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16. Northern analysis of RNA derived from M1, TF-1, and B9 cells was done with human or rodent probes for gp130, LIFRβ, and CNTFRα, revealing the expression patterns described in the text. In growth or survival assays done on B9 cells, substitution of LIF, CNTF, or the combination of CNTF and sCNTFRα for IL-6 resulted in cell death within 48 hours.
17. Two different methods were used to produce sCNTFRα. One method consisted of the transfection of COS cells with an expression vector that contained CNTFRα. Transfected cells were then treated with PI-PLC to release the receptor from the cells, and the supernatants were used as a source of soluble receptor. In the second method, CNTFRα without the signal sequence and the COOH-terminal GPI anchor (terminated at amino acid 349) was purified from bacteria expressing the recombinant protein (N.P., Regeneron Pharmaceuticals). Except as noted, bacterial sCNTFRα was used in all experiments.
18. The minor effects of sCNTFRα on LIF responses in TF-1 cells are perhaps consistent with a model in which CNTFRα acts as a nonessential component of the LIF receptor complex (N. Stahl *et al.*, unpublished results).
19. Protein immunoblot analysis of proteins derived from denervated muscle failed to detect an increased amount of CNTFRα compared with control samples. For the detection of the receptor in blood plasma samples, sCNTFRα was isolated by precipitation with streptavidin-agarose beads coated with biotinylated CNTF, and the resulting precipitates were subjected to protein immunoblotting with an antibody to CNTFRα. Because the size of the detected species is consistent with the PI-PLC–cleaved protein, the cleavage event that releases sCNTFRα could be in the GPI anchor. Alternatively, it could be a proteolytic cleavage that occurs close to the membrane.
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Multiple Defects of Immune Cell Function in Mice with Disrupted Interferon-γ Genes

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Interferon-γ (IFN-γ) is a pleiotrophic cytokine with immunomodulatory effects on a variety of immune cells. Mice with a targeted disruption of the IFN-γ gene were generated. These mice developed normally and were healthy in the absence of pathogens. However, mice deficient in IFN-γ had impaired production of macrophage antimicrobial products and reduced expression of macrophage major histocompatibility complex class II antigens. IFN-γ–deficient mice were killed by a sublethal dose of the intracellular pathogen *Mycobacterium bovis*. Splenocytes exhibited uncontrolled proliferation in response to mitogen and alloantigen. After a mixed lymphocyte reaction, T cell cytolytic activity was enhanced against allogeneic target cells. Resting splenic natural killer cell activity was reduced in IFN-γ–deficient mice. Thus, IFN-γ is essential for the function of several cell types of the murine immune system.

Interferon-γ, a cytokine secreted by activated T cells and natural killer (NK) cells, has immunomodulatory effects on several cell types (*1*). IFN-γ is probably the major cytokine responsible for the activation of macrophages, which are mediators of nonspecific, cell-mediated host defense (*2, 3*). Experiments that used neutralizing antibodies to IFN-γ have shown an in vivo requirement for IFN-γ in the activation of murine macrophages for microbicidal activity and in the induction of major histocompatibility complex (MHC) class II antigen on the surface of murine macrophages (*4*). IFN-γ primes murine macrophages for production of nitric oxide (*5–7*), although other cytokines, in addition to IFN-γ, can prime macrophages in vitro for production of reactive oxygen intermediates (*7, 8*). In addition, IFN-γ may regulate the proliferation and function of activated T lymphocytes (*9–11*). Finally, NK cells exhibit enhanced cytolytic activity in response to IFN-γ in vivo and in vitro (*12*). Although IFN-γ can profoundly affect immune responses in vitro and in vivo, it is not known to what extent this cytokine is essential for the normal development and function of the immune system. To gain a better understanding of the physiological role of IFN-γ we have generated mice with a targeted mutation of the IFN-γ gene.

Mice with a nonfunctional IFN-γ gene were generated by replacing one normal IFN-γ allele in mouse embryonic stem cells with a defective allele. The targeting vector (Fig. 1) has a 2-kb neomycin-resistance gene (*neo*^{r}) inserted into exon 2, which introduces a termination codon after the first 30 amino acids of the mature IFN-γ protein. The targeting vector was transfected into AB-1 embryonic stem cells and cells were selected for resistance to G418 and 1-[2-deoxy, 2-fluoro-β-δ-arabinofuranosyl]-5-iodouracil (FIAU) (*13*). Of 960 clones screened by polymerase chain reaction (PCR) in pools of eight for a replacement event, four were identified in which a normal allele was replaced by a disrupted allele (Fig. 1D).

