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Immune Response to Mouse Mammary Tumor Virus in Mice Lacking the Alpha/Beta Interferon or the Gamma Interferon Receptor

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Mouse mammary tumor virus (MMTV) is a retrovirus which induces a strong immune response and a dramatic increase in the number of infected cells through the expression of a superantigen (SAg). Many cytokines are likely to be involved in the interaction between MMTV and the immune system. In particular, alpha/beta interferon (IFN-α/β) and gamma interferon (IFN-γ) exert many antiviral and immunomodulatory activities and play a critical role in other viral infections. In this study, we have investigated the importance of interferons during MMTV infection by using mice with a disrupted IFN-α/β or IFN-γ receptor gene. We found that the SAg response to MMTV was not modified in IFN-α/β$R^{0/0}$ and IFN-γ$R^{0/0}$ mice. This was true both for the early expansion of B and T cells induced by the SAg and for the deletion of SAg-reactive cells at later stages of the infection. In addition, no increase in the amount of proviral DNA was detected in tissues of IFN-α/β$R^{0/0}$ and IFN-γ$R^{0/0}$ mice, suggesting that interferons are not essential antiviral defense mechanisms during MMTV infection. In contrast, IFN-γ$R^{0/0}$ mice had increased amounts of IL-4 mRNA and an altered usage of immunoglobulin isotypes with a reduced frequency of IgG2a- and IgG3-producing cells. This was associated with lower titers of virus-specific antibodies in serum early after infection, although efficient titers were reached later.

Mouse mammary tumor virus (MMTV) is a murine retrovirus which can be transmitted either as an infectious viral particle (exogenous MMTV) (6) or as an integrated provirus through the germ line (endogenous *Mtv* loci) (30). Transmission of exogenous MMTV occurs from the infected mother to the offspring upon ingestion of milk during the first days of life. The virus initially infects lymphocytes in the neonatal Peyer's patches (27) and later spreads to distant target organs most probably via cells of the immune system (56, 61). Viral particles are produced in large amounts by the lactating mammary gland, allowing virus transmission to the next generation of mice.

The overall efficiency of MMTV infection is critically dependent upon the interaction between the virus and the immune system (17, 20). In addition to the usual retroviral genes *gag*, *pol* and *env*, the viral genome contains within the 3′ long terminal repeat (LTR) (11, 12) an open reading frame (*orf* or *sag* gene) which has been shown to encode a superantigen (SAg) (3, 9). SAgs are defined by their ability to interact with a large number of T cells expressing specific variable domains in the T-cell receptor β chain (TCR Vβ domains) and need to be presented by major histocompatibility complex (MHC) class II molecules (25, 26, 36, 64). The encounter with a SAg leads first to the stimulation and then to the clonal deletion of reactive T cells (35, 62, 64). The virus makes use of these properties by initially infecting B cells and expressing its SAg at the B-cell surface in association with MHC class II molecules (18). SAg-reactive T cells accumulate locally and are stimulated, providing a potent help to infected B cells via cognate T-cell–B-cell interaction. During this process, the infected B cells increase dramatically in number and differentiate, providing a large reservoir of infected cells for the later stages of the viral life cycle (17, 20). SAg-reactive T cells are then eliminated by clonal deletion.

Many cytokines are likely to be involved in the interactions between MMTV and the immune system. In particular, we were interested in the role played by alpha/beta interferon (IFN-α/β) and gamma interferon (IFN-γ) in these interactions in vivo. IFN-α/β and IFN-γ are pleiotropic cytokines which were originally identified as antiviral molecules (24, 63) but which also have many other important functions. For example, both types of IFN modulate the expression of MHC molecules (28, 29, 38), increase the lytic potential of natural killer (NK) cells (42), and inhibit the proliferation of many cell types in culture (45). In addition, IFN-α/β was recently shown to drive the bystander proliferation of $CD8^+$ T cells during certain viral infections (55) whereas IFN-γ is known to activate macrophages (40), to induce the production of specific immunoglobulin (Ig) isotypes by B cells (14, 53), and to regulate the balance of cytokine production during immune responses (48).

Gene-disrupted mice proved to be very useful models to study the overall importance and effects of IFN-α/β and IFN-γ during viral infections in vivo (59). For example, mice lacking either the IFN-α/β or the IFN-γ receptor (IFN-α/β$R^{0/0}$ and IFN-γ$R^{0/0}$ mice) were shown to have a defective natural resistance to vaccinia virus, lymphocytic choriomeningitis virus, and Theiler's virus (13, 23, 39). In addition, IFN-α/β$R^{0/0}$ but not IFN-γ$R^{0/0}$ mice had an increased susceptibility to vesicular stomatitis virus (VSV) and Semliki forest virus (39), whereas

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no increase in viral replication was observed upon infection of IFN-$\gamma R^{0/0}$ mice with pseudorabies virus (47) and upon infection of IFN-$\gamma^{0/0}$ mice with Sendai virus or with murine gammaherpesvirus 68 (37, 46). However, little information is available so far on the role of IFN-α/β and IFN-γ during murine retroviral infections. In the murine AIDS (MAIDS) model, IFN-γ contributes to the development of disease in MAIDS-susceptible mouse strains as shown through the use of neutralizing antibodies to IFN-γ and of IFN-$\gamma^{0/0}$ mice (16, 57), while the ability to produce IFN-α/β has been linked to a MAIDS-resistant phenotype, although these data have not yet been corroborated by studies with IFN-$\alpha/\beta R^{0/0}$ mice (22). Furthermore, the addition of exogenous IFN in cell culture had a variable effect on different retroviruses, with effects on early steps of the viral life cycle in some cases (5, 15, 51) and on late events in others (1, 31, 43, 50).

In this article, we provide data on MMTV infection of mice lacking either the IFN-α/β or the IFN-γ receptor. Since the immune response induced by the MMTV SAg plays a critical role in the virus-host interaction, we have studied the viral SAg activity as a way to monitor the efficiency of the viral infection and correlated it with the direct semiquantitative detection of proviral DNA in infected tissues. In addition, we have studied the influence of a functional IFN-γ receptor gene on the isotype pattern of antibodies and on the balance of cytokine gene expression during the immune response to MMTV.

