Intralymphatic immunization enhances DNA vaccination

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Although DNA vaccines have been shown to elicit potent immune responses in animal models, initial clinical trials in humans have been disappointing, highlighting a need to optimize their immunogenicity. Naked DNA vaccines are usually administered either i.m. or intradermally. The current study shows that immunization with naked DNA by direct injection into a peripheral lymph node enhances immunogenicity by 100- to 1,000-fold, inducing strong and biologically relevant CD8$^+$ cytotoxic T lymphocyte responses. Because injection directly into a lymph node is a rapid and easy procedure in humans, these results have important clinical implications for DNA vaccination.

The discovery that administration of eukaryotic plasmid vectors could lead to the expression of cloned genes in mammalian tissues (1) led to the evaluation of such vectors as naked DNA vaccines. Potent and long-lived cell-mediated and humoral immune responses have been demonstrated after the injection of naked plasmid DNA into the dermis or muscle tissue of mice (2–4). Immune responses to DNA vaccines have been elicited in a number of species, including mice, chickens, cattle, and primates, against antigens from a variety of different pathogens, including influenza virus (2), rabies virus (5), hepatitis B virus (6), *Plasmodium* (7), *Mycobacterium tuberculosis* (8), and HIV (9). Protection has been observed in many different infectious disease models, including influenza, malaria, hepatitis B, and HIV (2, 6, 7, 9). DNA vaccination has also been successfully used to elicit antitumor immunity (10), and a recent report documents successful immunotherapy of established tuberculosis in mice by DNA vaccination (11). Thus, DNA vaccines have the potential to be used both prophylactically and therapeutically.

In contrast to the numerous reports of the potency of DNA vaccines in mice, initial results from clinical trials in humans have been disappointing (12, 13). Much higher doses of DNA vaccines were required to elicit detectable immune responses in humans than had been expected based on animal studies, and the magnitudes of human antibody and cytotoxic T lymphocyte (CTL) responses were considerably lower than those observed in mice (12, 13). Therefore there is clearly a need to optimize the immunogenicity of DNA vaccines to permit their effective use in humans.

One parameter that may influence the immunogenicity of any vaccine is the amount of antigen that is presented in organized lymphoid tissues. Although most vaccination schedules have administered naked DNA either by i.m. injection or by intradermal (i.d.) administration with the use of a gene gun, there is strong evidence that the immune responses elicited by DNA vaccination occur after presentation of antigen by professional bone marrow-derived antigen-presenting cells (14, 15), via direct transfection of local dendritic cells, which then migrate to the draining lymph nodes (10, 16, 17). Because naive T cells are restricted to recirculating between blood and secondary lymphoid tissues (18–20), the efficacy of priming of naive T cells correlates with the strength and duration of antigenic stimulus in secondary lymphoid organs (21). In fact, even large amounts of immunogenic antigens can be ignored by the immune system, as long as they remain outside organized lymphoid tissues (21, 22).

We have applied this concept in the context of DNA vaccination, by comparing conventional routes of immunization (i.m. or i.d.) with direct administration of naked DNA to secondary lymphoid organs. Our results show that immunization with a plasmid DNA vaccine directly into organized lymphoid tissues is 100- to 1,000-fold more efficient than immunization via conventional routes and suggest that intra-lymph node (i.ln.) administration is a potent means of optimizing the immunogenicity of DNA vaccines for human use.

Materials and Methods

Mice. C57BL/6 (H-2^b) mice and immunodeficient RAG1$^{-/-}$ (H-2^b) mice were obtained from the breeding colonies of the Institut für Zuchthygiene, Tierspital, Zürich, Switzerland, and were between 8 and 12 weeks of age when first used.

Viruses. Lymphocytic choriomeningitis virus (LCMV) isolate WE (23) was grown on L929 cells (ATCC CRL 1) with a low multiplicity of infection. Recombinant vaccinia virus expressing the LCMV glycoprotein (G) (Vacc-G2; ref. 24) was grown and plaqued on BSC 40 cells.

