2003).

**Efficient expansion of memory CD8\(^+\) T cells after systemic high-dose virus challenge**

Inoculation i.v. with high doses of LCMV clone 13, a fast-replicating strain, results in a chronic infection and
temporal exhaustion of the antiviral immune response (Zajac et al., 1998; Fuller et al., 2004; Bartholdy et al., 2004b). This challenge model has previously been evaluated using the same minimal β2-microglobulin-linked epitopes delivered as DNA vaccines. In that study, the primed CD8+ T cells were found to expand efficiently upon secondary challenge, and DNA-vaccinated mice were found to be protected against immune exhaustion and chronic infection, with clearance of the virus within 10–20 days of infection and a significant reduction of virus titres in lungs, but not spleen, 8 days after infection (Bartholdy et al., 2004b).

To evaluate the in vivo functionality of virus-specific CD8+ T cells generated in mice vaccinated with our modified construct, we analysed mice 8 days after i.v. infection with 10^6 p.f.u. LCMV clone 13 (Fig. 2). Mice vaccinated once 3 months previously had significantly lower viral titres in both spleen and lungs compared with the mice immunized with an MHC-mismatched epitope (Fig. 2a). Indeed, mice vaccinated with AdGP33 had barely detectable organ virus titres 8 days after challenge, and similar results were obtained in mice challenged 21 days after vaccination (not shown). Protection against chronic infection correlated with the ability of the generated memory CD8+ T cells to expand upon secondary challenge. Thus, analysing T-cell numbers in spleen and liver, we found a dramatic increase of about 100-fold in mice vaccinated with AdGP33 (Fig. 2b, c). Numbers of cells are consistently higher using detection of intracellular IFN-γ than by dextramer staining. This probably reflects a substantial contribution of H-2K^b GP34–41-restricted cells expanding upon challenge that are not detected with H-2D^b GP33–41 dextramer staining (Hudrisier et al., 1997). Moreover, at the level of the individual mouse, we noted a distinct inverse correlation between T-cell number and organ virus titres (data not shown). Control vaccinated mice achieved significantly smaller, but appreciable, numbers of CD8+ T cells. In any case, it should be noted that 8 days after infection is too early to expect immune exhaustion to be complete (Bartholdy et al., 2004b).

Adenovirus-induced responses to minimal MHC class I-coupled epitope, but not full-length protein, are CD4+ T-cell independent

The improved kinetics of the AdGP33 vaccine and the modest induction of CD4+ T-cell responses following vaccination with the AdGP vaccine prompted us to look at the role of CD4+ T-cell help in CD8+ T-cell expansion and immunity induced by the two vaccine candidates. To this end, we first evaluated the primary CD8+ T-cell expansion in wild-type (WT) and MHC-II^{-/-} mice vaccinated with AdGP or AdGP33. While the generation of mature IFN-γ-producing CD8+ T cells was totally dependent on CD4+ T-cell help after vaccination with AdGP, virus-specific IFN-γ-producing CD8+ T cells could be generated in AdGP33-vaccinated mice without CD4+ T-cell help (Fig. 3a), albeit at lower frequencies than in WT

Fig. 2. Robust CD8+ T-cell expansion after systemic high-dose virus challenge. Animals were vaccinated in the right footpad with 2×10^7 i.f.u. AdGP33 or control vaccine (AdNP118; MHC-mismatched) and left for 3 months. At this point, animals were infected i.v. with 10^6 p.f.u. LCMV clone 13 and 8 days later animals were sacrificed for analysis. (a) Viral content in the spleen and lungs of challenged animals as determined by plaque assay. Points represent individual animals; hatched line represents detection level of virus titration. Con, Control. (b) Total number of antigen-specific CD8+ T cells after challenge as determined by intracellular or dextramer staining (spleen, left panel) or dextramer staining only (liver, right panel). ND, Not done. (c) Representative plot of splenic GP33–41-specific CD8+ T cells determined by intracellular staining for IFN-γ (top panels) or dextramer staining (lower panel).
mice. Primary expansion of virus-specific CD8+ T cells in the absence of CD4+ T cells is not unusual, but memory CD8+ T-cell persistence and functionality in the absence of CD4+ T cells is (Thomsen et al., 1996; Sun & Bevan, 2003). Thus, to address persistence and functionality of primed CD8+ T cells generated in MHC-II-/- mice, we quantified total memory cells and mature IFN-γ-producing T cells 60 days after AdGP33 or AdGP vaccination and found memory CD8+ T-cell frequencies to be reduced, but still significant, in AdGP33-vaccinated mice deficient in CD4+ T cells (Fig. 3b).

