spleen cells. Conversely, addition of neutralizing antibody to IFN-γ (*21*) to the wild-type splenocytes led to increased [^{3}H]thymidine incorporation by wild-type cells.

On day 5 the surviving cells from the MLR were harvested and tested for cytolytic activity against ^{51}Cr-labeled P815 target cells (Fig. 4D). The gko effector cells had enhanced lytic activity against target cells compared to the wild-type effectors. Fewer gko effectors (75% less) than wild-type effectors were required to yield 41% lysis of target cells. Addition of IFN-γ at the beginning of the MLR decreased the lytic activity of the gko effectors to the activity of the wild-type effectors. Addition of IFN-γ–neutralizing antibody at the beginning of the MLR had little effect on gko cytolytic activity, but increased the cytolytic activity of the wild-type cells to that of the gko effector cells. Therefore, IFN-γ was not required for the generation of cytolytic effector cells during the MLR, but instead may suppress the response to allogeneic cells.

Natural killer cells both produce IFN-γ and respond to IFN-γ (*22*). We therefore determined whether the absence of IFN-γ affected the cytolytic activity of splenic NK cells. Although resting splenic NK activity was low in the wild-type mice, reflecting reduced exposure to infectious agents and inbred mouse strain differences in NK activity (*23*), the gko mice had significantly lower (P = 0.001) resting splenic NK activity at all effector to target ratios (Fig. 5A). Polyinosinic:polycytidylic acid [poly(I:C)]–treated gko and wild-type mice had equivalent NK cytolytic activity, with approximately 30% lysis of target cells at the highest effector to target ratio (Fig. 5B), suggesting that an induction of type I interferons (*24*) can compensate for the absence of IFN-γ in NK activation. Due to the genetic background of the mice with respect to NK cell surface markers (*25*), we cannot distinguish between a lower number of NK cells or lower resting NK activity per cell in the gko mice compared to wild-type. However, a lower number of NK cells in the gko mice seems unlikely because the poly(I:C)-stimulated NK activities of gko and wild-type cells were equivalent. The significantly reduced resting splenic NK cytolytic activity of the gko mice may be of biological importance during a nonviral infection.

A role for IFN-γ has been postulated for a number of infectious and autoimmune diseases. The study of such diseases in this model will help define the in vivo role of IFN-γ and promote rational strategies for the therapeutic use of this immunoregulatory cytokine.

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31. We thank J. Hooley, M. Rehse, L. DeGuzman, and B. Hultgren for technical assistance; W. Anstine, K. Andow, and M. Severy for manuscript preparation; R. Ward, G. Wong, M. Palladino, and K. Bauer for advice; and R. Schreiber for IFN-γ neutralizing antibody.

1 December 1992; accepted 19 February 1993

Immune Response in Mice That Lack the Interferon-γ Receptor

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Interferon-γ (IFN-γ) exerts pleiotropic effects, including antiviral activity, stimulation of macrophages and natural killer cells, and increased expression of major histocompatibility complex antigens. Mice without the IFN-γ receptor had no overt anomalies, and their immune system appeared to develop normally. However, mutant mice had a defective natural resistance; they had increased susceptibility to infection by *Listeria monocytogenes* and vaccinia virus despite normal cytotoxic and T helper cell responses. Immunoglobulin isotype analysis revealed that IFN-γ is necessary for a normal antigen-specific immunoglobulin G2a response. These mutant mice offer the possibility for the further elucidation of IFN-γ–mediated functions by transgenic cell- or tissue-specific reconstitution of a functional receptor.

The IFN-γ receptor (IFN-γR) is a unique cell surface receptor that appears to be expressed almost ubiquitously (*1*, *2*). To inactivate the murine IFN-γR gene, we transfected embryonic stem (ES) cells with a replacement vector that contained 11 kb of the murine IFN-γR gene (Fig. 1A). We disrupted the gene by inserting the neomycin resistance gene into exon V, which encodes an extracellular, membrane-proximal portion of the receptor. Homologous integration occurred with a frequency of 1 out of 400 G418-resistant colonies. Chimeric founder males were mated with C57BL/6 or 129/Ev/Sv females to yield heterozygous (129/Sv/Ev × C57BL/6)F_1 or (129/Sv/Ev × 129/Sv/Ev)F_1 offspring. These were interbred to yield wild-type and IFN-γR$^{0/0}$ (129/Sv/Ev × C57BL/6)F_2 or (129/Sv/Ev × 129/Sv/Ev)F_2 offspring. Mice homozygous for the null mutation of the IFN-γR gene (Fig. 1B) were viable and had no apparent phenotypic anomalies by 12 months. Splenocytes obtained from 6-week-old mutant mice displayed no detectable binding sites for ^{125}I-labeled IFN-γ (Fig. 2A). The disrupted gene

