

## Protective CD4<sup>+</sup> and CD8<sup>+</sup> T Cells against Influenza Virus Induced by Vaccination with Nucleoprotein DNA

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DNA vaccination is an effective means of eliciting both humoral and cellular immunity, including cytotoxic T lymphocytes (CTL). Using an influenza virus model, we previously demonstrated that injection of DNA encoding influenza virus nucleoprotein (NP) induced major histocompatibility complex class I-restricted CTL and cross-strain protection from lethal virus challenge in mice (J. B. Ulmer et al., *Science* 259:1745–1749, 1993). In the present study, we have characterized in more detail the cellular immune responses induced by NP DNA, which included robust lymphoproliferation and Th1-type cytokine secretion (high levels of gamma interferon and interleukin-2 [IL-2], with little IL-4 or IL-10) in response to antigen-specific restimulation of splenocytes *in vitro*. These responses were mediated by CD4<sup>+</sup> T cells, as shown by *in vitro* depletion of T-cell subsets. Taken together, these results indicate that immunization with NP DNA primes both cytolytic CD8<sup>+</sup> T cells and cytokine-secreting CD4<sup>+</sup> T cells. Further, we demonstrate by adoptive transfer and *in vivo* depletion of T-cell subsets that both of these types of T cells act as effectors in protective immunity against influenza virus challenge conferred by NP DNA.

Cellular immune responses play an important role in protection from disease caused by infectious pathogens, such as viruses and certain bacteria (e.g., *Mycobacterium tuberculosis*). The specific T cells involved in conferring immunity can include both CD4<sup>+</sup> and CD8<sup>+</sup> T cells, often through the action of secreted cytokines and cytolytic activity, respectively. Certain types of vaccines, such as subunit proteins and whole or partially purified preparations of inactivated organisms, in general induce CD4<sup>+</sup> T-cell responses but not CD8<sup>+</sup> cytotoxic T lymphocytes (CTL). In contrast, live attenuated organisms and subunit proteins formulated with certain experimental adjuvants can induce both types of responses. Recently, a different approach consisting of direct immunization with plasmid DNA expression vectors (i.e., DNA vaccines) has shown promise as a viable means of inducing broad-spectrum T-cell responses. The effectiveness of DNA vaccines in animal models is likely due, at least in part, to expression of antigens *in situ* (35), leading to the induction of CTL (29), antibodies (3, 4, 10, 21, 22, 32), and cytokine-secreting lymphocyte responses (12, 36). During the past 5 years, many reports have been published on the immunogenicity of DNA vaccines encoding various antigens in several animal models, thereby illustrating the applicability of the technology to many pathogens (for a review, see reference 6). However, in only a few instances has the nature of the effector cells responsible for protective immunity been described (7, 16). In the present study, we have analyzed in detail the cellular immune responses induced by influenza virus nucleoprotein (NP) DNA and have established that both CD4<sup>+</sup> T cells secreting Th1-type cytokines and CD8<sup>+</sup> cytotoxic T cells play important effector roles in heterosubtypic protective immunity against lethal influenza virus challenge in mice.

### MATERIALS AND METHODS

**Vaccination of animals.** Female BALB/c mice (4 to 6 weeks old) were purchased from Charles River Laboratories (Raleigh, N.C.). Animals were housed in an American Association for the Accreditation of Laboratory Animal Care-accredited facility and cared for in accordance with the "Guide for the Care and Use of Laboratory Animals." The plasmid DNA expression vector containing the NP gene cloned from the A/PR/8/34 influenza virus strain was prepared as previously described (17, 29).

**Measurement of lymphoproliferation and cytokines.** Single-cell suspensions of spleen cells from DNA-vaccinated animals were depleted of erythrocytes in ACK lysis buffer (Gibco) and stimulated with recombinant NP (10 µg/ml) *in vitro* in round-bottom microwell plates at 5 × 10<sup>5</sup> cells/ml in RPMI 1640 medium supplemented with HEPES, glutamine, 10% fetal calf serum, and 50 µM 2-mercaptoethanol. Cells were cultured for 5 days, and [<sup>3</sup>H]thymidine was added at 1 µCi/well during the last 24 h. Cells were harvested onto glass fiber filter mats by using a Tomtek cell harvester, and radioactivity was measured in a liquid scintillation counter (Betaplate; Wallac).

For analysis of cytokine secretion, culture supernatants from restimulated spleen cells (see above) were harvested on day 4. Interleukin-2 (IL-2), IL-4, IL-10, granulocyte-macrophage colony-stimulating factor, and gamma interferon (IFN-γ) levels were measured by an enzyme-linked immunosorbent assay (ELISA) according to kit instructions (Endogen and Genzyme).

