

## Response to Influenza Infection in Mice with a Targeted Disruption in the Interferon $\gamma$ Gene

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### Summary

Interferon  $\gamma$  (IFN- $\gamma$ ) is a pleiotropic cytokine secreted by T lymphocytes and natural killer (NK) cells and has been noted to be a first line of host defense in the control of viral infections. To examine further the role of this cytokine in the control of viral infections, mice with a targeted mutation in the *IFN- $\gamma$*  gene were infected with influenza virus, and the in vivo antibody and cell-mediated immune response to viral infection were examined. In addition, cell lines and clones were derived from the immunized animals and the in vitro cytokine production and cytotoxic T lymphocyte (CTL) response were analyzed. The absence of IFN- $\gamma$  led to increased production of influenza-specific IgG1, IL-4, and IL-5 as compared to wild-type littermate control animals. In contrast, there was no difference noted in the development of an effective CTL response between IFN- $\gamma$ -deficient and wild-type animals. In this model of experimental influenza infection, IFN- $\gamma$  is not necessary for the development of an effective humoral or cellular immune response to challenge with this respiratory virus.

IFN- $\gamma$ , also known as immune or type II interferon, is a pleiotropic cytokine secreted by T lymphocytes and NK cells. It has long been believed to be a first line of host defense in the control of viral infections. Its effects are multifaceted and include both direct and indirect activities. The direct antiviral activity attributed to IFN- $\gamma$ , as well as to IFN- $\alpha$  and IFN- $\beta$ , includes the induction of proteins and enzymes that inhibit viral multiplication by impairing accumulation of viral-specific mRNA, double-stranded RNA, and proteins (1). In addition, class I MHC-restricted CTL have been shown to secrete IFN- $\gamma$  upon antigenic stimulation (2–4) and the differentiation of precursors to activated CTL has been reported to be dependent on the action of IFN- $\gamma$  (5, 6). The indirect effects of IFN- $\gamma$  include inhibition of cell growth and up-regulation of class I and II MHC molecules on APC, specifically macrophages (7, 8). IFN- $\gamma$  may also regulate antigen processing by augmenting the proteolysis and peptide transport machinery in APC (9, 10).

The role of CTL in the host defense to influenza infection has been examined by a number of investigators (11–16). Both heterogeneous populations of immune spleen cells and cloned influenza-specific CTL have been shown to lyse virally infected, MHC-matched target cells in vitro and to reduce lung virus titers and promote survival after adoptive transfer to influenza virus-infected mice in vivo. The role of secreted cytokines by activated CTL, most notably IFN- $\gamma$ , in the ob-

served in vitro and in vivo antiviral effects has been unclear, and the relative importance of direct cytolysis versus soluble factors released by CTL has not been definitively determined. Although it has been suggested that CTL mediate their antiviral protective effects in vivo via direct cytolysis of virus-infected cells (16), the potential role of soluble mediators released by the clones in high local concentrations could not be ruled out as playing a significant role in recovery from influenza infection. The role that IFN- $\gamma$  plays in clearance of influenza virus mediated by CD4<sup>+</sup> MHC class II-restricted cytotoxic T cells is even less clear. In general, CD4<sup>+</sup> cytotoxic T cells that have been shown to exhibit cytolytic effector functions in vitro and in vivo have belonged to the Th1 subset of murine helper T cell clones (17, 18, and Graham, M. B., unpublished observations) which secrete IFN- $\gamma$  and IL-2 upon stimulation (19). These MHC class II-restricted CTL have been shown to promote virus clearance when adoptively transferred into influenza-infected animals.

Recently, it has become possible to directly examine the role of cytokines in immune and inflammatory responses using gene disruption strategies (20–22). In this report, we examine the in vivo cellular and humoral immune response to infection with type A influenza virus in mice with a targeted mutation in the *IFN- $\gamma$*  gene. In addition, the in vitro and in vivo antiviral effector activities of virus-specific cytolytic T lymphocytes derived from these animals are compared with

those of wild-type (WT)<sup>1</sup> littermates for antiviral activity in adoptive transfer. We show that IFN- $\gamma$  is not necessary for either recovery from experimental influenza infection or for the *in vivo* effector activity of influenza-specific CD4<sup>+</sup> or CD8<sup>+</sup> cytotoxic T cells which are cytolytic *in vitro* and protective *in vivo*.

## Materials and Methods

**Animals.** Male (8–9 wk of age) and female (6–7 wk of age) mice homozygous for a targeted disruption of the IFN- $\gamma$  gene (IFN- $\gamma^{\text{KO}}$ , H-2<sup>b</sup>) and WT littermates (IFN- $\gamma^{\text{wt/wt}}$ , H-2<sup>b</sup>) were derived as previously described (23). Murine pathogen-free male and female C57Bl/6 (H-2<sup>b</sup>) 4–6-wk-old mice were purchased from Taconic Farms, Inc. (Germantown, NY) and used at 6–9 wk of age.

**Viruses.** Influenza virus strain A/JAP/57 (A/Japan/305/57 [H<sub>2</sub>N<sub>2</sub>]) was grown in the allantoic cavity of 10-d-old embryonated hen eggs and stored as infectious allantoic fluid as previously described (24). Determination of virus titer, expressed as hemagglutinating units (HAU), was done as previously described (24).

**Peptide.** The peptide corresponding to residues 366–379 of the influenza A/Japan/305/57 nucleoprotein (NP 366–379) was produced manually by the rapid amino acid multiple peptide synthesizer system (DuPont Co., Wilmington, DE) and dissolved in DMSO (Sigma Chemical Co., St. Louis, MO) before final dilution in tissue culture medium.

