Fusion of a viral antigen to invariant chain leads to augmented T-cell immunity and improved protection in gene-gun DNA-vaccinated mice

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It has recently been demonstrated that a recombinant replication-deficient human adenovirus 5 (Ad5) vector expressing lymphocytic choriomeningitis virus (LCMV) glycoprotein (GP) fused to the p31 invariant (Ii) chain confers broad, long-lasting T-cell immunity that completely protects C57BL/6 mice against lethal peripheral challenge. The current study questioned whether the same strategy, i.e. linkage of GP to an Ii chain, could be applied to a naked DNA vaccine. Following gene-gun immunization with the linked construct (DNA–IiGP), GP-specific CD4+ T cells could not be detected by flow cytometry. However, inclusion of the Ii chain augmented the priming of GP-specific CD8+ T cells directed towards both immunodominant (GP 33–41) and subdominant (GP 276–286 and GP 92–101) epitopes, and vaccination with DNA–IiGP conferred significantly improved protection against systemic LCMV infection compared with the unlinked construct. In contrast, substantial protection against peripheral challenge was not observed. Additional experiments with T-cell subset-depleted or perforin-deficient mice revealed that virus control in vaccinated mice depends critically on cytotoxic CD8+ T cells. Finally, priming with the naked DNA vaccine was shown to augment the immune response raised by subsequent immunization with the Ad5 vector. In conclusion, this study showed that the immunoenhancing effect of Ii chain linkage is not limited to the Ad5 vector, but is also relevant with a DNA platform. Furthermore, given the fact that the Ii chain enhances the presentation of more than one epitope, this suggests that Ii-chain-based DNA vaccines may be promising candidates for various heterologous prime–boost regimes.

INTRODUCTION

The purpose of vaccination is to generate stable immunological memory, which protects against subsequent challenge with the same or antigenically related infectious agents. In terms of structure/form, vaccines can be classified into three groups: (i) killed/inactivated pathogens or a pathogen subunit, (ii) live attenuated/recombinant vectors encoding a pathogen subunit, and (iii) plasmids encoding a pathogen subunit, i.e. DNA vaccines. Many current licensed human vaccines belong to the first group and work by inducing long-lived antibody responses and B-cell memory (Zinkernagel & Hengartner, 2006). However, proper protection against several important intracellular pathogens, e.g. human immunodeficiency virus (HIV), Mycobacterium tuberculosis, hepatitis C virus, human T-lymphotropic virus and malaria, seems to require potent cell-mediated immunity (Cohen, 2005; Grakoui et al., 2003; Vanniasinkam & Ertl, 2005). One way of priming T cells is through the use of DNA-based vaccines. Delivery of antigens by DNA plasmids has been found to induce protective immunity in several animal models of infectious disease (Bartholdy et al., 2004; Dahl et al., 2004; Gurunathan et al., 2000; Hasset et al., 2000; Ulmer et al., 1995), and recently two veterinary DNA vaccines have been licensed for clinical use. Unfortunately, DNA vaccination has shown limited immunogenicity in humans (MacGregor et al., 1998, 2002; Roy et al., 2000), although recent results suggest that this can be improved significantly (Kwissa et al., 2007). Nonetheless, because of the many benefits involved (e.g. uncomplicated procedure, low cost of production, storage at room temperature, safety and the absence of anti-vector immunity), DNA-based vaccines remain an important tool in the development of a preventative vaccine for many viruses such as HIV (Graham et al., 2006).

The aim of the present study was to evaluate a new DNA vaccine strategy, using plasmid pACCMV.pLpA expressing the vaccine antigen linked to the p31 invariant (II) chain. The reasons for testing this strategy were twofold. First, several groups have recently used fusion of vaccine antigen to the Ii chain as a means of targeting the major histocompatibility complex (MHC) class II presentation pathway, and demonstrated increased priming of antigen-specific CD4+ T cells in vitro and in vivo (Diebold et al.,

Received 14 March 2008
Accepted 30 September 2008
CD4+ T cells may themselves serve as antiviral effector cells and, perhaps more importantly, they appear to be critical to the development and maintenance of virus-specific CD8+ T-cell memory (Janssen et al., 2003; Kalams & Walker, 1998; Kristensen et al., 2002; Sun et al., 2004; Thomsen et al., 1996). Second, and more importantly, our group recently found that adenoviral vectors in which the vaccine antigen is tethered to the Ii chain showed markedly improved immunogenicity in relation to both CD4+ and CD8+ T cells. Whilst improved presentation to CD4+ T cells was expected, it was surprising to find that a major effect was mediated independently of CD4+ T cells and through augmented presentation of MHC class I-restricted epitopes (Holst et al., 2008). However, whether the latter immunoenhancing effect is a specific phenomenon of antigen presentation in the context of an adenoviral vector or whether a similar effect may be obtained using other means of vaccination was not clear from that study.

