IFN-γ Receptor-Deficient Mice Generate Antiviral Th1-Characteristic Cytokine Profiles but Altered Antibody Responses[1]

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The lymphokine IFN-γ is a pleiotropic immunomodulator and possesses intrinsic antiviral activity. We studied its significance in the development of antiviral immune responses by using IFN-γ receptor-deficient (IFN-γR$^{-/-}$) mice. After inoculation with live attenuated pseudorabies virus (PRV), the mutant mice showed no infectivity titers in various tissues, and transient viral Ag expression only in the spleen, similar as in wild-type mice. However, the absence of the IFN-γR resulted in increased proliferative splenocyte responses. The PRV-immune animals showed a normal IFN-γ and IL-2 production, without detectable IL-4, and with decreased IL-10 secretion in response to viral Ag or Con A. Immunohistochemically, an increased ratio of IFN-γ:IL-4-producing spleen cells was found. After immunization with either live attenuated or inactivated PRV, IFN-γR$^{-/-}$ mice produced significantly less antiviral Ab, and more succumbed to challenge infection than the intact control animals. The reduction in Ab titers in the mutant mice correlated with lower protection by their sera in transfer experiments. Our data demonstrate that ablation of the IFN-γ receptor surprisingly does not inhibit the generation of antiviral Th1-type and increase Th2-type cytokine responses. However, it profoundly impairs the generation of protective antiviral Ab. *The Journal of Immunology*, 1994, 153: 2029.

IFN-γ possesses antiviral activity and exerts pleiotropic immunomodulatory activities, including activation of macrophages and NK cells and enhancement of MHC class I and II expression (for a review, see Ref. 1). Mice with a deficient IFN-γ receptor (IFN-γR$^{-/-}$)[3] provide an excellent in vivo model with a nonfunctional IFN-γ system (2). They develop a normal immune system, possess IFN-γ-independent macrophage and NK cell activity, and constitutively express MHC Ag.

Previous in vivo studies have shown that the role of IFN-γ in antiviral immune responses varies with the type and conditions of infection. IFN-γR$^{-/-}$ mice exhibit increased susceptibility to vaccinia virus (VV) infection, whereas the course of a vesicular stomatitis virus (VSV) or Semliki forest virus infection is unaltered (2, unpublished data). In addition, treatment with anti-IFN-γ Ab enhances the sensitivity to infection with herpes simplex virus (3), ectromelia virus (4), and mouse hepatitis virus (5), and specifically abrogates Th cell-mediated murine cytomegalovirus clearance in salivary glands (6). The increased viral replication probably results from failures in natural immunity, because a role for the lymphokine in antiviral T and B cell responses could not be demonstrated. Thus, IFN-γR$^{-/-}$ mice display normal CTL activity and Th responses (2). Similarly, anti-IFN-γ Ab treatment does not affect the generation of CTL responses to VSV, VV (7), and murine cytomegalovirus (6). Only in lymphocytic choriomeningitis virus infection has this treatment been seen to impair virus-specific CTL activity, which, however, may be caused by increased virus replication (7–9). Moreover, anti-IFN-γ does not affect the humoral immune

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[3] Abbreviations used in this paper: IFN-γR$^{-/-}$, IFN-γ receptor-deficient; VV, vaccinia virus; VSV, vesicular stomatitis virus; PRV, pseudorabies virus; Tk, thymidine kinase; gI, glycoprotein I; $TCID_{50}$, 50% tissue-culture infective dose; PFU, plaque-forming unit.

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responses to VSV, VV (7), or murine cytomegalovirus (6). In addition, the characteristic IgG2a-isotype restriction of murine Abs elicited by most replicating viruses (10) could not be ablated by several anti-IFN-γ Abs (11). In contrast, IFN-γ inhibits the production of IgG1 and IgE, but stimulates IgG2a production in vitro (12) and in goat anti-mouse IgD-treated mice (13). In mice with a disruption in the IFN-γ gene, the generation of influenza virus-specific IgG2a, cytolytic T cells, or resistance to challenge infection was unaffected (14).

IFN-γ is a key regulatory cytokine in Th1-driven immune responses that is necessary for the elimination of intracellular pathogens, including viruses (15). In vitro, it promotes the differentiation of $CD4^+$ T cells into Th1 cells, both in the human (16) and murine system (17). Administration of anti-IFN-γ mAb during *Leishmania major* or *Listeria monocytogenes* infection of mice leads to disease progression (18–20) and to reversion of Th1 into Th2 responses (18, 19). In mice with a disrupted IFN-γ gene, *Mycobacterium bovis* infection leads to increased susceptibility (21). In these mice, an influenza or *L. major* infection leads to increased IL-4 and IL-5 production (14, 22). The effect of IFN-γ neutralization on antiviral cytokine generation in other virus infections is unknown.

We used the pseudorabies virus (PRV) infection of IFN-$\gamma R^{-/-}$ mice to study the involvement of IFN-γ in antiviral immune parameters, including T cell, cytokine, and B cell activity. PRV (synonyms: Aujeszky's disease virus and suis herpesvirus type 1) is an alphaherpesvirus related to herpes simplex virus. It has a broad host range, including most domestic and wild animals. In the mouse, virulent PRV is highly neurotropic and produces lytic infections of cells in the central nervous system, leading to fatal encephalitis. Genetically engineered PRV mutants deleted in the genes encoding thymidine kinase (Tk) and glycoprotein I (gI) show a reduced virulence and lack neurotropism. Immunization with the live attenuated (Tk−, gI−) PRV leads to protective immunity in swine and mice (23).