MATERIALS AND METHODS

Mice. BALB/c mice were obtained from Harlan Olac Ltd. (Bicester, United Kingdom). 129Sv/Ev IFN-$\alpha/\beta R^{0/0}$ and IFN-$\gamma R^{0/0}$ mice have been generated and described previously (23, 39) and were kindly provided by M. Aguet (Institute of Molecular Biology, Zürich, Switzerland). IFN-$\alpha/\beta R^{0/0}$ or IFN-$\gamma R^{0/0}$ BALB/c mice were obtained by backcrossing the disrupted gene into the BALB/c background for 6 or 10 generations, respectively.

The genotype of the mice was determined by analysis of tail DNA. Southern blot analysis was used for the IFN-$\alpha/\beta R^{0/0}$ mice, as described previously (39). Disruption of the IFN-γR gene was verified by PCR with the oligonucleotides 5′ CCCATTTAGATCCTACATACGAAACATACGG and 3′ TTTCTGTCATCA TGGAAAGGAGGGATACAG. In the presence of a wild-type allele, these primers amplify a 189-bp fragment. With a disrupted allele, the amplification encompasses the inserted Neo^r gene and results in a 1,282-bp fragment.

Virus. We used MMTV(SW), an exogenous MMTV encoding a Vβ6-specific SAg (19). MMTV(SW)-infected mice were derived from mice originally purchased from IFFA Credo (L'Arbresle, France). Milk was collected from lactating virus-infected mothers by suction, as described previously (19). It was diluted 1:3 in phosphate-buffered saline (PBS), centrifuged at $600 \times g$ for 10 min to skim, and stored in aliquots at −70°C.

Injections and samplings. In all experiments, female or male mice were used at 6 to 12 weeks of age. Mouse milk containing MMTV(SW) was diluted in PBS, and 20 μl was injected subcutaneously into the hind footpad of naive IFN-$\alpha/\beta R^{0/0}$ BALB/c mice, IFN-$\gamma R^{0/0}$ BALB/c mice, or control heterozygous littermates. The milk was diluted to obtain a reproducible threefold increase, from about 12% to about 36%, in the percentage of $V\beta6^+$ cells among $CD4^+$ T cells on day 4 after footpad injection, as verified by titer determinations. This dose was chosen to give a strong but not maximal stimulation of SAg-reactive T cells, so that we could detect both an potential increase and potential decrease in the response of the knockout mice. At various time points after injection, the draining popliteal lymph node was isolated. Alternatively, mice were tail bled and leukocytes were recovered from heparinized blood samples by centrifugation through a Ficoll cushion.

Flow cytometric analysis. The following monoclonal antibodies were used: fluorescein isothiocyanate (FITC)-labeled anti-TCR Vβ6 (44.22.1) (41), FITC-labeled anti-B220 (RA3-3A1) (Caltag, San Francisco, Calif.), phycoerythrin (PE)-labeled anti-CD4 (H129.19), and PE-labeled anti-CD8 (53-6.7) (Boehringer, Mannheim, Germany). Lymph node or peripheral blood lymphocytes were stained in one step with a mixture of FITC-labeled and PE-labeled antibodies. Analysis was performed with a FACScan (Becton Dickinson, Mountain View, Calif.). Dead cells were excluded by a combination of forward- and side-scatter characteristics.

PCR. DNA was isolated from 10^6 lymph node cells after digestion at 52°C in 50 mM Tris-HCl (pH 8)–100 mM EDTA–0.5% sodium dodecyl sulfate–100 μg of proteinase K per ml. The DNA was extracted, precipitated, and resuspended at a DNA equivalent of 10^6 cells per 50 μl in 10 mM Tris-HCl–0.1 mM EDTA; 10% of this, corresponding to approximately 0.5 μg of genomic DNA, was used per PCR.

Semiquantitative PCR amplification of proviral DNA was performed with primers matching the conserved regions of the LTR, as described previously with some modifications (20). DNA extracted from mixtures of cells from BALB.D2 (*Mtv-6*, *Mtv-7*, *Mtv-8*, and *Mtv-9*) and BALB/c mice (*Mtv-6*, *Mtv-8*, and *Mtv-9*) at variable ratios was included as a control titer determination since *Mtv-7* is closely related to MMTV(SW) in the target region for amplification (19). The PCR was performed with 1× PCR buffer containing 67 mM Tris-HCl (pH 8.8), 16 mM $(NH_4)_2SO_4$, 0.01% Tween 20, 2.5 mM $MgCl_2$, 200 μM deoxynucleoside triphosphates, 2 μCi of [α-^{32}P]dATP, 1.5 U of *Taq* polymerase (Eurobiotaq; Eurobio, Les Vlis, France), and 200 nM each oligonucleotide (5′ oligonucleotide CTCA GGAAGAAAAAGACGACAT, 3′ oligonucleotide CAAACCAAGTCAGAAA CCACTTG). The cycling conditions were 5 min at 94°C followed by 28 cycles of 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C, with a final 10 min at 72°C. The PCR products were separated on an 8 M urea–6% polyacrylamide gel. Quantification was performed with an Instant Imager (Packard, Meriden, Conn.).

Competitive reverse transcription PCR (RT-PCR) analysis of IL-4 gene expression. Total RNA was extracted from cells of the draining popliteal lymph node with the Trizol reagent (Gibco, Basel, Switzerland) on day 5 or 6 after injection of MMTV. First-strand cDNA synthesis was performed with a kit as specified by the manufacturer (Pharmacia, Uppsala, Sweden). The cytokine polycompetitor plasmid pQRS was used to quantitate the amounts of transcripts originating from the interleukin-4 (IL-4) and the constitutively expressed hypoxanthine guanine phosphoribosyltransferase (HPRT) genes, with the primers and PCR conditions as described previously (44). Briefly, the cDNA was used as a template in the presence of serial fivefold dilutions of pQRS. After separation of the PCR products by agarose gel electrophoresis, the ratio of IL-4 to HPRT transcripts was calculated. The results are shown as the fold increase in the amount of IL-4 mRNA in mice infected with MMTV with respect to uninfected mice with the same genotype.