Plasmids. pEGFPL33A was constructed from the pEGFPN3 vector (CLONTECH) as described (25). pEGFPL33A contains a DNA insert coding for the immunodominant CTL epitope from the LCMV-G (gp33; amino acids 33–41), flanked N-terminally by three leucines and C-terminally by four alanines, fused to the enhanced green fluorescent protein. The plasmid has the cytomegalovirus promoter and a *kan/neo* resistance gene. The plasmid was used to transfect competent *Escherichia coli*, and positive colonies were selected with the use of LB containing kanamycin. Plasmid DNA was isolated with the use of a CONCERT maxi-prep kit (GIBCO/BRL) according to the manufacturer's instructions.

Immunizations. Mice were immunized 1–6 times with the indicated doses of pEGFPL33A DNA or pEGFPN3 DNA diluted in PBS, in the following volumes: i.m., 50 μl into quadriceps muscle in rear leg; i.d., 25 μl into abdominal skin; intrasplenic (i.spl.), 10 μl; i.ln., 10 μl into inguinal lymph node. For multiple immunization schedules, the opposite inguinal lymph node or quadriceps muscle was used after the third injection. Positive control mice received 500 plaque-forming units (pfu) LCMV i.v.

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Abbreviations: LCMV, lymphocytic choriomeningitis virus; G, glycoprotein; Vacc-G2, recombinant vaccinia virus expressing LCMV-G; i.d., intradermal; i.spl., intrasplenic; i.ln., intralymph node; pfu, plaque-forming units; CTL, cytotoxic T lymphocyte.

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CTL Assays. CTL responses specific for the gp33 CTL epitope were measured with the use of a standard ^{51}Cr release assay and EL4 target cells that had been pulsed with 10^{-6} M LCMV-G peptide gp33 (KAVYNFATM) as described (26). Secondary CTL responses were assessed after *in vitro* restimulation with irradiated LCMV-infected peritoneal exudate macrophages as stimulator cells as described (27). The specificity of CTL activity was assessed by examining the lysis of unpulsed EL4 target cells.

Assessment of Antiviral Immunity *in Vivo*. To assess systemic antiviral immunity, mice were infected with 5×10^4 pfu LCMV-WE, and 4 days later spleens were isolated and LCMV titers were determined by a LCMV infectious focus assay as described (28).

To assess antiviral immunity in peripheral organs, female mice were infected i.p. with 5×10^6 pfu of Vacc-G2. Ovaries were harvested 5 days later, and the vaccinia titers were determined on BSC 40 monolayers as described (29).

Assessment of Antitumor Immunity *in Vivo*. The EL4–33 cell line was generated by subcloning part of the LCMV-G (encoding amino acids 1–60) into a cytomegalovirus-driven eukaryotic expression vector containing the geneticin resistance gene (A. Ochsenbein *et al.*, personal communication). After electroporation into EL4 (H-2^b) cells, stable lines were selected with G418 (0.8 mg/ml), and gp33 expression was confirmed by analysis in a ^{51}Cr release assay. EL4–33 tumor cells (10^6) were injected s.c. into the flank of immunodeficient RAG$1^{-/-}$ mice (H-2 ^{b}), and 2 weeks later solid tumors were removed and dissected into small pieces ($2 \times 2 \times 2$ mm). The tumor pieces were transplanted into the flanks of C57BL/6 mice that had previously been immunized with pEGFPL33A DNA or pEGFPN3 DNA as described above. Tumor size was assessed every 3–4 days, and tumor volume was calculated by the formula $V = abc/6$, where a, b, and c are the orthogonal diameters (22).

Results

i.ln. Immunization Is the Most Efficient Way to Induce CTL Responses. To quantitatively compare the $CD8^+$ CTL responses induced by different routes of immunization, we used a plasmid DNA vaccine (pEGFPL33A) containing a well-characterized immunodominant CTL epitope from the lymphocytic choriomeningitis virus glycoprotein (LCMV-G) (gp33; amino acids 33–41) (25), as this system allows a comprehensive assessment of antiviral CTL responses (30). Mice were immunized with titrated doses (200–0.02 μg) of pEGFPL33A DNA or of control plasmid pEGFP-N3, administered i.m., i.d., i.spl., or i.ln. Ten days after immunization spleen cells were isolated, and gp33-specific CTL activity was determined after secondary *in* vitro restimulation (30). As shown in Fig. 1, i.m. or i.d. immunization induced weakly detectable CTL responses only when high doses of pEFGPL33A DNA (200 μg) were administered. In contrast, potent gp33-specific CTL responses were elicited by immunization with only 2 μg pEFGPL33A DNA i.spl. and with as little as 0.2 μg pEFGPL33A DNA given i.ln. (Fig. 1). Immunization with the control pEGFP-N3 DNA did not elicit any detectable gp33-specific CTL responses (data not shown).