might still encode a soluble, extracellular portion of the receptor. This protein would not be functional, because it lacks the cytoplasmic domain needed for signaling (3) and because the insertion was upstream of a cysteine residue that is essential for ligand binding (4). Functional inactivation of the IFN-γR gene was confirmed in an antiviral assay with wild-type or IFN-γR$^{0/0}$ primary embryonic fibroblasts (Fig. 2B). Cells from IFN-γR$^{0/0}$ embryos were insensitive to the antiviral activity of IFN-γ, whereas the response to IFN-α/β (type I IFNs) in both cultures was indistinguishable. Peritoneal macrophages of 6-week-old mutant and wild-type mice were assayed for nitrite release (5) upon stimulation with either IFN-γ or IFN-α/β (Fig. 2C). Macrophages from IFN-γR$^{0/0}$ mice proved insensitive to IFN-γ, whereas their response to IFN-α/β was the same as for macrophages from wild-type controls. Thus, IFN-γ and IFN-α/β utilize structurally and functionally independent receptor systems even though they may share some of the downstream signaling pathways (6, 7).

The effect of a nonfunctional IFN-γ system on major lymphocyte subpopulations was evaluated in 6- to 12-week-old IFN-γR–deficient and wild-type mice by comparative cytofluorometry. No differences in the expression of CD3, CD4, CD8, cell surface immunoglobulin M (IgM) (thymocytes and splenocytes), and major histocompatibility complex (MHC) class I and class II antigens (thymocytes, splenocytes, and peritoneal macrophages) were observed (8). The activity of natural killer (NK) cells in the spleens of animals pretreated with IFN-α/β was indistinguishable in wild-type and IFN-γR$^{0/0}$ mice, whereas IFN-γ was unable to stimulate NK cells in mutant mice (8). Thus, IFN-γ does not interfere with the normal development of lymphocyte subsets and is not limiting for constitutive MHC antigen expression.

Interferon-γ is crucial in the early defense against intracellular parasites such as *Listeria monocytogenes* (9, 10). IFN-γR–deficient and control mice were infected intravenously with 10^4 colony-forming units (CFU) of *L. monocytogenes*, a sublethal dose for wild-type mice. This inoculum proved lethal for most mutant mice (Fig. 3A). In comparison to titers in controls, bacterial titers increased approximately 100-fold in the livers and about 10-fold in the spleens of mutant mice.

To assess the consequences of IFN-γR ablation on antiviral T and B cell response, we infected mutant and control mice with vaccinia virus, lymphocytic choriomeningitis virus (LCMV), or vesicular stomatitis virus (VSV). We monitored replication of vaccinia virus (11) and specific immune parameters, including cytotoxic T cell

