Vaccination with DNA encoding internal proteins of influenza virus does not require CD8+ cytotoxic T lymphocytes: either CD4+ or CD8+ T cells can promote survival and recovery after challenge

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Abstract

DNA vaccination offers the advantages of viral gene expression within host cells without the risks of infectious virus. Like viral vaccines, DNA vaccines encoding internal influenza virus proteins can induce immunity to conserved epitopes and so may defend the host against a broad range of viral variants. CD8+ cytotoxic T lymphocytes (CTL) have been described as essential effectors in protection by influenza nucleoprotein (NP), although a lesser role of CD4+ cells has been reported. We immunized mice with plasmids encoding influenza virus NP and matrix (M). NP + M DNA allowed B6 mice to survive otherwise lethal challenge infection, but did not protect B6-β2m(−/−) mice defective in CD8+ CTL. However, this does not prove CTL are required, because β2m(−/−) mice have multiple immune abnormalities. We used acute T cell depletion in vivo to identify effectors critical for defense against challenge infection. Since lung lymphocytes are relevant to virus clearance, surface phenotypes and cytolytic activity of lung lymphocytes were analyzed in depleted animals, along with lethal challenge studies. Depletion of either CD4+ or CD8+ T cells in NP + M DNA-immunized BALB/c mice during the challenge period did not significantly decrease survival, while simultaneous depletion of CD4+ and CD8+ cells or depletion of all CD90+ cells completely abrogated survival. We conclude that T cell immunity induced by NP + M DNA vaccination is responsible for immune defense, but CD8+ T cells are not essential in the active response to this vaccination. Either CD4+ or CD8+ T cells can promote survival and recovery in the absence of the other subset.
Introduction

Influenza vaccines in current use require annual updating in order to induce antibody responses specific for prevalent viral strains. A vaccine relying on conserved viral epitopes might induce responses active against a broad range of influenza viruses. Broadly reactive responses could improve protection in years when the vaccine does not match circulating strains, perhaps even against unexpected pandemic strains.

In efforts to induce immunity to conserved influenza antigens, much attention has focussed on induction of CD8\(^+\) cytotoxic T lymphocyte (CTL) responses by multiple antigens in humans (1) and by nucleoprotein (NP) in the mouse (2), although mice do make CTL responses specific for multiple other influenza antigens (3). Similarly, CTL responses have also been the subject of intense study in vaccine development efforts for HIV (4,5), because anti-HIV neutralizing antibodies are often too narrow in strain specificity to protect against the range of viral variants. In influenza, CD8\(^+\) CTL are prominent in the infected lungs (6) and clearly play an important role in clearance of existing infection (7–11). Plasmid DNA and resulting endogenous antigen expression could induce immune responses without the risks of live virus exposure, and perhaps maintain CTL activity for longer periods than Children immune responses without the risks of live virus exposure, from pT3PR8M (a gift from Yoshihiro Kawaoka, St Jude Children’s Research Hospital, Memphis, TN) by PCR. The insert contained the full-length M gene segment with both the M1 and M2 open reading frames and perhaps even against unexpected pandemic strains. The plasmid VR1012 was obtained from Vical (San Diego, CA) and might induce responses active against a broad range of viral strains. A vaccine relying on conserved viral epitopes resulting endogenous antigen expression could induce immune responses without the risks of live virus exposure, and perhaps maintain CTL activity for longer periods than a viral infection which is rapidly cleared. However, a realistic assessment of these potential advantages or of the possible economic and logistic advantages of DNA vaccines has not been made.

When DNA vaccines for influenza were first studied, a construct expressing NP was shown to induce both antibody and CTL responses, as well as protection against lethal challenge (12). Another report confirmed these findings and suggested that the mechanism of protection was likely CD8\(^+\), MHC class I-restricted CTL (13). However, while such CTL were induced, their role in protection was not initially evaluated. Further studies of DNA vaccination to influenza have used hemagglutinin (HA) as well as genes for internal components and have analyzed antibody as well as T cell immunity (20–24). Regarding protection by conserved internal components, a study using NP DNA vaccination reported that depletion of CD8\(^+\) cells just prior to challenge abrogated protection against lethal challenge completely, while depletion of CD4\(^+\) cells had a partial effect (25).