Injection of the 48D clone into C57BL/6J blastocysts generated chimeric mice that transmitted the mutation through the germline. Heterozygous offspring of the chimeras were intercrossed to generate mice homozygous for the targeted mutation of the IFN-γ gene (gamma knock-out or gko) (Fig. 1E). To verify inactivation of IFN-γ gene function, an IFN-γ–specific ELISA (*14*) was used to measure IFN-γ protein in

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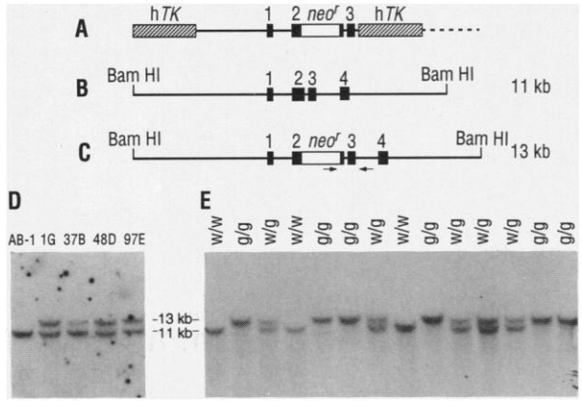

Fig. 1. Production and identification of gko mice. (**A**) Structure of the IFN-γ targeting vector. A 2.0-kb neomycin-resistance gene (*neo*^{r}) was inserted into exon 2. The *neo*^{r}-cassette is flanked 5′ by 5 kb and 3′ by 937 base pairs of IFN-γ genomic DNA. Exons 1 and 3 are also present. The IFN-γ sequences were flanked by hSV thymidine kinase genes (h*TK*). (**B**) IFN-γ gene structure on an 11-kb Bam H1 genomic fragment. (**C**) Structure of the disrupted IFN-γ gene. The insertion of the 2-kb *neo*^{r} gene into exon 2 results in a 13-kb Bam H1 IFN-γ gene. The targeting vector was linearized at a unique site for electroporation into AB-1 embryonic stem cells (*13*). Arrows, positions of PCR primers. (**D**) Southern hybridization analysis of genomic DNA derived from parental (AB-1) and four targeted embryonic stem cell clones (1G, 37B, 48D, and 97E). DNA was digested with Bam H1 and the filter probed with a DNA fragment from exon 4 to confirm the identity of ES cell clones that had one disrupted allele. (**E**) Southern blot of Bam H1–digested genomic DNA derived from progeny of an F_1 intercross. Wild-type (w) fragments were 11 kb, and gko (g) fragments were 13 kb. Chimeric mice, produced by injecting ES cells into C57BL/6 blastocysts, were developed to term in foster mothers. Chimeric males were bred to C57BL/6 females to identify offspring with agouti coat color derived from the injected ES cells. Mice used in our studies were (129/Sv × C57BL/6)F_2.

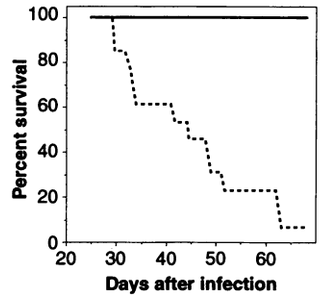

Fig. 3. Survival of mice infected with BCG. Wild-type (13) and 13 gko mice were infected by intravenous route with 10^7 colony-forming units of BCG (Pasteur). Mice were checked daily for moribund condition or death. Percent survival of wild-type (—) and gko (- - - -) is shown.

the supernatants of splenocytes cultured with the T cell mitogen, concanavalin A (Con A). Supernatants from the splenocytes of wild-type mice had from 5,547 to 27,765 pg/ml of IFN-γ protein, whereas supernatants from gko splenocytes had no detectable (<15.6 pg/ml) IFN-γ. In addition, splenocyte supernatants from the gko mice had no detectable IFN-γ activity in a bioassay measuring protection against cytocidal infection of L929 cells with encephalomyocarditis virus (*15*). Thus, the targeted mutation renders gko mice deficient for biologically active IFN-γ protein.