**Measurement of antibody responses.** For measurement of anti-NP antibodies, an ELISA was used as previously described (29). To detect specific immunoglobulin isotypes, peroxidase-conjugated rabbit anti-mouse immunoglobulin G1 (IgG1), IgG2a, IgG2b, and IgG3 (Zymed) were used as detailed elsewhere (5). For determination of geometric mean titer, samples below the limit of detection were assigned a value of 50, since the serum samples were diluted in 10-fold increments.

***In vitro* depletion of T-cell subsets.** Spleen cells were depleted of specific T-cell subsets by using two methods. First, R&D Systems murine CD4 or CD8 Subset Column kits were used according to the instructions provided. Briefly, 2.0 × 10<sup>8</sup> cells in 1 ml of sterile 1× column buffer were gently mixed with the contents of 1 ml of monoclonal anti-CD4 or anti-CD8 cocktail and incubated at room temperature for 15 min. The cells were washed and sedimented twice with 10 ml of 1× column buffer. The columns were washed with 10 ml of 1× column buffer, and the antibody-treated cells were applied to a column, allowed to enter into the column, and then incubated at room temperature for 10 min. The cells were eluted with column buffer and then sedimented prior to resuspension in culture medium for antigen restimulation.

Second, T-subset purification columns (Biotex Laboratories, Inc., Edmonton, Alberta, Canada) were used as instructed by the manufacturer. Briefly, splenocyte suspensions from mice immunized with DNA were washed and incubated with the monoclonal antibody (MAb) cocktails. These cocktail preparations consist of MAbs directed against surface marker antigens of B cells and of the T-cell subset which is intended to be depleted. The cells were then passed through a column of glass beads coated with anti-mouse IgG which bound the

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cells coated with MAbs. The unbound cells, the majority of which were the desired T-cell subset, were eluted from the column and collected. These enriched subsets of CD4<sup>+</sup> or CD8<sup>+</sup> lymphocytes contained <5% of the depleted cell population, as estimated by fluorescence-activated cell sorting (FACS) analysis. The lymphocytes were cultured and restimulated for 7 days with syngeneic cells that had been infected with A/PR/8/34 virus or pulsed with human immunodeficiency virus (HIV) Gag synthetic peptide 193-212. IL-2 (10 U/ml; Cellular Products Inc., Buffalo, N.Y.) was added on the second day of culture. Lymphocytes were washed three times with, and resuspended at desired concentrations in, phosphate-buffered saline.

To assess the purity of cell populations, cells were stained with fluorescein isothiocyanate-labeled anti-CD4 clone RM4-4 (PharMingen) and phycoerythrin-labeled anti-CD8b clone 53-5.8 (PharMingen), and flow cytometry analysis was performed with a FACScan (Becton Dickinson).

**In vivo depletion of T-cell subsets.** The regimen described previously by Wofsy and Seaman (34) was used to deplete T-cell subsets in vivo. Two separate experiments involving groups of 10 female BALB/c mice were injected with NP DNA (200 µg) on weeks 0, 3 and 6 and then challenged with live influenza virus on week 9. Starting 3 days before viral challenge, mice were given daily injections (100 µg each) of either normal rat IgG (Sigma), rat anti-mouse CD4 MAb (clone RM4-5; PharMingen), or rat anti-mouse-CD8a MAb (clone 53-6.7; PharMingen). Cell depletion was monitored by staining peripheral blood lymphocytes with antibodies specific for different epitopes on CD4 and CD8. Briefly, peripheral blood was collected from the tail veins of individual mice into tubes containing 10 ml of 0.85% saline. Cells were pelleted at 1,200 rpm for 10 min and then washed twice with Tris-ammonium chloride buffer (2) to lyse erythrocytes. Cells were stained and analyzed by flow cytometry as described above.

**Adoptive transfer of T cells.** The adoptive transfer protocol was modified from a method described previously (9, 33). The recipient mice, 4 h after challenge infection with influenza virus A/HK/68, received 0.2-ml volumes of lymphocytes through the tail veins.

**Influenza virus challenge model.** Challenge with live influenza virus A/HK/68 was performed essentially as previously described (29). Briefly, virus was administered by intranasal instillation of 20 µl containing 10<sup>3</sup> 50% tissue culture infective doses (TCID<sub>50</sub>) onto the nares of anesthetized mice, which in this study led to a rapid lung infection that was lethal to approximately 50% of nonimmunized mice. Individual mice were monitored daily for weight loss and survival. Data were calculated as average individual weight in a group, or as a percentage of group prechallenge weight, versus days after challenge. Statistical analyses were performed by using the *t* test for independent samples.