Therefore, in the present study, we compared two DNA vaccines, plasmid pACCMV.pLPa expressing lymphocytic choriomeningitis virus (LCMV) glycoprotein (GP) (DNA–GP) or expressing GP linked to the p31 Ii chain (DNA–IiGP). As previously seen with adenoviral vectors (Holst et al., 2008), insertion of the Ii chain before GP resulted in augmented priming of antigen-specific CD8+ T cells directed against both dominant and subdominant epitopes. We also demonstrated that DNA–IiGP-vaccinated mice were significantly better protected against systemic infection than DNA–GP-vaccinated animals. However, unlike vaccination with the matching adenoviral construct, the improved vaccine did not provide protection against lethal LCMV-induced meningoencephalitis. Nevertheless, priming with DNA–IiGP vaccine was demonstrated to significantly enhance the CD8+ T-cell response induced by the same gene construct delivered in an adenovirus serotype 5 vector (Ad5–IiGP), providing a strong argument for the inclusion of Ii-chain-based DNA vaccines in future heterologous immunization (‘prime-boost’) protocols.

**METHODS**

**Mice.** C57BL/6 (B6) wild-type mice were obtained from Taconic M&B. Perforin-deficient B6 mice were bred locally from breeder pairs originally obtained from The Jackson Laboratory. Seven- to 10-week-old mice were used in all experiments, and animals from outside sources were always allowed to acclimatize to the local environment for at least 1 week before use. All animals were housed under specific-pathogen-free conditions as validated by the screening of sentinel mice. All animal experiments were conducted according to national guidelines.

**DNA vaccine construction and immunization procedures.** The DNA vaccines were produced using the eukaryotic expression vector pACCMV.pLPa containing either the murine Ii chain followed by GP of LCMV or LCMV GP alone. The constructs were generated as described recently (Holst et al., 2008). *Escherichia coli* strain XL1-Blue (Stratagene) was transformed with the constructs by electroporation. DNA sequencing using cycle sequencing and an ABI Prism 310 Genetic Analyzer and Big Dye Terminator Cycle Sequencing kit identified positive clones. Primers were obtained from TAG Copenhagen. Large-scale DNA preparations were produced using a Qiagen Maxi Prep kit.

**Gene-gun immunization.** DNA was coated onto 1.6 nm gold particles at a concentration of 2 μg DNA (mg gold)−1. The DNA–gold complexes were 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) (Bartholdy et al., 2003). Mice were immunized into the abdominal skin using a hand-held gene-gun device using compressed helium (400 p.s.i.) as the particle-motive force. Unless otherwise indicated, mice were immunized twice at an interval of 3–4 weeks and then allowed to rest for 3 weeks before further challenge/investigation.

**Virus.** LCMV of the Armstrong strain clone 13 was used in most experiments. Unless otherwise stated, infected mice received a dose of 105 p.f.u. clone 13 as an intravenous (i.v.) injection of 0.3 ml, or 20 p.f.u. in 0.03 ml in the right hind footpad. For intracerebral (i.c.) injection, mice received 20 p.f.u. neurotropic LCMV Armstrong clone 53b in a volume of 0.03 ml. Replication-deficient adenovirus 5 encoding Ii-chain-linked GP (Ad5–IiGP) was produced and titrated as described recently (Holst et al., 2008).

**Virus titration.** Organ virus titres were assayed by an immune focus assay as described previously (Battegay et al., 1991).