Successful development and use of new vaccines will be aided if their mechanisms of action are understood. We tested the roles of T cell subsets in protective immunity induced by DNA vaccination, using constructs encoding conserved internal proteins (NP and M) of influenza virus and a lethal challenge model. The mice immunized had defined, partial immune deficiencies, due either to targeted disruption of the β2-microglobulin (β2m) gene or to acute \textit{in vivo} depletion of one or more T cell subsets during the challenge period. Since lymphocytes in the lungs may be particularly relevant to virus clearance, we focussed attention on them. Lymphocyte subpopulations in the infected lungs were monitored by flow cytometry and CTL activity was assayed in fresh lung lymphocytes without re-stimulation. Outcomes in our challenge experiments showed balanced contributions of the CD4\(^+\) or the CD8\(^+\) subsets to protection by NP + M DNA vaccination, with either T cell subset alone sufficient to promote survival and recovery.

Methods

Plasmids

The plasmid VR1012 was obtained from Vical (San Diego, CA) under a Materials Transfer Agreement. Full-length influenza genes were prepared and inserted into VR1012 as follows. Restriction enzyme cleavage sites are indicated by italics and arrows.

\( pA/NP. \) The influenza A/PR/8/34 NP gene was subcloned by PCR from the vector CMV-Int-PRNP obtained from Dr Suezanne Parker, Vical, under a Materials Transfer Agreement. Primers were synthesized with the following sequences: forward primer 5\(-\)gcg gct g\(\text{↓}\)gcg gac aag aag ttc ata-3\) (Sall site indicated) and reverse primer 5\(-\)aag tag tgc atg aac aag gtt-3\) (XbaI site indicated).

\( pA/M. \) The influenza A/PR/8/34 matrix gene was subcloned from pT3PR8M (a gift from Yoshihiro Kawaoka, St Jude Children’s Research Hospital, Memphis, TN) by PCR. The insert contained the full-length M gene segment with both the M1 and M2 open reading frames and flanking non-coding regions. Primers were designed as follows: forward primer 5\(-\)gcg gct g\(\text{↓}\)gcg gac aag aag ttc ata\(\text{↓}\) (XbaI site indicated) and reverse primer 5\(-\)aag tag tgc atg aac aag gtt-3\) (BglII site indicated).

The nucleotide sequences of the inserts were confirmed and bulk preparations of plasmid DNA were prepared by BioServe (Laurel, MD) by fermentation in \textit{Escherichia coli} and two rounds of CsCl purification. Quality control testing by restriction digestion and agarose gel electrophoresis confirmed plasmid size and purity. All plasmid lots were tested for endotoxin levels by CBER’s Division of Product Quality Control using the chromogenic LAL assay, in order to avoid non-specific polyclonal effects \textit{in vivo}. Endotoxin levels were <1 EU/100 μg dose.

Viruses

Influenza virus strains used were A/PR/8/34 (H1N1) obtained from Dr Jonathan Yewdell, (NIAID, NIH, Bethesda, MD), A/Udorn/307/72 (H3N2) and B/Ann Arbor/1/86 obtained from Dr Roland Levandowski (CBER, FDA, Bethesda, MD). A/Phil is a reassortant virus (27) with the HA and NA genes of A/Philippine/2/82 origin, and the NP and M genes of A/PR/8/34 origin (28). Thus it is not fully heterosubtypic compared to A/PR/8 in that genes for other influenza antigens (3). Similarly, CTL responses have also been the subject of intense study in vaccine development efforts for HIV (4,5), because anti-HIV neutralizing antibodies are often too narrow in strain specificity to protect against the range of viral variants. In influenza, CD8\(^+\) CTL are prominent in the infected lungs (6) and clearly play an important role in clearance of existing infection (7–11). Plasmid DNA and resulting endogenous antigen expression could induce immune responses without the risks of live virus exposure, and perhaps maintain CTL activity for longer periods than a viral infection which is rapidly cleared. However, a realistic assessment of these potential advantages or of the possible economic and logistic advantages of DNA vaccines has not been made.

When DNA vaccines for influenza were first studied, a construct expressing NP was shown to induce both antibody and CTL responses, as well as protection against lethal challenge (18). Another report confirmed these findings and suggested that the mechanism of protection was likely CD8\(^+\), MHC class I-restricted CTL (19). However, while such CTL were induced, their role in protection was not initially evaluated. Further studies of DNA vaccination to influenza have used hemagglutinin (HA) as well as genes for internal components and have analyzed antibody as well as T cell immunity (20–24). Regarding protection by conserved internal components, a study using NP DNA vaccination reported that depletion of CD8\(^+\) cells just prior to challenge abrogated protection against lethal challenge completely, while depletion of CD4\(^+\) cells had a partial effect (25).