**Cell Lines.** The EL-4 (H-2<sup>b</sup>) thymoma was maintained in RPMI (GIBCO BRL, Gaithersburg, MD) supplemented with 10% (vol/vol) heat-inactivated FCS (HIFCS; Hyclone Laboratories, Logan, UT), 1% glutamine (GIBCO BRL) and antibiotics (10 U/ml penicillin G and 10  $\mu$ g/ml streptomycin sulfate; GIBCO BRL). The LB15.13 (H-2<sup>b,d</sup>) B cell hybridoma was maintained in high glucose DME, 10% HIFCS, 1% glutamine, 1% nonessential amino acids, 0.5% sodium pyruvate (all from GIBCO BRL), 5  $\times$  10<sup>-5</sup> M 2-ME, and antibiotics.

**Immunization and Collection of Serum.** Six 8-wk-old female IFN- $\gamma^{\text{KO}}$  mice and six age-matched WT littermate female mice were bled via tail veins before immunization intraperitoneally with a 1:10 dilution of A/JAP/57 allantoic fluid (5  $\times$  10<sup>2</sup> HAU) in PBS. Mice were then bled on days 7, 14, 21, and 28 after immunization. Two mice from each group were killed on day 7 for analysis of *in vivo* primary cytolytic activity (see Results). The remaining four mice from each group were killed two at a time on days 21 and 28.

**Establishment and Maintenance of T Lymphocyte Bulk Cultures and Clones.** The procedures developed to establish and maintain influenza-specific bulk cultures clones are described in detail elsewhere (25) with a few minor modifications. Briefly, spleen cells from immunized IFN- $\gamma^{\text{KO}}$  and IFN- $\gamma^{\text{wt/wt}}$  (described above) were harvested 1, 3, or 4 wk after immunization. *In vitro* secondary bulk cultures from individual mice were established by infecting one third of the autologous spleen cells with influenza A/JAP/57, combining these cells with the two-thirds uninfected splenocytes, and culturing the cells at a density of 5  $\times$  10<sup>6</sup> cells per ml for 14 d in IMDM (GIBCO BRL), 10% HIFCS, 1% glutamine, 5  $\times$  10<sup>-5</sup> M 2-ME, and antibiotics (complete media). Subsequently, the bulk cultures were stimulated *in vitro* with influenza A/JAP/57 virus-infected,  $\gamma$ -irradiated (2,000 rad) C57Bl/6 spleen cells every 14 d with 10 U/ml human recombinant IL-2 (huRIL-2; Biosource In-

ternational, Inc., Camarillo, CA) in complete media. Clones were derived by limiting dilution of viable cells from *in vitro* secondary bulk cultures. Clones were selected, expanded, and maintained in the presence of influenza A/JAP/57 virus-infected,  $\gamma$ -irradiated C57Bl/6 spleen cells in complete media with 10 U/ml huRIL-2. The clones were restimulated every 7 d in six-well cluster tissue culture plates (model 3506; Costar Corp., Cambridge, MA) containing 10<sup>5</sup>/ml clone cells, 5  $\times$  10<sup>5</sup>/ml infected, irradiated C57Bl/6 spleen cells, and 10 U/ml huRIL-2 in complete media.

**Cytokine Production.** Supernatants were collected from *in vitro* secondary bulk cultures 48 h after stimulation and frozen at -20°C. Supernatants from clones were harvested 48 h after stimulation in the absence of huRIL-2 and frozen at -20°C. Production of IFN- $\gamma$  was assayed by ELISA as previously described (26) using Immulon 96-well flat-bottom plates (Dynatech Laboratories, Inc., Chantilly, VA). Production of IL-2, IL-4, and IL-5 was determined using murine cytokine immunoassay kits (Intertest-2X mouse IL-2 ELISA kit from Genzyme Corp., Cambridge, MA; and murine IL-4 ELISA and murine IL-5 ELISA kits from Endogen, Inc., Boston, MA). ELISA plates were read using an automated microplate reader (model EL 340; Bio Tek Instruments, Inc., Winooski, VT).

**Serum Ig Production.** ELISA plates were prepared by coating Immulon 96-well flat-bottom plates (Dynatech Laboratories, Inc.) with 10<sup>4</sup> HAU influenza A/JAP/57 allantoic fluid in 100  $\mu$ l PBS (pH 7.3) per well. Plates were covered and stored overnight at 4°C. The plates were then washed three times with 1  $\times$  ELISA wash buffer (0.02 M NaH<sub>2</sub>PO<sub>4</sub>, 0.1 M Na<sub>2</sub>HPO<sub>4</sub>, 1.5 M NaCl, and 0.5% Tween 20, pH 7.4). Plates were blocked for 1 h with ELISA working and blocking buffer (2% BSA and 0.1% Tween 20 in 1  $\times$  PBS, pH 7.3). Plates were washed three times, and serial dilutions of sera from each animal (diluted in working buffer) were added to the plate and incubated for 2 h at 37°C. Plates were washed three times, a 1:1,000 dilution of alkaline phosphatase-conjugated goat anti-mouse IgG1 or IgG2a (Southern Biotechnology Associates, Birmingham, AL) was added to each well, and plates were incubated for 2 h at 37°C. The plates were washed a final three times, 1 mg/ml *p*-nitrophenyl phosphate disodium (Sigma Chemical Co.) in substrate buffer (1 M diethanolamine, 0.1 mg/ml MgCl<sub>2</sub>, and 0.2% NaN<sub>3</sub>) was added to each well, and a color change was allowed to develop for 1 h. OD read at 415 nm on an ELISA microplate reader (Bio Tek Instruments, Inc.).

**Assays for Cell-mediated Cytotoxicity.** The <sup>51</sup>Cr-release cytotoxicity assay was carried out as previously described (24). Briefly, 10<sup>6</sup> target cells, either EL-4 or LB15.13, were either left uninfected, infected with influenza A/JAP/57, or pulsed with 1.0  $\mu$ g/ml NP 366–379 in the presence of <sup>51</sup>Cr for 1.5 h at 37°C. Targets were then washed three times and plated at 5  $\times$  10<sup>3</sup> cells per well in 96-well, flat-bottom, microtiter tissue culture plates in 0.1-ml vol of RPMI plus 10% HIFCS. Effector cells were added in a 0.1-ml vol of RPMI plus 10% HIFCS to appropriate wells in quadruplicate. E/T ratios ranged from 5:1 to 100:1, depending on the assay. The plates were incubated at 37°C in 10% CO<sub>2</sub> for 3 h. From each well, 0.1 ml of supernatant was removed and counted on a gamma counter (Isomedic; ICN Biomedicals, Inc., Costa Mesa, CA). The percent specific lysis was determined as previously described (16). SEM were always <5% of the mean value and are omitted.