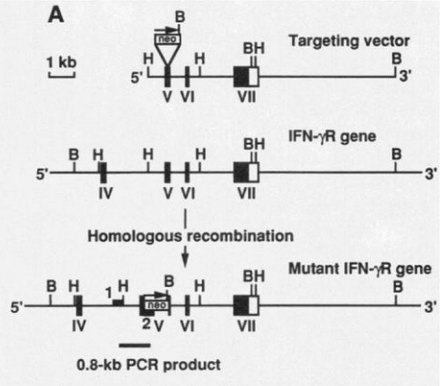

Fig. 1. Gene targeting of the murine IFN-γR gene and germline transmission of the disrupted allele. (**A**) Using the murine IFN-γR cDNA (31) as a probe, we isolated partially overlapping 15- and 17.5-kb fragments that encompassed the entire gene (approximately 25 kb) from an EMBL3 genomic library of brain DNA from C57BL/6 mice. The replacement vector encompassed 10 kb of the gene and contained exons V to VII. Exon VI encodes the transmembrane portion of the receptor. We disrupted the gene by inserting a blunted Xho I–Sal I fragment of pMC1neopA (Stratagene) into the blunted Aat II site of exon V. Exons are indicated by black boxes. H, Hind III; B, Bam HI. Identification of AB-1 ES cell clones (32) with homologous insertion of the replacement vector was done as described (33), with the following specific modifications: polymerase chain reaction (PCR) primer 1, derived from a genomic wild-type sequence immediately 5′ of the targeting construct, and primer 2, derived from the thymidine kinase promoter of the neomycin resistance gene, were designed to generate a 0.8-kb fragment in lysates from successfully targeted cells. Homologous recombination events were verified by Southern (DNA) blot analysis of Bam HI– or Hind III–digested genomic DNA. Bam HI digestion would generate a 4.7-kb Bam HI fragment after hybridization with the random oligonucleotide-labeled 2.0-kb Hind III fragment containing exon IV, if homologous insertion had disrupted exon V. (**B**) Southern blot analysis of Bam HI–digested genomic tail DNA of offspring from C57BL/6 females mated with chimeric founder males. Probe: 2.0-kb Hind III fragment containing exon IV. Lane 1, IFN-γR$^{+/0}$ heterozygote; lane 2, wild-type; lane 3, IFN-γR$^{0/0}$ homozygote. No additional integration events were detected with the 3.1-kb Hind III fragment that contained the disrupted exon V as a probe.

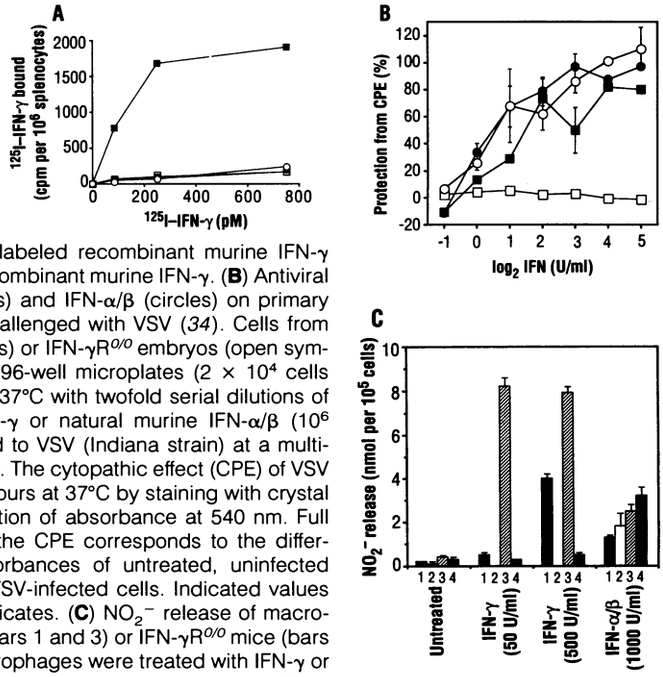

Fig. 2. (**A**) The binding of ^{125}I-labeled recombinant murine IFN-γ to splenocytes from IFN-γR$^{0/0}$ (circles) or wild-type mice (squares) at 4°C (1). Nonspecific binding (open symbols) was determined by simultaneous addition of a 100-fold excess of unlabeled recombinant murine IFN-γ (10^7 U/mg) to labeled recombinant murine IFN-γ. (**B**) Antiviral activity of IFN-γ (squares) and IFN-α/β (circles) on primary embryonic fibroblasts challenged with VSV (34). Cells from wild-type (closed symbols) or IFN-γR$^{0/0}$ embryos (open symbols) were incubated in 96-well microplates (2×10^4 cells per well) for 24 hours at 37°C with twofold serial dilutions of recombinant murine IFN-γ or natural murine IFN-α/β (10^6 U/mg) and then exposed to VSV (Indiana strain) at a multiplicity of infection of 10^{-3}. The cytopathic effect (CPE) of VSV was quantified after 36 hours at 37°C by staining with crystal violet and the determination of absorbance at 540 nm. Full protection (100%) from the CPE corresponds to the difference between the absorbances of untreated, uninfected cells and of untreated, VSV-infected cells. Indicated values are means ± SD of duplicates. (**C**) NO_2^- release of macrophages from wild-type (bars 1 and 3) or IFN-γR$^{0/0}$ mice (bars 2 and 4). Peritoneal macrophages were treated with IFN-γ or IFN-α/β for 48 hours in the absence (bars 1 and 2) or presence (bars 3 and 4) of lipopolysaccharide (100 ng/ml). Indicated values are means ± SD of triplicate determinations (5, 35).

