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Long-term CD8^{+} T cell memory to Sendai virus elicited by DNA vaccination

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The capacity of DNA vaccines to prime CD8^{+} T cells makes them excellent candidates for vaccines that are designed to emphasize cellular immunity. However, the long-term stability of CD8^{+} T cell memory induced by DNA vaccination is poorly characterized. Here, the quality of CD8^{+} T cell recall responses in mice was investigated more than 1 year after DNA vaccination with the Sendai virus nucleoprotein gene. Cytotoxic T lymphocyte (CTL) activity specific for both dominant and subdominant epitopes could be recalled readily 1 year after vaccination and the frequencies of CTL precursors specific for both of these epitopes were relatively high. These CTL responded strongly to subsequent Sendai virus infection in terms of their ability to migrate to the lung and to differentiate into effector cells. In addition, the recall response to virus infection, as determined by CTL activity in the lungs and IFN-γ responses in the spleen, was both faster and greater in magnitude than that in control-immunized mice. Significantly, virus titres were reduced at least 100-fold in the lungs of mice that were immunized more than 1 year before infection, as compared with control mice. These data demonstrate that CD8^{+} T cell memory elicited by DNA vaccination is functionally relevant and persists for at least 1 year.

Introduction

DNA vaccination has been shown to prime potent cytotoxic T lymphocyte (CTL) responses specific for a variety of viral antigens in animal models (Boyer *et al.*, 1997; Cardoso *et al.*, 1996; Chen *et al.*, 1998; Davis *et al.*, 1995; Gonzalez Armas *et al.*, 1996; Lagging *et al.*, 1995; Lu *et al.*, 1996; Manning *et al.*, 1997; Ulmer *et al.*, 1993; Yasutomi *et al.*, 1996; Yokoyama *et al.*, 1995; Zarozinski *et al.*, 1995). This priming is surprisingly broad, inasmuch as CTL precursors (CTLp) are induced for both dominant and subdominant epitopes (Chen *et al.*, 1998; Fu *et al.*, 1997). It is currently believed that viral antigens encoded by DNA vaccines are expressed intracellularly *in vivo*, thus gaining access to the MHC class I pathway to elicit CTL activity. This property of DNA vaccines offers a substantial advantage over subunit vaccines and inactivated virus vaccines in that they can elicit CD8^{+} T cell responses in addition to humoral responses. Most experiments analysing the CTL response elicited by DNA vaccines have been performed within weeks of DNA immunization, and reports on long-term memory T cell responses induced by DNA immunization are very limited. It has been demonstrated that intramuscular (i.m.) or intradermal injection of DNA encoding influenza virus nucleoprotein (NP) can prime CTL that can be recalled more than 1 year after vaccination (Raz *et al.*, 1994; Yankauckas *et al.*, 1993) and that CTLp specific against Env or Gag of SIVmac were detectable in macaques almost 6 months after i.m. or gene gun immunization (Lu *et al.*, 1996). However, there have been no studies on the quality of long-lived memory CTLp induced by DNA vaccination. For example, it is not clear whether the frequencies of CTLp primed by DNA vaccines remain stable over time or whether long-lived CTLp specific for both dominant and subdominant epitopes retain the capacity to mediate a functional recall response. In addition, there is no information on the capacity of long-lived CTLp induced by DNA vaccination to clear virus *in vivo*.

We have previously described an extensive analysis of the induction of protective CD8^{+} T cell memory to Sendai virus by a DNA vaccine encoding the NP gene. These studies focussed

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on the quality of memory induced within 2 months of vaccination. In the current report, we analysed the quality of memory CTLp induced by the same protocol more than 1 year after vaccination. The data show that gene gun-mediated vaccination with DNA encoding Sendai virus NP elicited long-lasting memory CTL specific to both dominant and sub-dominant epitopes. The CTLp frequency remained relatively high more than 1 year after immunization. These memory T cells functioned vigorously *in vivo* and reduced the virus titre in the lungs greatly following virus infection.

Methods

■ **Mice.** Female C57BL/6 (K^b/D^b) mice and B6.C-H-2^{bm1} (K^{bm1}/D^b) mice were purchased from Jackson Laboratories. All mice were held under specific pathogen-free conditions until virus infection.

■ **Cell lines and culture conditions.** The L929-K^b, L929-D^b and SVBM1 (K^{bm1}/D^b) cells have been described previously (Ostrand-Rosenberg *et al.*, 1991; Whitmore & Gooding, 1981). All cell lines were cultured in complete medium (CTM) (Kappler *et al.*, 1981) at 37 °C in the presence of 10% CO_2. L929-D^b cells were cultured in CTM with 0·5 mg/ml G418. Restimulation of $NP_{324–332}$-specific T cells *in vitro* was performed as described previously (Chen *et al.*, 1998). Briefly, spleen cells obtained from DNA-vaccinated mice were processed with cell strainers and depleted of erythrocytes with Gey's solution. Spleen cells ($1{\cdot}2 \times 10^7$) were incubated with $1{\cdot}2 \times 10^7$ irradiated syngeneic splenocytes in 4 ml CTM supplemented with 0·5 μg/ml $NP_{324–332}$ and 10 U human recombinant IL-2 (rIL-2) in a 12-well tissue culture plate for 5–6 days.