To address in vivo functionality during a recall response, AdGP33- and AdGP-vaccinated MHC-II-/- and WT mice were infected with 10^8 p.f.u. LCMV clone 13 and analysed for CD8+ T-cell expansion and virus control 5 days after challenge, since we hoped analysis at this time would allow better discrimination between groups with varying levels of protective efficacy (Fig. 2c, d). Enumeration of virus-specific T cells after challenge with live virus revealed that memory CD8+ T cells generated in the absence of CD4+ T cells expanded in AdGP33-vaccinated MHC-II-/- mice, but expanded only marginally in AdGP-vaccinated MHC-II-/- mice (Fig. 3c). Similarly, spleen and lung viral titres were high in MHC-II-/- mice vaccinated with AdGP, but were lower in MHC-II-/- mice vaccinated with AdGP33. The same pattern was repeated in vaccinated WT mice, yet here AdGP-vaccinated mice had a titre approaching that seen in AdGP33-vaccinated MHC-II-/- mice, and AdGP33 mice had lower titres still (Fig. 3d). All differences commented upon reached the chosen level of statistical significance (P<0.05 with Mann–Whitney rank sum test).

**Long-term protection from lethal i.c. challenge after a single adenovirus immunization**

To evaluate protection not only against i.v. challenge, but also against viral infection in a solid organ, animals were challenged i.c. 7, 14, 21, 60–80 and 365 days after immunization with a lethal dose (20 p.f.u.) of neurotropic LCMV Armstrong 53b (Fig. 4). The AdGP33 vaccine already conferred potent protection at challenge 7 days after vaccination, and this protection then seemed to stabilize and ended at 40% after 1 year. Full-length protein induced significant, but delayed and transient, protection, and no vaccinated mice survived when challenged more
than 21 days after vaccination. The peak in protection observed when AdGP-vaccinated mice are challenged 14 days and not 21 days after vaccination is consistent with previous observations, showing that protective CD8\(^+\) T-cell responses first need to be present at 5–6 days after challenge in order to protect mice from lethal infection (Thomsen et al., 1979). All animals vaccinated with an MHC-mismatched vaccine as well as mock-vaccinated mice died within 8 days of challenge (not shown).

Several epitopes are immunogenic when covalently linked to \(\beta_2\)-microglobulin

It is evident from the data presented above that the AdGP33 vaccine has qualities that are superior to the AdGP vaccine. However, as the linkage to \(\beta_2\)-microglobulin presents a peptide with a C-terminal extension, it could not be excluded that responses to other epitopes or epitopes restricted to other MHC genes would be negatively affected. To test the reliability of the responses to other minimal epitopes linked to \(\beta_2\)-microglobulin, C57BL/6 mice were vaccinated with adenoviruses expressing \(\beta_2\)-microglobulin-linked NP396–404 (AdNP396) and BALB/c mice with adenoviruses expressing \(\beta_2\)-microglobulin-linked NP118–126 (AdNP118). NP396–404 is H-2Db restricted, whereas NP118–126 is H-2Ld restricted. For comparison, C57BL/6 mice vaccinated with AdGP33 were included. Twenty-one or 60–90 days after vaccination, vaccinated animals were sacrificed for analysis of epitope-specific CD8\(^+\) T-cell responses. Both AdNP396 and AdNP118 were found to induce antigen-specific IFN-\(\gamma\)-producing CD8\(^+\) T cells at least as efficiently as the AdGP33 vaccine (Fig. 5a), and primed cells were also distributed to non-lymphoid organs, as evidenced by the detection of these cells in the liver (Fig. 5b).