In this study, we demonstrate that endogenous IFN-γ does influence specific immune parameters, including T and B cell responses, but leaves the generation of cytokine profiles in response to PRV unaffected.

Materials and Methods

Mice

The mutant (129/Sv/Ev) mouse strain deficient in expression of the IFN-γ receptor (IFN-$\gamma R^{-/-}$) was generated by gene targeting in murine embryonic stem cells (2). In the experiments, we used IFN-$\gamma R^{-/-}$, wild-type 129/Sv/Ev or (129/Sv/Ev × C57BL/6) F2 mice of both sexes, or female C57BL/6 mice (purchased from the breeding facilities of the Central Animal Laboratory, Utrecht, The Netherlands). All animals were housed in filter-top cages and immunized at 4 to 5 wk of age. The animal experiments had been approved by the Institutional Animal Welfare Committee, Utrecht, The Netherlands.

Virus

Virulent wild-type PRV (strain NIA-3) was obtained from the Central Veterinary Institute (Lelystad, The Netherlands). The live attenuated (Tk−, gI−) PRV and the binary ethyleneimine-inactivated (gI−) PRV preparation (antigenic mass corresponding to $10^{8.8}$ 50% tissue-culture infective dose ($TCID_{50}$)/ml) were kindly provided by Dr. N. Visser (Intervet International, Boxmeer, The Netherlands); the gI protein is not essential for virus replication (24). Inactivation was confirmed by the absence of cytopathic effect and viral Ag expression, tested by indirect immunofluorescence, in PRV-permissive baby hamster kidney (BHK) cells, and by inoculation of a 10-ml vaccine preparation into highly PRV-sensitive rabbits.

Infectious virus in the organs of immunized mice was quantitated by plaque titration of 10% (w/v) tissue homogenates on National Institutes of Health (Bethesda, MD) 3T3 cell monolayers; the detection limit was 50 plaque-forming units (PFU) per gram of tissue.

Immunohistologic detection of viral Ag in tissues was performed on acetone-fixed cryostat sections (8-μm) by using polyclonal PRV-specific rabbit serum (overnight incubation in a 1/50 dilution, after blocking of endogenous peroxidase activity by 0.5% H_2O_2 in acetone). After washing in PBS and after 1-h incubation with peroxidase-conjugated goat anti-rabbit Ab (Dakopatts, Glostrup, Denmark), peroxidase activity was visualized by using 0.015% H_2O_2 and 3-amino-9-ethylcarbazole. PRV-positive and -negative sections and irrelevant Abs were included in each experiment.

Experimental protocol

Mice were immunized with live attenuated or inactivated PRV via the i.p. route. Seven days later, the spleens were removed, and one-third of each organ was snap-frozen in liquid nitrogen and stored at −70°C for immunohistochemistry. The remaining part of the spleen was used for in vitro cultures. Single cell suspensions of splenocytes were tested for PRV-specific cytokine production and proliferative activity. Other groups of mice were bled from the retro-orbital plexus at day 27; their sera were analyzed for the levels of virus-specific Ig isotypes and for protective activity in transfer experiments (per recipient, 380 μl pooled serum were injected i.p. 30 min before infection). All mice were challenge-infected via the i.p. route with 250 PFU (corresponding to 250 LD_{100}) of virulent PRV. For temporal depletion of endogenous IFN-γ, 1 mg mAb DB-1 (25) or an isotype-matched irrelevant control mAb (8B7G9H9, specific for chloramphenicol) was injected i.p. 30 min before challenge.

Proliferative splenocyte responses

Seven days after immunization, erythrocyte-depleted single cell suspensions were prepared from the spleens. Splenocytes were cultured in 96-well round-bottom plates at a density of 10^5 cells per well in RPMI 1640 medium containing 10% FCS, L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Purified inactivated PRV or Con A was added at concentrations of 1.25 μg/ml and 5 μg/ml, respectively. After 72-h or 120-h incubation at 37°C in a 5% CO_2 atmosphere, the cultures were pulsed with 1.0 μCi [^{3}H]thymidine (Amersham, Den Bosch, The Netherlands) for another 16 h. Cells were harvested on glass filters, and the incorporated radioactivity was measured in a Betaplate scintillation counter (LKB-Wallac). The data represent the mean values ± SEM of [^{3}H]thymidine uptake of triplicate cultures determined in three mice per group.

In vitro analysis of cytokine production

Splenocytes (10^6 cell/ml) were cultured in 24-well plates (Nunc, Breda, The Netherlands) for analysis of cytokine production. Supernatants of stimulated cultures were harvested at 24 h for IL-2 measurements, and at 48 h for IFN-γ, IL-4, and IL-10 detection, and stored at −20°C until use. IL-2 levels were quantitated by measuring [^{3}H]thymidine incorporation in an IL-2-dependent CTLL-16 proliferation assay (26). IFN-γ, IL-4, and IL-10 were measured in two-site ELISAs (Holland Biotechnology, Leiden, The Netherlands, and PharMingen, San Diego, CA). The cytokine concentrations were determined from a standard curve that was established with known amounts of murine rIFN-γ (kindly provided by Dr. H. Heremans, Leuven, Belgium), rIL-4, rIL-10 (Genzyme Corp., Cambridge, MA), and human rIL-2 (EuroCetus, Amsterdam, The Netherlands).