## RESULTS

**Induction of cellular immune responses.** Previous studies have demonstrated that injection of NP DNA into mice resulted in the induction of IgG anti-NP antibodies and CD8-restricted CTL (29), the latter of which were detected up to 1 to 2 years after injection (30, 37). These data suggest that a helper T-cell response against NP was also induced, resulting in a source of cytokines that facilitated switching of the immunoglobulin isotype and priming of a memory CTL response. Indeed, spleen cells from mice that were injected with NP DNA showed robust lymphoproliferative responses upon restimulation (Fig. 1). The magnitude of these responses from NP DNA-injected mice was greater than that induced by live influenza virus infection or vaccination with formalin-inactivated virus, possibly due to potential immunostimulatory effects of DNA or longevity of NP expression after DNA vaccination (6). Lymphoproliferative responses have been detected in spleen cells from mice as soon as 2 weeks and as late as 1 year after injection with NP DNA (not shown). Certain cytokines also were secreted from these spleen cells during antigen restimulation in vitro. The profile of cytokines secreted was indicative of a Th1 type of helper T-cell response, with high levels of IFN-γ and IL-2 (Fig. 2), but little or no IL-4 or IL-10 secreted into the culture supernatants of restimulated cells (not shown). In addition, granulocyte-macrophage colony-stimulating factor was detectable in the culture supernatants, but at modest levels (not shown). As might be expected from this Th1 type of response, the immunoglobulin subtype profile of anti-NP antibodies was predominated by IgG2a and IgG2b, with lesser amounts of IgG1 (Fig. 3).

**Analysis of T-cell subsets in vitro.** To ascertain the type of T cells responsible for lymphoproliferation and cytokine secre-

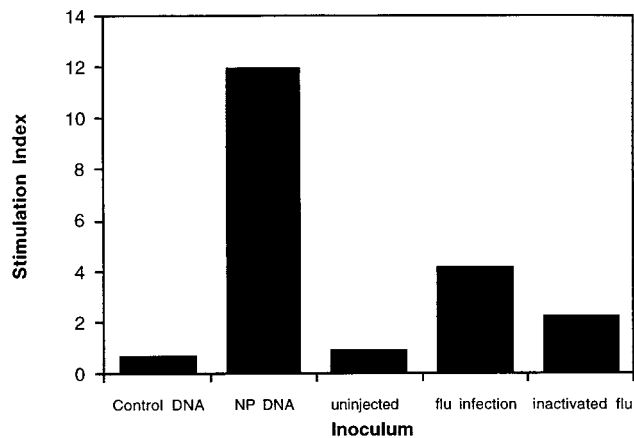


FIG. 1. Lymphoproliferative responses after NP DNA vaccination. Female BALB/c mice were uninjected or injected with NP DNA (50 µg), control DNA (50 µg), or inactivated influenza virus (A/PR/8/34) (flu; 15 µg) on weeks 0 and 3 or were infected awake with 1,000 TCID<sub>50</sub> of influenza virus (A/PR/8/34) on week 0. Spleens were collected and pooled from three mice per group on week 7 and restimulated in vitro with NP. Lymphoproliferation data are presented as a stimulation index.

tion in vitro, T cells were depleted of either CD4<sup>+</sup> or CD8<sup>+</sup> T cells prior to restimulation with antigen. In three separate experiments, depletion of CD4<sup>+</sup> T cells resulted in preparations containing 0.3 to 0.6% CD4<sup>+</sup> and 63 to 82% CD8<sup>+</sup> cells, while depletion of CD8<sup>+</sup> T cells resulted in preparations containing 80 to 85% CD4<sup>+</sup> and 0.05 to 0.3% CD8<sup>+</sup> cells, as quantified by FACS analysis. Unseparated populations consisted of 20 to 22% CD4<sup>+</sup> and 8 to 10% CD8<sup>+</sup> cells. The relative proportion of cells did not change appreciably during the 5-day restimulation period. Measurement of proliferation in these separated T-cell populations indicated that under these conditions most, if not all, lymphoproliferation was due to CD4<sup>+</sup> T cells (Fig. 4A). The higher level of proliferation in the CD8-depleted population, compared to unseparated