To determine the protective efficacy of the AdNP396 and AdNP118 vaccines, vaccinated mice were challenged i.c. with 20 p.f.u. LCMV Armstrong 53b; mice vaccinated with MHC-mismatched epitopes served as controls. Both AdNP396 and AdNP118 induced good protection at 21 days post-vaccination, and significant protection was maintained at 60–80 days post-vaccination (Fig. 6). On the basis of these findings, we conclude that efficient responses towards adenovirus-expressing minimal epitopes

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**Fig. 4.** Protection from lethal i.c. challenge after a single adenovirus immunization. Percentages of AdGP33- or AdGP-vaccinated animals (2×10\(^7\) i.f.u. in the right footpad) surviving i.c. challenge with 20 p.f.u. LCMV Armstrong clone 53b when challenged at 7, 14, 21, 60–80 and 365 days after vaccination are shown. *, \(P<0.05\) when compared with AdGP-vaccinated mice.

**Fig. 5.** Adenovirus immunization induces long-lived CD8\(^+\) T-cell memory in spleen and liver. Animals were vaccinated in the right footpad with 2×10\(^7\) i.f.u. of either AdGP33 or AdNP396 (C57BL/6) or AdNP118 (BALB/c). On day 21 post-vaccination, splenocytes (a, top) and liver lymphocytes (b) and 2–3 month post-vaccination splenocytes (a, bottom) were analysed for the presence of antigen-specific CD8\(^+\) T cells either by staining for intracellular IFN-\(\gamma\) or by dextramer staining. Controls are animals vaccinated with irrelevant epitope. All results shown are means±SD of four to ten animals in each group.
were infected i.c. with 20 p.f.u. LCMV Armstrong clone 53b and AdNP118. Twenty-one and 60–80 days post-vaccination, mice

**DISCUSSION**

Our initial experiments demonstrated that a primary expansion of CD8+ T cells could be induced more efficiently with an adenviral vector encoding a minimal epitope covalently fused to β2-microglobulin compared with a conventional full-length viral glycoprotein. This is in agreement with similar experiments using gene-gun immunizations with DNA plasmids encoding conventional minigenes and β2-microglobulin-linked epitopes (Bartholdy et al., 2003). We saw this efficient CD8+ T-cell generation with other epitopes and in two different strains of mice, although it should be noted that we have not shown whether covalent epitope linkage to β2-microglobulin is the factor responsible for the enhanced CD8+ T-cell responses, as we have not performed analysis of unlinked minigenes, fusions of full-length antigen to β2-microglobulin or co-expression of vaccine validation. Thus, the adenovirus-based vaccines described represent one of a few examples in which long-lived antiviral protection can be obtained based solely on CD8+ T cells (Klavinskis et al., 1989, 1990; Oldstone et al., 1993; Slikfka et al., 1996), and this immunity is effective even after a single immunization with a non-replicative vector. Furthermore, we have demonstrated that CD4+ T cells are essential to CD8+ T-cell maturation after vaccination with adenvirus encoding a full-length protein, whereas CD4+ T cells are less essential to CD8+ T-cell maturation after vaccination with our β2-microglobulin-linked epitope constructs. The reasons for this difference are not absolutely clear. However, we observe a nice correlation between clonal burst size on the one hand and memory levels and recall responses on the other, suggesting that the difference is induced during priming and reflects a difference in initial T-cell activation. Perhaps increased expression of antigen on the surface of antigen-presenting cells bypasses the essential requirement for CD4+ T-cell help (Kundig et al., 1996). To the best of our knowledge, this is the first study to describe a direct dependence on CD4+ T-cell help for primary CD8+ T-cell activation after vaccination with an adenvirus-encoded recombinant antigen. The late CTL generation and CD4+ T-cell dependence of the AdGP-induced response is not unique to the LCMV glycoprotein as antigen; similar results have been observed using β-galactosidase as a model antigen (unpublished results). Our observation that modification of the epitope-encoding insert can reduce this dependence offers prospects in treatment of human disease. Most obviously, the CD4+ T-cell deficiency of AIDS patients renders them susceptible to opportunistic viral infections such as cytomegalovirus and Epstein–Barr virus that are normally believed to be controlled by CD8+ T cells (Kovacs & Masur, 2000). A system in which antiviral immunity can be induced without CD4+ T-cell help offers the prospect of vaccines that can be used in these patients. Moreover, many patients with chronic viral infections are deficient in virus-specific CD4+ T-cell help, and it is possible that a vaccine inducing a CD4+ T-cell-independent CD8+ T-cell immune response could be used for therapeutic vaccination (Gandhi & Walker, 2002; Bowen & Walker, 2005). A potential drawback of our strategy is the need to match the epitopes to the MHC haplotype of a given recipient. Another potential problem is the induction of a highly focused response with the β2-microglobulin minimal epitope-linked vaccines. However, in our case, this does not lead to loss of significant responses to other epitopes when compared with full-length antigen, and we have demonstrated previously that a highly focused response may favour the generation of a broadly reactive response during viral challenge by controlling the level of early virus replication (Bartholdy et al., 2004b). It should be noted that we have not shown whether covalent epitope linkage to β2-microglobulin is the factor responsible for the enhanced CD8+ T-cell responses, as we have not performed analysis of unlinked minigenes, fusions of full-length antigen to β2-microglobulin or co-expression of