In situ analysis of cytokine production

The number of cytokine-producing spleen cells was determined immunohistochemically, as described by Van den Eertwegh et al. (27). Briefly, acetone-fixed cryostat sections (8-μm) from normal and mutant mice were incubated with biotinylated rat anti-mouse IFN-γ mAb (AN-18.17.24) and peroxidase-conjugated mouse anti-murine IFN-γ mAb (DB-1), and rat anti-mouse IL-2 mAb (S4B6) or rat anti-mouse IL-4 mAb (11B11) for 1 h. Subsequently, the sections were washed, and when necessary, incubated with secondary peroxidase-conjugated rabbit anti-rat (Dakopatts) or biotinylated rabbit anti-rat Ab, followed by peroxidase-labeled streptavidin (Nordic, Tilburg, The Netherlands). Peroxidase activity was visualized by using 0.015% H_2O_2 and 3-amino-9-ethylcarbazole. Spleen sections of trinitrophenyl-Ficoll-injected and nonimmunized mice were picked up on the same glass slide, and served as positive and negative controls, respectively (27). The numbers of cytokine-producing cells were enumerated by light microscopy in three mice/group in six spleen sections/mouse of equal surface areas.

PRV specific serum Ab isotypes

PRV-specific Ab isotype levels were determined in an ELISA; 96-well flat-bottom plates were incubated overnight at 4°C with inactivated PRV in $NaHCO_3$ (0.05 M, pH 9.6), washed with tap water, and saturated with 1% BSA (Sigma Chemical Co., St. Louis, MO) in PBS. Twofold serum dilutions (100 μl/well; in PBS containing 0.05% Tween 80 and 0.1% BSA) were added and incubated for 1 h at 37°C. After washing with tap water, a 1/6 400 dilution of isotype-specific peroxidase-coupled goat anti-mouse Ig Ab (Southern Biotechnology Associates Inc., Birmingham, AL) was added. After incubation for 1 h at 37°C and another wash, the substrate was developed with tetramethylbenzidine for 10 min at room temperature. The reaction was stopped with 2 M sulphuric acid and read at an optical density of 450 nm in a Titertek Multiskan MC. The titer was defined as the reciprocal of the highest dilution at which the absorbance was equal to two times the background value.

Statistical analysis

Evaluation of statistical differences between data obtained from mutant and wild-type mice was performed by using the Wilcoxon-Mann-Whitney test.

Results

Replication of live attenuated PRV is not increased in IFN-γR$^{-/-}$ mice

Mice lacking the IFN-γR are more susceptible to infections with *L. monocytogenes* and VV, as evidenced by increased bacterial and viral titers in organs (2). However, the course of infections with VSV and Semliki forest virus in mutant mice is normal (2, M. Aguet, unpublished observation). Similarly, inoculation of IFN-γR$^{-/-}$ mice with live attenuated PRV led neither to overt symptoms nor to recovery of infectious virus from various tissues, including the lungs, liver, kidneys, and spleen, at 1, 3, and 7 days after infection. In contrast to virulent PRV, which rapidly spreads to the central nervous system from the site of inoculation, live attenuated (Tk−, gI−) PRV is not neurotropic, and replicates only poorly in vivo. Viral Ag was detectable only in the spleen at day 3 after inoculation in both wild-type and IFN-γR$^{-/-}$ mice, with similar expression levels in mice of both genotypes. The absence of infectious virus in mutant mice demonstrates that physiologic IFN-γ has no major role in controlling the replication of live attenuated PRV in vivo. The virtually equal amounts of Ag in IFN-γR$^{-/-}$ and wild-type mice allow comparisons of PRV-specific cellular and humoral immune responses, without the complicating effects of increased virus replication.

IFN-γR$^{-/-}$ splenocytes exhibit increased proliferative responses

As a measure of T cell function we determined proliferative responses of IFN-γR$^{-/-}$ and normal splenocytes. In response to Con A, nonimmune IFN-γR$^{-/-}$ splenocytes proliferated to a normal extent in the first 72 to 88 h, but showed a three-fold increase in proliferation rate at 120 to 136 h after plating. Splenocytes from immunized mutant mice cultured for 72 to 88 h showed slightly increased proliferation rates when cultured in the presence or absence of Con A (Fig. 1, *A* and *B*). Addition of 1 to 10 μg Ag/ml did not further enhance the proliferation rates. Between 120 to 136 h of culture when [^{3}H]thymidine incorporation had declined in wild-type splenocytes, the IFN-γR$^{-/-}$ cells showed a twofold to threefold higher proliferation when cultured either without Ag (Fig. 1*C*) or in the presence of Con A (Fig. 1*D*). PRV-immune splenocytes of both genotypes displayed no detectable specific cytolytic activity when assayed directly on PRV-infected EL-4 cells (not shown). This is in fact not surprising, because PRV-specific cytolytic T cell activity probably requires a 3-day culture period after isolation of the Ag-specific lymphoid cells (28).