Mice homozygous for the mutation in the IFN-γ gene were housed in a barrier animal facility and were free of specific mouse pathogens. Under these conditions, the gko mice are normal, healthy, and fertile. No gross or histological abnormalities of the lymphoid (or other) organs are apparent in young mice (less than 4 months). No significant difference was observed in the total number of cells in the spleens and thymuses of the gko compared to wild-type mice, nor were there any alterations of splenic and thymic cell populations or peripheral blood cells with respect to CD3, B220, CD4, and CD8 surface markers. Thus, IFN-γ was not essential for development of the immune system, with respect to the above parameters, and was not required for survival of the mice under pathogen-free conditions.

Infection of mice with *Mycobacterium bovis* (strain bacillus Calmette Guérin or BCG) generates macrophages with enhanced MHC class II on the cell surface and enhanced capacity to kill microorganisms (*3*, *16*). To investigate the in vivo role of IFN-γ in macrophage activation, adherent peritoneal cells (consisting of primarily macrophages by morphological criteria) that were derived from BCG-infected wild-type and gko mice were assayed for production of nitric oxide and superoxide anion.

In response to lipopolysaccharide (LPS) stimulation, macrophages from BCG-infected wild-type mice produced large quantities of nitric oxide. In contrast, identically treated macrophages from BCG-infected gko mice produced little detectable nitric oxide above unstimulated controls. This defect in nitric oxide production was reversed by treating the gko cells in vitro with murine IFN-γ for 48 hours (Fig. 2A). Thus, IFN-γ is required, during infection with BCG, to prime macrophages for production of nitric oxide.

Production of reactive oxygen intermediates is controlled by pathways independent of the induction of nitric oxide (*7*). Macrophages from the BCG-infected wild-type mice produced superoxide anion in response to phorbal myristate acetate (PMA). However,

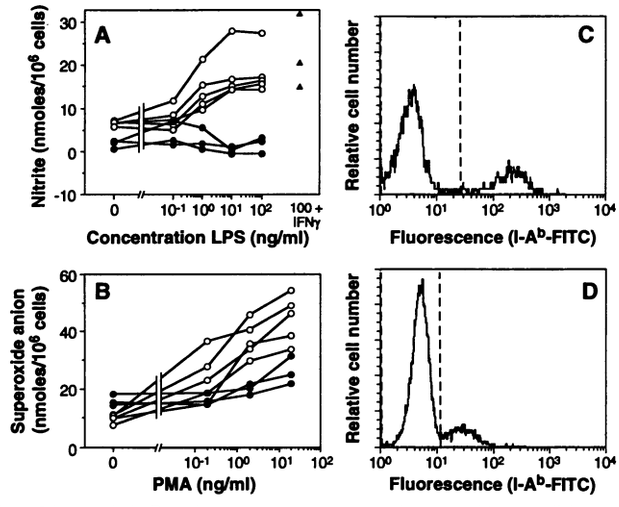

Fig. 2. Macrophage activation in peritoneal cells from BCG infected mice. (**A**) Nitric oxide production in adherent peritoneal exudate cells (PECs) from BCG infected (*26*) wild-type (○) and gko (●) mice. Most adherent cells were identified as macrophages by morphological criteria. Nitric oxide produced by macrophages after 24 hours of LPS stimulation was assayed using the Griess reagent to measure nitrite generated from nitric oxide (*27*). N^G-monomethyl-1-arginine at 250 μM completely inhibited nitric oxide production. Values for gko cells cultured with 500 U/ml of murine IFN-γ for 48 hours (▲). Results represent two experiments with ten wild-type and six gko mice. (**B**) Superoxide production in macrophages from BCG infected wild-type (○) and gko (●) mice. Macrophages were treated with PMA, and superoxide production was determined by reduction of ferricytochrome C (*28*). Results are representative of two experiments with 10 wild-type and 6 gko mutant mice. (**C**) Expression of MHC class II antigen (I-A^b) on the surface of BCG-infected wild-type or (**D**) gko macrophages as determined by flow cytometry. PECs were stained with phycoerythrin (PE)-conjugated rat antibody to mouse Mac-1 (clone M1/70HL), then stained with fluorescein isothiocyanate (FITC)–conjugated antibody to mouse I-A^b. Analysis was done on the monocyte-macrophages, gated by light scatter characteristics, from Mac-1–stained population of PECs. Cellular fluorescence of I-A^b–FITC was quantitated using a FACscan. The percentage of I-A^b positive macrophages was variable, with mean value of approximately 25% in both wild-type and gko mice. Results represent two experiments with ten wild-type and six gko mutant mice.