Similar thresholds for CTL detection were observed when a different readout system was used. Mice were immunized with titrated doses of pEFGPL33A DNA as above and were challenged 10 days later with LCMV i.v. Four days after challenge spleen cells were isolated, and *ex vivo* CTL activity was assayed. This time point is too early to detect any primary CTL response to LCMV infection in naive mice (Fig. 2, Controls), but it allows the detection of anamnestic CTL responses in mice that have previously been immunized (Fig. 2, LCMV). As before, mice immunized with 200 μg i.m. showed only weak anamnestic CTL responses after LCMV challenge, which were not detectable when lower immunizing doses of DNA were used (Fig. 2). Those

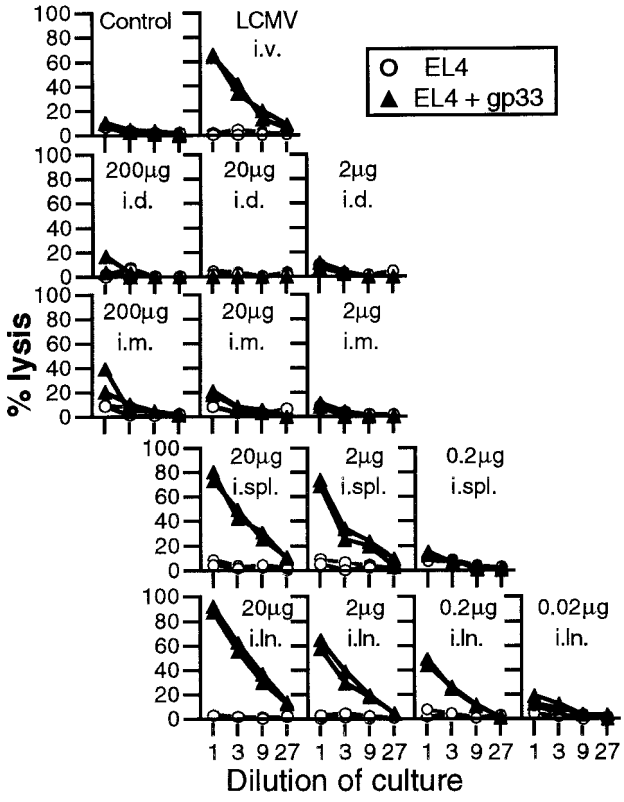

Fig. 1. i.ln. immunization is the most efficient way to induce antiviral CTL responses. Groups of two C57BL/6 mice were immunized once with pEGFPL33A (0.02–200 μg) given i.d. or i.m. or i.spl. or i.ln. Positive control mice received 500 pfu LCMV i.v. Ten days after immunization spleen cells were isolated, and gp33-specific CTL activity was determined after secondary *in vitro* restimulation. Symbols represent individual mice; one of three similar experiments is shown.

immunized by the i.spl. route showed strong anamnestic CTL responses that titered out at an immunizing dose of 2 μg pEFGPL33A DNA, whereas the i.ln. route of immunization was again more efficient, with anamnestic CTL responses detectable when only 0.2 μg pEFGPL33A DNA was administered (Fig. 2). These results clearly demonstrate that administration of plasmid DNA directly into lymphoid tissues is 100- to 1,000-fold more efficient than i.d. or i.m. routes for the induction of CTL responses. In addition, they show that the i.l.n. route is around 10-fold more efficient than the i.spl. route.

