DNA immunization induces antibodies to the encoded protein, which indicates that the protein must gain access to the extracellular milieu, allowing it to interact with naïve B lymphocytes. It has been suggested that antigen release may be effected by cytotoxic-T-lymphocyte-mediated lysis of transfected antigen-expressing cells; this might be particularly important for the induction of responses to a noncytopathic, cytosolic protein. Here we show that the induction of antibody responses to one such DNA-encoded protein required neither perforin nor CD8\(^+\) T cells. In addition, there was no skewing of the immunoglobulin G isotypes in the absence of perforin.

DNA immunization is unique in that antigenic proteins are synthesized within the transfected cell in the absence of any associated infectious agent, and the vaccine itself contains no soluble protein that could initiate humoral immune responses. Thus, if the immunizing plasmid expresses a cytoplasmic protein which is relatively noncytopathic and is unable to be effectively processed endogenously by the major histocompatibility complex (MHC) class II antigen presentation pathway, then it might be ineffective in inducing humoral immune responses. The nucleoprotein (NP) of lymphocytic choriomeningitis virus (LCMV) meets these criteria, yet intramuscular injection with a plasmid expressing the NP (pCMV-NP) has been shown by us and others to induce antibody responses in mice (13, 23, 24). Antibodies are also induced following DNA immunization with plasmids expressing other cytoplasmic antigens, such as the measles virus and influenza virus NPs (4, 18, 21). How might such antigens be released from the transfected cell? We hypothesized that the development of CD8\(^+\) antigen-specific cytotoxic T lymphocytes (CTL) could lead to the recognition and lysis of cells expressing plasmid-derived NP, resulting in the liberation of protein into the extracellular milieu, where it can then interact with B cells and antigen-presenting cells (APCs). CTL-mediated release of LCMV NP occurs during virus infection (12), and the possibility that it occurs following DNA immunization was strengthened by our observations of a profound myositis following intramuscular inoculation of pCMV-NP into LCMV-infected or -immune mice; the peak of the inflammatory infiltrate in acutely infected mice coincided with the development of anti-LCMV CTL, and destruction of muscle cells occurred (8, 22). Professional APCs, which are known to be a source of plasmid-expressed antigens (5) and which appear to be the cell type responsible for initiating immune responses following DNA immunization (6, 7), can also be recognized and lysed by CTL. Dendritic cells infected with human immunodeficiency virus are susceptible to lysis in vitro by CTL (11), and there is evidence to suggest that CD8\(^+\) T cells can limit the immune response by lysing APCs in vivo (1–3). Therefore, the possibility of lytic release of protein is not limited to NP-expressing myocytes but extends to most transfected somatic cells, including APCs. To assess what effect such lysis may have on the generation or maintenance of B-cell responses following DNA immunization, we analyzed humoral immune responses in DNA-immunized mice that lacked the cytolytic protein perforin (10, 20). Although antigen-specific CD8\(^+\)-T-cell responses were induced in these mice by vaccination with pCMV-NP, they were unable to lyse NP-expressing cells in a perforin-dependent manner (data not shown).