**Intranasal Influenza Virus Inoculation.** Intranasal inoculation of mice was performed as previously described (16). The procedure was modified from that described by using light anesthesia from methoxyflurane (Pitman-Moore, Mundelein, IL). To evaluate a dose-response to intranasal virus, animals received serial 10-fold dilutions of allantoic fluid in cold PBS ranging from 10<sup>-2</sup> to 10<sup>-6</sup>,

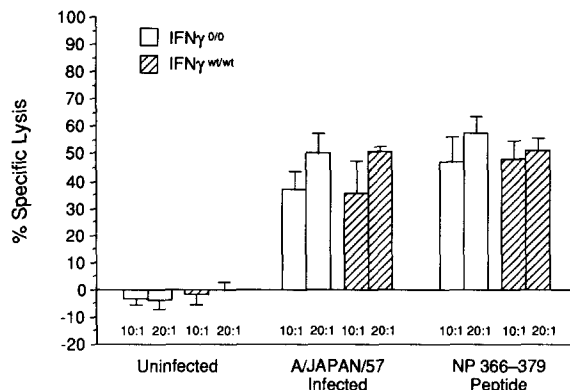
<sup>1</sup> Abbreviations used in this paper: HAU, hemagglutinating unit; HIFCS, heat-inactivated FCS; huRIL-2, human recombinant IL-2; NP, nucleoprotein; WT, wild type.

and animals were watched daily for morbidity and/or mortality. LD<sub>50</sub> values were calculated according to a modified Spearman and Karber method (27).

**Adoptive Transfer Procedure.** Adoptive transfer of day 6 viable cloned cells was performed as previously described (16). 9-wk-old male C57Bl/6 mice were intranasally inoculated with 10 LD<sub>50</sub> influenza A/JAP/57 virus and within 30 min, 10<sup>7</sup> clone cells in 0.5 ml IMDM (GIBCO BRL) were injected intravenously. Control mice were injected intravenously with 0.5 ml IMDM alone. Mice were watched daily for 21 d for morbidity and/or mortality.

## Results

**Ability of IFN- $\gamma$ -deficient Mice and WT Littermates to Generate a Cytotoxic T Cell Response to Challenge with Influenza.** To evaluate the role of IFN- $\gamma$  in the murine host response to infectious influenza virus, we immunized H-2<sup>b</sup> haplotype mice generated with a targeted mutation in the IFN- $\gamma$  gene, and with no demonstrable IFN- $\gamma$  production, and their WT littermates. Six female mice homozygous for the mutation in the IFN- $\gamma$  gene, designated IFN- $\gamma$  deficient, and six WT female littermates were initially analyzed for the ability to mount a CTL response to immunization with influenza A/JAP/57 virus. In vitro secondary bulk cultures of immune splenocytes from both IFN- $\gamma$  deficient and WT animals were able to lyse virally infected targets with similar efficiencies (Fig. 1). In addition, MHC class II-negative, H-2D<sup>b</sup>-expressing EL4 cells treated with a synthetic peptide corresponding to type common influenza NP were also recognized equally well by IFN- $\gamma$  deficient and WT bulk cultures. This peptide (amino acid 366-379) mimics an immunodominant epitope on the NP recognized by class I MHC-restricted T lymphocytes from H-2<sup>b</sup> haplotype mice in association with H-2D<sup>b</sup> (28). Analysis of in vivo primary CTL responses by standard chromium release assay did not reveal demonstrable influenza-



**Figure 1.** Recognition of target cells by in vitro secondary bulk splenocyte cultures derived from IFN- $\gamma$ -deficient (IFN- $\gamma$ <sup>o/o</sup>) and wild-type (IFN- $\gamma$ <sup>wt/wt</sup>) littermate mice. Secondary bulk cultures derived from IFN- $\gamma$ <sup>o/o</sup> mice (open columns) or from IFN- $\gamma$ <sup>wt/wt</sup> mice (hatched columns) were tested for reactivity on mock (uninfected), A/JAPAN/57-infected, or NP<sub>366-379</sub> peptide-treated (1  $\mu$ g/ml) EL-4 target cells. E/T ratios are shown under each column. SEM depicted by error bars. Spontaneous release from mock- and peptide-treated EL-4 cells was <15%. Spontaneous release from A/JAPAN/57-infected EL-4 cells was <20%.

specific CTL from either IFN- $\gamma$  deficient or WT littermates (data not shown).

**Cytokine Production by In Vitro Secondary Bulk Cultures from IFN- $\gamma$  Deficient and WT Mice.** To ensure that splenocytes from immunized IFN- $\gamma$ -deficient mice did not produce any measurable IFN- $\gamma$ , supernatants from in vitro secondary bulk cultures were analyzed for the production of IFN- $\gamma$ . As expected, IFN- $\gamma$ -deficient bulk cultures released no detectable IFN- $\gamma$ , whereas bulk cultures from WT animals produced variable amounts of IFN- $\gamma$  (Table 1). The same supernatants were then analyzed for the production of IL-2, IL-4, and IL-5 (Table 1). In the absence of IFN- $\gamma$ , the production of IL-4 and IL-5 was significantly higher than that in WT bulk cultures. There was no statistical difference in the amounts of IL-2 produced by the IFN- $\gamma$ -deficient bulk cultures versus those from WT animals. Subsequent stimulation of bulk cultures with irradiated, virus-infected splenocytes from WT mice yielded identical results (data not shown). Stimulation of IFN- $\gamma$ -deficient cultures with irradiated, infected splenocytes from normal mice that have an intact IFN- $\gamma$  gene, did not alter the cytokine production profile of the cultures.