**In vivo depletion of CD4+ and CD8+ T cells.** Anti-CD4 (clone GK1.5) and anti-CD8 (clone 53.6.72) monoclonal antibodies (mAbs) were used. Mice in which cells were depleted received a dose of 200 μg in a volume of 0.3 ml PBS intraperitoneally on days −1 and 0 relative to infection; for mock treatment purified rat IgG (Jackson ImmunoResearch) was used instead. The efficiency of cell depletion was verified by flow cytometry.

**Survival study.** Mortality was used to evaluate the clinical severity of acute LCMV-induced meningoencephalitis. Mice were checked twice daily for a period of 14 days or until 100% mortality was reached.

**Assay of LCMV-specific footpad swelling reaction.** Mice were infected locally in the right hind footpad as described above, and the local swelling reaction was followed until day 14 post-infection (p.i.). Footpad thickness was measured with a dial caliper (Mitutoyo 7309; Mitutoyo Co.), and virus-specific swelling was determined as the difference in thickness between the infected right and the uninfected left foot (Christensen et al., 1994).

**Cell preparations.** Spleens from mice were removed aseptically and transferred to Hanks’ balanced salt solution (HBSS). Single-cell suspensions were obtained by pressing the organs through a fine sterile steel mesh. The cells were washed twice with HBSS, and the cell concentration was adjusted in RPMI 1640 containing 10% fetal calf serum and supplemented with 2-mercaptoethanol, L-glutamine and penicillin/streptomycin.

**Flow cytometric analysis.** The following mAbs were purchased from PharMingen as rat anti-mouse antibodies: fluorescein isothiocyanate-conjugated anti-CD44, Cy-Chrome-conjugated anti-CD8α, Cy-Chrome conjugated anti-CD4 and phycoerythrin-conjugated anti-gamma interferon (IFN-γ).

For visualization of LCMV-specific (IFN-γ-producing) CD8+ T cells, 1×106 splenocytes were resuspended in 0.2 ml complete RPMI supplemented with 10 U murine recombinant interleukin-2 (R&D Systems Europe), 3 μM monensin (Sigma Chemical Co.) and 1 μg relevant peptide ml−1, and incubated for 5 h at 37 °C. The
RESULTS AND DISCUSSION

Fusion of LCMV GP to p31 Ii chain augments GP-specific CD8+ T-cell responses

With the aim of improving antigen-specific CD4+ T-cell responses, several groups have used targeting of the vaccine antigen into the MHC class II presentation pathway with the help of the Ii chain. Quite surprisingly, our group recently provided evidence that tethering of the vaccine antigen to the Ii chain increases not only CD4+ T-cell responses, but also – and to an even higher degree – CD8+ T-cell responses upon vaccination with replication-deficient Ad5 expressing LCMV GP fused to the p31 Ii chain (Ad5–IiGP) (Holst et al., 2008).

In the current study, we wanted to test whether the aforementioned plasmid could be used by itself to induce more efficient priming of GP-specific CD4+ and/or CD8+ T cells in C57BL/6 mice.

To evaluate the contribution of the Ii chain to enhancing GP presentation, we compared two DNA vaccines, plasmid–IiGP (DNA–IiGP) and plasmid–GP (DNA–GP). Mice were vaccinated through the abdominal skin using a gene gun (1 μg DNA per shot) and splenocytes from vaccinated mice were analysed for antigen-specific, IFN-γ-producing CD4+ and CD8+ T cells according to standard protocols for intracellular cytokine staining (ICCS). In accordance with previous results (Bartholdy et al., 2003), immunizing the mice twice did not induce detectable levels of epitope-specific T cells, when analysed 7 days after the last immunization (data not shown). Therefore, in order to get enough DNA-primed cells from vaccinated mice for analysis directly ex vivo, we used an intensified immunization protocol (Bartholdy et al., 2003). Thus, the mice were immunized four times at weekly intervals with 1 μg DNA, and 1 week after the last immunization, spleen cells were stimulated for 5 h with peptides representing known LCMV epitopes, surface stained for CD44, CD8 and CD4, and after permeabilization were stained for intracellular IFN-γ. One week after the last immunization, splenocytes were stimulated for 5 h with peptides representing known LCMV epitopes, surface stained for CD44, CD8 and CD4, and after permeabilization were stained for intracellular IFN-γ.