IFN-γR$^{-/-}$ mice generate antiviral Th1-type cytokine responses

IFN-γ is essential for the induction of Th1 development and inhibition of Th2 function in *L. monocytogenes* and *Leishmania* infections (18, 19, 29). In addition, in viral infection it is viewed as a key regulator of Th1 characteristic responses (15), although this has never been shown. We determined whether a defective IFN-γR function leads to alterations in cytokine profiles 7 days after immunization with live attenuated PRV. Cultured spleen cells of both normal and IFN-γR$^{-/-}$ mice, when restimulated with PRV Ag, produced a Th1-characteristic cytokine pattern with normal IFN-γ and IL-2 secretion, but no detectable IL-4 and reduced IL-10 levels (Fig. 2, *panels A*). Similar results were obtained in cultures of splenocytes isolated 3 days after immunization (not shown). The increased proliferative activity in these Th1 type-cultures (see Fig. 1) suggests a proliferation-inhibitory activity of IFN-γ for the Th1 subset. In the presence of Con A, wild-type and mutant splenocytes of both immunized and nonimmunized mice produced a Th1-type pattern (Fig. 2, *right panels*), with even increased levels of IFN-γ produced by the mutant splenocytes. The IFN-γ produced by the mutant mice could have been secreted by NK cells stimulated by T

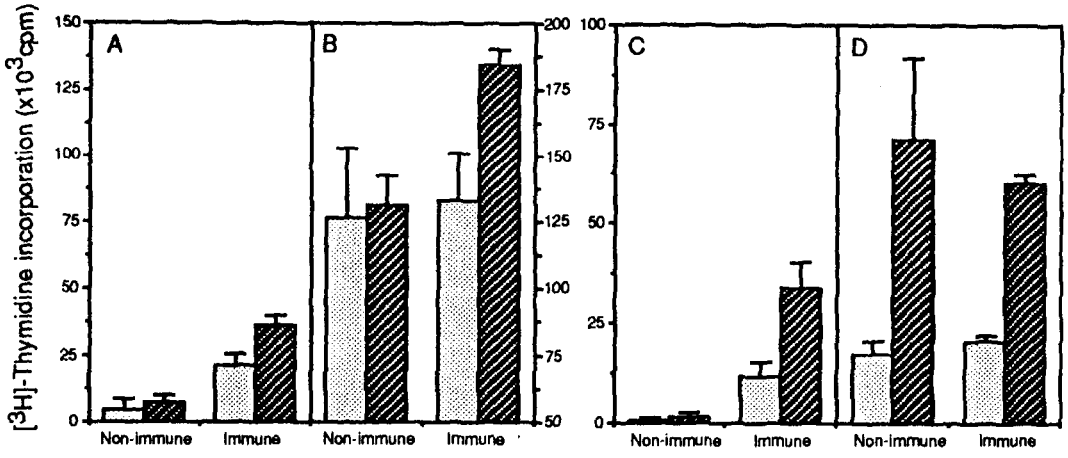

FIGURE 1. Proliferative responses of wild-type (stippled bars) and IFN-γ$^{-/-}$ (hatched bars) splenocytes, cultured in medium only (*panels A, C*) or in the presence of Con A (*panels B, D*) for 72 to 88 h (*A, B*) or 120 to 136 h (*C, D*). The splenocytes were isolated from naive (nonimmune) mice or immune mice inoculated 7 days before with 10^5 $TCID_{50}$ live attenuated PRV. Mean values ± SEM in duplicate cultures of three individual mice are shown.

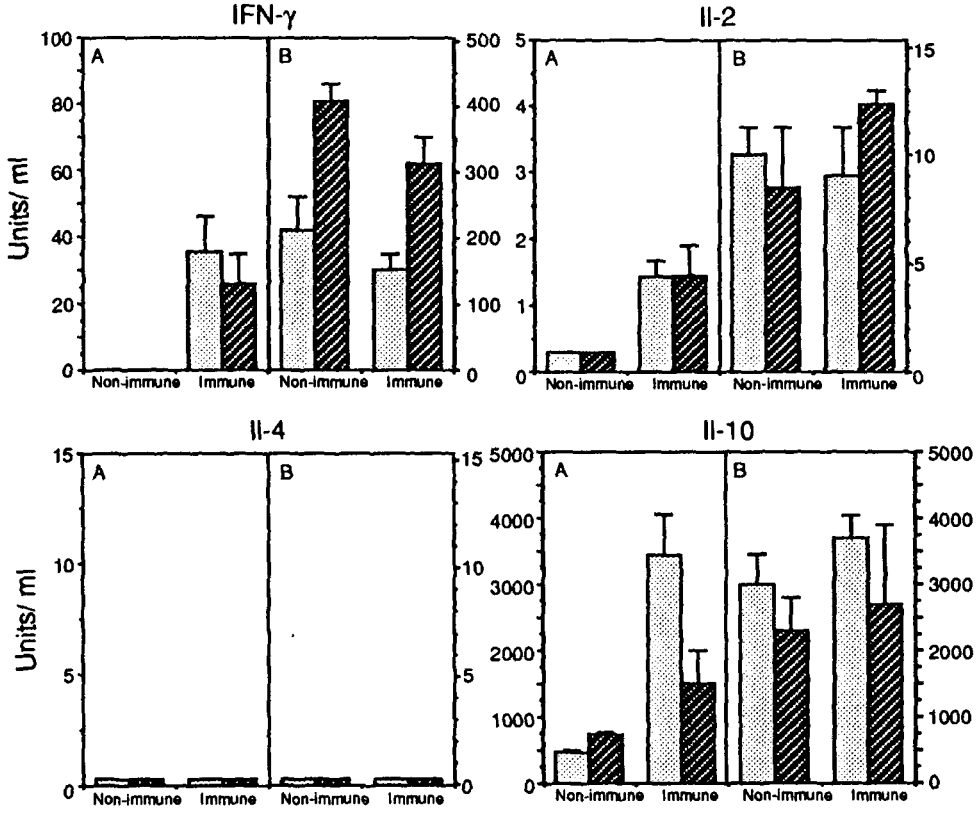

FIGURE 2. In vitro cytokine production by wild-type (stippled bars) and IFN-γR$^{-/-}$ splenocytes (hatched bars) after stimulation with 1.25 μg/ml PRV Ag (*panels A*) or Con A (*panels B*). Splenocytes isolated from naive (nonimmune) mice and (immune) mice inoculated 7 days before with 10^5 $TCID_{50}$ live attenuated PRV were cultured, and their supernatants were harvested at 24 h for IL-2 determination, and at 48 h for IFN-γ, IL-4, and IL-10 detection. Mean cytokine concentrations ± SEM in duplicate cultures of three individual mice are shown.