**Humoral Immune Response to Immunization with Influenza Virus in IFN- $\gamma$ -deficient and WT Mice.** IFN- $\gamma$  has been reported to be crucial in antibody class switch during the maturation of a humoral immune response (29, 30). To assess the impact of IFN- $\gamma$  in determining the humoral immune response of the animals to immunization with influenza, mice were inoculated parenterally with influenza virus, and sera from serial bleeds of the immunized animals was assayed for influenza-specific IgG1 and IgG2a antibodies (Table 2). In mice lacking IFN- $\gamma$ , the IgG1 antibody response to type A influenza was significantly higher than the response of the WT animals. This finding is in agreement with previous studies (29) that have shown that IFN- $\gamma$  blocks the ability of B cells to respond to IL-4, thereby decreasing synthesis of IgG1 and IgE. Thus, in the absence of IFN- $\gamma$  and an increased production of IL-4 (Table 1), antibody class switch

**Table 1.** Cytokine Production by In Vitro Secondary Bulk Splenocyte Cultures Derived from IFN- $\gamma$ -deficient (IFN- $\gamma$ <sup>o/o</sup>) and Wild Type (IFN- $\gamma$ <sup>wt/wt</sup>) Littermate Mice

Cytokine produced	IFN- $\gamma$ <sup>o/o</sup>	IFN- $\gamma$ <sup>wt/wt</sup>
IFN- $\gamma$ (U/ml)*	None detectable	154.8 (72.3) <sup>‡</sup>
IL-2 (pg/ml)	408.3 (279.4)	150.0 (75)
IL-4 (pg/ml)	342.2 (64.8) <sup>§</sup>	66.8 (31.1)
IL-5 (pg/ml)	1,846.7 (611.6) <sup>§</sup>	605 (140.75)

\* 48-h culture supernatants from in vitro secondary bulk splenocyte cultures were assayed on at least two separate occasions for cytokine production by ELISA.

† Values equal the average amount of each cytokine produced by six separate IFN- $\gamma$ <sup>o/o</sup> bulk cultures and six separate IFN- $\gamma$ <sup>wt/wt</sup> bulk cultures. SEM for each set of data are shown in parentheses.

§ Data represent significantly higher levels of cytokine produced.

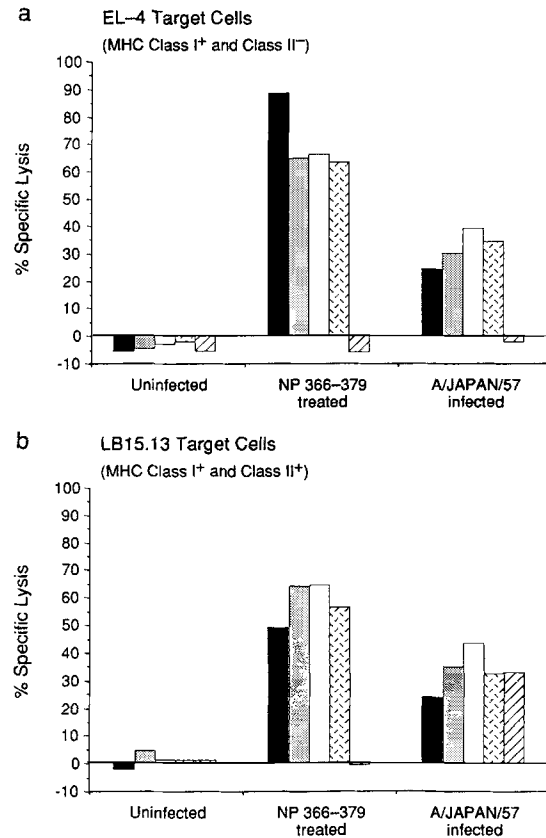
**Table 2.** Influenza Virus-specific Antibody Production in IFN- $\gamma$  Deficient and WT Littermate Mice

Ab production (OD <sub>415</sub> )	Serum dilution	WT group	IFN- $\gamma$ -deficient group
IgG1*	1:100 <sup>†</sup>	0.51 (0.14) <sup>§</sup>	0.99 (0.13)
	1:200	0.42 (0.08)	0.96 (0.10)
	1:400	0.38 (0.06)	0.87 (0.08)
	1:800	0.33 (0.04)	0.70 (0.08)
	1:1600	0.25 (0.03)	0.50 (0.06)
	1:3200	0.16 (0.02)	0.30 (0.04)
IgG2a	1:100	1.51 (0.22) <sup>§</sup>	1.08 (0.19)
	1:200	1.45 (0.20)	1.01 (0.21)
	1:400	1.30 (0.17)	0.86 (0.20)
	1:800	1.10 (0.15)	0.69 (0.17)
	1:1600	0.82 (0.11)	0.50 (0.14)
	1:3200	0.54 (0.07)	0.33 (0.09)

\* *P*-value for comparison of the two groups for IgG1 production: 0.005. *P*-value for comparison of the two groups for IgG2a production: 0.14. <sup>†</sup> All sera collected on day 14 after immunization. <sup>§</sup> Values represent the mean OD of four animals from each group with SEM in parentheses.

to IgG1 is favored. Although the production of IgG2a was slightly diminished in the IFN- $\gamma$ -deficient animals, the level was not significantly lower than the amount made by the WT animals. This result is in keeping with previous *in vitro* studies which examined the effect of neutralizing anti-IFN- $\gamma$  antibody on isotype switch during humoral immune responses (31). Analysis of preimmune serum samples from these animals revealed no detectable levels of influenza-specific IgG1 or IgG2a (data not shown).