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NP-VAC, a vaccinia virus (WR strain) recombinant expressing NP of influenza A/PR/8/34 (29), was obtained from Drs Bernard Moss and Jonathan Yewdell (NIAID, NIH, Bethesda, MD). It was grown by the method of Earl (30) in HeLa cells obtained from the ATCC (Rockville, MD) and was harvested on day 3.

**Mice**

BALB/c and C57BL/6 (B6) female mice were purchased from the Division of Cancer Treatment, National Cancer Institute. B6-β2m(-/-) mice of both sexes were obtained and bred in our colony as described previously (28). Protocols for in vivo experiments were approved by the CBER Animal Care and Use Committee. The name β2m(-/-) mice used in the text includes β2m knockout mice on other genetic backgrounds from The Binding Site (Birmingham, UK), and all doses were from Drs Bernard Moss and Jonathan Yewdell (NIAID, NIH, Bethesda, MD) (37). In some experiments, aliquots of cell extracts were made from HeLa cells infected with a vaccinia virus expressing NP of influenza previously (28), except that cells were stained with FITC-conjugated anti-mouse CD90, previously coated with the injected mAb but not deleted. No construct expressing the HN protein of parainfluenza virus intranasally (i.n.) under Metofane anesthesia. They were monitored for body wt and mortality until survivors were regaining weight (at least 2 weeks) or were sacrificed for in vitro titration of lung homogenates. Differences in survival were compared by Fisher’s exact test using SigmaPlot software from SPSS. Differences in survival kinetics were subjected to Kaplan–Meier statistical comparisons using the JMP program from SAS Institute, Inc.

In preliminary studies, vaccine-induced defense against lethal challenge was not improved by use of sucrose or bupivacaine as diluents compared to saline (data not shown), so low-endotoxin saline or PBS was used in further studies, with comparable in vivo results. Vaccine-induced protection could be overwhelmed by higher challenge doses (data not shown), so the dose chosen was the lowest dose lethal to most unimmunized mice, consistent with the use of LD90 challenge by Rhodes et al. (18). The challenge viruses were titrated in vivo in each mouse strain, because susceptibility differs. A challenge dose of 10^3 TCID50/mouse of A/Phil was chosen for B6 mice, while 1–2×10^3 TCID50/mouse was chosen for BALB/c mice.

**ELISA for antibodies to influenza NP**

Antibodies to NP were assayed on plates coated with extracts of HeLa cells infected with NP-VAC, as described previously (28). The cells were harvested, subjected to three cycles of freezing–thawing and then spun down at 1250 g. Control extracts were made from HeLa cells infected with a vaccinia construct expressing the HN protein of parainfluenza virus. Plates coated with this extract showed no antibody activity in NP or NP + M immunized mice.

**Virus quantitation assays**

Influenza virus was quantitated by titration on Madin-Darby canine kidney cells using cytopathic effect as the indicator of presence of virus and titers was expressed as tissue culture infectious dose50 either TCID50/ml or TCID50/g lung tissue, as specified.

**In vivo depletion of T cell subsets**

The mAb used for in vivo depletion were: GK1.5 specific for mouse CD4 (31), 2.43 specific for mouse CD8 (32), SFR3-DRS specific for human leukocyte antigen, used as a negative control, and 30-H12 specific for mouse CD90, previously called Thy-1.2 (33), a clone that does not activate T cells (34). Ascites fluid was prepared by Harlan Bioproducts for Science (Madison, WI). All mAb are rat IgG2b antibodies and were used as delipidated ascites. The concentration of rat IgG2b was measured by radial immunodiffusion, using kits from The Binding Site (Birmingham, UK), and all doses were 1 mg/mouse of each antibody. The acute depletion protocol has been reported previously (28).