Enzyme-linked immunospot (ELISPOT) assay. The numbers of Ig-secreting cells in the draining lymph node were determined as described previously (49) with some modifications. Briefly, 96-well plates (Nunc Maxisorb; Gibco, Basel, Switzerland) were coated with 2 μg of goat anti-mouse IgG plus IgM (Tago, Burlingame, Calif.) per ml and blocked with PBS–1% bovine serum albumin. Freshly isolated lymph node cells were washed and resuspended in Dulbecco modified Eagle medium with 5% fetal calf serum, 1.7 mM L-glutamine, 10 mM HEPES, and 50 μM β-mercaptoethanol. Cells were added to the plates as duplicates and in twofold titer determination series starting with 10^5 cells/well. After a 5-h incubation at 37°C, the plates were washed with PBS–0.1% Tween 20 and incubated overnight at 4°C with biotinylated goat anti-mouse IgM, IgG1, IgG2a, IgG2b, or IgG3 (Caltag). After incubation with a streptavidin-alkaline phosphatase conjugate (Boehringer Mannheim), the plates were developed with the substrate 5-bromo-4-chloro-3-indolyl phosphate (Sigma Chemical Co., St. Louis, Mo.) at 1 mg/ml in a buffer of 9% 2-amino-2-methyl-1-propanol (Sigma). The spots were counted by projection on an overhead projector.

Enzyme-linked immunosorbent assay (ELISA). Serum was collected by tail bleeding of infected mice. For this experiment, 96-well plates (Nunc Maxisorp) were coated with 6 μg of bacterial recombinant envelope protein gp52 per ml and blocked with PBS–1% bovine serum albumin. The serum samples were added and incubated at room temperature for 3 to 4 h. Specific IgG antibodies were detected with biotinylated antibodies against IgG isotypes (Caltag) followed by a streptavidin-alkaline phosphatase conjugate (Boehringer). The reaction was developed with *p*-nitrophenyl phosphate (pNPP) substrate (Sigma), and the optical density at 405 nm was read. A standard curve was prepared with the gp52-specific mouse monoclonal antibodies 2B5 (IgG1), H149.5 and H141.16 (IgG2a), and VE7 (IgG3). Serum titers were calculated from the serum dilution giving an optical density at 405 nm of 0.5.

In vivo neutralization assay. After depletion of complement by heating for 30 min at 56°C, the serum was mixed with virus and with PBS in equal volumes and was incubated on ice for 1 h. The mixture was injected into the hind footpad of naive BALB/c mice, and the draining popliteal lymph node was removed after 4 days. The percentage of $V\beta6^+$ cells among the $CD4^+$ T cells was measured by flow cytometry. Virus neutralization by the serum was determined from the reduction in the level of SAg-reactive $V\beta6^+$ cells in comparison to the level reached after injection of the virus with a control serum from an uninfected mouse, as described previously (33). The results are shown as a percentage of neutralization by comparison with a titer determination curve obtained after injection of serial dilutions of the virus preparation with control serum.

RESULTS

Backcrossing of the disrupted IFN receptor genes to the BALB/c mouse strain. The susceptibility to MMTV infection differs widely among different mouse strains (2). In particular, efficient presentation of the viral SAg is critical for the establishment of a high-level infection. MHC class II molecules differ in their ability to present the MMTV SAgs, and I-E molecules are the best presenters of all MMTV SAgs (2, 21). Since the disruption of the IFN-α/βR or of the IFN-γR gene

was performed in 129Sv/Ev mice ($H\text{-}2^b$, I-E$^-$), a mouse strain which is not highly susceptible to MMTV and has not been used as a classical model of MMTV infection, we decided to backcross the disrupted genes to the susceptible BALB/c background ($H\text{-}2^d$, I-E$^+$). Backcrossing of the disrupted IFN-α/βR or IFN-γR gene was performed for 6 or 10 generations, respectively, and heterozygous littermates were included as controls in all experiments, since heterozygous mice have not been shown to differ from wild-type animals in other studies (3a).

Early immune response induced by the viral SAg. To study the early immune response to MMTV in the presence or absence of a functional IFN-α/β or IFN-γ receptor, we injected MMTV(SW) into the footpad of adult IFN-α/βR$^{0/0}$, IFN-α/βR$^{+/0}$, IFN-γR$^{0/0}$, or IFN-γR$^{+/0}$ BALB/c mice and used flow cytometry to study the response in the draining popliteal lymph node at various time points after injection. Such an infection protocol has been shown previously to mimic the natural infection and to lead to a SAg-induced immune response in the draining lymph node, which is much easier to study than the small neonatal Peyer's patches (19, 27). All groups of mice showed a large increase in the percentage of SAg-reactive Vβ6$^+$ CD4$^+$ T cells, which peaked on day 4 after infection (Fig. 1A and B). During the same period, the percentage of control SAg-unreactive Vβ populations did not change or showed only slight compensatory decreases (data not shown). No significant difference was observed between IFN-α/βR$^{0/0}$ or IFN-γR$^{0/0}$ mice and their respective control heterozygous littermates. Since the overall efficiency of MMTV infection ultimately depends upon the absolute magnitude of this early T-cell response, in particular upon the increase in the number of infected B cells resulting from this response, we also studied the absolute increase in the number of cells in the draining lymph node at various time points after infection. The number of B cells increased more than 10-fold during the response, with a peak on days 4 to 6 after infection (Fig. 1C and D). Here again, although the variability was higher, no significant difference in the absolute number of B cells (Fig. 1C and D) or T cells (data not shown) could be observed among the various groups. Finally, a dose-response experiment was performed to study the SAg-induced immune response after injection of increasing amounts of virus (Fig. 1E and F). Maximal doses of MMTV(SW) induced an increase in the percentage of Vβ6$^+$ cells among CD4$^+$ T cells to 40 to 48% in all groups of mice. Importantly, no significant shift was observed in the dose-response curve of IFN-α/βR$^{0/0}$ or IFN-γR$^{0/0}$ BALB/c mice in comparison to their respective control littermates, showing that disruption of the IFN receptor does not cause an increased sensitivity to small amounts of MMTV, in contrast to the data reported for other viruses (23, 39).