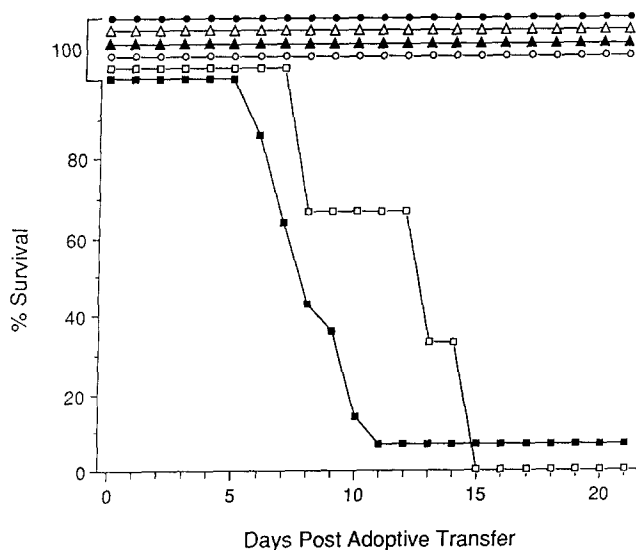
**Evaluation of *In Vitro* and *In Vivo* Effector Function of Clones from IFN- $\gamma$ -deficient and WT Animals.** To investigate further the role of IFN- $\gamma$  in determining *in vitro* and *in vivo* function of T cells derived from immunized IFN- $\gamma$  deficient and WT animals, T cell clones were derived by limiting dilution from *in vitro* secondary bulk cultures (see Materials and Methods). A total of 38 clones from IFN- $\gamma$ -deficient WT animals were analyzed for cytolytic potential, antigen specificity, and MHC restriction. Five representative clones were chosen for further *in vitro* and *in vivo* analyses, one CD4<sup>+</sup> MHC class II restricted and two CD8<sup>+</sup> MHC class I restricted clones from the IFN- $\gamma$ -deficient bulk cultures and two CD8<sup>+</sup> MHC class I restricted clones from the WT bulks were selected on the basis of their ability to lyse virally infected H-2<sup>b</sup> MHC class I or II expressing target cells. IFN- $\gamma$ -deficient clones G-11D6 and G-11E4, and WT clones W-16G8 and B1.11 express CD8 as assessed by flow cytometry (data not shown) and lyse virally infected EL-4 cells and EL-4 cells treated with the D<sup>b</sup>-restricted NP<sub>366-379</sub> peptide (Fig. 2). EL-4 cells express only class I MHC molecules of



**Figure 2.** Recognition of target cells by clones derived from IFN- $\gamma$ -deficient and WT littermate mice. (a) EL-4 or (b) LB15.13 target cells were <sup>51</sup>Cr labeled and either left uninfected, treated with NP 366-379 (1  $\mu$ g/ml), or infected with A/JAPAN/57. Clones B1.11 (■), W-16G8 (▨), G-11D6 (▩), G-11E4 (□), and G-4D7 (▤) were added for a final E/T ratio of 5:1. Spontaneous release for all target cells was <15%. Data is representative of multiple assays performed with these cells.

the H-2<sup>b</sup> haplotype. The IFN- $\gamma$  deficient clone G-4D7 expresses CD4 as assessed by flow cytometry (data not shown). This T lymphocyte clone was unable to lyse virally infected or peptide-treated EL-4 targets, but did lyse virally infected LB15.13 target cells which express both MHC class I and II for H-2<sup>b</sup> haplotype (Fig. 2). All of these clones have been stable in culture for more than 6 mo. None of the clones from IFN- $\gamma$ -deficient mice secreted measurable levels of IFN- $\gamma$ , whereas clones W-16G8 and B1.11 produced 64 U/ml and 72 U/ml IFN- $\gamma$  upon stimulation, respectively. Clones G-11E4, G-11D6, W-16G8, and B1.11 had no detectable levels of IL-2, IL-4, or IL-5, whereas the CD4<sup>+</sup> IFN- $\gamma$ -deficient clone G-4D7 secreted measurable levels of IL-2, IL-4, and IL-5 (159, 59, and 960 pg/ml, respectively).

Our laboratory has previously examined the ability of cloned CTL to promote recovery after transfer into lethally infected animals by adoptive transfer experiments (16). To assess the function of CTL clones lacking a functional IFN- $\gamma$  gene, cloned T cells from the mutant and WT mice were adoptively transferred into histocompatible C57Bl/6 mice challenged with a lethal intranasal inoculation of influenza (Fig. 3). The three IFN- $\gamma$ -deficient clones G-4D7, G-11D6, and



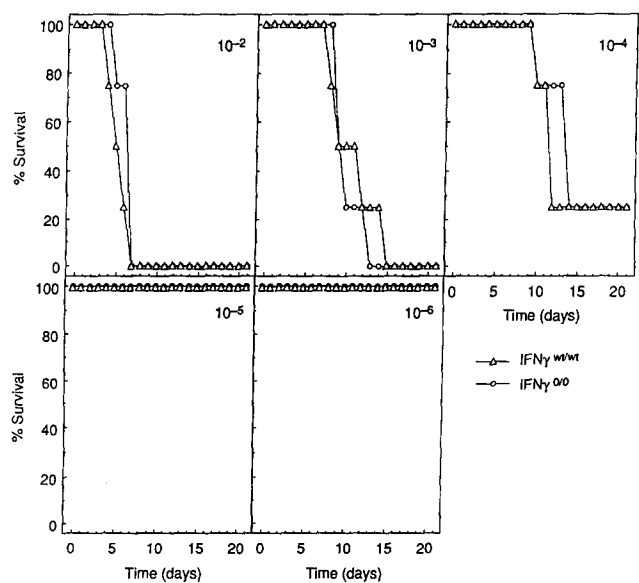
**Figure 3.** Adoptive transfer of clones derived from IFN- $\gamma$ -deficient and WT littermate mice into lethally challenged C57Bl/6 mice. Within 30 min of intranasal inoculation of a lethal dose of A/JAPAN/57 into C57Bl/6 mice,  $10^7$  clone cells (day 6 after stimulation) were injected intravenously. Mice were observed for 21 d for morbidity and mortality. Clones G-4D7 (●), G-11D6 ( $\Delta$ ), G-11E4 ( $\blacktriangle$ ), and B1.11 (○) were injected into groups of six animals each. Clone W-16G8 ( $\square$ ) was injected into three animals, and 14 control animals ( $\blacksquare$ ) received no clone.