**Flow cytometry**

Flow cytometry was used to confirm completeness of in vivo T cell depletion within each experiment. Spleen, lung and lymph node cell suspensions were prepared by mincing the tissue, straining through nylon mesh, and lysing red blood cells with ammonium chloride–potassium buffer. Single-color analysis utilized the stains and procedures described previously (28), except that cells were stained with 7-aminotestosterone D (Sigma, St Louis, MO) in PBS for at least 30 min. Also, in some cases apoptotic cells were excluded by staining with 7-aminotestosterone D (Sigma) (35). In early experiments, single-color flow cytometry analyses of spleen cells showed that simultaneous depletion of CD4+ and CD8+ T cells left some cells staining for CD90. Addition of anti-CD90 to the depletion mixture reduced numbers of CD90+ cells to very low levels, but did not appear to further reduce the number of CD4+ cells (data not shown).

In order to assess only T cells, two-color analyses were performed with staining for CD3 versus CD4, CD8 or CD90. Cells were preincubated with DNase I (Sigma) at 0.01 mg/ml for 5 min at room temperature. Staining used phycoerythrin (PE) conjugated anti-mouse CD3ε (‘anti-mouse CD3ε’; PharMingen, San Diego, CA) along with FITC-conjugated anti-T cell antibodies as follows: anti-mouse L3T4 (CD4), RM4.4 (CD4) and anti-mouse Ly3.2 (CD8b.2) (all from PharMingen); anti-mouse Thy1.2 (CD90), anti-mouse Lyt 2 (CD8a) and anti-human Leu2a as a negative control (all from Collaborative Biomedical Products, Bedford, MA). PE and FITC stains were added to the cells in the presence of 2.4G2, a rat mAb to the mouse Fc receptor derived and generously distributed by Dr Jay Unkeless (36), a gift of Dr Elizabeth Shores (CBER, FDA, Bethesda, MD) (37). In some experiments, aliquots of cells were stained with FITC–anti-rat Ig absorbed against mouse serum proteins (Southern Biotechnology Associates, Birmingham, AL) to detect any residual cells that might have been coated with the injected mAb but not deleted. No staining above background was seen with anti-rat Ig, while this reagent stained strongly cells intentionally coated with GK1.5 as a control (data not shown). After staining cells were washed 3 times, fixed as above and washed 3 times before analysis. Analysis was performed on a Becton Dickinson (Mountain

**Roles of T cells in DNA vaccination to influenza**

Data not shown.)
Roles of T cells in DNA vaccination to influenza

View, CA) FACScan used in conjunction with the FACSMate. The instrument is calibrated bi-annually by service representatives utilizing Becton Dickinson’s CaliBRITE beads. Calibration is also maintained several times weekly using beads or cells and Becton Dickinson’s FACSComp program. The sample cells are positioned for viability and compensation, based on operator experience and training.

There were 15,000 live gated events per sample collected. Data analysis was performed utilizing the program CellQuest (Becton Dickinson). Gating by forward and side scatter used a much larger range than is typical for small lymphocytes, in order to include large activated lymphocytes, which were especially frequent in infected lungs.

**CTL analysis**

Detection of CTL activity without in vitro re-stimulation was performed as described previously (17,38). In order to obtain sufficient numbers of effector cells for analysis, cells from six mice per group were pooled. P815 cells as targets were infected with indicated viruses at 1000 HAU/ml for 4 h before labeling with ⁵¹Cr. Note that P815 does not express class II MHC antigens, so class II-restricted cytolysis would not be detected. Assays used 10,000 target cells/well, with E:T ratios titrated in 2-fold steps. Effectors were incubated with targets for 6 h before harvest. Results are expressed as percent specific ⁵¹Cr release, according to the formula: [(experimental release – medium release)/(total release – medium release)]x100.

**Results**

**Vaccination with DNA expressing influenza virus proteins**

Mice were immunized with plasmids i.m., using a schedule, DNA dose and diluent established in preliminary experiments. Immunization used DNA expressing NP and in most cases M as indicated or plasmid VR1012 without an insert to control for non-specific immune stimulation due to CpG motifs (39), of which there are a number in the plasmid backbone. Note that the H-2[β] (B6) and H-2[d] (BALB/c) haplotypes are both responders to NP (40), and both strains made antibodies to NP. Antibody responses in B6 mice are analyzed below; IgG antibodies in BALB/c mice had mean titers of ~4000 on ELISA plates coated with NP.