![Graph showing protection from lethal i.c. challenge after a single adenvirus immunization.](image-url)
antigen with \( \beta_2 \)-microglobulin. The decision to proceed with the constructs described here is, however, based upon experiments with DNA immunizations that showed epitopes covalently linked to \( \beta_2 \)-microglobulin to be superior to other variants, but also that minigene immunogenicity benefited from co-expression with \( \beta_2 \)-microglobulin (Bartholdy et al., 2003, 2004a; A. Stryhn, unpublished).

We also performed a more detailed analysis of the adenovirus-induced immunity in WT mice and found robust protection against chronic infection as well as partial protection from acute i.c. challenge that was still significant 1 year after vaccination. This adenovirus-induced immunity is superior to that induced by gene-gun delivery of DNA plasmids, even when DNA-vaccinated mice have been challenged shortly after a quadruple boost regime (Bartholdy et al., 2003). Notably, in a previous report, mice vaccinated using intramuscular injection of 50 mg plasmid DNA encoding LCMV nucleoprotein were found to achieve almost at high a frequency of antigen-specific CD8\(^+\) T cells, but only short-term protection from lethal challenge was reported (Hassett et al., 2000). Thus, this study is proof of concept that CD8\(^+\) T-cell memory, induced by a single adenovirus immunization, suffices to induce protection against chronic viral infection, and, to a lesser extent, acute lethal infection. The data also indirectly demonstrate the feasibility of boosting the achieved CD8\(^+\) T-cell immunity through secondary vaccination, as described previously using heterologous adenoviruses (Pinto et al., 2003).

In summary, we have here made the first adenovirus-based vaccine formulation against the natural mouse pathogen LCMV. Our adenovirus-based vaccine is superior to hitherto-tested DNA- and lentivirus-based vaccine strategies, but has similar potency to vaccinia- and listeria-based strategies (Klavinskis et al., 1989, 1990; Oldstone et al., 1993; Slikf et al., 1996; Zarei et al., 2004; Bartholdy et al., 2004b; Wherry et al., 2005). Perhaps more important, we have demonstrated strategies to improve the generation and maintenance of efficient CD8\(^+\) T-cell responses and extended such responses to include immunodeficient hosts. Our results support and strengthen the emerging concept of adenovirus vectors as potent vaccine vehicles for both acute and chronic infections and offer new prospects for vaccination of immunodeficient patients.

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