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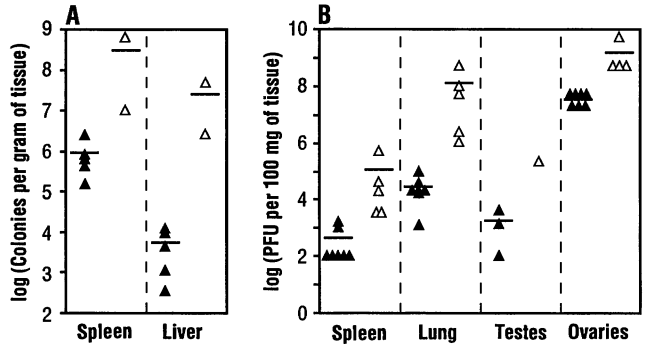

Fig. 3. Defense of IFN-$\gamma R^{0/0}$ (open symbols) and wild-type mice (closed symbols) against infection by *L. monocytogenes* (**A**) and vaccinia virus (**B**). In (A), five wild-type or IFN-$\gamma R^{0/0}$ mice were infected intravenously with 10^4 CFU of *L. monocytogenes* on day 0. The number of bacteria recovered from spleens and livers on day 5 was determined by colony formation assay on trypticase-soy agar (*36*). The values for individual mice are shown. In this experiment, three out of five mutant mice died before day 5, whereas all control mice survived. Horizontal bars indicate mean values. In (B), 11 wild-type or IFN-$\gamma R^{0/0}$ mice were infected intravenously with 10^6 PFU of vaccinia virus (WR strain) on day 0. Six of 11 IFN-$\gamma R^{0/0}$ mice died on days 3 and 4, whereas all control mice survived. The number of vaccinia virus PFU recovered from the various organs on day 5 was determined (*11*). The values from three independent experiments are compiled and shown for individual mice. Horizontal bars indicate mean values.

(CTL) responses to vaccinia virus and LCMV (*12*), footpad swelling in response to inoculation of LCMV (*13*), and production of neutralizing T cell help–independent IgM and T cell help–dependent IgG antibodies after VSV infection (*14*). The early defense against vaccinia virus, which replicates only poorly in mice, was severely defective because within the first few days of infection this virus replicated to titers 10^2- to 10^3-fold greater than those found in wild-type mice and caused lethal pathology (Fig. 3B). Still, mutant mice mounted a normal CTL response against vaccinia virus (Fig. 4A). Like mice treated with antibody to IFN-γ (*15*), mutant mice mounted a slightly reduced CTL response against LCMV (*8*), probably as a result of enhanced virus replication. Mutant mice generated a potent anti-LCMV T cell response in vivo (Fig. 4B) because they had slightly increased, strictly $CD8^+$ T cell–dependent early footpad swelling upon local infection with LCMV and a prolonged $CD4^+$ T cell–dependent second phase of the swelling reaction 10 days after infection (*13*) (Fig. 4B), which is consistent with enhanced viral replication. The generated CTLs were able to cause lethal immunopathology (*16*) because four of four mutant mice infected intracerebrally with 100 plaque-forming units (PFU) of LCMV (Armstrong) died after 6 days of $CD8^+$ T cell–mediated choriomeningitis, whereas three of three mutant mice treated with antisera to CD8 (*17*) survived. The course of infection with VSV was unaltered in IFN-$\gamma R^{0/0}$ mice, and neutralizing antibody titers to VSV were as in controls (*8*).

Interferon-γ is involved in Ig isotype regulation (*18*). Mutant mice had decreased total serum IgG2a concentrations (*8*). At day 12 after immunization with trinitrophenyl-conjugated ovalbumin, mutant mice also had decreased titers of hapten-specific IgG2a and, to a lesser extent, of IgG3 antibodies (Fig. 4C). A similar isotype pattern was observed at day 21 after immunization (*8*), which indicates that in mutant mice the response was not merely delayed. IgE antibodies were undetectable in this primary response. Highly reduced IgG2a titers were also observed in IFN-$\gamma R^{0/0}$ mice immunized with live, attenuated pseudorabies virus (*19*). Thus, IFN-γ not only enhances (*18*, *20*) but is necessary for the generation of a normal IgG2a response, even though it seems nonessential for switching to this isotype.