Immunization of B6 mice with NP plasmid caused only a modest reduction in challenge virus replication compared to controls (Table 1) at day 4 post-challenge, chosen as being around the peak of virus replication in the lungs. This result is consistent with the previous studies of purified NP protein or NP-expressing vaccinia recombinants cited above. In BALB/c mice, lung virus titers were again reduced only partially after immunization with a mixture of both NP and M constructs (Table 1), a mixture used by other investigators (21) to enhance immunization by providing multiple antigens just as virus does. Modest reductions in lung virus often correlate with improved survival (28,41,42), and these lung titers are compatible with accelerated virus clearance and significant protective effects.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Geometric mean lung virus titer [TCID₅₀ U/ml (SEM)]</th>
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<tbody>
<tr>
<td>B6 mice</td>
<td></td>
</tr>
<tr>
<td>pA/NP</td>
<td>1.3 x 10⁶ (1.6)²</td>
</tr>
<tr>
<td>VR1012</td>
<td>6.7 x 10⁶ (1.6)</td>
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<tr>
<td>BALB/c mice</td>
<td></td>
</tr>
<tr>
<td>pA/NP + pA/M</td>
<td>2.2 x 10⁶ (1.5)³</td>
</tr>
<tr>
<td>VR1012</td>
<td>3.5 x 10⁶ (1.2)</td>
</tr>
</tbody>
</table>

Mice, eight per group for B6 and 10 per group for BALB/c, were immunized 3 times with 100 µg of the indicated plasmids, i.m., and challenged 2 weeks after the last immunization. B6 mice were challenged with A/PR8, 10³ TCID₅₀/mouse. BALB/c mice were challenged with A/Phil 2 x 10⁷ TCID₅₀/mouse. Lungs were harvested at day 4.

**DNA vaccination in B6 and B6-β₂m(−/−) mice**

As a first approach to assessing the role of CTL in the outcome of vaccination and challenge, we immunized B6-β₂m(−/−) mice and B6 mice as congenic controls. The B6-β₂m(−/−) mice lack normal MHC class I complexes and thus produce few CD₈+ anti-viral CTL (43) (see also Discussion). Still, they can clear primary influenza virus infection (44,45), and when immunized can control homologous (46) and heterosubtypic (47) challenge infections.

As shown in Fig. 1(A), B6 mice immunized with NP + M DNA recovered from a challenge that was lethal to mice given control DNA lacking an insert. In contrast (Fig. 1B), B6-β₂m(−/−) mice immunized with NP + M DNA succumbed to challenge infection. This result might suggest that class I-restricted CTL are needed for vaccine-induced promotion of recovery. However, further analysis of immune responses in B6-β₂m(−/−) mice shows that one cannot draw that conclusion (following and Discussion). As shown in Table 1, anti-NP titers of the IgG2a isotype induced by DNA or NP-expressing vaccinia recombinants cited above. In BALB/c mice immunized with NP plasmid and BALB/c mice lacking normal MHC class I complexes and thus produce few CD₈+ anti-viral CTL (43) (see also Discussion).

β₂m(−/−) mice have defective antibody responses to a variety of antigens (46,48). While antibodies to internal proteins such as NP do not neutralize influenza virus and are not protective upon passive transfer (19), we analyzed them in order to assess whether the antibody response defect in β₂m(−/−) mice extended to DNA vaccination. Serum IgG1 and IgG2a antibody responses to NP in sera of the immunized mice were analyzed by ELISA. As shown in Fig. 2, anti-NP titers of the IgG2a isotype induced by DNA vaccination were significantly higher in B6 mice than in B6-β₂m(−/−) mice (P = 0.0014, Student’s t-test). IgG1 titers were lower and more variable in both mouse strains (data not shown). The predominance of IgG2a is consistent with reports of others for i.m. administration of DNA (25,49). The reduced titers in B6-β₂m(−/−) mice show that their responses to DNA vaccination are defective not only in CTL but also in other immune effectors.
Roles of T cells in DNA vaccination to influenza

Fig. 2. IgG2a anti-NP antibodies in B6-β2m(−/−) and B6 mice after DNA vaccination. The same mice in the experiment in Fig. 1 were bled 1 week after the third immunization. Sera were analyzed by ELISA on plates coated with extracts of cells infected with NP-VAC. Each bar represents serum from one mouse (eight per group except five B6-β2m(−/−) given NP/H11001 M DNA). Responses in the two mouse strains differed significantly, \( P = 0.0014 \) by Student's t-test.