■ **DNA vaccine and immunization of mice.** Plasmids pJW4303 and pJW-SNP, which contains the cDNA for Sendai virus NP, have been described previously (Chen *et al.*, 1998) and were purified with a Qiagen Maxiprep Kit. Eight- to 12-week-old C57BL/6 and B6.C-H-2^{bm1} mice were vaccinated twice with either pJW4303 or pJW-SNP at 3-week intervals by using an Accell gene gun as described previously (Chen *et al.*, 1998). Briefly, 40 μg pJW-SNP or pJW4303 was coated onto 25 mg 2·1 μm-diameter gold beads in the presence of 100 μl 1 M $CaCl_2$ and 100 μl 0·05 M spermidine. The beads were then washed three times with ethanol and resuspended in 3·6 ml ethanol in the presence of 0·1 mg/ml polyvinylpyrrolidone. The DNA–gold was loaded onto the interior surface of a piece of Tefzel tubing by using a tube loader. The tubing was then cut into 1·27 cm segments, each of which contained approximately 0·4–0·6 μg DNA. Mice received DNA from a single tube segment on a shaved region of the abdomen with each vaccination. The mice used in these experiments were vaccinated at the same time as the mice used for our previous study (Chen *et al.*, 1998) and were maintained under specific pathogen-free conditions until sacrificed for tissue analysis or infected with Sendai virus.

■ **Synthetic peptides.** $NP_{324–332}$ from Sendai virus NP (Kast *et al.*, 1991) and $NP_{366–374}$ from influenza A virus NP (Townsend *et al.*, 1986) were synthesized at St Jude Children's Research Hospital Molecular Resources Center by fast fMOC chemistry on an ABI model 431A peptide synthesizer. The peptides were diluted in PBS at 1 mM.

■ **Virus infection and collection of bronchoalveolar lavage (BAL).** The Enders strain of Sendai virus was prepared and stored as described previously (Cole *et al.*, 1994; Hou *et al.*, 1992). Mice were anaesthetized by intraperitoneal injection with avertin and infected intranasally (i.n.) with 330–660 50% egg infectious doses (EID_{50}) of Sendai virus. BAL cells were collected and pooled from between six and ten mice per group as described previously (Chen *et al.*, 1998). The cells were then allowed to adhere to a T75 flask for 1 h at room temperature to remove adherent cells and the erythrocytes were lysed with Gey's solution.

■ **Titration of virus in the lungs.** Lungs from individual mice were excised 6 days after infection i.n. with 660 EID_{50} Sendai virus and homogenized in 1 ml PBS. Three mice were used per group. The homogenate was diluted serially in antibiotic solution containing 5×10^3 U/ml polymyxin B sulphate, 5×10^4 U/ml penicillin G (potassium salt) and 10 μg/ml streptomycin sulphate in PBS and inoculated into embryonated hens' eggs (0·1 ml per egg). The eggs were incubated at 37 °C for 2 days and then stored at 4 °C overnight. Allantoic fluid from each egg was incubated with an equal volume of 0·5% (w/v) chicken erythrocytes in 96-well, round-bottomed tissue culture plates. The presence of Sendai virus was indicated by the agglutination of erythrocytes after 45 min at room temperature.

■ **Cytotoxicity assay.** Cytotoxic activity was determined as described previously (Chen *et al.*, 1998). Briefly, various target cells labelled with $Na^{51}CrO_4$ were either infected with Sendai virus or incubated with the $NP_{324–332}$ peptide. Target cells were incubated with graded numbers of effector cells in 96-well plates at 37 °C for 4 h. Supernatants (80–100 μl) were collected from each well and ^{51}Cr-release was measured with a gamma-counter. The percentage of specific release was calculated as 100 × (experimental release − spontaneous release)/(maximum release − spontaneous release). Spontaneous release was typically less than 20% of the total release induced by 1% Triton X-100.

■ **Limiting dilution analysis (LDA).** CTLp frequencies were measured by LDA as described previously (Allan *et al.*, 1990; Cole *et al.*, 1997; Hou *et al.*, 1993). Briefly, spleen cells pooled from three vaccinated mice were diluted serially and co-cultured with 5×10^4 irradiated C57BL/6 splenocytes in the presence of 0·5 μg/ml $NP_{324–332}$ peptide and 10 U/ml human rIL-2. The cultures were set up in a final volume of 200 μl in 96-well round-bottomed tissue culture plates. After 7 days, cells from individual wells were tested in a 6 h cytotoxicity assay on 2×10^3 ^{51}Cr-labelled L929-K^b cells and L929-D^b cells pulsed with or without the $NP_{324–332}$ peptide. Minimal estimates of CTLp frequency were determined according to the Poisson equation using χ^2 analysis. This gives rise to the minimal frequency estimate and 95% confidence intervals (Allan *et al.*, 1990; Hou *et al.*, 1993).