cell-derived IL-2. However, similar analysis in the *L. major* model demonstrated that IFN-γR$^{-/-}$ T cells are capable of IFN-γ production (J. Louis, personal communication). Remarkably, wild-type naive splenocytes, when precultured for 4 days in the presence of IL-4 (600 U/ml) and restimulated for 24 h in fresh medium containing Con

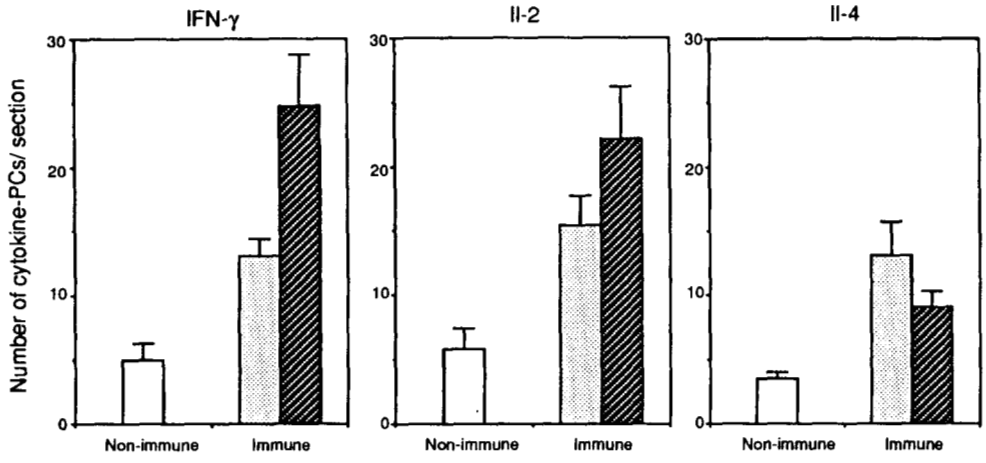

FIGURE 3. In situ cytokine production in the spleens of wild-type (stippled bars) or IFN-$\gamma R^{-/-}$ mice (hatched bars). The animals were immunized 7 days before with 10^5 $TCID_{50}$ live attenuated PRV. Nonimmune animals of both genotypes (open bars) served as controls. The mean numbers of cytokine-producing cells/section ± SEM of three mice per group are depicted.

A, also secreted a Th1-like profile with <0.5 U IL-4/ml and high amounts of IFN-γ (225 U/ml); in contrast, naive IFN-$\gamma R^{-/-}$ splenocytes produced a Th2-like response with detectable IL-4 secretion (10–15 U/ml), but normal high IFN-γ synthesis (200 U/ml).

Immunohistochemical analysis of in situ cytokine production at 7 days after immunization revealed significantly increased numbers of IFN-γ-producing IFN-$\gamma R^{-/-}$ spleen cells ($p = 0.023$), normal numbers of IL-2-producing cells and slightly decreased numbers of IL-4-producing IFN-$\gamma R^{-/-}$ splenocytes (Fig. 3). Most cytokine-producing cells were localized in the periarteriolar lymphocytic sheaths and around terminal arterioles. Together with the cytokine profiles in vitro, these data indicate that ablation of the IFN-γR does not necessarily result in increased Th2-type responses, but rather in an increased IFN-γ:IL-4 ratio. Moreover, IFN-γ is not required to mount a Th1-characteristic response. Similar results were obtained by using mouse hepatitis virus (strain MHV-A59) infection (unpublished observations). In addition, *L. major*-infected IFN-$\gamma R^{-/-}$ mice exhibited a Th1 cytokine profile, despite increased susceptibility (J. Louis, Lausanne, Switzerland, personal communication).

IFN-$\gamma R^{-/-}$ mice produce markedly less antiviral Abs

IFN-γ is postulated to regulate Ab isotype generation (30). We therefore determined antiviral Ig-isotype responses in IFN-$\gamma R^{-/-}$ mice 27 days after immunization with live attenuated and (to exclude any interference by viral replication) inactivated PRV. In the serum of IFN-$\gamma R^{-/-}$ mice inoculated with 10^4 or 10^5 $TCID_{50}$ live attenuated PRV, the levels of virus-specific total IgG (heavy and light chain) were reduced significantly 7.4- to 9.2-fold ($p = 0.0002$ for both), and specific IgG2a was decreased 9.8- to 26-fold ($p = 0.0004$ and $p = 0.0001$, respectively) (Fig. 4). IgG1 levels were increased 4.6-fold ($p = 0.01$) in mutant mice immunized with 10^5 $TCID_{50}$ attenuated PRV. The reduced Ab responses in IFN-$\gamma R^{-/-}$ mice correlated with less and smaller germinal centers in the spleens (not shown).