Fig. 1. Protection by NP + M DNA against H3N2 influenza virus challenge in B6 mice but not B6-β2m(−/−) mice. Eight mice per group were immunized i.m. 3 times at intervals of 2 weeks. Each dose consisted of 100 μg each of NP and M DNA or 200 μg of VR1012 DNA with no insert in control mice. Mice were challenged 2 weeks after the third immunization with 10^6 TCID<sub>50</sub> mouse of A/H3N2 given i.n. under anesthesia. The difference in mortality in the control groups was not significant (\( P = 0.2 \), Fisher's exact test). The kinetic difference seen in the B6-β2m(−/−) mice (NP + M immunized mice dying faster than controls) was not reproduced in a another experiment.

Acute T cell depletion for analysis of immune effector phase: role of T cells in promoting recovery

Given the limitations on interpretation of experiments in β2m knockout mice, we turned to acute depletion of T cells to analyze their contributions to survival and recovery. After vaccination with NP + M plasmid DNA, T cell subsets were depleted in immunocompetent mice during the challenge period. As shown in Fig. 3, intact BALB/c mice vaccinated with NP + M DNA survived challenge that was lethal to naive mice or to mice given control DNA. Unexpectedly, depletion of CD8<sup>+</sup> cells during the challenge period had only an insignificant effect on survival, compared to controls. This indicates that CD8<sup>+</sup> CTL were not solely responsible for survival and furthermore that they were not even necessary. Similarly, depletion of CD4<sup>+</sup> cells did not affect survival significantly. Mice depleted of both CD4<sup>+</sup> and CD8<sup>+</sup> T cells, with or without additional depletion by anti-CD90, did not survive, indicating that effector T cells of these two subsets account for the immune defense against disease. In one experiment, CD4,CD8,CD90-depleted mice died ~3 days earlier than CD4,CD8-depleted mice, but this was not significant (\( P = 0.18 \), Kaplan–Meier analysis) unless one outlier was excluded (then \( P = 0.001 \)). The kinetic difference was not seen in a second experiment (\( P = 0.42 \)). Thus there was not sufficient evidence to show a role of double-negative T cells.

Phenotypic analysis of T cell-depleted mice

Effectiveness of the T cell depletion procedure was examined by flow cytometry. In addition to spleens, lymphocytes isolated from infected lungs were analyzed, since this population is particularly relevant to viral clearance. To maximize detection, analyses were performed at the height of the response after viral challenge, when any residual T cells could have been re-expanded. Counter-staining with anti-CD3 assured that only T cells were analyzed.

As shown in Fig. 4, CD4 depletion alone or CD8 depletion alone left very few residual T cells of the respective subset in the lungs. Depletion of both T cell subsets by injection of a mixture of anti-CD4 and anti-CD8 also left very few residual cells of the corresponding phenotypes, but some double-
in lungs and spleen, was incomplete in lymph nodes, with 10% of residual cells staining brightly for CD90 in the cervical lymph node and 15% in the mediastinal lymph node after CD4, CD8, CD90 depletion in vivo.

Functional depletion: CTL activity

The effectiveness of T cell depletion in eliminating CTL activity was analyzed in DNA vaccinated mice, as it has been in previous studies of mice immunized with live virus (38, 50). We measured MHC class I-restricted CTL activity at the height of the in vivo recall response 7 days after challenge, without in vitro re-stimulation.

As expected, both A/PR/8- and A/Phil-infected targets were lysed by lung CTL, since the NP and M genes of the two viruses are the same (Fig. 5). Very strong CTL activity was seen in the lungs of immunized and challenged mice not depleted of any T cells, with some CTL activity seen even at E:T ratios of 1:1. Lytic capacity on targets infected with either A/PR/8 or A/Phil was reduced at least 64-fold by CD8-depletion, compared to control mice not depleted of T cells. The modest CTL activity seen in the spleens (data not shown) was specific for influenza A-infected targets and was completely absent in CD8-depleted mice. In a separate depletion experiment, CTL activity in lung cells of CD8- or CD4, CD8-depleted mice was also drastically reduced (~32-fold reduction in lytic units; data not shown). Thus, cytolytic activity was abrogated in mice depleted of CD8+ T cells, even when measured by the most stringent test of activity in the infected lungs after challenge.