Repetitive Immunization with Plasmid DNA Induces Specific CTL Irrespective of the Route of Immunization. In the next series of experiments, we attempted to determine whether our plasmid DNA vaccine was able to induce specific CTL responses after repetitive immunization by various routes. Mice were immunized three times with pEGFPL33A DNA or with the control plasmid pEGFP-N3, administered i.m. (200 μg per immunization), i.spl. (20 μg per immunization), or i.ln (20 μg per immunization). Seven days after the final immunization spleen cells were isolated, and gp33-specific CTL activity was determined after secondary *in vitro* restimulation. As shown in Fig. 3, after repetitive immunization with pEGFPL33A DNA, gp33-specific CTL responses were detected by all routes of immunization. As expected, repetitive immunization with the control pEGFP-N3 DNA did not elicit any detectable CTL responses (Fig. 3).

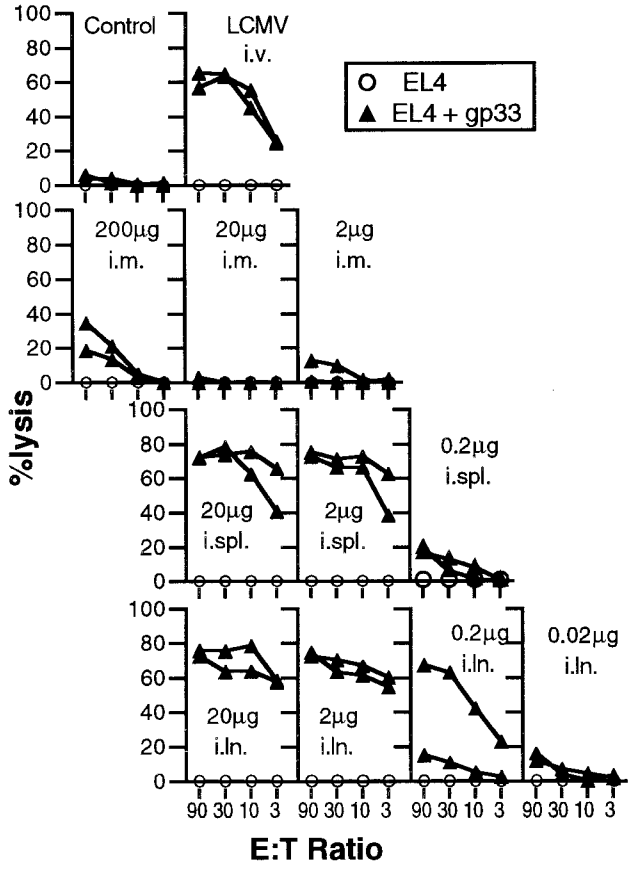

Fig. 2. i.ln. immunization is the most efficient way to induce antiviral anamnestic CTL responses. Groups of two C57BL/6 mice were immunized once with pEGFPL33A (0.2–200 μg) given i.d. or i.m. or i.spl. or i.ln. Positive control mice received 500 pfu LCMV i.v. Ten days after immunization mice were challenged with 5×10^4 pfu LCMV i.v., and 4 days later spleen cells were isolated and *ex vivo* CTL activity was assayed. Symbols represent individual mice; one of two similar experiments is shown.