In pigs, IFN-γ is constitutively expressed by the trophectoderm of implanting embryos: thus, IFN-γ may participate in feto-maternal interaction (*21*). However, our results show that, at least in mice, IFN-γ is not required for normal embryonic development, the development of the immune system, or the generation of a specific immune response. In contrast, IFN-γ is crucial in early defense against infectious agents such as *L. monocytogenes* and vaccinia virus. Although the precise nature of this defect remains unclear, it is reasonable to speculate that small amounts of endogenous IFN-γ regulate basic microbicidal activities of macrophages or neutrophils (*10*). It is unclear whether the surprisingly high susceptibility of mutant mice to infection by vaccinia virus was a result of a lack of direct antiviral activity of IFN-γ, as has been proposed to explain enhanced replication of LCMV in mice treated with antibodies to IFN-γ (*15*, *22*). We could not confirm a proposed limiting role for IFN-γ in the generation and maturation of virus-specific CTLs (*23*). In IFN-$\gamma R^{0/0}$ mice pretreated with IFN-α, vaccinia virus replicat-

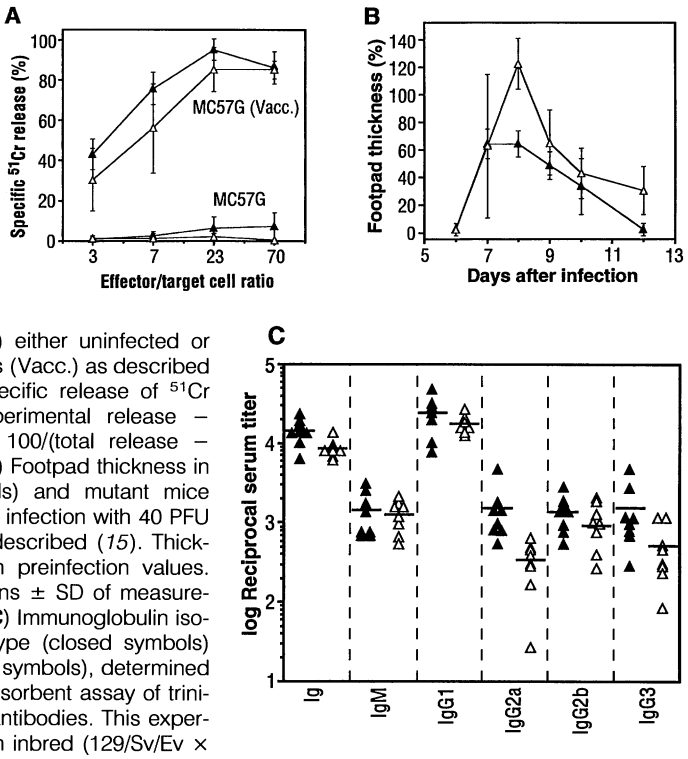

Fig. 4. Specific immune response in IFN-$\gamma R^{0/0}$ and wild-type mice. (**A**) Cytotoxic activities of T cells (*H-2*^{b}) from spleens of wild-type (closed symbols) and IFN-$\gamma R^{0/0}$ mice (open symbols) challenged in vivo with 2×10^6 PFU of vaccinia virus, determined on day 5 after infection. Target cells were MC57G cells (*H-2*^{b}) either uninfected or infected with vaccinia virus (Vacc.) as described (*12*). The percentage specific release of ^{51}Cr was calculated as [(experimental release − spontaneous release) × 100/(total release − spontaneous release)]. (**B**) Footpad thickness in wild-type (closed symbols) and mutant mice (open symbols) after local infection with 40 PFU of LCMV (WE strain) as described (*15*). Thickness was compared with preinfection values. Indicated values are means ± SD of measurements on four footpads. (**C**) Immunoglobulin isotype distribution in wild-type (closed symbols) and IFN-$\gamma R^{0/0}$ mice (open symbols), determined by enzyme-linked immunosorbent assay of trinitrophenyl (TNP)-specific antibodies. This experiment was carried out with inbred (129/Sv/Ev × 129/Sv/Ev)F_2 mice. Eight 6- to 12-week-old mice were immunized by injection at the base of the tail with 100 μg of TNP-ovalbumin in complete Freund's adjuvant (Difco). Hapten-specific serum antibody titers were determined after 12 days with plates coated with TNP–bovine serum albumin and isotype-specific, alkaline phosphatase–conjugated antibodies (Southern Biotechnology Associates). Antibody titers were determined by twofold serial dilutions and are shown for individual mice. Horizontal bars indicate mean values. Preimmune titers were below 2^{-5}.

ed 10- to 100-fold less than in untreated mice but still at least 10-fold more than in IFN-α–treated control mice (*8*). These preliminary results indicate that in vivo, IFN-γ and IFN-α/β may exert their antiviral activity through different, in part nonredundant, pathways.