These results show that the early SAg-induced immune response is not modified in the absence of a functional IFN-α/β or IFN-γ system.

Detection of proviral DNA by PCR. A semi-quantitative PCR assay was used to correlate the viral SAg activity with a more direct estimate of the viral load in infected tissues (Fig. 2). PCR amplification was performed on genomic DNA extracted from infected lymph nodes with oligonucleotides matching conserved regions of the LTR and amplifying the variable 3′ end of the *sag* gene (20). In such a PCR, the MMTV(SW) proviral DNA is amplified together with the DNA of endogenous *Mtv* (*Mtv-6*, *Mtv-8*, and *Mtv-9* in BALB/c mice) and the PCR products are separated according to their size. The endogenous *Mtv* can be considered natural internal controls of the reaction. DNA extracted from mixtures of cells from BALB.D2 mice (*Mtv-6*, *Mtv-7*, *Mtv-8*, and *Mtv-9*) and BALB/c mice (*Mtv-6*, *Mtv-8*, and *Mtv-9*) at variable ratios was included as a titer determination, since *Mtv-7* is closely related to MMTV(SW) and gives a PCR product of approximately the same size (Fig. 2A). The results are shown for samples of the draining lymph node on day 6 after injection of the virus, a time point when a peak of proviral DNA signal was demonstrated previously (18). No significant difference could be observed among the different groups of mice, as shown by a representative set of PCR products (Fig. 2B) or by the quan-

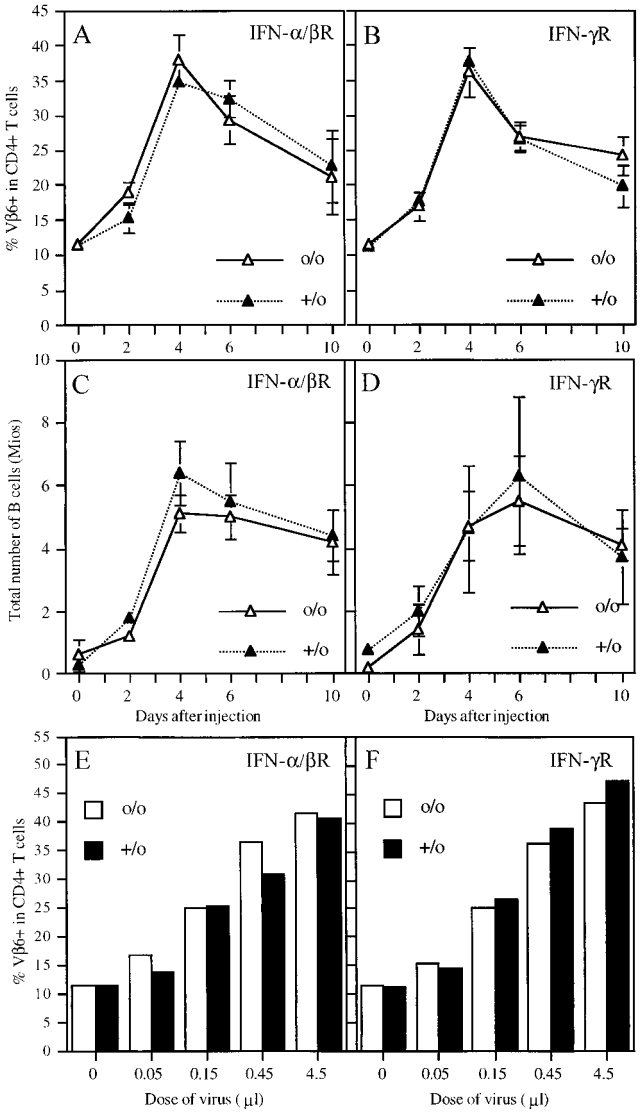

FIG. 1. Early immune response induced by the MMTV superantigen in mice lacking either the IFN-α/β receptor (IFN-α/βR) or the IFN-γ receptor (IFN-γR). MMTV(SW) was injected into the hind footpad of BALB/c IFN-α/βR$^{0/0}$ or IFN-γR$^{0/0}$ mice or control heterozygous littermates. Cells of the draining popliteal lymph node were studied by flow cytometry at various time points after injection. (A and B) Percentage of SAg-reactive Vβ6$^+$ cells among CD4$^+$ T cells in IFN-α/βR$^{0/0}$ and IFN-α/βR$^{+/0}$ mice (A) or IFN-γR$^{0/0}$ and IFN-γR$^{+/0}$ mice (B). (C and D) Absolute number of B cells (B220$^+$ CD4$^-$ CD8$^-$) in the draining popliteal lymph node of IFN-α/βR$^{0/0}$ and IFN-α/βR$^{+/0}$ mice (C) or IFN-γR$^{0/0}$ and IFN-γR$^{+/0}$ mice (D). Data in panels A to D are shown as means ± 2 standard errors (SE) for groups of three to six mice per time point. (E and F) Dose-response experiment. IFN-α/βR$^{0/0}$ and IFN-α/βR$^{+/0}$ mice (E) or IFN-γR$^{0/0}$ and IFN-γR$^{+/0}$ mice (F) were infected with increasing doses of MMTV (SW), as indicated by the amount of purified milk (microliters) present in the 20 μl injected in the footpad. The percentage of Vβ6$^+$ cells among CD4$^+$ T cells in the draining lymph node is shown on day 4 after injection (mean for two or three mice per dose).