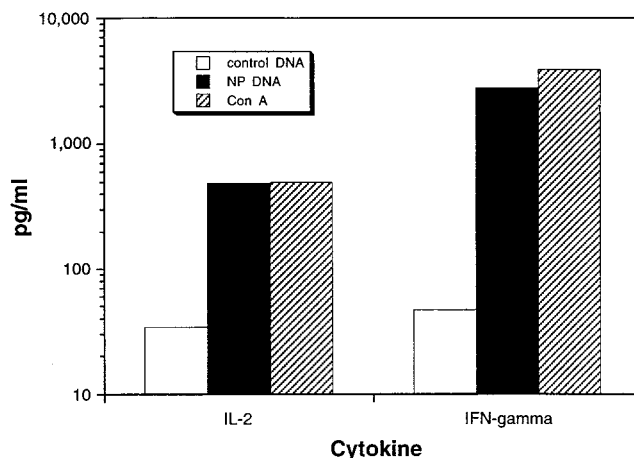


FIG. 2. Cytokine secretion from restimulated spleen cells. Female BALB/c mice were injected with NP DNA (50 µg) or control DNA (50 µg) on weeks 0 and 3, and spleens were collected and pooled from three mice per group on week 7. Cells from DNA-injected mice were restimulated in vitro specifically with recombinant NP protein, and cells from NP DNA-injected mice were nonspecifically activated with the mitogen concanavalin A (Con A). Cytokines secreted into the culture supernatant were detected by ELISA and are presented as picograms/milliliter of culture supernatant.

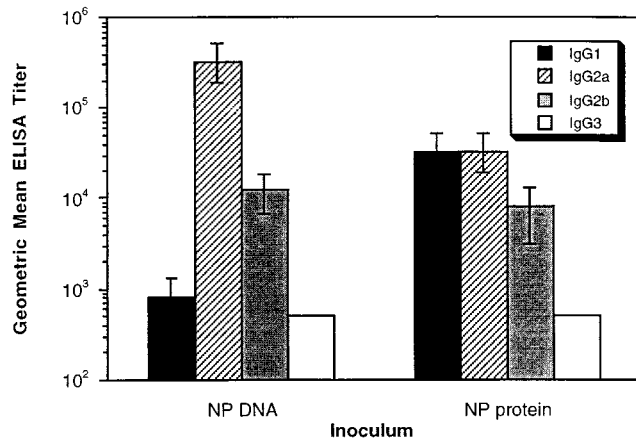


FIG. 3. Anti-NP immunoglobulin subtype profile. Female BALB/c mice were injected with NP DNA (50  $\mu$ g) or NP protein (10  $\mu$ g) on weeks 0 and 3, and sera were collected on week 5. Anti-NP antibody subtypes were measured by ELISA as described in Materials and Methods. Data are presented as geometric mean ELISA titers  $\pm$  standard errors of the means for groups of five mice.

spleen cells, was likely due to the three- to fourfold enrichment in CD4<sup>+</sup> cells. Similarly, detectable cytokine (IFN- $\gamma$  and IL-2) secretion upon restimulation was mediated solely by CD4<sup>+</sup> T cells (Fig. 4B and C). However, it is possible that the NP-specific CD8<sup>+</sup> T cells can undergo lymphoproliferation and cytokine secretion after restimulation of an unseparated spleen cell population. Regardless, the data show that, in addition to CD8<sup>+</sup> CTL (26), Th1-type cytokine-secreting helper T cells were induced by vaccination with NP DNA.

**Determination of effector cells.** Elucidation of the NP-specific effector cells responsible for protection from lethal influenza virus challenge of NP DNA-vaccinated mice was accomplished in two complementary ways. First, specific T-cell subsets were depleted *in vivo* in mice that had been inoculated with NP DNA. Mice were given injections of anti-CD4 or anti-CD8 antibodies on 3 successive days prior to challenge with influenza virus. Based on FACS analysis of cells from blood drawn on day 0 or 7 after challenge, these mice were substantially depleted of CD4 (<4.2%) or CD8 (<0.6%) T cells. Similar treatment with isotype control antibodies did not affect the levels of CD4 or CD8 cells. To ensure that such antibody treatment did not have an effect on the influenza virus challenge model, unimmunized mice were treated with anti-CD4, anti-CD8, or control antibodies, then challenged with virus, and monitored for survival and weight loss. Neither survival nor weight loss was discernibly affected after challenge with  $\sim$ 1 50% lethal dose of virus (data not shown). Similarly, tail vein bleeding of the mice on the day of challenge had no effect on survival or weight loss. This latter issue was important, since every mouse was bled on the day of virus challenge for determination of levels of circulating CD4<sup>+</sup> and CD8<sup>+</sup> T cells. Groups of mice were vaccinated with NP DNA or control DNA not encoding a protein and then challenged with virus. Mice that had received NP DNA and were untreated prior to challenge were completely protected from death (Fig. 5A) and showed minimal weight loss after challenge (Fig. 5B), as did NP DNA-vaccinated mice that were treated with control antibody. However, protection was abrogated in NP DNA-vaccinated mice that were depleted of CD8<sup>+</sup> T cells prior to challenge, as measured by survival ( $P < 0.0001$ ) and weight loss ( $P < 0.05$ ). Depletion of CD4<sup>+</sup> T cells also decreased the level of protection in NP DNA-vaccinated mice but not to the same