Similarly, immunization of IFN-$\gamma R^{-/-}$ mice with inactivated PRV resulted in reduced antiviral Ab responses that were 2.8-fold for total IgG ($p = 0.0001$), 6.5-fold for IgG2a ($p = 0.0017$), 7.5-fold for IgG3 ($p = 0.023$), and 3.5-fold for IgA ($p = 0.009$) (Fig. 5).

The significant drop in serum levels of total IgG, IgG2a, IgG2b, IgG3, and IgA in IFN-$\gamma R^{-/-}$ mice indicates a positive regulatory role for IFN-γ in Ab generation. No evidence was obtained for a difference in kinetics of Ig-isotype production; sera of animals surviving the challenge infection showed similar Ig ratios between mutant and wild-type mice.

Immunized IFN-$\gamma R^{-/-}$ mice exhibit reduced resistance to challenge infection

To examine the significance of IFN-γ for resistance to virus infection, we tested the effect of IFN-γR gene disruption on the ability of mice to survive a lethal PRV challenge 4 wk after immunization. As compared with normal littermates, protective immunity was impaired in IFN-$\gamma R^{-/-}$ mice. Immunization with 10^4 $TCID_{50}$ attenuated PRV, followed by an otherwise lethal challenge infection (250 PFU of virulent PRV), resulted in increased mortality among the mutant mice; only two of 10 IFN-γR-deficient mice (20%) survived as compared with 10 survivors among 17 wild-type mice (58%; five of nine 129/Sv/Ev and five of eight C57BL/6; Fig. 6*A*). Immunization with inactivated PRV ($0.25 \times 10^{8.8}$ $TCID_{50}$ antigenic mass) completely protected normal mice ($n = 7$) against the lethal challenge infection, whereas among IFN-$\gamma R^{-/-}$ mice, only two of seven survived (Fig. 6*B*).

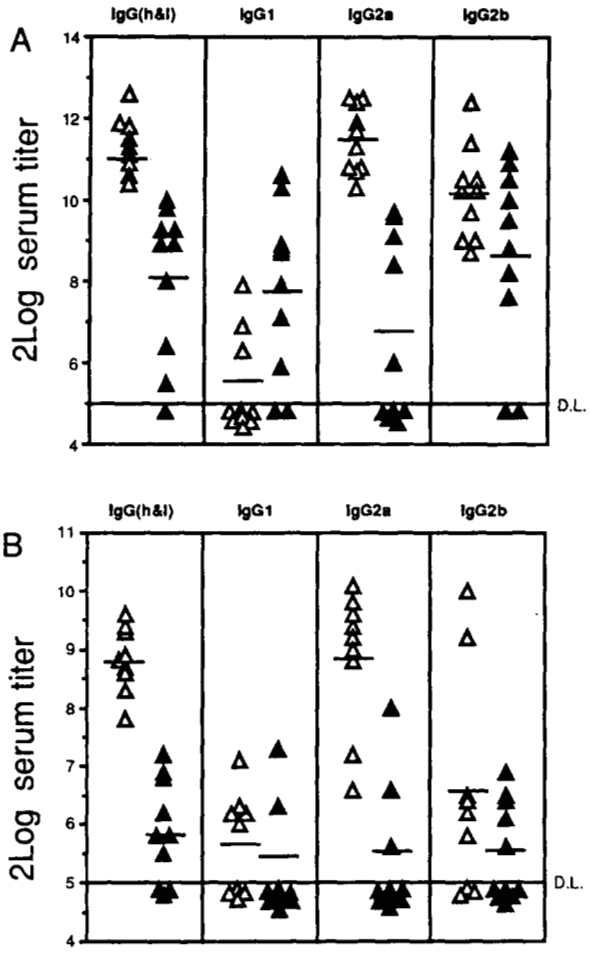

FIGURE 4. PRV-specific serum Ig-isotype distribution in wild-type (open symbols) and IFN-$\gamma R^{-/-}$ (closed symbols) mice. The animals were immunized at *day 0* with 10^5 $TCID_{50}$ (*panel A*) or 10^4 $TCID_{50}$ (*panel B*) live attenuated PRV. *Day 27* Ab titers shown for individual mice were determined by twofold serial dilutions in an ELISA. Mean values per group are represented by horizontal bars.

The impaired resistance in IFN-$\gamma R^{-/-}$ mice can be explained either by the lower Ab levels of certain isotypes or by the requirement of IFN-γ for effector mechanisms operative after challenge infection. However, i.p. injection of the well-characterized IFN-γ-neutralizing mAb DB-1 (31, 32) 1 mg/mouse, with a half-life of 7 days in the circulation of normal mice (25), 30 min before a lethal challenge infection did not affect protection elicited by both PRV preparations in groups of five mice (not shown) and neither did an isotype-matched irrelevant control mAb. These data indicate an IFN-γ-independent mechanism, and suggest an immunomodulatory role of the lymphokine in the generation of protective immunity.