■ **Intracellular cytokine staining.** Splenocytes from individual vaccinated mice were depleted of B cells by panning in flasks coated with AffiniPure goat anti-mouse IgG (H+L) (Jackson ImmunoResearch). Three mice were used per group. The B cell-depleted splenocytes were cultured in 96-well round-bottomed tissue culture plates at 5×10^5 per well in 200 μl CTM supplemented with 10 μg/ml brefeldin A (BFA) and 10 U/ml human rIL2. Sendai virus $NP_{324–332}$ or influenza virus $NP_{366–374}$ peptides were then added at final concentrations of 1 μg/ml and the cells were incubated for 5 h at 37 °C. Positive controls were set up in parallel in which phorbol myristic acetate and ionomycin were added to final concentrations of 50 nM and 500 nM, respectively. After incubation, the cells were washed twice with staining wash buffer (0·2% foetal calf serum and 0·1% sodium azide in PBS) supplemented with 10 μl/ml BFA prior to incubation with 50 μl anti-Fc-γIII/II receptor and anti-mouse CD16/32 (Pharmingen) antibodies for 10 min on ice. The cells were then stained with rat anti-mouse Tri-color-conjugated CD8α (Caltag) in staining wash buffer supplemented with 10 μl/ml BFA for 20 min on ice. Cells were fixed with 1% formaldehyde in PBS for 20 min at room temperature and then permeabilized by incubation with 0·5% (w/v) saponin in staining wash buffer for 10 min at room temperature. Finally, permeabilized cells

were incubated with phycoerythrin-conjugated anti-IFN-γ antibody (Pharmingen) or phycoerythrin-conjugated anti-IgG1 isotype control diluted in staining wash buffer supplemented with 0·5% (w/v) saponin. After 20 min on ice, the cells were washed and analysed with a FACScan flow cytometer and CELLQuest software (Becton Dickinson).

Results

DNA immunization induces long-term memory CTLp specific for both dominant and subdominant epitopes

We have shown previously that gene gun immunization of C57BL/6 mice with the Sendai virus NP gene induced memory CTLp specific for both dominant ($NP_{324-332}/K^b$) and subdominant ($NP_{324-332}/D^b$) epitopes (Chen *et al.*, 1998). However, the CTL responses in these experiments were measured only a few weeks after immunization. To investigate the longevity of DNA vaccine-primed memory CTLp, we analysed C57BL/6 mice that had been immunized more than 1 year earlier with either pJW-SNP or pJW4303. This was the same cohort of mice used in the previous report (Chen *et al.*, 1998) and had been vaccinated at the same time. Spleen cells from individual mice were restimulated *in vitro* with the $NP_{324-332}$ peptide and tested for their lytic activity against $NP_{324-332}$-pulsed L929-K^b and L929-D^b cells. As expected, antigen-specific CTL activity was not recovered from the spleen cells of control pJW4303-immunized mice (Fig. 1A). In contrast, potent CTL activity specific for both dominant $NP_{324-332}/K^b$ and subdominant $NP_{324-332}/D^b$ epitopes was readily detected in pJW-SNP-immunized mice (Fig. 1B). These cells were also strongly lytic against L929-K^b and L929-D^b target cells that had been infected with Sendai virus. To quantify this recall CTL response, we measured the frequencies of CTLp specific for these two epitopes by LDA. As shown in Table 1, relatively high frequencies of CTLp specific for both dominant and subdominant epitopes (in the order of 1:40000 and 1:60000 spleen cells, respectively) were maintained more than 1 year after the final DNA vaccination. These frequencies are only 2–3-fold lower than the frequencies in spleen cells from mice immunized with pJW-SNP less than 8 weeks before (approximately 1:20000 spleen cells or 1:2000 $CD8^+$ T cells) (Chen *et al.*, 1998; Cole *et al.*, 1997). These data demonstrate that memory CTLp primed by DNA vaccination persist long after DNA vaccination.

Long-term memory CTLp primed by DNA vaccination are functional *in vivo*

We next asked whether the long-lived memory CTLp primed by DNA vaccination remained functional *in vivo*, in terms of their ability to respond to an infection in the lung. To address this issue, we took advantage of a system that assesses the migration of memory $CD8^+$ T cells to the site of virus infection (Cole *et al.*, 1997). Previous studies have shown that $CD8^+$ T cells specific for subdominant Sendai virus epitopes constitute only a minor proportion of the acute CTL response

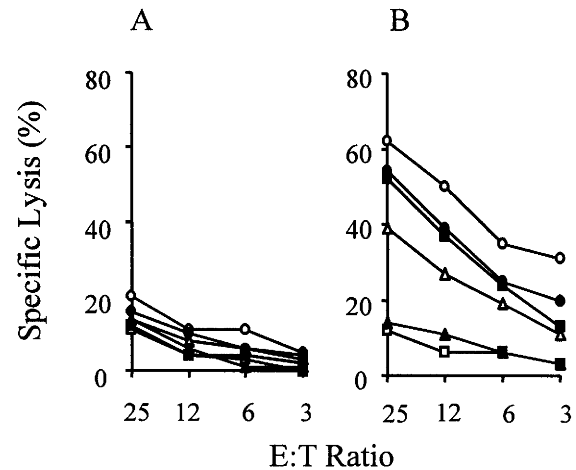

Fig. 1. DNA immunization induces long-term memory CTLp specific for both dominant and subdominant epitopes. Spleen cells from C57BL/6 (H-2^b) mice immunized 12 months before with pJW4303 (A) or pJW-SNP (B) were restimulated *in vitro* and tested for lysis of L-D^b cells (□), $NP_{324-332}$-pulsed L-D^b cells (△), Sendai virus-infected L-D^b cells (■), L-K^b cells (▲), $NP_{324-332}$-pulsed L-K^b cells (●) or Sendai virus-infected L-K^b cells (○). The data are representative of three independent experiments. E:T, effector:target.