Interferon-γ has been proposed to regulate antigen processing at the level of both proteolytic generation (*24*) and transport of MHC class I–binding peptides (*25*). The finding that IFN-γR–deficient mice mounted normal T or B cell responses suggests that these IFN-γ effects are not essential. Still, several questions regarding the role of IFN-γ in modulating certain immune reactions remain to be answered. Thus, the balance between T helper cell subsets (*26*) known to determine the immune response to parasites such as *Leishmania* (*27*) may be affected in IFN-γR$^{0/0}$ mice. IFN-γ has been implicated in autoimmunity (*28–30*). Investigating the consequences of a genetic IFN-γR defect in these immune reactions will require backcrossing of the mutation into appropriate genetic backgrounds. In addition to its direct analytical potential, the approach of genetically inactivating the IFN-γ receptor offers the possibility of cell- or tissue-specific reconstitution of a functional receptor to further elucidate IFN-γ–mediated functions. Moreover, combining the IFN-γR defect with other cytokine deficiencies through appropriate breeding of mice may help to reveal interactions of various cytokines in vivo.

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34. We obtained primary embryonic fibroblasts by dissecting and trypsinizing 12-day-old embryos and cultured them in Dulbecco's minimum essential medium (Gibco) and 10% fetal calf serum.
35. Macrophages were collected from the peritoneal cavity of mice 4 days after intraperitoneal injection of 2 ml of 4% thioglycollate broth. Adherent cells were incubated at 37°C with or without lipopolysaccharide (from *Escherichia coli* 0127:88, Sigma), recombinant murine IFN-γ (10^7 U/mg, Genzyme, Cambridge, MA), or natural murine IFN-α/β (9.8×10^6 U/mg, Lee Biomolecular, San Diego, CA). After 48 hours of incubation, the NO_2^- concentration was determined.
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37. We thank W. Wille for the C57BL/6 genomic library; A. Bradley for advice and for the AB-1 ES cells; M. Evans for 129/Sv/Ev mice; G. Adolf for recombinant murine IFN-γ; I. Gresser for natural murine IFN-α/β; H. Hengartner and H. P. Pircher for antibodies to lymphocyte surface antigens; H. R. Büeler, J. Le, L. Reis, S. Alkan, and L. Glimcher for advice and help with the various assays; E. Horvath and G. Stark for excellent technical assistance; and C. Weissmann for support and suggestions. Supported in part by the Kanton of Zürich, by grants 31-28642.90 and 31-32195.91 from the Swiss National Science Foundation (M.A. and R.M.Z., respectively), by grants from the Human Frontier Science Program (M.A. and J.V.), by grant CA 49731 from the National Cancer Institute (United States) (J.V.), by the European Molecular Biology Organization (W.H.), and by F. Hoffmann–La Roche AG, Basel.

22 September 1992; accepted 4 January 1993

Heterologous Protection Against Influenza by Injection of DNA Encoding a Viral Protein

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Cytotoxic T lymphocytes (CTLs) specific for conserved viral antigens can respond to different strains of virus, in contrast to antibodies, which are generally strain-specific. The generation of such CTLs in vivo usually requires endogenous expression of the antigen, as occurs in the case of virus infection. To generate a viral antigen for presentation to the immune system without the limitations of direct peptide delivery or viral vectors, plasmid DNA encoding influenza A nucleoprotein was injected into the quadriceps of BALB/c mice. This resulted in the generation of nucleoprotein-specific CTLs and protection from a subsequent challenge with a heterologous strain of influenza A virus, as measured by decreased viral lung titers, inhibition of mass loss, and increased survival.

A challenge to the development of vaccines against viruses such as influenza A or human immunodeficiency virus (HIV), against which neutralizing antibodies are generated, is the diversity of the viral envelope proteins among different isolates or strains. Because CTLs in both mice and humans are capable of recognizing epitopes derived from conserved internal viral proteins (*1*) and are thought to be important in the immune response against viruses (*2*), efforts have been directed toward the development of CTL vaccines capable of providing heterologous protection against different viral strains. $CD8^+$ CTLs kill virally infected cells when their T cell receptors recognize viral peptides associated with major histocompatibility complex (MHC) class I molecules (*3*). These peptides are derived from endogenously synthesized viral proteins, regardless of the protein's location or function in the virus. Thus, by recognition of epitopes from conserved viral proteins, CTLs may provide cross-strain protection. Peptides capable of associating with MHC class I molecules for CTL recognition originate from proteins that are present in or pass through the cytoplasm or endoplasmic reticulum (*4*). Therefore, in general, exogenous proteins,

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