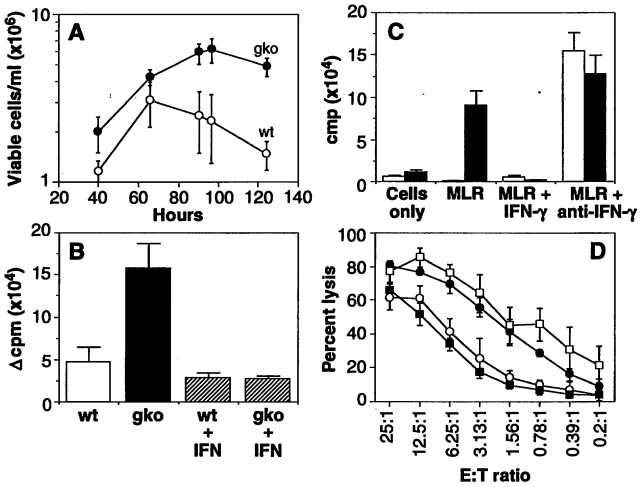

Fig. 4. Proliferation in response to Con A; proliferation and cytolytic activity in response to allogeneic cells. (**A**) Kinetic analysis of viable cell numbers in Con A–stimulated splenocyte cultures from wild-type (○) and gko mice (●). Unfractionated spleen cells from wild-type or gko mice were plated at 2 × 10^6 cells per milliliter in medium with Con A (3 μg/ml). Viable cells were manually counted in trypan blue dye. Mean and SEM of three wild-type and three gko mice are shown. (**B**) [^{3}H]thymidine uptake, between 96 and 108 hours, by spleen cells from wild-type (open bar) and gko mutant (closed bar) mice, grown with and without Con A. Mean change (Δ) in cpm and SEM for wild-type (wt) and gko mice (four per group) are given. Parallel cultures grown in the presence of 1000 U/ml of IFN-γ (hatched bars). Results are representative of four experiments using 12 mice per group. (**C**) [^{3}H]thymidine uptake, between 108 and 120 hours, of wild-type (open bar) and gko (closed bar) splenocytes cultured with irradiated BALB/c stimulators (*H-2^b* versus *H-2^d*) in an MLR. Splenocytes from wild-type and gko mice were cultured at 2.5 × 10^6 cells per milliliter, alone, or with equal numbers of irradiated (2000 rads) BALB/c spleen cells (MLR). Interferon-γ (1000 U/ml) and neutralizing antibody to IFN-γ (anti–IFN-γ) (*21*) were added to parallel cultures. Mean cpm and SEM are shown for groups of eight mice. (**D**) Cytolytic activity of effector cells from an MLR. Wild-type effectors (○), gko effectors (●), wild-type effectors from MLR with neutralizing antibody to IFN-γ (□), gko effectors from MLR with 1000 U/ml IFN-γ (■). On day 5 of the MLR, unfractionated cells from the MLR, adjusted to 10^7 viable cells per milliliter, were tested for cytolytic activity against ^{51}Cr-labeled P815 target cells (*H-2^d*) (*29*). Mean percent lysis and SEM of three mice per group. Results are representative of three separate experiments with a total of ten wild-type and ten gko mice.