Fig. 1. Increased numbers of GP-specific IFN-γ+ CD8+ T cells in DNA–IiGP-vaccinated mice. C57BL/6 mice were gene-gun vaccinated four times at weekly intervals; at each immunization, the mice received 1 μg plasmid DNA, DNA–GP or DNA–IiGP. One week after the last immunization, splenocytes were stimulated for 5 h with peptides representing known LCMV epitopes, surface stained for CD44, CD8 and CD4, and after permeabilization were stained for intracellular IFN-γ. (a) Numbers of epitope-specific IFN-γ+ CD8+ (GP33–41, GP276–286, GP118–125 and GP92–101 plus NP396–404 for control) or IFN-γ+ CD4+ T cells (GP61–80) presented as means ± SEM (n = 4 mice), from DNA–GP- or DNA–IiGP-vaccinated mice. *, P<0.05 relative to DNA–GP-vaccinated mice (Mann–Whitney rank-sum test). Results are representative of two independent experiments. d.l., Detection limit based on staining with isotype-control antibody. (b) Representative dot plots of GP33–41-stimulated splenocytes, gated for CD8+ T cells, from DNA–GP- or DNA–IiGP-vaccinated mice. Numbers refer to the percentage of IFN-γ+ CD8+ T cells.

The following peptides were used: for CD8+ T cells, GP33–41, GP276–286, GP92–101 and GP118–125, and NP396–404 as a control; for CD4+ T cells, GP61–80. After incubation, cells were surface stained, washed, permeabilized and stained with IFN-γ-specific mAb as described previously (Andreassen et al., 2000; Christensen et al., 2003). Isotype-matched antibody served as a control for non-specific staining. Cells were analysed using a FACS Calibur (Becton Dickinson), and at least 105 live cells were gated using a combination of low angle and side scatter to exclude dead cells and debris. Data analysis was conducted using CellQuest software.

would undergo rapid, secondary expansion and therefore be visualized prior to day 8 p.i., which represents the time point at which maximal frequencies of virus-specific T cells are found in naïve mice. Therefore, DNA-vaccinated (1 μg DNA twice, 3 weeks apart) mice were inoculated with 103 p.f.u. LCMV clone 53b 3 weeks after the last immunization, and 5 days later splenocytes were analysed by flow cytometry after ICCS. Even under these conditions, the numbers of GP61–80-specific CD4+ T cells in DNA–IiGP-vaccinated mice were low and not detectably higher than in DNA–GP-vaccinated mice (results not shown). However, this may reflect the efficiency with which virus is eliminated (see below), because, when similar analyses were carried out on DNA–IiGP-vaccinated perforin-
deficient mice, we found a distinct population of GP-specific CD4+ T cells (see Fig. 3b).

In contrast to the situation for CD4+ T cells, direct ex vivo flow cytometric analysis of spleen cells from intensively vaccinated mice revealed a substantially increased GP-specific CD8+ T-cell response in mice immunized with the fused construct (Fig. 1). Thus, we found that, in DNA–IiGP-vaccinated mice, approximately 3.5% of splenic fused construct (Fig. 1). Thus, we found that, in DNA–GP-vaccinated mice there was three- to sevenfold more GP33–41-, GP276–286- and GP92–101 epitope specificity, DNA–IiGP-vaccinated mice produced three- to sevenfold more GP33–41-, GP276–286- and GP92–101-specific IFN-γ+ CD8+ T cells and DNA–GP-vaccinated mice. The frequency of GP118–125-specific IFN-γ+ CD8+ T cells was very low in both groups of mice and did not exceed our negative control of NP396–404-pulsed CD8+ T cells.

Overall, the above results demonstrated that inclusion of the Ii chain before LCMV GP improved the immunogenicity of the DNA vaccine, and this improvement seemed primarily to be reflected in an increased priming of MHC class I-restricted T cells (cf. Holst et al., 2008). A previous in vitro study (Diebold et al., 2001) revealed that a construct coding for Ii chain fused to a model antigen stimulated MHC class II-restricted but also class I-restricted T cells; furthermore, MHC class I presentation was more efficient. The present results seem to be in line with these observations, extending those results to the in vivo situation. One possible explanation for an increased loading of MHC class I molecules with GP peptides, taken from Diebold et al. (2001), is that the compound Ii–GP molecule synthesized in the endoplasmic reticulum is misfolded and therefore transported to the cytosol. Here, it could be degraded by the proteosomes and subsequently fed into the MHC class I presentation pathway. Whether our construct also affects MHC class II presentation and priming of CD4+ T cells is difficult to say based on the above results. Although we could not detect any difference in numbers of GP-specific CD4+ T cells following inclusion of the Ii chain, this could simply reflect a lesser expansion of CD4+ T cells. As the frequency of antigen-specific CD4+ T cells bordered on the level of detection, a small but still significant difference might not be detectable above background noise.