IFN-$\gamma R^{-/-}$ mice generate less protective antiviral Ab

Virus-specific Ab can protect against PRV infection (33, 24). Furthermore, immunization with both live attenuated or inactivated PRV elicits a dose-dependent Ab response (see Fig. 4) that correlates with protection (not shown). Thus, the decline in antiviral Ab titers in IFN-$\gamma R^{-/-}$ mice could explain the impaired immunity. To determine their protective activity we transferred immune sera from wild-type and mutant mice (individual Ig-isotype levels are shown in Fig. 5) into C57BL/6 recipients (380 μl/mouse) which were challenged subsequently with virulent PRV. Sera from the wild-type donors immunized with the inactivated PRV, which all resisted a lethal challenge infection (Fig. 6*B*), protected all five recipients whereas sera from the IFN-$\gamma R^{-/-}$ mice, of which only two of seven survived after challenge (Fig. 6*B*), protected only two of five recipients. Control mice ($n = 5$) receiving normal mouse serum died within 3 to 4 days after challenge infection. Furthermore, passively transferred hyperimmune mouse serum protected all four lethally infected wild-type mice and five of five IFN-$\gamma R^{-/-}$ mice, indicating that Ab can confer protection by an IFN-γ-independent mechanism. These data demonstrate that the reduced PRV Ab titers in IFN-$\gamma R^{-/-}$ mice correlate both with impaired resistance to challenge and less protective activity in serum recipients. The declines in Ab titers were pronounced even more in mutant mice immunized with live attenuated PRV (Fig. 4), despite a smaller reduction in resistance (Fig. 6*A*). This phenomenon can be explained by compensatory or IFN-γ-independent virus-inhibitory mechanism(s) evoked by immunization with the live attenuated virus.

Discussion

Our studies have demonstrated that IFN-γR ablation does not affect antiviral Th1-characteristic cytokine production during PRV infection. Unaltered cytokine profiles also were observed during a mouse hepatitis virus (strain MHV-A59) infection (unpublished observations). Similarly, *L. major*-infected IFN-$\gamma R^{-/-}$ mice exhibited a Th1 cytokine profile, despite increased susceptibility (J. Louis, Lausanne, personal communication). In contrast, previous in vivo studies reported that anti-IFN-γ Abs promote Th2 and impair Th1 activity (18, 29). In addition, mice with a disruption in the IFN-γ gene show increased influenza virus and *L. major* Ag-specific IL-4 and IL-5 production (14, 22). The observed discrepancies with our data obtained in genetically IFN-γ-unresponsive mice may be explained by the involvement of other cytokines, the modulatory activity of non-Th populations, and genetic background differences. There is no evidence for a compensation of the IFN-γR function by other (cytokine) receptors, because treatment with IFN-γ-neutralizing Ab did not alter the Th1- characteristic cytokine responses in IFN-$\gamma R^{-/-}$ and IFN-$\gamma R^{+/+}$ mice after *L. major* infection (K. Swihart and J. Louis, personal communication). Furthermore, the attenuated PRV, as many other herpes viruses, may encode host-response modifier genes. In accordance with our data is the observation of Flynn and coworkers that splenocytes

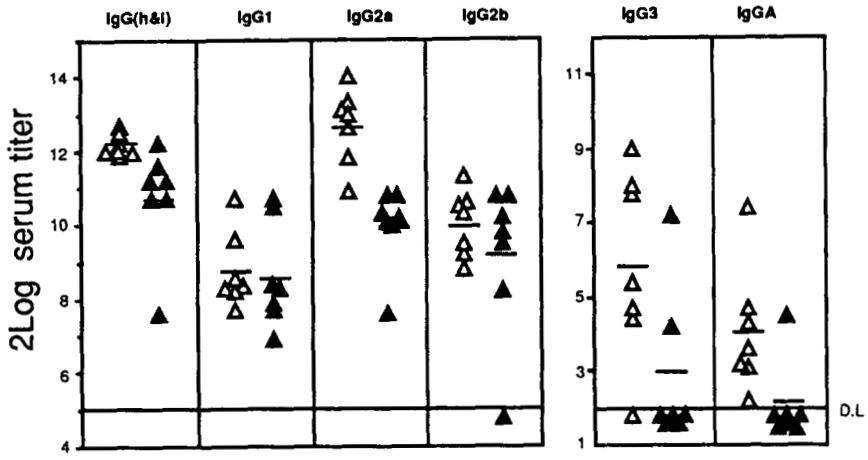

FIGURE 5. PRV-specific serum Ig-isotype distribution in wild-type (open symbols) and IFN-$\gamma R^{-/-}$ (closed symbols) mice. The animals were immunized at *day 0* with an antigenic mass of $0.25 \times 10^{8.8}$ $TCID_{50}$-inactivated PRV/mouse. Day 27 Ab titers shown for individual mice were determined by twofold serial dilutions in an ELISA. Mean values per group are represented by horizontal bars.

of *Mycobacterium tuberculosis*-infected mice lacking the IFN-γ gene produce no increased amounts of IL-4 after antigenic stimulation (34). Recently, IL-12 has been demonstrated to promote Th1-like responses (35). Moreover, Manetti et al. (36) demonstrated that in cultures of human $CD4^+$ T cell clones, neutralizing Abs to IFN-γ failed to overcome the IL-12-mediated suppression of IL-4-producing cell development, which suggests that IL-12 may stimulate Th1 responses independently from IFN-γ (36). Similarly, Seder et al. showed that anti-IFN-γ Ab did not reduce IL-12-induced priming for IFN-γ production in an accessory cell-dependent system (37). In addition, IL-12-induced Th1 development in C57BL/6 mice is not prevented by IFN-γ-neutralizing Ab treatment (38). In contrast, cytokines such as TNF-α and IFN-α (39–41), of which the release is strain-dependent (42), can inhibit Th2 activity. In other model systems, addition of Con A alone or Con A plus either IL-4 or anti-IFN-γ Ab to cultured spleen cells permitted a clear Th2-type cytokine profile (43, 44). We could confirm that Con A-stimulated splenocytes of BALB/c mice produced detectable amounts of IL-4 (not shown), whereas those of wild-type 129/Sv/Ev mice failed to do so, even when precultured in the presence of IL-4. Our data therefore suggest that the wild-type 129/Sv/Ev strain is Th1-type predisposed. Other host factors or cytokines apparently can replace or dominate over IFN-γ to induce Th1-type responses and inhibit those of the Th2-type. This may be revealed after backcrossing of the mutation into different genetic backgrounds or breeding with other cytokine receptor-deficient mice.