to Sendai virus infection. However, if $CD8^+$ T cells specific for subdominant epitopes are first exclusively primed by vaccination, these cells then dominate the response to a subsequent Sendai virus infection in the lung, reversing the immunodominance pattern. The CTL response to Sendai virus infection in the lungs of B6.C.H-2^{bm1} mice is directed predominantly against a haemagglutinin-derived epitope, HN_{58-66}/K^{bm1}, with little or no detectable effector response to the subdominant $NP_{324-332}/D^b$ epitope. Thus, mice were vaccinated with pJW4303 (control) or with pJW-SNP, which specifically primes $CD8^+$ T cells specific for the subdominant $NP_{324-332}/D^b$ epitope but not the dominant HN/K^{bm1} epitope. Thirty-five weeks after the last immunization, mice were infected i.n. with Sendai virus and the BAL was collected 8 days later and assayed for the presence of CTL specific for the subdominant $NP_{324-332}/D^b$ epitope. As shown in Fig. 2, no $NP_{324-332}/D^b$-specific lytic activity was detected in the BAL of mice that had been immunized with pJW4303. In contrast, strong $NP_{324-332}/D^b$-specific lytic activity was detected in the BAL of mice that had been immunized with pJW-SNP. These data confirm that the long-lived memory CTLp induced by DNA vaccination were functional, in terms of their ability to mediate a recall effector response to Sendai virus infection *in vivo*.

Long-term memory CTLp elicited by DNA vaccination respond rapidly to Sendai virus infection

We next investigated whether DNA-primed CTLp could mediate a secondary CTL response at the site of virus infection *in vivo* by analysing the kinetics of the CTL response to Sendai virus in two groups of C57BL/6 mice immunized 13 months before with either pJW4303 or pJW-SNP. On different days

Table 1. Frequency of $NP_{324-332}/K^b$- and $NP_{324-332}/D^b$-specific CTLp induced by DNA vaccination with pJW-SNP

Experiment	Time since last immunization* (months)	Reciprocal of CTLp frequency (95% confidence limits)	
		$NP_{324-332}/K^b$	$NP_{324-332}/D^b$
1	13	53874 (48770–59504)	85699 (77979–94368)
2	12	33069 (25581–42089)	68845 (43547–111583)
3	12	27738 (23573–32367)	37408 (33584–41577)

* The primary immunization was followed 4 months later by two immunizations at 3 week intervals.

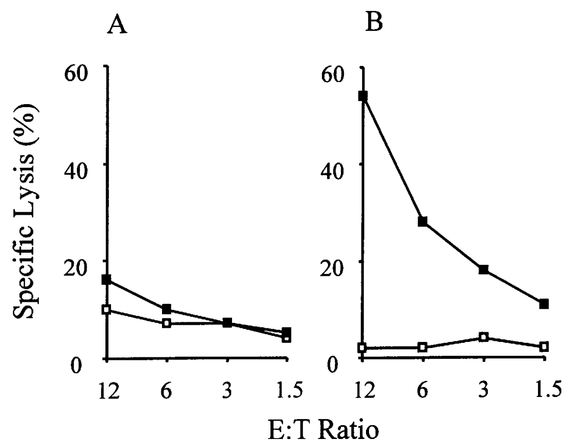

Fig. 2. Long-term memory CD8 T cells specific for the subdominant epitope migrate to the lung after Sendai virus infection. B6-H-2^{bm1} mice immunized with pJW4303 (A) or pJW-SNP (B) 35 weeks before and infected with Sendai virus (330 EID_{50}). Eight days after infection, pooled BAL cells from each group of mice were tested for lysis of L-D^b cells (□) and $NP_{324-332}$-pulsed L-D^b cells (■). The data are representative of two independent experiments. E:T, effector:target.