DNA–IiGP vaccination augments protection against systemic infection

The value of an antiviral T-cell-based vaccine obviously lies in its ability to accelerate T-cell responses and the associated virus control. Therefore, to evaluate the capacity of DNA-primed CD8+ T cells to expand following viral challenge, mice were vaccinated twice, 3 weeks apart, and 3 weeks later inoculated with a high dose (10^5 p.f.u.) of rapidly invasive and viscerotropic LCMV Armstrong clone 13. Five days after virus infection, splenocytes were analysed using ICCS and flow cytometry. In both groups of vaccinated mice, we found that up to 40% of activated CD8+ T cells were GP-specific IFN-γ+ CD8+ T cells. We also observed essentially the same differences in the number of antigen-specific IFN-γ+ CD8+ T cells (Fig. 2a) that we had previously seen in vaccinated but uninfected mice (Fig. 1a). Thus, significantly higher numbers of GP33–41-, GP276–286- and GP92–101-specific IFN-γ+ CD8+ T cells could be recovered from the spleens of virus-infected, DNA–IiGP-vaccinated animals compared with similar infected, DNA–GP-vaccinated mice. Notably, no significant difference between the two vaccinated groups was found with regard to the level of NP396–404-specific CD8+ T cells, demonstrating that inclusion of the Ii chain in the plasmid did not lead to non-specific augmentation of immunoreactivity in vaccinated mice (NP is strongly expressed during LCMV replication, but not by the vaccine construct).

Next, we evaluated the protective capacity of the memory response induced by both vaccines. For this purpose, we titrated the amount of virus present in spleens and lungs from mice infected 5 days earlier. As expected from the evaluation of T-cell numbers, we found 2–3 logs less infectious virus in DNA–IiGP-vaccinated mice than in organs from the DNA–GP-vaccinated group (Fig. 2b). Furthermore, on day 8 p.i., no infectious virus could be detected in organs from DNA–IiGP-vaccinated mice, whereas the DNA–GP-vaccinated group still contained large amounts of virus in both lymphoid and non-lymphoid organs (data not shown). Together, these results showed clearly that: (i) the DNA–IiGP vaccine protects against systemic infection, and (ii) the DNA–IiGP vaccine provides substantially better protection than DNA–GP.

Clearance of LCMV in DNA–IiGP-vaccinated mice depends critically on cytolytic CD8+ T cells

Next, we wanted to define the effector mechanism(s) underlying the efficient virus control in DNA–IiGP-vaccinated mice. Additional effectors besides GP-specific CD8+ T cells could be GP-specific CD4+ T cells (although below our detection level). Moreover, besides T cells, GP-specific antibodies could play a role. Thus, many studies have demonstrated that DNA vaccines are quite efficient inducers of strong antibody responses (Gurunathan et al., 2000). Therefore, to evaluate the role of CD4+ and CD8+ T cells during the antiviral effector phase, DNA–IiGP-vaccinated mice were depleted of CD4+ or CD8+ T cells immediately prior to i.v. challenge with 10^5 p.f.u. LCMV Armstrong clone 13. On day 5 p.i., all of the animals were sacrificed and their spleens removed for analysis. As can be seen in Fig. 3(a), elimination of CD8+ T cells completely abolished the capacity of vaccinated mice to rapidly reduce spleen virus levels, whilst depletion of CD4+ T cells had absolutely no effect.
To pinpoint further the effector mechanism involved in vaccine-induced virus control, we used perforin-deficient mice; in these mice, clearance of LCMV-infected cells by virus-specific CD8+ T cells is normally very inefficient. Consequently, IiGP-primed and unvaccinated perforin-deficient mice were challenged i.v. with 10^5 p.f.u. LCMV Armstrong clone 13. Five days later, the mice were sacrificed, the spleens and lungs were removed for evaluation of the virus content, and the splenocytes were analysed as described in Fig. 1. (a) Numbers of epitope-specific IFN-γ+ CD8+ T cells, presented as means ± SEM (n=10 mice per group), from DNA–GP- or DNA–IiGP-vaccinated mice. *, P<0.05 or 0.01 relative to DNA–GP-vaccinated mice (Mann–Whitney rank-sum test). Results were pooled from two independent experiments. (b) Virus titres in the spleens and lungs of unvaccinated and the same DNA-vaccinated mice as in (a). Points represent individual mice, and group medians are indicated by horizontal bars. *, P<0.01 relative to unvaccinated mice (Mann–Whitney rank-sum test). Results are representative of two independent experiments. d.l., Detection limit.