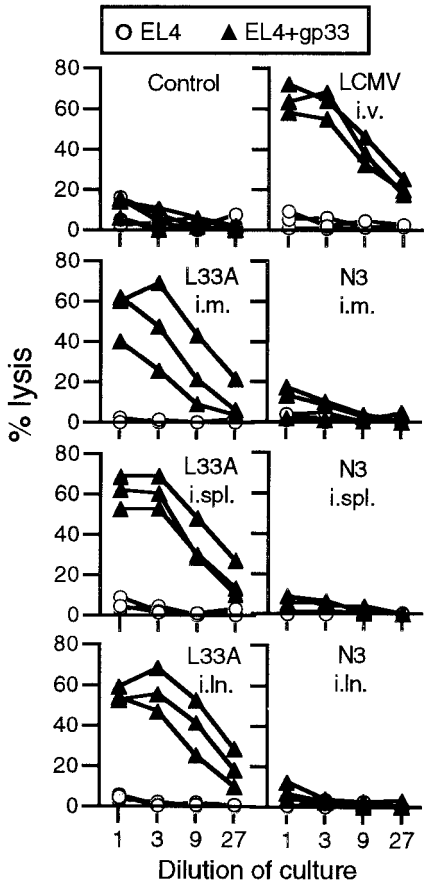

Fig. 3. Repetitive i.m. immunization with pEGFPL33A plasmid DNA induces gp33-specific CTL. Groups of three C57BL/6 mice were immunized three times (on days 0, 7, and 14) with pEGFPL33A or with the control plasmid pEGFP-N3, given i.m. (200 μg per immunization) or i.spl. (20 μg per immunization) or i.ln. (20 μg per immunization). Seven days after the final immunization spleen cells were isolated, and gp33-specific CTL activity was determined after secondary *in vitro* restimulation. Positive control mice received 500 pfu LCMV i.v. Symbols represent individual mice; one of two similar experiments is shown.

i.ln. Immunization Can Elicit Protection Against Systemic and Peripheral Virus Infection. To determine whether the enhanced CTL responses elicited after i.ln. immunization with plasmid DNA were able to qualitatively influence antiviral immunity, we used challenge infections with LCMV or with Vacc-G2 as models of systemic and peripheral virus infection, respectively. When systemic antiviral immunity was assessed by challenging the immunized mice with a high dose of LCMV i.v., mice that had been immunized once with 200 μg pEGFPL33A DNA i.m. showed only partial and incomplete protection against systemic LCMV challenge, whereas those that had received 20 μg of pEFGPL33A DNA by the i.spl. or i.ln. routes were completely protected (Fig. 4*A*).

Eradication of Vacc-G2 infection from peripheral organs such as ovaries depends on the presence of high levels of recently activated effector $CD8^+$ T cells (31, 32). Mice were immunized four times at 6-day intervals with pEFGPL33A DNA administered either i.m. (100 μg per immunization) or i.ln. (10 μg per immunization). Five days after the last immunization they were challenged with 5×10^6 pfu Vacc-G2 i.p., and vaccinia titers in ovaries were assessed after an additional 5 days. Repeated i.m. immunization with pEFGPL33A DNA had no influence on the growth of Vacc-G2 in peripheral tissues (Fig. 4*B*). In contrast, mice that were repetitively immunized with pEFGPL33A DNA by the i.ln. route were completely protected against peripheral infection with Vacc-G2 (Fig. 4*B*). These results illustrate that although repeated i.m. immunization with naked DNA induced detectable CTL responses, these were never of sufficient magnitude to offer protection against virus infection. In contrast, immunization with 10-fold lower amounts of DNA directly into lymphoid organs elicited quantitatively and qualitatively stronger CTL responses, which gave complete protection against systemic or peripheral virus challenge.

i.ln. Immunization Elicits Enhanced Antitumor Immunity. Last, we attempted to determine whether the potent CTL responses elicited after i.ln. immunization were able to confer protection against peripheral tumors. Mice were immunized three times at 6-day intervals with 10 μg of pEFGPL33A DNA or control pEGFP-N3 DNA. Five days after the last immunization small pieces of solid tumors expressing the gp33 epitope (EL4–33; A. Ochsenbein *et al.*, personal communication) were transplanted s.c. into both flanks. Whereas the EL4–33 tumors grew well in mice that had been repetitively immunized with control pEGFP-N3 DNA (Fig. 5), mice that had been immunized with pEFGPL33A DNA i.ln. rapidly eradicated the peripheral EL4–33 tumors (Fig. 5).

Discussion

The results presented here demonstrate that direct administration of plasmid DNA vaccine into secondary lymphoid tissues is