**Strong antibody responses are induced by DNA immunization of PKO mice.** To determine if a lack of perforin-mediated lysis by antigen-specific CTL resulted in an alteration in the temporal appearance or maintenance of antiviral serum antibodies, antibody levels were measured in PKO and C57BL/6 mice at 0, 2, 4, and 6 weeks after they received a single 50-μg intramuscular injection of pCMV-NP DNA. Serum immunoglobulin G (IgG) levels in individual mice were measured by enzyme-linked immunosorbent assay (ELISA), and the average for each group was calculated based upon the optical density measurement at a dilution of 1:200; the results are shown in Fig. 1A. Within 2 weeks of vaccination, anti-LCMV antibodies were demonstrable in both PKO and C57BL/6 mice, and the average levels in both groups were similar. The slight drop in antibody levels in the C57BL/6 mice at 4 weeks after DNA immunization is not statistically significant, but at 6 weeks, the difference is highly significant. In mice, the average half-life of an IgG molecule is approximately 6 to 10 days (17, 19). Therefore, the high level of antibodies present at 6 weeks postimmunization implies ongoing synthesis of NP-specific IgG in PKO mice, whereas the drop in antibody levels indicates decreased IgG synthesis in C57BL/6 mice. A representative analysis of the antibody response at 6 weeks postimmunization is presented in Fig. 1B. Anti-LCMV serum antibody levels were measured by ELISA in individual perforin-positive (C57BL/6, n = 6) or perforin-negative (PKO, n = 8) animals 6 weeks postvaccination. LCMV DNA-vaccinated C57BL/6 and PKO mice both produced anti-LCMV IgG. Together, these data clearly show that perforin-mediated release of plasmid-expressed LCMV NP is not required for the induction of humoral responses following intramuscular DNA injection.
Isotype responses are unaltered in DNA-immunized perforin-deficient mice. IgG responses to the LCMV NP require CD4\(^+\) T-cell help, and unlike the LCMV glycoprotein, endogenously synthesized NP cannot gain access to the MHC class II antigen presentation pathway (15). Thus, the CD4\(^+\) T cells required for immunoglobulin isotype class switching must be primed by APCs which have acquired extracellular NP. To determine if the isotype pattern was skewed in the absence of perforin, titers of LCMV-specific IgG, IgG1, IgG2a, and IgG2b were determined by ELISA in individual PKO and C57BL/6 mice 6 weeks after DNA immunization (Fig. 2). At this time point, all of the pCMV-NP DNA-immunized mice contained virus-specific immunoglobulin in their serum. Anti-LCMV IgG endpoint titers among PKO mice ranged from 1:4,000 to 1:15,000, with a geometric mean titer (Fig. 2) of 1:6,825. In contrast, among the C57BL/6 vaccines, the total IgG titers ranged from 1:1,700 to 1:3,400, with a geometric mean of 1:2,455. Detectable IgG1 and IgG2a were present in the sera of all mice of both strains with the exception of a single PKO mouse which lacked demonstrable levels of anti-LCMV IgG1. Therefore, the vaccinated PKO mice showed no skewing of their isotype classes, suggesting that CD4\(^+\) T-cell responses are appropriately induced in these mice and providing further evidence that perforin is not required for the release of NP from transfected cells.

CD8\(^+\) T cells play no part in controlling humoral responses to DNA-encoded antigens. Our data show that perforin is not required to prime B-cell responses against a plasmid-expressed, cytoplasmic antigen. Perforin is expressed by CD8\(^+\) CTL, some CD4\(^+\) T cells, and natural killer (NK) cells; we can therefore conclude that perforin-mediated lysis by any of these cell populations is not required for DNA-mediated antibody induction. However, other CD8\(^+\) T-cell functions, such as the Fas pathway (16) or the secretion of cytotoxic cytokines, can mediate target cell death and thus could liberate intracellular antigens (9). Our observation that PKO mice had elevated antibody titers compared to those of C57BL/6 mice (Fig. 1 and 2) also raised the possibility that perforin-mediated lysis might actually depress humoral responses to DNA immunization by killing cells expressing foreign plasmid-encoded antigens. Consistent with this idea, CD8\(^+\) T-cell-mediated lysis of APCs has been shown to suppress immune responses (1–3), and perforin has been shown to play a role in regulating immunity (14). If this hypothesis is correct, the absence of perforin might result in elevated and/or prolonged responses to DNA-encoded immunogens. To determine whether CD8\(^+\) T cells might play a perforin-independent role in antigen release and antibody induction, CD8\(^+\) T cells were removed from C57BL/6 mice by immunodepletion prior to and throughout the course of a DNA immunization experiment. C57BL/6 mice were treated with either an anti-mouse CD8\(^+\) monoclonal antibody or saline daily for 3 days prior to immunization with pCMV-NP. Nondepleted PKO mice served as controls. Mice were bled biweekly, and the average IgG response for each group is shown in Fig. 3A. Antibody responses were easily detected in CD8-depleted mice, showing that CD8\(^+\) T-cell functions are not required to release soluble NP for the initiation of humoral immune responses. Furthermore, at all
MHC class II antigen presentation can elicit strong IgG responses which are relatively noncytotoxic and incapable of endogenous cell–induced.

In conclusion, it is clear that plasmids encoding proteins which are relatively nontoxic and incapable of endogenous MHC class II antigen presentation can elicit strong IgG responses in the absence of CD8 T cells and perforin. Thus, the mechanism of antigen release and B-cell stimulation remains to be determined. It is important that we identify the underlying mechanisms to permit the rational optimization of DNA vaccines.

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