**Fig. 2.** Numbers of splenic antigen-specific IFN-γ+ CD8+ T cells and virus titres in the spleen and lungs of DNA-vaccinated and unvaccinated mice 5 days after systemic infection with a high dose of LCMV. C57BL/6 mice were gene-gun vaccinated twice, 3 weeks apart. Three weeks after the last immunization, vaccinated and matched unvaccinated mice were challenged i.v. with 10^5 p.f.u. LCMV Armstrong clone 13. Five days later, the mice were sacrificed, the spleens and lungs were removed for evaluation of the virus content, and the splenocytes were analysed as described in Fig. 1. (a) Numbers of epitope-specific IFN-γ+ CD8+ T cells, presented as means ± SEM (n=10 mice per group), from DNA–GP- or DNA–IiGP-vaccinated mice. *, P<0.05 or 0.01 relative to DNA–GP-vaccinated mice (Mann–Whitney rank-sum test). Results were pooled from two independent experiments. (b) Virus titres in the spleens and lungs of unvaccinated and the same DNA-vaccinated mice as in (a). Points represent individual mice, and group medians are indicated by horizontal bars. *, P<0.01 relative to unvaccinated mice (Mann–Whitney rank-sum test). Results are representative of two independent experiments. d.l., Detection limit.

**Fig. 3.** Virus control in DNA–IiGP-immunized mice is primarily mediated by cytolytic CD8+ T cells. Wild-type and perforin-deficient C57BL/6 mice were immunized with DNA–IiGP as described in Fig. 2. Three weeks after the last immunization, vaccinated and matched, unvaccinated mice were challenged i.v. with 10^5 p.f.u. LCMV Armstrong clone 13. Some of the vaccinated wild-type mice were depleted of CD4+ or CD8+ T cells prior to virus challenge. On day 5 p.i., all of the mice were sacrificed and spleens were removed for evaluation of virus load and for flow cytometric analysis. (a) Spleen virus titres from unvaccinated and DNA-vaccinated, antibody-treated or mock-treated wild-type mice. (b) Representative dot plots of splenic IFN-γ+ CD4+ and CD8+ T cells, stimulated with GP 61–80 or GP 33–41, respectively, from unvaccinated or DNA–IiGP-vaccinated, perforin-deficient mice. Numbers refer to the percentage of IFN-γ+ CD8+ or CD4+ T cells; numbers in parentheses represent the range (n=4 mice per group). (c) Spleen virus titres from the same unvaccinated and DNA-vaccinated, perforin-deficient mice as in (b). Points represent individual mice. d.l., Detection limit.
deficient mice were challenged with LCMV, and on day 5 p.i. the animals were sacrificed and their spleens removed for analysis.

First, to confirm efficient vaccination of perforin-deficient mice, splenocytes were analysed for IFN-γ-producing cells by ICCS. In comparison with unvaccinated mice, a massive expansion of CD8⁺ T cells was detected in DNA–IiGP-vaccinated mice. In the latter group, activated CD8⁺ T cells represented approximately 76% of splenic cells, and approximately 40% of these activated CD8⁺ T cells were GP₃₃₋₄₁-specific IFN-γ⁺ CD8⁺ T cells (Fig. 3b). In contrast, in unvaccinated mice about 12% of splenocytes were activated CD8⁺ T cells, and only 3% of the activated CD8⁺ T cells were GP₃₃₋₄₁-specific IFN-γ⁺ CD8⁺ T cells (Fig. 3b). In this case, we also found clear expansion of virus-specific CD4⁺ T cells in the vaccinated mice (Fig. 3b); these cells might be involved in antibody synthesis. However, despite this markedly augmented CD4⁺ and CD8⁺ T-cell response in vaccinated perforin-deficient mice, our analysis of virus content in the spleen did not reveal any differences between vaccinated and unvaccinated perforin-deficient mice (Fig. 3c), strongly indicating that the clearance of LCMV in DNA–IiGP-vaccinated mice depends primarily on cytolytic GP-specific CD8⁺ T cells.