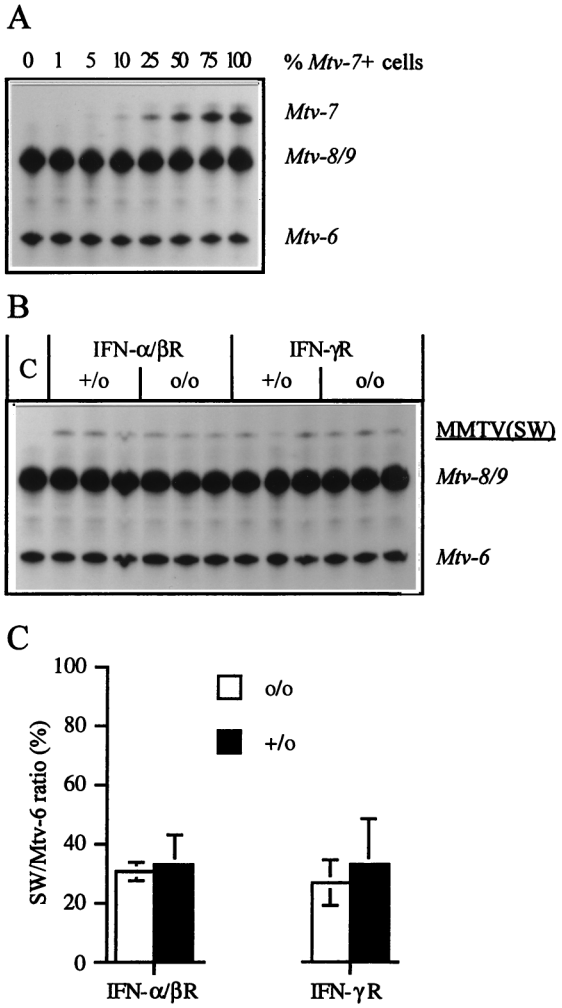

FIG. 2. Semiquantitative PCR analysis of proviral DNA from infected lymph node cells. DNA was extracted from cells of the draining popliteal lymph node on day 6 after footpad injection of MMTV(SW) into BALB/c IFN-α/βR$^{0/0}$ or IFN-γR$^{0/0}$ mice or control heterozygous littermates. PCR amplification was performed with primers matching conserved regions of the LTR. PCR products from MMTV(SW) provirus and from endogenous *Mtv* were separated according to their size. Endogenous *Mtv* are natural internal controls of the reaction. (A) Control titer determination with mixtures of cells from BALB/c (*Mtv-6*, *Mtv-8*, and *Mtv-9*) or BALB.D2 mice (*Mtv-6*, *Mtv-8*, and *Mtv-9* + *Mtv-7*). (B) Representative set of samples from BALB/c IFN-α/βR$^{0/0}$ or IFN-γR$^{0/0}$ mice or control heterozygous littermates. C, control uninfected BALB/c mouse. (C) Quantification of a large number of samples (8 to 12 independent draining lymph nodes per group). The results are shown as a ratio between the MMTV(SW) and the *Mtv-6* PCR products (mean ± 2 SE).

tification of a larger number of samples (Fig. 2C). Similar results were obtained on days 4 and 10 after infection. These data correlate with measurements of the viral SAg activity and argue against a substantial antiretroviral effect of IFNs during the early immune response to MMTV.

Long-term deletion of SAg-reactive cells. The MMTV infection was monitored by studying the viral SAg activity at later time points. A classical feature of SAgs is the clonal deletion of reactive T cells after the initial phase of stimulation (35, 62, 64). Such a deletion can be easily monitored in peripheral blood lymphocytes of infected mice. Furthermore, the kinetics and magnitude of the deletion correlate with the efficiency of the SAg presentation and with the initial dose of virus (18). A progressive decrease in the percentage of Vβ6$^+$ CD4$^+$ T cells was observed in IFN-α/βR$^{0/0}$ mice, IFN-γR$^{0/0}$ mice, and control heterozygous littermates, reaching an almost complete deletion after 15 weeks (Fig. 3). During this period, the percentage of control SAg-unreactive Vβ populations did not change or showed only slight compensatory increases (data not shown). Here again, no significant difference was seen between knockout mice and their respective control littermates. In addition, no increase in the amount of MMTV(SW) proviral DNA was seen in a set of target organs, including the spleen and mammary gland, during the late phase of the infection, and all groups of mice transmitted the virus with a similar efficiency to the first litter of the next generation of mice (data not shown). These results show that the two types of IFNs do not seem to play a significant antiviral role in MMTV infection. If minor differences cannot be excluded at late stages of the infection, they would be irrelevant, since they would not result in any change in the completion of the viral life cycle.

Balance of cytokine gene expression during the SAg-induced immune response. In addition to its antiviral activity, IFN-γ is known to modulate the production of several cytokines and, in particular, to play a central role in the regulation of Th1-Th2 CD4$^+$ T-cell subsets. To study the importance of IFN-γ on the expression of cytokine genes during the strong immune response triggered by the MMTV SAg, we extracted RNA from infected lymph nodes of IFN-γR$^{0/0}$ or IFN-γR$^{+/0}$ mice on day 5 or 6 after injection of MMTV(SW) and used a semiquantitative competitive RT-PCR assay for IL-4 mRNA. The basal

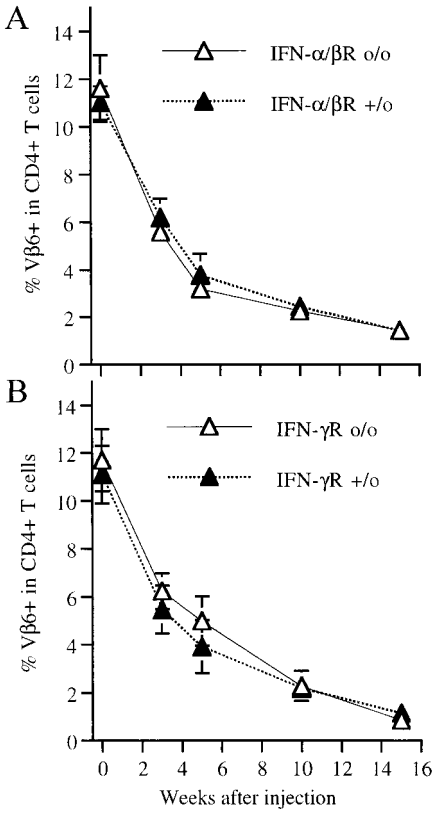

FIG. 3. Kinetics of the deletion of SAg-reactive cells after infection with MMTV. BALB/c IFN-α/βR$^{0/0}$ (A) or IFN-γR$^{0/0}$ (B) mice or control heterozygous littermates were injected in the hind footpad with MMTV(SW). Peripheral blood lymphocytes were isolated at various time points after infection, and the percentage of SAg-reactive Vβ6$^+$ cells among CD4$^+$ T lymphocytes was determined by flow cytometry. The results are shown as mean ± 2 SE for at least five mice per group.