In contrast to other murine models of infection with ectromelia virus, vaccinia virus, mouse hepatitis virus, and the PRV-related herpes simplex virus (2–5), our data show that the control of PRV is not IFN-γ-dependent, similar to Semliki forest virus and VSV infection (Ref. 2, and our unpublished data). This is surprising in view of the efficient protection against lethal PRV infection by recombinant IFN-γ administration (45), and the strong inhibitory activity of IFN-γ against PRV replication in vitro (46). In

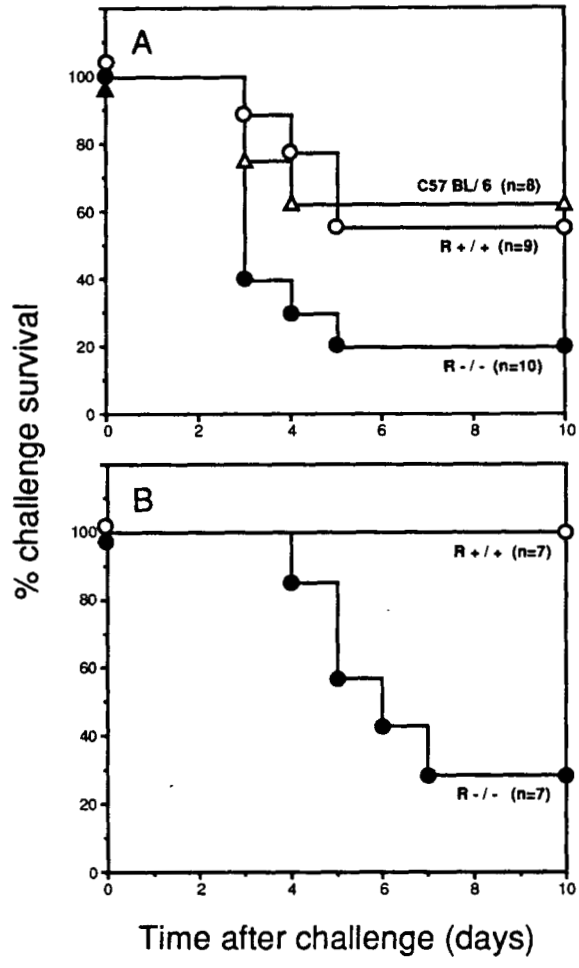

FIGURE 6. Survival of IFN-$\gamma R^{-/-}$ (closed symbols) and wild-type 129/Sv/Ev or C57BL/6 mice (open symbols) after lethal challenge infection with 250 PFU virulent PRV. Twenty-eight days before challenge infection, groups of animals were immunized with either 10^4 $TCID_{50}$ live attenuated PRV/mouse (*panel A*) or an antigenic mass of $0.25 \times 10^{8.8}$ $TCID_{50}$ inactivated PRV/mouse (*panel B*). Nonimmunized wild-type mice died within 3 to 4 days of infection.

most virus infections, no role for IFN-γ in antiviral T and B cell responses could be demonstrated (6, 7). Our data, however, demonstrate that the lymphokine mediates specific antiviral immune functions; although it inhibits T cell proliferation, endogenous IFN-γ is an important mediator for the generation of protective antiviral Ab. The increased proliferation rates of IFN-γR$^{-/-}$ splenocytes resemble those found in splenocyte cultures of mice with a disrupted IFN-γ gene (21); they could be explained by either the antiproliferative and/or apoptotic effects of IFN-γ (47, 48) or the reduced production of inhibitory cytokines such as IL-10 (Fig. 2) and TGF-β (unpublished data). The significant drop in serum levels of total IgG, IgG2a, IgG2b, IgG3, and IgA in IFN-γR$^{-/-}$ mice indicates a positive regulatory role for IFN-γ in Ab generation. These data are in line with its proliferation- and differentiation-enhancing activity on resting B cells (49, 50), and the strong enhancing effect of exogenous IFN-γ on rabies virus- and PRV-specific IgG responses (51, unpublished data). The observed drop in IgG3 is in agreement with the reported induction by IFN-γ of IgG3 class switching (52). Our findings are at variance with the failure of anti-IFN-γ Ab to inhibit virus infection-associated IgG2a-isotype production (11). In addition, immunization of IFN-γR$^{-/-}$ mice with VSV evoked normal neutralizing Ab titers, whereas their isotype pattern after immunization with trinitrophenyl-conjugated OVA showed clear decreases in the IgG2a levels only (2). Our experiments revealed that IFN-γR ablation substantially diminishes the acquisition of resistance to PRV challenge infection that corresponds with reduced Ab production, whereas lymphokine production remains surprisingly unaffected. In future studies, IFN-γR$^{-/-}$ mice will provide a useful system to determine the indispensable requirement or redundancy of IFN-γ in vivo in many other immune responses.

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