Depletion of only CD4+ cells at the time of challenge had no effect on cytolyis; in another experiment in NP + M DNA immunized mice, lysis was just as high as in mice given control ascites (data not shown). Apparently any major helper dependence of the CTL response occurs at the priming phase, but CD4+ helper cells are no longer needed at the time of challenge and CTL reactivation. A small difference in CTL activity is shown in Fig. 5 between mice subject to CD8 depletion alone and to CD4, CD8 depletion. However, part of the minor lytic activity in the CD8-depleted mice was also seen on uninfected targets or flu B-infected targets and is thus non-specific, and was reduced in CD4, CD8-depleted mice (data not shown).

Discussion

Vaccination with DNA encoding internal proteins from an influenza A virus of the H1N1 subtype protected mice against lethal challenge with a virus of the H3N2 subtype, as has been reported by other investigators (19). However, such immune defense against challenge viruses that belong to different subtypes, termed heterosubtypic immunity, is also observed in animals following immunization with live virus and is not a novel property of DNA vaccines. A long literature beginning in the 1960s reports and analyzes such findings in mice and ferrets (28, 41, 50–52), using widely divergent virus strains. Heterosubtypic immunity has occasionally been reported in humans (53, 54), but its potency and duration are limited. It remains to be seen if DNA vaccines will be more successful at inducing broad cross-protection in humans than live virus or if once again cross-protection will be more
pronounced in animals than in humans. Even in animals, a DNA vaccine expressing NP appears less effective than prior virus infection at inducing heterosubtypic immunity (55).

We investigated the responses of B6-β2m(−/−) mice because earlier reports of heterosubtypic immunity induced by NP DNA suggested a likely role of CD8+ CTL. Disruption of the β2m gene confers a defect in class I MHC expression and thus in development of most CD8+, class I-restricted CTL (unless β2m protein is provided in serum-containing culture medium or on injected tumor cells or adoptively transferred lymphocytes (56–58)). The low level of class I heavy chain on the cell surface without β2m does not usually function in antigen presentation of viral peptides or lead to induction of class I-restricted CTL. An exception is presentation of a few peptides in association with H-2Ld (59,60). However, these do not include influenza antigens;
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\[ \beta_2m(-/-) \text{ mice display cytolytic activity to influenza antigens only on class II^+ targets and this activity is due to CD4^+ cells.} \]

Under the conditions studied, B6-\( \beta_2m(-/-) \) mice were not protected against lethal virus challenge by NP + M DNA vaccination. While this result suggests a requirement for CTL in survival, alternative explanations are possible due to defects in other immune compartments. We found that DNA vaccination elicited lower NP-specific antibody titers in these mice than in normal mice, as previously reported for viral and protein antigens (43,46,48). Other immune defects in \( \beta_2m(-/-) \) mice include abnormalities in NK cells, NKT cells, a subset of γδ T cells and possibly CD4^+ cells (43), and in IgG homeostasis (62), which may explain the lower IgG antibody titers. These findings indicate the pleiotropic nature of the immune defects resulting from disruption of the \( \beta_2m \) gene. It is possible that some of the additional cellular effectors play roles in protection by DNA vaccination. Thus, the ineffectiveness of NP + M DNA vaccination in \( \beta_2m(-/-) \) mice does not accurately determine the role CTL play in defense against challenge infection.

Acute T cell depletion in normal mice during the challenge period provided an alternative approach. Depletion of either CD4^+ or CD8^+ T cells had only insignificant effects on survival. Simultaneous depletion of both CD4^+ and CD8^+ T cells with or without addition of anti-CD90, however, dramatically reduced survival. This suggests that function by either CD4^+ or CD8^+ T cells can suffice for immune defense against challenge infection. It should be noted that CD8^+ T cell responses can enhance survival by clearing virus, but they can also produce immunopathological damage to the lungs. The competing contributions of these two effects were studied in detail in BALB/c versus nude mice by Wells et al. (63). BALB/c mice succumbed early or else cleared the virus and survived long term, while nude mice survived the early period, presumably because they were not subject to tissue damage by T cells, but could not clear virus and eventually had higher overall mortality.