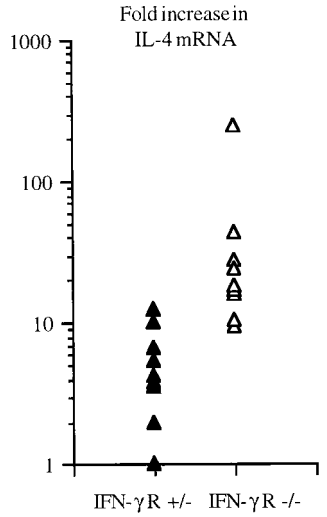

FIG. 4. Increase in IL-4 mRNA levels in the draining lymph node after infection with MMTV. BALB/c IFN-α/βR$^{0/0}$ or IFN-γR$^{0/0}$ mice or control heterozygous littermates were injected in the hind footpad with MMTV(SW). Total RNA was isolated from the draining popliteal lymph node on day 5 or 6 after infection. A semiquantitative competitive RT-PCR analysis of IL-4 transcripts was performed with HPRT mRNA to normalize the results. The data are shown as the fold increase in cytokine mRNA in the infected lymph node in comparison to a control lymph node from an uninfected mouse.

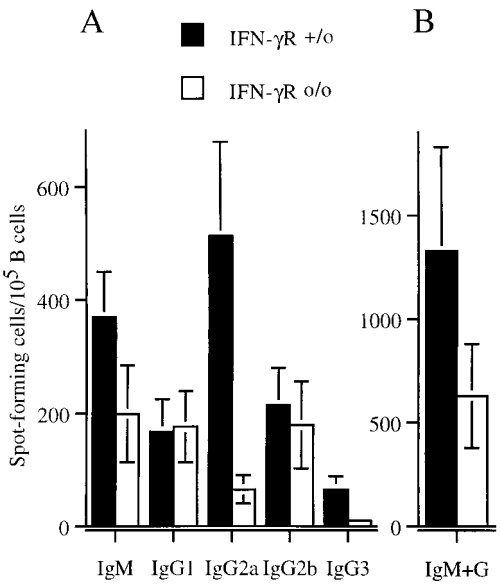

FIG. 5. Quantification of the number of B cells producing Igs in the draining lymph node after infection with MMTV. BALB/c IFN-γR$^{0/0}$ mice or control heterozygous littermates were injected in the hind footpad with MMTV(SW). Cells of the draining popliteal lymph node were isolated on day 6 after infection and used in an ELISPOT assay. The results are shown as the number of spot-forming cells per 10^5 B cells (mean ± SE for four mice per group) for individual Ig isotypes (A) or for all IgM- and IgG-producing cells (B).

levels of IL-4 mRNA in uninjected mice were very low and did not differ by more than twofold (data not shown). Despite variations in the results for individual mice, the amounts of IL-4 mRNA after injection of MMTV were significantly larger in the draining lymph node in the absence of a functional IFN-γ receptor (Fig. 4). These results show that IFN-γ negatively regulates the production of IL-4 during the early immune response to MMTV. Using the same technique, we also assessed the levels of IFN-γ mRNA in the same samples (data not shown). The fold increase in the level of IFN-γ mRNA after MMTV infection was smaller in IFN-γR$^{0/0}$ mice than in control heterozygous littermates. However, the basal values were also higher in IFN-γR$^{0/0}$ mice and the peak levels of IFN-γ mRNA were thus similar in the presence and absence of IFN-γ receptor.

Antibody secretion during the SAg-induced immune response. In the course of the early immune response to MMTV, the T-cell–B-cell interaction induced by the viral SAg leads to the activation of the B cells, which differentiate and secrete large amounts of antibodies with a peak production on day 6 after infection (18, 34). We used an ELISPOT assay to study the isotype pattern of antibodies produced by cells of the draining lymph node after footpad injection of MMTV(SW). Dramatic differences were observed between IFN-γR$^{0/0}$ mice and control littermates (Fig. 5A), whereas no changes were seen in IFN-α/βR$^{0/0}$ mice (data not shown). In the absence of a functional IFN-γ receptor, at least 10-fold-fewer cells produced IgG2a at the peak of secretion on day 6. The number of cells secreting IgG3 was also significantly reduced. In contrast, the number of cells producing IgG1 was not increased and the number of cells producing IgM was even slightly reduced in the IFN-γR$^{0/0}$ mice. As a result, the total number of IgM- and IgG-producing cells was smaller in the absence of IFN-γ receptor (Fig. 5B) even if the total number of B cells in the draining lymph node was similar (Fig. 1D). These data confirm that IFN-γ plays a critical role in inducing the switch to IgG2a and IgG3 production in vivo. However, the lack of increase in the number of IgG1-producing cells in IFN-γR$^{0/0}$ mice is surprising in view of the increased amounts of IL-4 mRNA detected by RT-PCR (Fig. 4), since IL-4 has been shown to upregulate the production of IgG1 reciprocally to IFN-γ (14, 53).