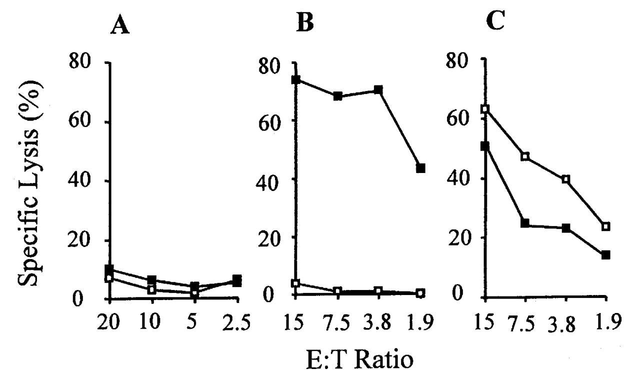

Fig. 3. DNA immunization induces long-term memory CD8 T cells that respond rapidly to virus infection. C57BL/6 mice immunized 13 months before with pJW-SNP (■) or pJW4303 (□) were infected with Sendai virus. The lytic activity of pooled BAL cells was tested on $NP_{324-332}$-pulsed L-K^b cells on day 5·5 (A), day 6·5 (B) and day 8·5 (C) after infection. E:T, effector:target.

after Sendai virus infection, the BAL was collected and lytic activity was tested on $NP_{324-332}$-pulsed L-K^b cells. As shown in Fig. 3, the kinetics of CTL induction in the lungs of the two groups of mice were remarkably different. Consistent with previous studies, the BAL cells collected from control mice showed little lytic activity on $NP_{324-332}$-pulsed L-K^b cells until day 8·5 post-infection. In contrast, the CTL activity of BAL cells from pJW-SNP-immunized mice peaked 2 days earlier than control mice, around day 6·5 after infection. This was consistent with studies of an influenza virus model, which have shown an accelerated secondary $CD8^+$ T cell response to virus infection (Flynn *et al.*, 1998).

To quantify $NP_{324-332}/K^b$-specific $CD8^+$ T cells present after DNA vaccination, we used intracellular staining to assess the number of cells that could synthesize IFN-γ rapidly after antigen stimulation *in vitro*. Thus, C57BL/6 mice were vaccinated with either pJW-SNP or pJW4303 and then infected with Sendai virus 16 months later. Before virus infection, the percentage of $CD8^+$ T cells producing IFN-γ in response to the dominant $NP_{324-332}$ peptide was at background levels in both pJW4303- and pJW-SNP-vaccinated mice (Table 2). In contrast, 7 days after i.n. Sendai virus infection, up to 10% of $CD8^+$ T cells from spleens of mice immunized with pJW-SNP produced IFN-γ in response to the $NP_{324-332}$ peptide. There was a minimal increase in the percentage of specific $CD8^+$ T cells from blank vector-immunized mice at this time after infection. These data indicate that mice vaccinated more than 16 months before retained long-lasting memory CTLp that could be recalled quickly in the presence of virus infection.

Control of Sendai virus infection after long-term DNA vaccination

We next wanted to determine whether long-term DNA vaccination conferred some form of protection against Sendai virus infection. The normal approach would be to determine whether vaccination protected the animals against a lethal dose of Sendai virus. However, this is not feasible in mice over 1 year old, as the LD_{50} of Sendai virus increases substantially with age. Therefore, as an alternative, we assessed virus clearance from the lungs of vaccinated and control mice

Table 2. Percentage of IFN-γ-positive $CD8^+$ cells in spleens of immunized mice

C57BL/6 mice were immunized with pJW4303 or pJW-SNP twice at 3 week intervals. Approximately 16 months after the last immunization, spleen cells from uninfected mice or mice infected i.n. 7 days earlier with 660 EID_{50} Sendai virus were stained for IFN-γ. Three animals were used for each combination of vaccination and infection (12 animals in total). The data are presented for each individual animal. Peptides were Sendai virus (SV) $NP_{324-332}$ and influenza virus (Flu) $NP_{366-374}$. ND, Not done.

Treatment	Peptide	pJW4303			pJW-SNP		
		#1	#2	#3	#1	#2	#3
Uninfected	SV $NP_{324-332}$	0·4	0·6	ND	1·2	0·5	1·0
	Flu $NP_{366-374}$	0·4	0·0	ND	0·4	0·4	0·3
Sendai virus-infected	SV $NP_{324-332}$	0·1	0·2	0·3	6·7*	5·7*	10·9*
	Flu $NP_{366-374}$	0·2	0·6	0·0	1·0	0·2	1·7

* The IFN-γ response to SV $NP_{324-332}$ was significantly different from the response in control vaccinated animals or uninfected animals ($P < 0·05$ by Student's t test).

Table 3. Virus titres in lungs from Sendai virus-infected mice

C57BL/6 mice were immunized with pJW4303 or pJW-SNP twice at 3 week intervals. A year after the final vaccination, mice were infected i.n. with 660 EID_{50} Sendai virus and lungs were collected 6 days later. Ten-fold serial dilutions of lung homogenate were inoculated into hens' eggs and the presence of virus was detected by a haemagglutination test. The virus titre given (as $\log_{10}$) is the highest dilution to give haemagglutination in at least two of three eggs. The data are from three individual mice and are significantly different in vaccinated versus control animals ($P < 0·05$ by Student's t test).

Mouse	pJW4303	pJW-SNP
#1	4	< 2
#2	7	< 2
#3	4	2

directly. Mice were immunized with pJW4303 or pJW-SNP and were infected i.n. with Sendai virus 12·5 months later. Six days post-infection, the lungs were removed and serial dilutions of lung homogenates were inoculated into the allantoic cavity of eggs. As shown in Table 3, the virus titres in lungs from pJW-SNP-vaccinated mice were reduced 10–100-fold compared with control mice. These data demonstrated that long-term DNA vaccination resulted in accelerated virus clearance.