A study by Ulmer et al. of DNA vaccination to influenza showed complete abrogation of protective immunity by CD8 depletion and partial abrogation by CD4 depletion (25). We saw little effect of either depletion alone and saw substantial abrogation of immune defense only when both subsets were depleted. The difference in the two studies is interesting because the experimental conditions were similar in many ways (BALB/c mice, plasmids constructed with NP genes from A/PR/8, H3N2 virus challenge, similar DNA doses and immunization schedule, and in vivo CD8 depletion with a mAb specific for CD8α). Differences in effectiveness of in vivo depletion do not seem to explain the outcomes. Our analysis showing only very low numbers of residual CD8^+ cells after depletion was more rigorous, in that we stained lung lymphocytes at the height of challenge infection when residual cells would be expanded, while in the other study, peripheral blood cells taken before challenge were stained. In addition, we performed functional studies of CTL activity in the lungs of depleted animals, as the site most relevant to viral clearance, and observed depletion of activity.

Possible explanations for the differing outcomes include the difference in challenge dose (50% lethality in the Ulmer study, 100% in ours), the specific challenge virus strains (A/HK/68 in the Ulmer study, A/Phil in ours) and our use of M in addition to NP DNA. We are further analyzing effector mechanisms induced by M DNA to address one of these possibilities. Previous studies have shown that T cells specific for M can provide potent help (64), and our M plasmid given alone promotes survival and recovery (Benton et al., unpublished data). Regarding the challenge virus strains, A/Phil is a reassortant H3N2 virus with genes for internal viral proteins derived from A/PR/8. A/HK/68 is not a reassortant and so the A/PR/8 NP gene in the DNA vaccine differs from that of the A/HK/68 virus. The NP sequence of A/PR/8 differs by ~30 amino acids from NP of each of four different H3N2 viruses (65), so one or more sites recognized in A/PR/8 NP
by CD4+ T cells could be mutated in A/HK/68. A study by Brett et al. of T cell proliferation in response to NP peptides showed functional effects of mutations in NP (66). If a significant part of the CD4+ response was ineffective against A/HK/68 challenge virus, that could perhaps explain why the CD8+ response would be required.

Ulm et al. reported that CD4+ or CD8+ cells induced by DNA vaccination were able to transfer resistance to mild challenge, although details of the purity of the enriched cells with respect to residual cells of the other subset was not reported (25). Influenza-specific CD8+ cells (67,68) and Tc1 but not Tc2 CD4+ cells (69) had been shown previously to confer anti-viral immunity upon adoptive transfer. However, it should be emphasized that adoptive transfer experiments can only test the capability of a particular cell type to perform a function such as viral clearance, not the magnitude of the role it in fact plays in the active, composite response of animals or the necessity of its role. Thus in vivo depletion studies address a different question.

The relative inefficiency of CD4+ cells in mediating protective immunity has been emphasized in interpreting some studies of influenza infection (69–71), while more balanced contributions of CD4+ and CD8+ cells have been noted in our study of Ig-/- mice (38) and in studies of immunity to some other pathogens (72,73). CD4+ T cells also appear to play significant roles in HIV infection (74) and in a transgenic mouse model of hepatitis (75). Possible mechanisms for immune protection by CD4+ cells in the absence of CD8+ cells include cytokine secretion, cytolyis of MHC class II-expressing cells and help for augmented or accelerated antibody responses to challenge virus. We are currently investigating these possibilities in the mouse influenza model.

Our challenge studies in T cell-depleted mice extend the finding of balanced redundancy to DNA vaccination, with CD4+ or CD8+ cells each able to promote survival and recovery of vaccinated animals in the absence of the other subset. The reported requirement for MHC class I-restricted, CD8+ CTL in protection by DNA vaccination to NP alone (25) did not extend to the combination of influenza antigens and conditions we studied. Thus, under some circumstances CD4+ T cells may make quite an important contribution to protective immunity even when acting as the sole T cell responders rather than as help for CTL responses. Vaccine design and development should seek to evoke strong CD4+ responses in addition to other effectors, and monitoring of preclinical and clinical immunizations for them would be valuable.

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Abbreviations

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<tr>
<th>Abbreviation</th>
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<tbody>
<tr>
<td>A/Phil</td>
<td>A/Philippines/2/82/X-79</td>
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<tr>
<td>B6</td>
<td>C57BL/6</td>
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<tr>
<td>β2-m</td>
<td>β2-microglobulin</td>
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<td>CTL</td>
<td>cytotoxic T lymphocyte</td>
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<td>HA</td>
<td>hemagglutinin</td>
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<td>i.n</td>
<td>intranasal</td>
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<td>M</td>
<td>matrix</td>
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<td>NP</td>
<td>nucleoprotein</td>
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Reference

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