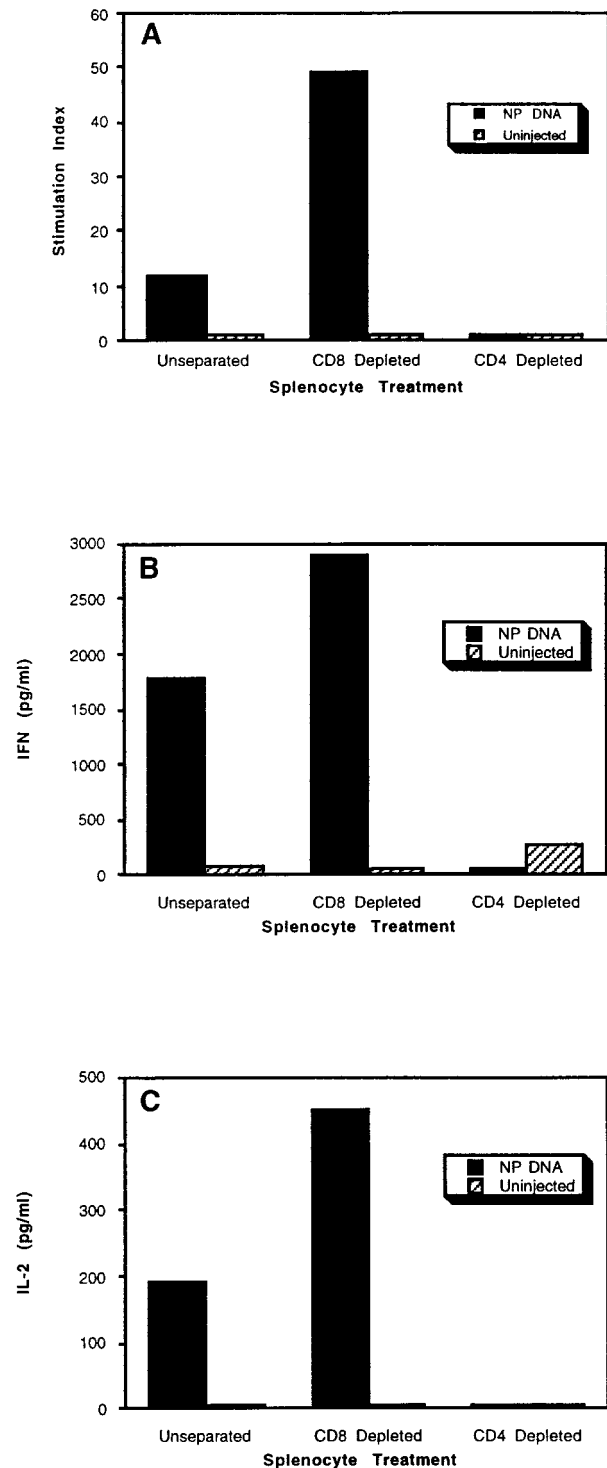


FIG. 4. *In vitro* depletion of T-cell subsets. Female BALB/c mice were injected with NP DNA (200  $\mu$ g) on weeks 0, 3, and 6, and spleens were collected and pooled from groups of three mice on week 23. T-cell subsets were prepared and restimulated as described in Materials and Methods. Cells from NP DNA-injected and uninjected mice were restimulated with NP protein and analyzed for proliferation plotted as a stimulation index (A) and secretion of IFN- $\gamma$  (B) or IL-2 (C), as measured by ELISA and plotted as picograms/milliliter of culture supernatant.