G-11E4, and the WT clone B1.11 each promoted recovery from lethal viral challenge with influenza A/JAP/57. Although IFN- $\gamma$  has been noted to have potent antiviral activity, and has been suggested to play a critical role in the function of virus-specific CTL (2, 4), production of IFN- $\gamma$  by either CD4<sup>+</sup> or CD8<sup>+</sup> cytotoxic T cells is not required for expression of antiviral effector activity in vivo. WT clone W-16G8 did not demonstrate any in vivo protective capability despite its ability to lyse virally infected target cells and produce IFN- $\gamma$  in vitro. The defect in clone W-16G8 is currently being evaluated.

**In Vivo Response to Influenza Infection in IFN- $\gamma$ -deficient and WT Mice.** Since either CD4<sup>+</sup> or CD8<sup>+</sup> IFN- $\gamma$ -deficient T lymphocyte clones promoted recovery from lethal influenza infection upon adoptive transfer, we next examined the response of IFN- $\gamma$ -deficient mice to pulmonary infection with influenza virus. To assess the impact of IFN- $\gamma$  expression on susceptibility to infection with influenza virus, WT and IFN- $\gamma$ -deficient mice were inoculated intranasally with varying doses of infectious mouse-adapted influenza virus. Although at the highest doses of virus given, the WT animals died in a slightly faster time frame (ranging from 24 to 48 h) during the first 7 d after infection, both the time to death and minimal lethal dose of virus were identical for both the IFN- $\gamma$ -deficient and WT animals (Fig. 4).

## Discussion

IFN- $\gamma$  is a pleiotropic cytokine with wide-ranging immunomodulatory effector functions. In this model of ex-



**Figure 4.** In vivo response to influenza infection in IFN- $\gamma$ -deficient and WT littermate mice. Groups of four 9–11-wk-old IFN- $\gamma$ -deficient (○) and WT littermate ( $\Delta$ ) mice were intranasally inoculated with varying concentrations of mouse-adapted A/JAPAN/57 influenza virus diluted in PBS. Mice were observed for 21 d for morbidity and mortality.

perimental influenza infection, its absence did lead to differential antibody response and cytokine production as compared with controls, but did not affect the development of an effective cell-mediated immune response to a respiratory virus. The role that IFN- $\gamma$  plays in differentiation of precursors into CTL, thereby influencing the cell-mediated immune response, has been controversial. Originally it was thought that IFN- $\gamma$  was an essential cofactor in these precursors and was necessary for CD8<sup>+</sup> T cells to acquire cytolytic activity (5, 6). Other studies have indicated that IFN- $\gamma$  is not necessary for the induction of CTL in MLC and that IL-2 is the prime cytokine needed for growth and differentiation of CTL (32, 33). Additional lines of investigation have also implicated IL-4 as a prime inducer of CTL (34, 35). In the bulk cultures generated from IFN- $\gamma$ -deficient mice, variable amounts of both IL-2 and IL-4 were produced, thus potentially playing a significant role in the generation of cytotoxic T cells in these populations in the absence of IFN- $\gamma$ .

The results of the adoptive transfer experiment are consistent with speculations made previously by our laboratory that in experimental influenza infection, direct cytolysis of virally infected cells may be the primary mechanism by which clonal populations of CD8<sup>+</sup> CTL express their antiviral effector activity in vivo (16). Similarly, IFN- $\gamma$  deficiency did not have a significant impact on recovery from infection in this experimental model for influenza infection. As shown previously, IFN- $\gamma$ -deficient mice are unable to control infection with the intracellular mycobacterium, *Mycobacterium bovis* (BCG strain) (23). Also, IFN- $\gamma$  receptor-deficient mice have been shown to be unable to control infection with the intracellular bacterium, *Listeria monocytogenes* (36). Furthermore, in experimental vaccinia virus infection, vaccinia virus is le-

thal in IFN- $\gamma$  receptor-deficient mice, suggesting that in this viral infection, IFN- $\gamma$  may play a critical modulating role (36). It seems likely, therefore, that the importance of IFN- $\gamma$  in clearance of a pathogen and recovery will vary among microorganisms.

Previous studies (17) from this laboratory have demonstrated that influenza-specific CD4<sup>+</sup> T lymphocytes can eliminate infectious virus and promote recovery from lethal experimental infection when CD4<sup>+</sup> T cell clones are adoptively transferred in vivo. An interesting feature of these early studies was the finding that protective CD4<sup>+</sup> T cell populations express specific cytolytic activity similar to influenza-specific CD8<sup>+</sup> T lymphocytes. In addition, these protective CD4<sup>+</sup> T lymphocyte populations secrete a constellation of cytokines upon antigenic stimulation, including IL-2 and IFN- $\gamma$ , and fall into the subset of Th1 T cells. In the present report, clone G-4D7, derived from an IFN- $\gamma$ -deficient animal, was able to lyse virally infected MHC class II expressing targets in vitro and promote recovery from lethal viral challenge in vivo. The mechanisms by which CD4<sup>+</sup> T cells may be involved in promoting recovery from viral infection include both humoral and cellular immune responses. It is well established that in experimental influenza infection, CD4<sup>+</sup> T cells can support antigen-specific B cell responses with production of influenza-specific antibodies (37). The mechanism(s) by which clone G-4D7 acts could include specific antibody production as a result of B cell help and cytokine production, in addition to direct cytolytic effector activity on virally infected respiratory epithelial cells. In this model, IFN- $\gamma$  does not appear to be necessary for either the in vitro or in vivo effector function of CD4<sup>+</sup>, influenza-specific, cytolytic clones.