Virus-specific antibody response. In parallel to the polyclonal antibody production triggered by the viral SAg, a viral envelope-specific humoral response can be observed quickly after MMTV infection, appearing on day 3 to 4 and persisting for life (32, 33). We were interested to study the influence of IFN-γ on the generation and maintenance of this virus-specific response. The presence of MMTV-specific antibodies in the serum of infected mice was studied by an ELISA with recombinant viral envelope protein gp52 (Fig. 6). The results are shown for *env*-specific IgG of all isotypes (Fig. 6A) and of the IgG1 and IgG2a isotype only (Fig. 6B and C). Both IFN-γR$^{0/0}$ and IFN-γR$^{+/0}$ mice generated significant titers of virus-specific IgGs, especially at late time points after infection. However, the IgG titers tended to be lower in IFN-γR$^{0/0}$ mice early after infection. In particular, the levels of *env*-specific IgG2a antibodies were markedly reduced and even undetectable on day 8 in mice lacking the IFN-γ receptor. This reduction was no longer seen on day 200. Interestingly, titers of virus-specific IgG1 antibodies were similar but not increased in the absence of the IFN-γ receptor, in accordance with the results of the ELISPOT assay (Fig. 5).

To assess these antibodies functionally, an in vivo neutralization test was performed (Fig. 7), as described previously (33). A high neutralizing potential was observed for sera taken on day 8 after infection, even in IFN-γR$^{0/0}$ mice with low IgG titers, probably because of the presence of neutralizing IgM antibodies. The serum neutralization potential was also not significantly different between IFN-γR$^{0/0}$ and IFN-γR$^{+/0}$ mice when the serum was used at higher dilutions (data not shown). In contrast, the virus neutralization observed with day 24 and day 50 sera from IFN-γR$^{0/0}$ mice seemed to be less efficient

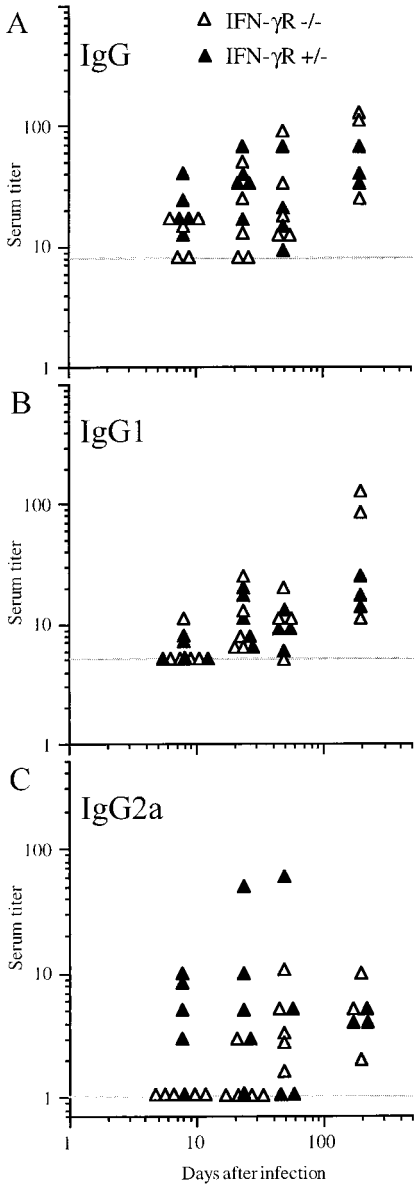

FIG. 6. Virus-specific antibody response in the serum of BALB/c IFN-$\gamma R^{0/0}$ mice or control heterozygous littermates after infection with MMTV. Serum was collected at various time points after injection of MMTV(SW) and analyzed by ELISA for gp52-specific total IgG (A), IgG1 (B), or IgG2a (C). The results are shown for individual mice as the reciprocal serum dilution giving an optical density at 405 nm of 0.5. The horizontal line indicates the value obtained with control serum from an uninfected mouse.

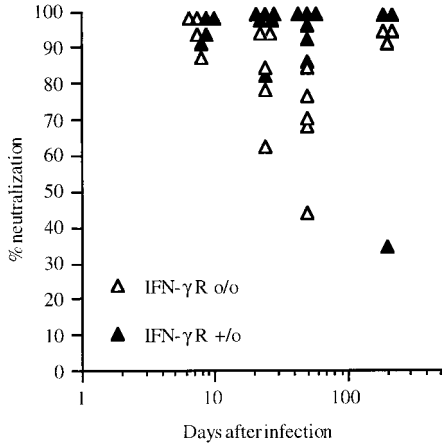

FIG. 7. Virus-neutralizing activity in the serum of BALB/c IFN-$\gamma R^{0/0}$ mice or control heterozygous littermates after infection with MMTV as assessed by an in vivo neutralization test. Serum was collected at various time points after injection of MMTV(SW), coincubated with a virus preparation, and injected into naive BALB/c mice. Neutralization of the virus by the serum resulted in a smaller increase in the percentage of SAg-reactive $CD4^+$ T cells in the draining lymph node on day 4 after infection. The results are shown for individual mice as a percentage of virus neutralization by using a standard curve obtained by injection of serial dilutions of the virus preparation.

than with sera from IFN-$\gamma R^{+/0}$ mice, although it was far from being completely abolished. Finally, all sera collected on day 200 were comparably neutralizing, with one exception. In summary, IFN-γ seems to contribute to the generation of an early neutralizing IgG antibody titer after MMTV infection. Mice lacking the IFN-γ receptor have a delayed increase in the titer of MMTV-specific IgG antibodies but eventually reach similar antibody titers to those of heterozygous littermates.

DISCUSSION

We have used mice with a disrupted IFN-α/β or IFN-γ receptor gene to study the overall effects of these IFNs during MMTV infection in vivo. The MMTV SAg activity was taken as a measure of the viral infection, since there are no conventional methods available to measure MMTV titers in infected organs. The amplitude of the SAg response was shown previously to differ according to the dose of injected virus, with greater amplitudes of response for higher doses of virus (18). We therefore assumed that the SAg response would allow us to assess indirectly the level of viral replication in vivo and in particular to determine whether IFNs have a significant antiviral activity during MMTV infection.