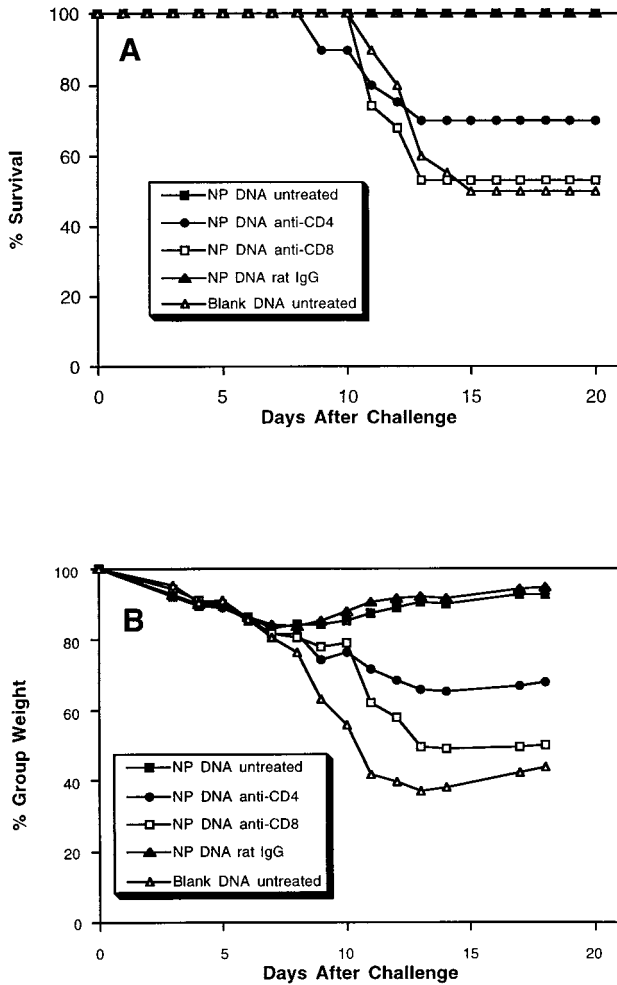


FIG. 5. In vivo depletion of T-cell subsets. Groups of 10 female BALB/c mice were injected with NP DNA (200  $\mu$ g) on weeks 0, 3, and 6 and then were untreated or treated with anti-CD4, anti-CD8, or control (rat IgG) antibody on week 9. As a negative control, mice were injected three times with control DNA (200  $\mu$ g). All groups were challenged under anesthesia with 1,000 TCID<sub>50</sub> of influenza virus A/HK/68 and monitored for survival (A) and weight loss (B). The results of two experiments were similar, and the data were combined in Fig. 5 to achieve an *n* of 20 per data point.

degree as that seen with CD8 depletion, as reduction was significant when measured by survival ( $P < 0.001$ ) but not when measured by weight loss ( $P > 0.05$ ). Therefore, both CD4<sup>+</sup> and CD8<sup>+</sup> T cells appear to play a role in protection induced by NP DNA.

The ability of the mice depleted of T cell subsets to mount antibody responses was also investigated. Mice were vaccinated with hepatitis B surface antigen (HBsAg) after the 3-day antibody treatment and then monitored for the development of anti-HBsAg antibodies. As expected, mice depleted of CD4 cells were severely limited in the ability to generate anti-HBsAg antibodies, while no such impairment was seen in mice depleted of CD8 cells (data not shown). In NP DNA-immunized mice subsequently challenged with influenza virus A/HK/68, antibody responses to the challenge virus were assessed, as measured by hemagglutination-inhibiting antibodies. In mice depleted of CD4 cells, the postchallenge hemagglutination inhibition titers were lower than in undepleted mice or in mice depleted of CD8 cells (data not shown), indicating that the