Discussion

One of the key properties of DNA vaccines is that they are able to elicit strong and protective populations of memory CTLp. However, the stability and function of long-term CTLp generated by DNA vaccines are poorly characterized. Here, we performed a detailed analysis of the quality of the CTLp that persisted more than 1 year after the final vaccination. The data show that the frequency of CTLp specific for both dominant and subdominant epitopes from spleens of mice vaccinated more than a year before remained relatively stable, with only a 2–3-fold reduction relative to recently vaccinated mice. Moreover, these cells were able to respond rapidly to infection, in terms of their ability to migrate to the site of infection and to differentiate into effector cells capable of lysing virus-infected cells and secreting IFN-γ. Thus, vaccinated mice mounted a classical secondary response to Sendai virus infection that was very similar to that seen in secondary responses to other respiratory virus infections. These data therefore indicate that the $CD8^+$ T cell memory induced by DNA vaccination is highly functional and persists for a substantial period of time.

It is still the subject of controversy whether antigen is required to maintain memory CD8 T cells (Ahmed & Gray, 1996; Asano & Ahmed, 1996; Di Rosa & Matzinger, 1996; Gray & Matzinger, 1991; Hou *et al*., 1994; Lau *et al*., 1994). Although no studies have been done to address similar questions with respect to DNA vaccination, foreign proteins could be expressed *in vivo* long after the delivery of DNA vaccines. For example, influenza virus NP was detected in dermal tissues 1 month after intradermal injection (Raz *et al*., 1994). Similarly, luciferase remained active in muscle cells 19 months after i.m. injection of the corresponding plasmid DNA (Wolff *et al*., 1992). In the case of gene gun immunization, epidermal expression after immunization tends to be transient, due to the normal sloughing process (Pertmer *et al*., 1995; Torres *et al*., 1997), although transfected cells that migrate to the draining lymph nodes can express the encoded protein for a longer time. In contrast, Davis *et al*. (1997) showed that

transfected muscle fibres were destroyed by immune responses within 10 days of i.m. vaccination with DNA. Whether continuous protein expression is needed to maintain T cell memory elicited by a DNA vaccine is far from clear.

The mechanism by which gene gun-mediated DNA vaccination primes memory CTLp is poorly understood. It is known that gene gun bombardment mediates direct transfection of epidermal and dermal cells, leading to the expression of the encoded foreign proteins. Interestingly, the epidermis contains about 3–8% Langerhans' cells, which can migrate to the local lymph nodes and potentially function as professional antigen-presenting cells (Condon *et al*., 1996). In support of this general idea, it has been shown that vaccinated skin must be present for at least 3 days to elicit an optimal CTL response (Torres *et al*., 1997). Klinman *et al*. (1998) reported that induction of a primary T cell response requires that the transfected skin be present for at least 3 days, whereas only 5 h was needed for the development of a memory T cell response. Taken together, these data suggest that T cell priming requires either that enough antigen is expressed or that sufficient numbers of antigen-presenting cells migrate to the local lymph nodes.

More than five years after the first demonstration of the protective effect of DNA vaccines (Ulmer *et al*., 1993), the field of DNA vaccine biology has entered the clinical trial phase (Calarota *et al*., 1998; MacGregor *et al*., 1998). However, the long-term effects of DNA vaccination on the immune system are still unknown. This report analysed memory CTLp induced by a DNA vaccine and showed that the CTLp primed more than 1 year before could mount a vigorous response that accelerated virus clearance, suggesting that DNA vaccines could fulfil the requirement for long-lasting immunity. The data not only provide new information concerning memory CTLp elicited by gene gun immunization, but could also provide guidance for future experiments with DNA vaccines on larger animals.

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References

Ahmed, R. & Gray, D. (1996). Immunological memory and protective immunity: understanding their relation. *Science* **272**, 54–60.

Allan, W., Tabi, Z., Cleary, A. & Doherty, P. C. (1990). Cellular events in the lymph node and lung of mice with influenza. Consequences of depleting CD4+ T cells. *Journal of Immunology* **144**, 3980–3986.

Asano, M. S. & Ahmed, R. (1996). CD8 T cell memory in B cell-deficient mice. *Journal of Experimental Medicine* **183**, 2165–2174.

Boyer, J. D., Ugen, K. E., Wang, B., Agadjanyan, M., Gilbert, L., Bagarazzi, M. L., Chattergoon, M., Frost, P., Javadian, A., Williams, W. V., Refaeli, Y., Ciccarelli, R. B., McCallus, D., Coney, L. & Weiner, D. B. (1997). Protection of chimpanzees from high-dose heterologous HIV-1 challenge by DNA vaccination. *Nature Medicine* **3**, 526–532.

Calarota, S., Bratt, G., Nordlund, S., Hinkula, J., Leandersson, A. C., Sandstrom, E. & Wahren, B. (1998). Cellular cytotoxic response induced by DNA vaccination in HIV-1-infected patients. *Lancet* **351**, 1320–1325.