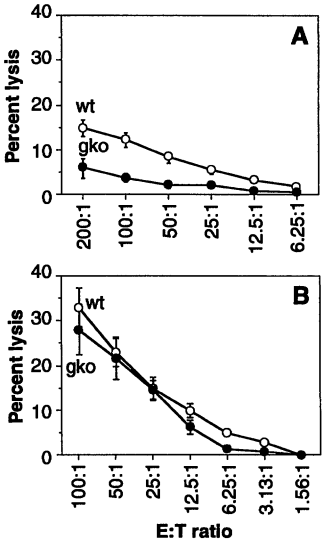

Fig. 5. Natural killer cytolytic activity of splenocytes for wild-type (wt) and mutant (gko) mice. (**A**) Resting NK cytolytic activity against ^{51}Cr-labeled YAC-1 cells. Effectors derived from wild-type (○) and gko mutant mice (●). Unfractionated spleen cells derived from untreated mice were resuspended at 10^7 cells per milliliter and tested at various effector to target ratios for spontaneous cytolytic activity against ^{51}Cr-labeled YAC-1 cells (*30*). Results shown are mean percent lysis and SEM obtained from two separate experiments with a total six mice per group. (**B**) Poly(I:C)-stimulated splenic NK cytolytic activity from wild-type (○) and gko mutant mice (●). Mice were injected intraperitoneally with 100 μg of poly(I:C) 24 hours prior to assay, then tested for NK activity. Results shown are mean percent lysis and SEM from two separate experiments with a total of six mice per group (student's t test used to determine statistical significance).

production was reduced, but not absent, in macrophages from gko mice (Fig. 2B). Thus, priming for the respiratory burst is not entirely dependent on IFN-γ. It is possible that other cytokines, such as granulocyte-macrophage colony-stimulating factor (GM-CSF), may compensate for the absence of IFN-γ (*17*).

To examine the in vivo role of IFN-γ in another aspect of macrophage activation, expression of MHC class II molecules on the surface of peritoneal macrophages from BCG-infected mice was determined by flow cytometry (Fig. 2, C and D). In contrast to the high expression, as determined by mean fluorescent intensity, of the MHC class II positive macrophages from wild-type infected mice, MHC class II expression of macrophages from the gko mice was reduced. Thus, we have confirmed the crucial role of IFN-γ in enhanced expression of macrophage MHC class II molecules during infection.

The profound defects in macrophage production of antimicrobial products and defects in expression of MHC class II antigen suggest that the course of infections involving cell-mediated immunity could be altered in gko mice. Indeed, infection of the mice with a sublethal dose of BCG led to significantly increased mortality of the gko mice (Fig. 3). On day 30 of the infection, the gko mice had 10 to 100 times more BCG colony-forming units, compared with wild-type mice, in lung, liver, and spleen (*18*).

The antiproliferative and cytotoxic effects of IFN-γ could participate in the regulation of T cell activation (*9*, *10*). To investigate T cell function, we measured the proliferation of gko splenocytes in response to Con A for 5 days. Although the gko cells and wild-type cells proliferated at the same rate early in the response, the gko cells showed enhanced proliferation late in the response. The number of viable cells in the wild-type cultures declined between 66 and 90 hours. At the same time, the number of viable cells in the gko cultures continued to increase (Fig. 4A). Between 96 and 108 hours of culture, the gko spleen cells incorporated significantly more thymidine than the wild-type cultures (Fig. 4B). Cultures of gko spleen cells continued to proliferate until they formed a dense, overgrown layer on the bottom of the wells. In contrast, wild-type cells seeded at the same density stopped proliferating before the wells were overgrown with cells. Addition of IFN-γ at the beginning of the culture period limited the proliferative response of the gko cultures to that of the wild-type cultures. Because IFN-γ has antiproliferative effects on a number of cells, including some T cells and B cells (*9*), increased growth of one or more of these populations may be responsible for the prolonged proliferation of Con A–stimulated splenocytes. In addition, IFN-γ has been implicated in the apoptotic cell death of an activated T cell clone (*10*). Consistent with this, we observed that late in the proliferative response to Con A, the gko cultures had more viable cells than the wild-type cultures.

Since the development of cytotoxic T cells during a mixed lymphocyte reaction (MLR) is accompanied by the release of IFN-γ by activated T cells (*19*), IFN-γ may be required for the development or function of allogeneic cytotoxic T cells (CTLs). However, two reports describing the effects of adding IFN-γ–neutralizing antibodies to the MLR have led to opposite conclusions about the role of IFN-γ (*11*). To further investigate the role of IFN-γ in development and function of allogeneic CTLs, proliferation and cytolytic activity were measured in responder spleen cells from gko and wild-type littermates after culture with irradiated allogeneic BALB/c stimulator cells.

On day 5 of the MLR, splenocytes in the wild-type cultures had stopped proliferating (Fig. 4C) (*20*). In contrast, the gko cultures incorporated large amounts of [^{3}H]thymidine. Addition of IFN-γ to the gko splenocytes reduced [^{3}H]thymidine incorporation by gko cells to the amount incorporated by wild-type

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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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