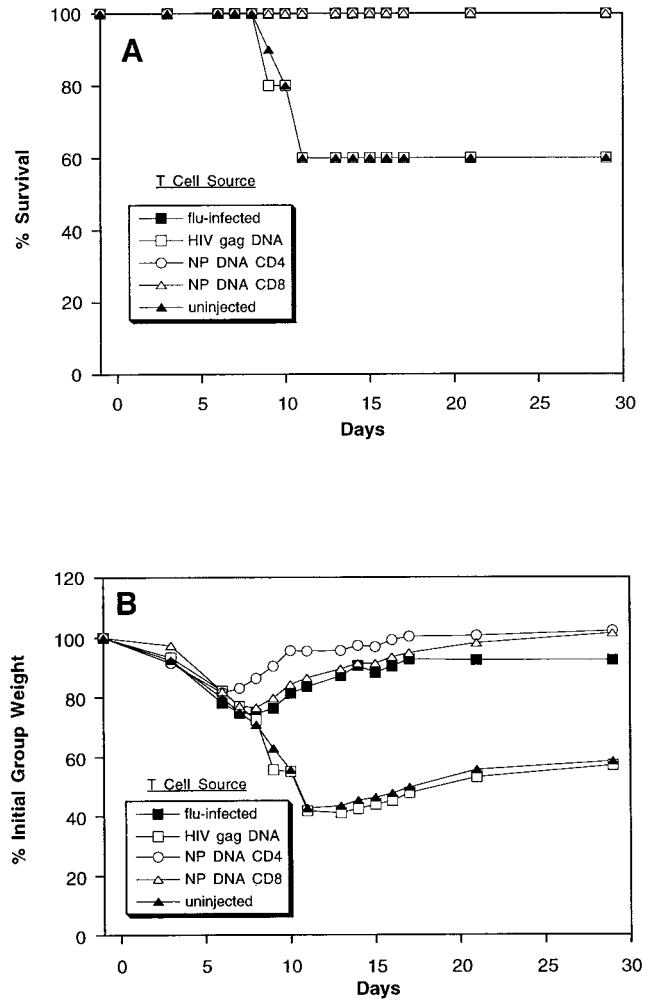


FIG. 6. Adoptive transfer of T-cell subsets. Spleen cells from uninjected mice (solid triangles) or mice primed with influenza virus A/PR/8/34 (flu-infected; solid squares), immunized with NP DNA, or injected with HIV Gag DNA (open squares) were harvested. The NP DNA-primed spleen cells were enriched for CD4<sup>+</sup> (open circles) or CD8<sup>+</sup> T lymphocytes (open triangles). Spleen cells from mice immunized with HIV Gag DNA were restimulated with syngeneic cells pulsed with Gag peptide 193-212, while cells from the remaining groups were restimulated in vitro for 7 days with syngeneic cells infected with A/PR/8/34. These lymphocytes were adoptively transferred into age-matched naive mice ( $2.5 \times 10^7$  cells/mouse for the groups denoted by open and solid squares;  $10^7$  for groups denoted by open circles and triangles) that had been intranasally challenged with A/HK/68 (H3N2) 4 h previously. Data are plotted as percent survival (A) and weight loss (B) versus days after challenge for groups of 10 mice.

absence of CD4 cells impaired the development of an antibody response against the challenge virus.

The second approach to assessing the nature of the effector cells after NP DNA vaccination was adoptive transfer of T-cell subsets. Spleen cells from groups of DNA-vaccinated or influenza virus-infected mice were enriched in CD4 or CD8 cells in vitro and then inoculated into naive mice. At 4 h after transfer, mice were challenged with ~1 50% lethal dose of virus and monitored for survival and weight loss. Recipients of unseparated spleen cells from influenza virus-infected mice were completely protected from death (Fig. 6A) and showed minimal weight loss (Fig. 6B). Similarly, transfer of either CD4<sup>+</sup> or CD8<sup>+</sup> T cells from NP DNA-vaccinated mice resulted in complete protection from death ( $P < 0.003$  compared to HIV Gag DNA-injected mice) and substantial protection from weight

loss ( $P < 0.01$  compared to HIV Gag DNA-injected mice). In contrast, mice that received cells from mice injected with HIV Gag DNA, which contained Gag-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cells (unpublished observations), were not protected. Therefore, these adoptive transfer studies demonstrate that NP-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cells can both independently act as effectors for protection from influenza virus challenge, thereby corroborating the results of the *in vivo* depletion studies. Further, vaccination of mice with NP DNA appears to prime T cells with approximately the same effectiveness as infection of mice with influenza virus.

## DISCUSSION

Induction of immune responses against influenza virus NP in mice can be accomplished by several means, including inoculation of recombinant protein (together with adjuvant) (26), live influenza virus (8, 15), recombinant live vectors such as vaccinia virus (14) and *Salmonella* (25) expressing NP, myoblasts expressing NP (31), and DNA vaccines (9, 19, 29, 37). Most of these modes of vaccination can confer heterosubtypic protection against influenza virus challenge (i.e., challenge with a different subtype of virus than that from which the vaccine was prepared). Such protection has long been thought to involve, at least in part, major histocompatibility complex (MHC) class I-restricted CTL (23). However, several lines of evidence suggest that other cells may also be involved in protection. For example, recombinant NP protein plus adjuvant (26) and NP-expressing *Salmonella* (25) protected mice from challenge despite the apparent lack of induction of MHC class I-restricted CTL. Also, beta-2-microglobulin (B<sub>2</sub>M)  $-/-$  mice, which are deficient in the ability to induce MHC class I-restricted CTL, can be protected when vaccinated with recombinant NP-expressing vaccinia virus or live influenza virus (1, 24). Finally, adoptive transfer of MHC class II-restricted, cytokine-secreting helper T cells can confer protection in normal (11, 27) and *nu/nu* (20) mice. These results strongly implicate cells other than MHC class I-restricted CTL as effector cells in protection from influenza virus challenge. However, analyses of the T-cell subsets that can act as effectors in protection from influenza virus challenge in immune mice have yielded conflicting results. *In vivo* depletion of CD4<sup>+</sup> or CD8<sup>+</sup> T cells was shown by Liang et al. (15) to result in partial abrogation of protection, as measured by virus shedding into the nasal cavity, while depletion of CD8<sup>+</sup> but not CD4<sup>+</sup> T cells led to a diminution of protection, as measured by virus titers in the lungs. Further, in the absence of B cells, CD4<sup>+</sup> T cells are inefficient in controlling an influenza virus infection in mice (18, 28). In contrast, Epstein et al. (8) demonstrated that neither depletion of CD4<sup>+</sup> T cells nor depletion of CD8<sup>+</sup> T cells had any effect on protection from virus challenge, as measured by lung virus titers after a sublethal dose of virus or survival after a lethal challenge. They did, however, find that CD4<sup>+</sup> T cells were necessary for protection in B<sub>2</sub>M  $-/-$  mice.