Cardoso, A. I., Blixenkrone-Moller, M., Fayolle, J., Liu, M., Buckland, R. & Wild, T. F. (1996). Immunization with plasmid DNA encoding for the measles virus hemagglutinin and nucleoprotein leads to humoral and cell-mediated immunity. *Virology* **225**, 293–299.

Chen, Y., Webster, R. G. & Woodland, D. L. (1998). Induction of CD8+ T cell responses to dominant and subdominant epitopes and protective immunity to Sendai virus infection by DNA vaccination. *Journal of Immunology* **160**, 2425–2432.

Cole, G. A., Katz, J. M., Hogg, T. L., Ryan, K. W., Portner, A. & Woodland, D. L. (1994). Analysis of the primary T-cell response to Sendai virus infection in C57BL/6 mice: CD4+ T-cell recognition is directed predominantly to the hemagglutinin–neuraminidase glycoprotein. *Journal of Virology* **68**, 6863–6870.

Cole, G. A., Hogg, T. L., Coppola, M. A. & Woodland, D. L. (1997). Efficient priming of CD8+ memory T cells specific for a subdominant epitope following Sendai virus infection. *Journal of Immunology* **158**, 4301–4309.

Condon, C., Watkins, S. C., Celluzzi, C. M., Thompson, K. & Falo, L. D., Jr (1996). DNA-based immunization by in vivo transfection of dendritic cells. *Nature Medicine* **2**, 1122–1128.

Davis, H. L., Schirmbeck, R., Reimann, J. & Whalen, R. G. (1995). DNA-mediated immunization in mice induces a potent MHC class I-restricted cytotoxic T lymphocyte response to the hepatitis B envelope protein. *Human Gene Therapy* **6**, 1447–1456.

Davis, H. L., Millan, C. L. & Watkins, S. C. (1997). Immune-mediated destruction of transfected muscle fibers after direct gene transfer with antigen-expressing plasmid DNA. *Gene Therapy* **4**, 181–188.

Di Rosa, F. & Matzinger, P. (1996). Long-lasting CD8 T cell memory in the absence of CD4 T cells or B cells. *Journal of Experimental Medicine* **183**, 2153–2163.

Flynn, K. J., Belz, G. T., Altman, J. D., Ahmed, R., Woodland, D. L. & Doherty, P. C. (1998). Virus-specific CD8+ T cells in primary and secondary influenza pneumonia. *Immunity* **8**, 683–691.

Fu, T. M., Friedman, A., Ulmer, J. B., Liu, M. A. & Donnelly, J. J. (1997). Protective cellular immunity: cytotoxic T-lymphocyte responses against dominant and recessive epitopes of influenza virus nucleoprotein induced by DNA immunization. *Journal of Virology* **71**, 2715–2721.

Gonzalez Armas, J. C., Morello, C. S., Cranmer, L. D. & Spector, D. H. (1996). DNA immunization confers protection against murine cytomegalovirus infection. *Journal of Virology* **70**, 7921–7928.

Gray, D. & Matzinger, P. (1991). T cell memory is short-lived in the absence of antigen. *Journal of Experimental Medicine* **174**, 969–974.

Hou, S., Doherty, P. C., Zijlstra, M., Jaenisch, R. & Katz, J. M. (1992). Delayed clearance of Sendai virus in mice lacking class I MHC-restricted CD8+ T cells. *Journal of Immunology* **149**, 1319–1325.

Hou, S., Fishman, M., Murti, K. G. & Doherty, P. C. (1993). Divergence between cytotoxic effector function and tumor necrosis factor alpha production for inflammatory CD4+ T cells from mice with Sendai virus pneumonia. *Journal of Virology* **67**, 6299–6302.

Hou, S., Hyland, L., Ryan, K. W., Portner, A. & Doherty, P. C. (1994). Virus-specific CD8+ T-cell memory determined by clonal burst size. *Nature* **369**, 652–654.

Kappler, J. W., Skidmore, B., White, J. & Marrack, P. (1981). Antigen-inducible, H-2-restricted, interleukin-2-producing T cell hybridomas. Lack of independent antigen and H-2 recognition. *Journal of Experimental Medicine* **153**, 1198–1214.

Kast, W. M., Roux, L., Curren, J., Blom, H. J., Voordouw, A. C., Meloen, R. H., Kolakofsky, D. & Melief, C. J. (1991). Protection against lethal Sendai virus infection by in vivo priming of virus-specific cytotoxic T lymphocytes with a free synthetic peptide. *Proceedings of the National Academy of Sciences, USA* **88**, 2283–2287.

Klinman, D. M., Sechler, J. M., Conover, J., Gu, M. & Rosenberg, A. S. (1998). Contribution of cells at the site of DNA vaccination to the generation of antigen-specific immunity and memory. *Journal of Immunology* **160**, 2388–2392.

Lagging, L. M., Meyer, K., Hoft, D., Houghton, M., Belshe, R. B. & Ray, R. (1995). Immune responses to plasmid DNA encoding the hepatitis C virus core protein. *Journal of Virology* **69**, 5859–5863.

Lau, L. L., Jamieson, B. D., Somasundaram, T. & Ahmed, R. (1994). Cytotoxic T-cell memory without antigen. *Nature* **369**, 648–652.