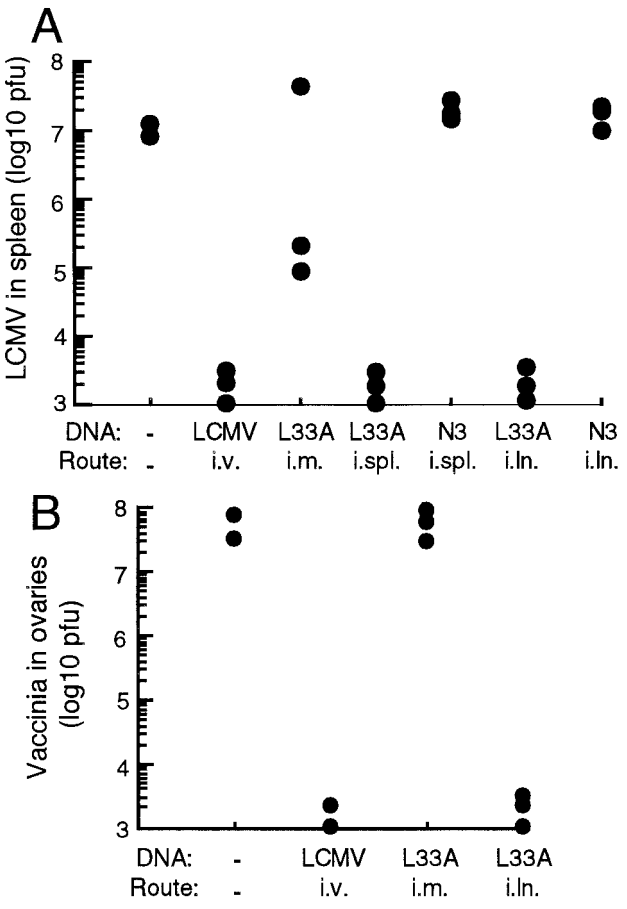

Fig. 4. i.ln. immunization elicits protective immunity against systemic and peripheral virus infection. (*A*) Groups of three C57BL/6 mice were immunized once with pEGFPL33A given i.m. (200 μg) or i.spl. (20 μg) or i.ln. (20 μg). Positive control mice received 500 pfu LCMV i.v. Ten days after immunization mice were challenged with 5×10^4 pfu LCMV i.v., and 4 days later spleens were isolated and LCMV titers were determined. Symbols represent individual mice; one of two similar experiments is shown. (*B*) Groups of three C57BL/6 mice were immunized four times at 6-day intervals with pEFGPL33A DNA administered either i.m. (100 μg per immunization) or i.ln. (10 μg per immunization). Five days after the last immunization they were challenged with 5×10^6 pfu Vacc-G2 i.p., and vaccinia titers in ovaries were assessed after a further 5 days. Symbols represent individual mice; one of two similar experiments is shown.

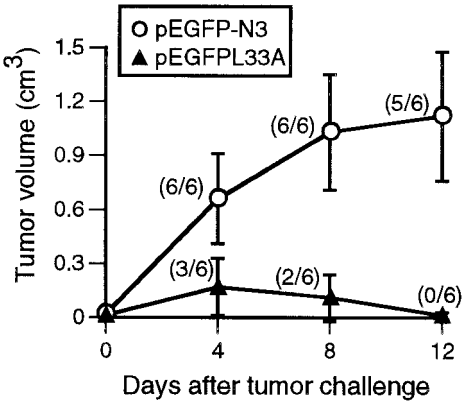

Fig. 5. i.ln. immunization elicits protective antitumor immunity. Groups of six C57BL/6 mice were immunized three times at 6-day intervals with 10 μg of pEFGPL33A DNA or control pEGFP-N3 DNA. Five days after the last immunization small pieces of solid EL4–33 tumors were transplanted s.c. into both flanks, and tumor growth was measured every 3–4 days. Mean tumor volumes $\pm$ 1 SD are shown, and numbers in brackets indicate the ratio of the number of mice with tumors to the total number of mice in a group. One of two similar experiments is shown.

a far more efficient means of generating antiviral CTL responses than immunizing via the currently used i.d. or i.m. routes. Around 100- to 1,000-fold fewer amounts of DNA were required to induce CTL responses when the DNA was administered directly into lymphoid organs. In particular, the i.ln. route of administration appeared to be the optimal method for the induction of CTL responses with a DNA vaccine.