Previously, we demonstrated that vaccination of mice with NP DNA induced robust MHC class I-restricted CTL and heterosubtypic protection and that this protection was not due to antibody responses against NP (29). In this study we sought to investigate the spectrum of cellular immune responses induced by NP DNA and to delineate which of these responses mediate heterosubtypic protection. Here we show that, in addition to MHC class I-restricted CTL, NP DNA induces helper T-cell responses, as measured by lymphoproliferation of CD4<sup>+</sup> T cells, with concomitant secretion of Th1-type cytokines. Furthermore, using the two separate approaches of *in vivo* depletion and adoptive transfer of T-cell subsets, both CD4<sup>+</sup> and

CD8<sup>+</sup> T cells were demonstrated to be capable of effector cell function in protection from influenza virus challenge. Based on the depletion studies, CD8<sup>+</sup> T cells generated by NP DNA vaccination are necessary for protection, while CD4<sup>+</sup> T cells may not be as critical (although they do appear to play a role, as evidenced by the partial abrogation of protection in their absence). However, based on the adoptive transfer experiments, either CD4<sup>+</sup> or CD8<sup>+</sup> T cells alone are sufficient to confer protection. This apparent contradiction in the necessity of CD4<sup>+</sup> or CD8<sup>+</sup> T cells for protection conferred by NP DNA could be a result of different levels of these cells present in NP DNA-vaccinated mice versus those levels in mice receiving a bolus inoculation of NP-specific T cells activated *in vitro*. For example, there may be higher levels of activated CD4<sup>+</sup> cells in naive recipient mice than in NP DNA-vaccinated mice that could overcome the necessity for CD8<sup>+</sup> T cells seen in vaccinated mice. This argument has been suggested by Epstein et al. (8) to account for differences in previous adoptive transfer (23) and depletion studies (8, 15). Regardless of the relative importance of CD4<sup>+</sup> and CD8<sup>+</sup> T cells, though, the data presented here are consistent with the hypothesis that both cell types are involved in protection conferred by NP DNA.

The precise nature of the NP-specific effector CD4<sup>+</sup> T cells induced by NP DNA is not known. Studies using adoptive transfer of T cells from influenza virus-infected mice into naive mice have demonstrated or implicated cytokine-secreting helper T cells as having an effector function (11, 20, 27), while studies of B<sub>2</sub>M  $-/-$  mice suggest that cytolytic CD4<sup>+</sup> T cells can also confer protection (1, 24). The CD4<sup>+</sup> T cells induced by NP DNA in our work were clearly of the Th1-type helper T-cell phenotype, as indicated by the profile of immunoglobulin subtypes of anti-NP antibodies and the cytokines secreted from CD4<sup>+</sup> T cells upon antigen restimulation *in vitro*. Several attempts to detect CD4-mediated cytolytic activity were unsuccessful (not shown), even in spleen cells of highly vaccinated mice and using A20-1.11 target cells that express high levels of MHC class II (13). Therefore, while the presence of cytolytic CD4<sup>+</sup> T cells in NP DNA-vaccinated mice cannot be ruled out, it is likely that the CD4<sup>+</sup> T-cell effectors induced by NP DNA mediated protection through secretion of Th1-type cytokines.

In conclusion, NP DNA vaccines are effective at inducing a broad spectrum of cellular immune responses, including MHC class I-restricted CTL and Th1-type cytokine-secreting helper T cells. Both of these types of cells appear to be important as effector cells for protection against challenge with influenza virus. Taken together with previously published reports, these results indicate that there may be overlapping levels of protection against influenza virus infection involving several types of immune mediators, including MHC class I-restricted CTL, cytokine-secreting helper T cells, and possibly other types of cells. Since the current inactivated virus vaccines are not thought to be efficient at inducing broad-based cellular immune responses, these results have implications for development of human vaccines against influenza virus infection.

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