In this study, there was no significant difference in the amounts of influenza-specific IgG2a antibody made by the IFN- $\gamma$ -deficient or WT animals. IFN- $\gamma$  has potent effects on B cells, stimulating B cell proliferation and active Ig secretion and isotype switch from IgM to IgG2a (29). IgG2a synthesis may be promoted by other cytokines, such as IL-2, since it has been shown that although IFN- $\gamma$  produced by Th1 cells promotes IgG2a synthesis, neutralization of IFN- $\gamma$

by the addition of anti-IFN- $\gamma$  antibody does not affect the IgG2a antibody production promoted by cytokines from CD4<sup>+</sup> Th1 T cells (31). In immune responses to viral infections with both RNA and DNA viruses, IgG2a is the dominant antibody response, followed by IgG1 (38). The IgG2a response to viral infection has been speculated to be advantageous because of its ability to fix complement and bind Fc receptors on both macrophages and NK cells, potentially enhancing the ability to combat intracellular infections (30). In these experiments, the viral-specific IgG1 antibody response was significantly higher in the IFN- $\gamma$ -deficient versus the WT animal, presumably reflecting increased production of IL-4 in the absence of IFN- $\gamma$  and therefore a greater switch to IgG1 production (20).

These observations differ from those made in mice lacking the IFN- $\gamma$  receptor (36), where there was no demonstrable difference in IgG1 antibody response to immunization with OVA, but a reduced IgG2a antibody response in the mutant animals. An intriguing explanation for this difference is that IFN- $\gamma$  receptor-deficient mice still produce IFN- $\gamma$ , and potentially a second receptor for IFN- $\gamma$  exists, such as was discovered for IL-1 (39). Alternatively, studies have shown that different cytokines can cross-compete for the same receptor (40) and may account for the lack of a difference in IgG2a production in our experimental model.

In conclusion, we have shown that animals lacking endogenous production of IFN- $\gamma$  do not differ significantly from mice that make IFN- $\gamma$  normally when challenged with influenza virus. Although deficient in IFN- $\gamma$ , these animals express functional IFN- $\alpha$  and IFN- $\beta$ . Activities of these potent antiviral effectors could potentially account for the lack of differences observed. In addition, cloned CD4<sup>+</sup> and CD8<sup>+</sup> influenza-specific T cells do not require IFN- $\gamma$  to lyse virally infected target cells in vitro or to clear influenza infection in vivo. The availability of cloned CTL from IFN- $\gamma$ -deficient mice with in vitro and in vivo functional activities will enable us to study further the role different cytokines play in determining these effector mechanisms.

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## References

1. Joklik, W.K. 1990. Interferons. In *Virology*. 2nd ed. B.N. Fields, and D.N. Knipe, editors. Raven Press, New York. 383-410.
2. Morris, A.G., Y.L. Lin, and B.A. Askonas. 1982. Immune interferon release when a cloned cytotoxic T cell line meets its correct influenza-infected target cell. *Nature (Lond.)* 295:150.