A recent study that compared the administration of a DNA vaccine by a number of traditional injection (including i.v., i.p., i.m., and i.d.) and noninvasive (including i.n., intrarectal, and intravaginal) routes found that the i.m., i.v., and i.d. routes were the most efficient, although direct administration of DNA into lymphoid tissues was not tested (33). However, our results concur with previous reports showing that repeated vaccination by the i.m. or i.d. routes with DNA encoding LCMV antigens induced only weak CTL responses, which conferred only partial antiviral protection against LCMV infection (25, 34–36). Similarly, we found that i.m. administration of pEFGPL33A DNA induced weak CTL responses only after immunization with high amounts of plasmid DNA (100–200 μg) and offered only partial protection against systemic LCMV infection. Thus DNA immunization by conventional routes is a suboptimal method of inducing protective immunity.

In contrast, administration of pEFGPL33A DNA directly into organized lymphoid tissues by i.spl. or i.ln. injection was a much more efficient means of inducing antiviral CTL responses. Titration of the immunizing dose showed that detectable CTL responses could be elicited by a single injection of only 2 μg pEFGPL33A DNA i.spl. and by as little as 0.2 μg pEFGPL33A DNA given i.ln. Furthermore, the CTL responses induced by immunization into organized lymphoid tissues were sufficient to fully protect recipients against a challenge infection with LCMV, indicating that these routes also generated qualitatively stronger antiviral CTL immunity. Our results show that immunogenicity of DNA vaccines can be enhanced by up to 1,000-fold over the i.m. and i.d. routes when DNA is administered directly into a peripheral lymph node. We consistently found that i.ln. immunization was around 10-fold more potent in inducing CTL responses than i.spl. immunization. The reasons for this difference are not clear, but it is possible that the higher perfusion rate of the spleen immediately washes out a large proportion of the injected DNA, thus lowering the dose available for cellular uptake. Alternatively, the smaller volume of the lymph node may result in higher local concentrations of naked DNA after injection, which could account for the enhanced efficacy of CTL priming.

Most vaccination schedules have administered naked DNA either by i.m. injection or by i.d. administration with a gene gun. Although muscle cells at the site of immunization have been shown to express the antigens encoded by DNA vaccines, there is strong evidence that the immune responses elicited by DNA vaccination are dependent on antigen presentation by local bone marrow-derived dendritic cells, which take up the DNA and then migrate to the draining lymph nodes (10, 14–17) However, there may be more than one mechanism involved, as there is also evidence that antigen-presenting cells may acquire and present antigens produced by other transfected cells (37). By using fluorescence-activated cell sorter analysis to detect green fluorescent protein expression, which was also encoded on our plasmid vaccine, we consistently observed that a small fraction (1%) of $CD11C^+$ lymph node cells expressed green fluorescent protein 24 h after i.ln. immunization with the pEFGPL33A DNA (data not shown), indicating that dendritic cells within the lymph

node acquire and express the naked DNA vaccine. Thus, direct administration of naked DNA into the lymph node may increase the presentation by dendritic cells, resulting in more efficient priming of T cell responses.

That qualitatively superior CTL responses were elicited by i.ln. immunization was illustrated by the fact that repeated immunization with pEFGPL33A DNA by the i.ln. route, but not by the i.m. route, was able to protect mice against a peripheral challenge infection with Vacc-G2. Eradication of Vacc-G2 infection from peripheral organs such as ovaries depends on the presence of high levels of recently activated effector $CD8^+$ T cells (29, 31, 32) and is an important biological measure of immunity because many infections or tumors are primarily located in peripheral tissues. The qualitatively enhanced CTL responses were confirmed by our assessment of antitumor immunity, where mice immunized i.ln. with pEFGPL33A DNA rapidly eradicated peripherally transplanted EL4–33 tumor pieces expressing the gp33 epitope. Thus intralymphatic immunization with a naked DNA vaccine elicited qualitatively superior CTL responses that protected against peripheral challenge with either virus or tumors.

In summary, our data clearly indicate that immunization directly into organized lymphoid tissues with a plasmid DNA vaccine elicited antiviral immunity that was qualitatively and quantitatively superior to what could be achieved by conventional inoculation routes and suggest that i.ln. administration could be a potent means of optimizing the immunogenicity of DNA vaccines. In humans, injection into a s.c. lymph node is readily feasible with the use of ultrasound guidance and is a simple procedure that takes only a few minutes (Koch *et al.*, personal communication). Therefore the presented data have important clinical implications for the prevention or therapeutic eradication of infectious diseases or tumors in humans.

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