Lu, S., Arthos, J., Montefiori, D. C., Yasutomi, Y., Manson, K., Mustafa, F., Johnson, E., Santoro, J. C., Wissink, J., Mullins, J. I., Haynes, J. R., Letvin, N. L., Wyand, M. & Robinson, H. L. (1996). Simian immunodeficiency virus DNA vaccine trial in macaques. *Journal of Virology* **70**, 3978–3991.

MacGregor, R. R., Boyer, J. D., Ugen, K. E., Lacy, K. E., Gluckman, S. J., Bagarazzi, M. L., Chattergoon, M. A., Baine, Y., Higgins, T. J., Ciccarelli, R. B., Coney, L. R., Ginsberg, R. S. & Weiner, D. B. (1998). First human trial of a DNA-based vaccine for treatment of human immunodeficiency virus type 1 infection: safety and host response. *Journal of Infectious Diseases* **178**, 92–100.

Manning, W. C., Paliard, X., Zhou, S., Pat Bland, M., Lee, A. Y., Hong, K., Walker, C. M., Escobedo, J. A. & Dwarki, V. (1997). Genetic immunization with adeno-associated virus vectors expressing herpes simplex virus type 2 glycoproteins B and D. *Journal of Virology* **71**, 7960–7962.

Ostrand-Rosenberg, S., Roby, C., Clements, V. K. & Cole, G. A. (1991). Tumor-specific immunity can be enhanced by transfection of tumor cells with syngeneic MHC-class-II genes or allogeneic MHC-class-I genes. *International Journal of Cancer* **Suppl. 6**, 61–68.

Pertmer, T. M., Eisenbraun, M. D., McCabe, D., Prayaga, S. K., Fuller, D. H. & Haynes, J. R. (1995). Gene gun-based nucleic acid immunization: elicitation of humoral and cytotoxic T lymphocyte responses following epidermal delivery of nanogram quantities of DNA. *Vaccine* **13**, 1427–1430.

Raz, E., Carson, D. A., Parker, S. E., Parr, T. B., Abai, A. M., Aichinger, G., Gromkowski, S. H., Singh, M., Lew, D., Yankauckas, M. A. and others (1994). Intradermal gene immunization: the possible role of DNA uptake in the induction of cellular immunity to viruses. *Proceedings of the National Academy of Sciences, USA* **91**, 9519–9523.

Torres, C. A., Iwasaki, A., Barber, B. H. & Robinson, H. L. (1997). Differential dependence on target site tissue for gene gun and intramuscular DNA immunizations. *Journal of Immunology* **158**, 4529–4532.

Townsend, A. R., Rothbard, J., Gotch, F. M., Bahadur, G., Wraith, D. & McMichael, A. J. (1986). The epitopes of influenza nucleoprotein recognized by cytotoxic T lymphocytes can be defined with short synthetic peptides. *Cell* **44**, 959–968.

Ulmer, J. B., Donnelly, J. J., Parker, S. E., Rhodes, G. H., Felgner, P. L., Dwarki, V. J., Gromkowski, S. H., Deck, R. R., DeWitt, C. M., Friedman, A. and others (1993). Heterologous protection against influenza by injection of DNA encoding a viral protein. *Science* **259**, 1745–1749.

Whitmore, A. C. & Gooding, L. R. (1981). Specificities of killing by T lymphocytes generated against syngeneic SV40 transformants: cross-reactivity of the H-2Kbm1 through Kbm4 H-2 mutant alleles in Kb-restricted SV40-specific killing. *Journal of Immunology* **127**, 1207–1211.

Wolff, J. A., Ludtke, J. J., Acsadi, G., Williams, P. & Jani, A. (1992). Long-term persistence of plasmid DNA and foreign gene expression in mouse muscle. *Human Molecular Genetics* **1**, 363–369.

Yankauckas, M. A., Morrow, J. E., Parker, S. E., Abai, A., Rhodes, G. H., Dwarki, V. J. & Gromkowski, S. H. (1993). Long-term anti-nucleoprotein cellular and humoral immunity is induced by intramuscular injection of plasmid DNA containing NP gene. *DNA Cell Biology* **12**, 771–776.

Yasutomi, Y., Robinson, H. L., Lu, S., Mustafa, F., Lekutis, C., Arthos, J., Mullins, J. I., Voss, G., Manson, K., Wyand, M. & Letvin, N. L. (1996). Simian immunodeficiency virus-specific cytotoxic T-lymphocyte induction through DNA vaccination of rhesus monkeys. *Journal of Virology* **70**, 678–681.

Yokoyama, M., Zhang, J. & Whitton, J. L. (1995). DNA immunization confers protection against lethal lymphocytic choriomeningitis virus infection. *Journal of Virology* **69**, 2684–2688.

Zarozinski, C. C., Fynan, E. F., Selin, L. K., Robinson, H. L. & Welsh, R. M. (1995). Protective CTL-dependent immunity and enhanced immunopathology in mice immunized by particle bombardment with DNA encoding an internal virion protein. *Journal of Immunology* **154**, 4010–4017.

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