**DNA–IiGP-immunized mice are partially protected from peripheral infection**

Finally, we addressed the question of how well the DNA–IiGP-vaccinated mice were protected against direct infection of solid, non-lymphoid organs. Experimental models of LCMV-induced inflammation, which is the result of virus-specific T-cell activity in infected tissues, are virus-induced footpad swelling and severe LCMV-induced meningitis (Bartholdy et al., 2003; Christensen et al., 1994). If vaccinated animals are protected from these consequences of peripheral infection, it is because virus-specific CD8⁺ T cells curtail the local infection quicker in these mice than in their unvaccinated counterparts. This accelerated CD8⁺ T-cell response is manifested by earlier and reduced inflammation of the footpad or by escape from the lethal meningitis (Hany et al., 1989; Thomsen et al., 1979).

To determine whether the DNA–IiGP vaccine conferred protection towards peripheral infection, we therefore inoculated DNA-vaccinated and unvaccinated mice with 20 p.f.u. LCMV Armstrong clone 13 in the right hind footpad, and footpad swelling was measured daily up to day 14 p.i. (Fig. 4a). We found a slightly accelerated reaction in DNA–IiGP-vaccinated mice compared with DNA–GP-vaccinated and unvaccinated mice. The inflammatory response in the former group peaked 2 days earlier than in the latter two groups. Moreover, overall inflammation (evaluated as the area below the curve) was reduced in DNA–IiGP-vaccinated mice.

In contrast, DNA–IiGP-vaccinated mice invariably succumbed to i.c. challenge with low doses (20 p.f.u.) of LCMV Armstrong clone 53b (Fig. 4b). In all groups, virtually all of the mice, whether unvaccinated or DNA vaccinated, had died by 9 days after i.c. LCMV infection. Similar to the pattern regarding footpad swelling, the group of DNA–IiGP-immunized mice tended to show an accelerated death.
pattern in comparison with DNA–GP-vaccinated and unvaccinated mice, indicating that DNA–IiGP-vaccinated mice responded more quickly than DNA–GP-vaccinated mice, but that this acceleration was insufficient to prevent virus spreading inside the brain (Thomsen et al., 1979), and therefore that it did not protect the animals from extensive cell damage and subsequent death. This result is different from what we have found previously in mice vaccinated with the fused antigen encoded by adenovirus (Holst et al., 2008), where all of the vaccinated mice survived i.c. challenge for up to at least 200 days post-vaccination. The difference, however, fits well with the much lower frequency of memory CD8+ T cells in DNA-vaccinated mice compared with mice vaccinated using the adenoviral constructs, and thus simply underscores the superior immunogenicity of the adenoviral vaccine platform.

**Fig. 5.** DNA priming with an li-chain-based naked DNA vaccine significantly augments the generation of virus-specific CD8+ T cells upon subsequent boosting with an optimized viral vector.