3. Klein, J.R., D.H. Raulet, M.S. Pasternack, and M.J. Bevan. 1982. Cytotoxic T lymphocytes produce immune interferon in response to antigen or mitogen. *J. Exp. Med.* 155:1198.
4. Zinkernagel, R.M., and A. Althage. 1977. Antiviral protection by virus-immune cytotoxic T lymphocytes: infected target cells are lysed before infectious virus progeny is assembled. *J. Exp. Med.* 145:644.
5. Simon, M.S., U. Hochgeschwender, U. Brugger, and S. Landolfo. 1986. Monoclonal antibodies to interferon- $\gamma$  inhibit interleukin 2-dependent induction of growth and maturation in lectin/antigen-reactive cytolytic T lymphocyte precursors. *J. Immunol.* 136:2755.
6. Maraskovsky, E., W.-F. Chen, and K. Shortman. 1989. IL-2 and IFN- $\gamma$  are two necessary lymphokines in the development of cytolytic T cells. *J. Immunol.* 143:1210.
7. Vilcek, J., P.W. Gray, E. Rinderknecht, and C.G. Stenvik. 1985. Interferon-gamma: a lymphokine for all seasons. *Lymphokines.* 11:1.
8. Fellous, M., U. Nir, D. Wallach, G. Merlin, M. Rubenstein, and M. Revel. 1982. Interferon-dependent induction of mRNA for the major histocompatibility antigens in human fibroblasts and lymphoblastoid cells. *Proc. Natl. Acad. Sci. USA.* 79:3082.
9. Restifo, N.P., F. Esquivel, Y. Kawakami, J.W. Yewdell, J.J. Mule, S.A. Rosenberg, and J.R. Bennink. 1993. Identification of human cancers deficient in antigen processing. *J. Exp. Med.* 177:265.
10. Yang, Y., J.B. Waters, K. Fruh, and P.A. Peterson. 1992. Proteasomes are regulated by interferon  $\gamma$ : implications for antigen processing. *Proc. Natl. Acad. Sci. USA.* 89:4928.
11. Yap, K.L., and G.L. Ada. 1978. Cytotoxic T cells in the lungs of mice infected with an influenza A virus. *Scand. J. Immunol.* 7:73.
12. Yap, K.L., and G.L. Ada. 1978. The recovery of mice from influenza virus infection: adoptive transfer of immunity with immune T lymphocytes. *Scand. J. Immunol.* 7:389.
13. Yap, K.L., T.J. Braciale, and G.L. Ada. 1979. Role of T cell function in recovery from murine influenza infection. *Cell. Immunol.* 43:341.
14. Lin, Y.-L., and B.A. Askonas. 1981. Biological properties of an influenza A virus-specific killer T cell clone. Inhibition of virus replication in vivo and induction of delayed-type hypersensitivity reactions. *J. Exp. Med.* 154:225.
15. Taylor, P.M., and B.A. Askonas. 1983. Diversity in the biological properties of anti-influenza cytotoxic T cell clones. *Eur. J. Immunol.* 13:707.
16. Lukacher, A.E., V.L. Braciale, and T.J. Braciale. 1984. In vivo effector function of influenza virus-specific cytotoxic T lymphocyte clones is highly specific. *J. Exp. Med.* 160:814.
17. Lukacher, A.E., L.A. Morrison, V.L. Braciale, and T.J. Braciale. 1986. T lymphocyte function in recovery from experimental viral infection: the influenza model. In *Mechanisms of Host Resistance to Infectious Agents, Tumors, and Allografts: A Conference in Recognition of the Trudeau Institute Centennial*. R.M. Steinman and R.J. North, editors. The Rockefeller University Press, New York. 233-254.
18. Taylor, P.M., F. Esquivel, and B.A. Askonas. 1990. Murine CD4<sup>+</sup> T cell clones vary in function in vitro and in influenza infection in vivo. *Int. Immunol.* 2:323.
19. Mosmann, T.R., H. Cherwinski, M.W. Bond, M.A. Giedlin, and R.L. Coffman. 1986. Two types of murine helper T cell clones. I. Definition according to profiles of lymphocyte activities and secreted proteins. *J. Immunol.* 136:2348.
20. Kuhn, R., K. Rajewsky, and W. Muller. 1991. Generation and analysis of interleukin-4 deficient mice. *Science (Wash. DC).* 254:707.
21. Kopf, M., G.L. Gros, M. Bachmann, M.C. Lamers, H. Bluethmann, and G. Kohler. 1993. Disruption of the murine IL-4 gene blocks Th2 cytokine responses. *Nature (Lond.).* 362:245.
22. Schorle, H., T. Holtschke, T. Hunig, A. Schimpl, and I. Horak. 1991. Development and function of T cells in mice rendered interleukin-2 deficient by gene targeting. *Nature (Lond.).* 352:621.
23. Dalton, D., S. Pitts-Meek, S. Keshav, I.S. Figari, A. Bradley, and T.A. Stewart. 1993. Multiple defects of immune cell function in mice with disrupted interferon- $\gamma$  genes. *Science (Wash. DC).* 259:1739.
24. Braciale, T.J. 1977. Immunologic recognition of influenza virus-infected cells. I. Generation of a virus strain-specific and a cross-reactive subpopulation of cytotoxic T cells in the response to type A influenza viruses of different subtypes. *Cell. Immunol.* 33:423.
25. Braciale, T.J., M.E. Andrew, and V.L. Braciale. 1981. Heterogeneity and specificity of cloned lines of influenza virus-specific cytotoxic T lymphocytes. *J. Exp. Med.* 153:910.
26. Schreiber, R.D. 1991. Measurement of mouse and human interferon  $\gamma$ . In *Current Protocols in Immunology*. J.E. Coligan, A.M. Krusbeek, D.H. Margulies, E.M. Shevach, and W. Strober, editors. John Wiley & Sons Inc., New York. Vol. 1, Chapter 6, Unit 6.8.
27. Irwin, J.O., and E.A. Cheeseman. 1939. On an appropriate method of determining the median effective dose and its error in the case of a quantal response. *J. Hyg.* 39:574.
28. Bastin, J., J. Rothbard, J. Davey, I. Jones, and A. Townsend. 1987. Use of synthetic peptides of influenza nucleoprotein to define epitopes recognized by class I-restricted cytotoxic T lymphocytes. *J. Exp. Med.* 165:1508.
29. Finkelman, F.D., I.M. Katona, T.R. Mosmann, and R.L. Coffman. 1988. IFN  $\gamma$  regulates the isotypes of Ig secreted during in vivo humoral immune responses. *J. Immunol.* 140:1022.
30. Teale, J.M., and D.M. Estes. 1990. Immunoglobulin isotype regulation. In *Cytokines and B Lymphocytes*. R.E. Callard, editor. Academic Press Inc., San Diego. 173-193.
31. Stevens, T.L., A. Bossie, V.M. Sanders, R. Fernandez-Botran, R.L. Coffman, T.R. Mosmann, and E.S. Vitetta. 1988. Regulation of antibody isotype secretion by subsets of antigen-specific helper T cells. *Nature (Lond.).* 334:255.
32. Erard, F., P. Corthesy, M. Nabholz, J.W. Lowenthal, P. Zaeck, G. Plaetinck, and H.R. MacDonald. 1985. Interleukin 2 is both necessary and sufficient for the growth and differentiation of lectin-stimulated cytolytic T lymphocyte precursors. *J. Immunol.* 134:1644.
33. Bucy, R.P., D.W. Hanto, E. Berens, and R.D. Schreiber. 1988. Lack of an obligate role for IFN- $\gamma$  in the primary in vitro mixed lymphocyte response. *J. Immunol.* 140:1148.
34. Widmer, M.B., and K.H. Grabstein. 1987. Regulation of cytolytic T-lymphocyte generation by B-cell stimulatory factor. *Nature (Lond.).* 326:795.
35. Trenn, G., H. Takayama, J. Hu-Li, W.E. Paul, and M.V. Sitovsky. 1988. B cell stimulatory factor 1 (IL-4) enhances the development of cytotoxic T cells from Lyt-2<sup>+</sup> resting murine T lymphocytes. *J. Immunol.* 140:1101.
36. Huang, S., W. Hendriks, A. Althage, S. Hemmi, H. Bluethmann, R. Kamijo, J. Vilcek, R.M. Zinkernagel, and M. Auger. 1993. Immune response in mice that lack the interferon- $\gamma$  receptor. *Science (Wash. DC).* 259:1742.

37. Scherle, P.A., and W. Gehard. 1986. Functional analysis of influenza-specific helper T cell clones in vivo. T cells specific for internal viral proteins provide cognate help for B cell responses to hemagglutinin. *J. Exp. Med.* 164:1114.
38. Coutelier, J.-P., J.T.M. Van Der Logt, F.W.A. Heessen, G. Warneir, and J.V. Snick. 1987. IgG2a restriction of murine antibodies elicited by viral infections. *J. Exp. Med.* 165:64.
39. Solari, R. 1990. Identification and distribution of two forms of the interleukin 1 receptor. *Cytokine.* 2:21.
40. Lopez, A.F., M.J. Elliot, J. Woodcock, and M.A. Vadas. 1992. GM-CSF, IL-3, and IL-5: cross competition on human haemopoietic cells. *Immunol. Today.* 13:495.