Based on the above findings, it is clear that, in order to achieve more complete protection, a larger pool of virus-specific CD8+ memory T cells needs to be generated. One way to improve the induced T-cell memory is through a heterologous prime–boost regime (Ramshaw & Ramsay, 2000), e.g. naked DNA priming followed by a vector boost. Thus, as we had the appropriate vector in our laboratory, i.e. replication-deficient adenovirus expressing LCMV GP fused to p31 li chain (Ad5–IiGP), this possibility was easy to test experimentally. First, we performed standard gene-gun DNA vaccination twice, 3 weeks apart, with DNA–IiGP or DNA–GP. Three weeks after the second DNA vaccination, both groups of mice and matched controls were immunized by inoculation of 2 × 10⁷ infectious units (i.u.) Ad5–IiGP in the right hind footpad, and 4 weeks later the number of virus-specific CD8+ T cells in the spleen was enumerated by ICCS for IFN-γ and flow cytometry. Mice primed with the fused DNA construct contained significantly more GP33–41- and GP276–286-specific IFN-γ+ CD8+ T cells than unprimed mice, and a similar trend was noted for NP92–101-specific cells, although in this case the difference was not statistically significant. In contrast, priming with naked DNA encoding GP in the absence of li had little effect on the level of GP-specific memory CD8+ T cells induced by subsequent immunization with Ad5–IiGP (Fig. 5). It should be noted that the observed effect of including li did not reflect non-specific augmentation of the immunoreactivity of vaccinated mice, as DNA priming with a vector including li, but no GP, had no effect on the level of GP-specific CD8+ T cells in mice subsequently inoculated with the adenoviral vector (data not shown).

**Concluding remarks**

The present study clearly demonstrates that fusion of the li chain to LCMV GP expressed by a naked DNA vaccine facilitates priming of GP-specific CD8+ T cells. Thus, our results confirm and extend previous findings regarding the improved CD8+ T-cell response and memory induced upon inclusion of the li chain in adenoviral vaccine vectors (Holst et al., 2008). Moreover, together these two reports suggest that the advantage of this strategy is independent of the vaccine platform to which it is applied. Notably, in a previous report, a lentiviral vector was used for delivery of a similar fused antigen construction with fairly limited success (Rowe et al., 2006). We believe that the previous finding is not the result of any peculiarity of lentiviruses as opposed to naked DNA or adenoviruses, but rather reflects those authors’ unfortunate choice of model antigen (ovalbumin). Thus, in the context of adenoviral vaccines, ovalbumin is one of the few antigens we have tested so far where fusion to li only marginally improves the induced CD8+ T-cell response (Holst et al., unpublished observations). Our results clearly demonstrated that cytolytic CD8+ T cells were the key mediators of the accelerated virus control in our vaccinated mice, whereas CD4+ T cells played no role following challenge, either as antiviral effector cells or as helpers of secondary CD8+ T-cell expansion and differentiation. Of course, this division of labour is more likely to represent a property of the chosen challenge model than the intrinsic qualities of the vector system. Nevertheless, this finding serves to underscore the fact that linkage to the li chain works efficiently as a vaccine strategy, even in situations where CD4+ T cells play no role during the recall response. The extent to which improved antigen presentation to CD4+ T cells is essential
as a precondition for optimal CD8+ T-cell priming and survival (Janssen et al., 2003; Sun et al., 2004) lies beyond the scope of the present study. However, preliminary studies using the adenoviral vector system indicate that much of the improved immunogenicity of the fused construct is preserved in MHC class II-deficient mice (Holst et al., unpublished observation), suggesting that increased MHC class I presentation represents a major mechanism underlying the remarkable efficiency of the fused construct. Interestingly, the response induced to LCMV GP covered a broad range of epitopes, both dominant and subdominant, which is important when attempting to control infections with rapidly mutating viruses, such as HIV (Frahm et al., 2006; Turnbull et al., 2006). Such a broad CD8+ T-cell memory response efficiently protected the vaccinated host against systemic infection with a high dose of virus, but the Ii-chain-based DNA vaccine did not prime for a substantial improvement in virus control following peripheral challenge. Finally, we showed that use of the improved DNA vector as part of a heterologous prime–boost regime may significantly augment the response induced by an already optimized viral vector (Holst et al., 2008). This strongly suggests that even highly immunogenic vector-based immunization may be improved further through initial priming of the host with an Ii-chain-based naked DNA vaccine. Overall, as Ii chain fusion to the antigen will lead to priming for a broad CD8+ T-cell response, Ii-chain-based DNA vaccines are likely to represent a clear advantage with regard to prevention strategies against rapidly mutating viruses as part of heterologous prime–boost regimes.

ACKNOWLEDGEMENTS

This work was supported in part by the Novo Nordisk Foundation, the Lundbeck Foundation, the Sophus C. E. Friis Foundation and the Danish Research Council. M.G. is the recipient of a postdoctoral fellowship from the Benzon Foundation, and P.J.H. is the recipient of research fellowships from the Faculty of Health Science, University of Copenhagen.

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