We found that the SAg response to MMTV(SW) was not affected by disruption of either the IFN-α/β or IFN-γ receptor. In addition, no increase in the amount of proviral DNA could be observed in the infected lymph nodes of IFN-α/β$R^{0/0}$ or IFN-$\gamma R^{0/0}$ mice. These observations imply that IFNs do not play an essential role in the immunological events triggered by the SAg. Furthermore, they suggest that IFNs are not part of the important defense mechanisms of the host during MMTV infection. Similar results were obtained in the original 129Sv/Ev IFN-α/β$R^{0/0}$ and IFN-$\gamma R^{0/0}$ mice, showing that the lack of a functional IFN-α/β or IFN-γ system does not convert a poorly susceptible mouse strain to a highly susceptible one (36a).

Our data do not necessarily imply that IFN-α/β or IFN-γ per se cannot have an effect on MMTV replication. It is possible that these molecules are produced in insufficient amounts and/or for too short a time to have a measurable antiviral effect in vivo. Such a paradox has already been reported for vesicular stomatitis virus (VSV), which does not replicate more effectively in IFN-$\gamma R^{0/0}$ mice but is usually sensitive to the addition of IFN-γ to the cell culture (23, 60). Indeed, the production of MMTV particles has been previously reported to be inhibited by the addition of IFN-α/β to infected mammary tumor cells in culture (4, 50). However, the effects observed upon addition of large doses of exogenous IFNs either in cell culture or in vivo might not be representative of the true role of these molecules in the course of a natural infection. IFNs have many effects on cell growth and metabolism which may exert profound changes on the replication of certain viruses, especially if they are

applied at high concentrations. Therefore, we believe that gene-disrupted mice are particularly useful in distinguishing relevant antiviral effects of interferons in vivo from the different phenomena observed in cell culture.

MMTV is a noncytopathic retrovirus which establishes a lifelong infection of the host and does not cause acute pathologic effects. The lack of an apparent antiviral effect of IFNs on MMTV infection in vivo must be interpreted in this context. Indeed, the effects of IFNs might be more relevant for the control of acutely pathogenic cytopathic viruses such as VSV or vaccinia virus (23, 39). In the future, IFN-$\alpha/\beta R^{0/0}$ and IFN-$\gamma R^{0/0}$ mice could be interesting models for use in studies of the course of infection with other retroviruses and for determinations of whether our observations with MMTV are representative of a general property of retroviruses or are specific for this virus. We already have preliminary evidence that IFNs might play a more important role in the course of infection with Moloney murine leukemia virus (36a).

The similar course of MMTV infection in IFN-$\gamma R^{0/0}$ BALB/c mice and heterozygous controls is also interesting, since IFN-γ might have been expected to play an important role in the activation of MMTV-infected B cells. Indeed, the SAg-mediated amplification of MMTV infection clearly relies on a T-cell-dependent B-cell activation, and IFN-γ might have been one of the signals mediating this interaction. However, the absolute increase in the number of B cells as well as in the amount of proviral DNA did not differ in IFN-$\gamma R^{0/0}$ BALB/c mice and control littermates, even though fewer antibody-producing cells were observed in these mice (Fig. 5B). Interestingly, there are data suggesting that cell-cell interactions (e.g., through CD40L-CD40 or CD28-B7 interaction) might play a more critical role than cytokine-mediated signals in the T-cell–B-cell interaction which is driving the amplification of the infected B cells during MMTV infection (7, 8).

Environmental and host genetic factors are known to influence the pattern of the Th1-Th2 $CD4^+$ T-cell response in several experimental systems (48). In our study, the increased amounts of IL-4 mRNA observed in IFN-$\gamma R^{0/0}$ mice during the MMTV SAg-induced immune response are consistent with a skewing of the $CD4^+$ T-cell response to a Th2 phenotype. In contrast, other groups have shown that IFN-$\gamma R^{0/0}$ mice failed to switch to a Th2 response when infected with pseudorabies virus or with the parasite *Leishmania major* (47, 54). These contrasting results might be explained by differences between the experimental systems or between mouse strains, since pseudorabies and *L. major* infections were studied in mice from the 129Sv/Ev background whereas we worked with IFN-$\gamma R^{0/0}$ mice backcrossed on the BALB/c background.

Studying the Ig isotype pattern is an indirect way to assess the Th1/Th2 cytokine balance, because the switch to IgG2a is known to be induced by IFN-γ secretion (Th1 response) and the switch to IgG1 is induced by IL-4 secretion (Th2 response) (14, 53). We observed an at least 10-fold reduction in the number of IgG2a-secreting cells in IFN-$\gamma R^{0/0}$ mice, confirming that IFN-γ is one of the key factors for the switch to this isotype in vivo. Interestingly, this reduction was most apparent in the polyclonal antibody response triggered by the viral SAg, as shown in the ELISPOT assay, and in the virus-specific antibody response at early time points after infection. At later time points, the titers of *env*-specific IgG2a also rose in the IFN-$\gamma R^{0/0}$ mice, indicating that other cytokines might ultimately compensate for the lack of IFN-γ-mediated signals in the induction of the switch to IgG2a production. Data from others suggest that IFN-α/β could have such an effect, since undetectable levels of specific IgG2a antibodies were observed during lymphocytic choriomeningitis virus infection in mice lacking both IFN-α/β and IFN-γ systems (58). In addition, the production of IgG3 antibodies was reduced in the IFN-$\gamma R^{0/0}$ mice, although to a lower extent than the production of IgG2a, in accordance with data from other groups (23, 52). In contrast, no significant increase in the number of IgG1-producing cells or in the virus-specific IgG1 titers were observed after infection. This finding is surprising in view of the increased amounts of IL-4 mRNA already detected by RT-PCR by days 5 and 6 after infection. It is possible, however, that the increased IL-4 production is not sufficiently high or sustained to have a measurable effect on the secretion of IgG1 in our experimental system.

Infection with MMTV induces the appearance of virus-specific neutralizing antibodies in the serum, which can be detected as early as 3 or 4 days after infection and persist for life (32, 33). Although this humoral response seems to be very efficient, its functional significance in the course of the viral life cycle remains unclear, as discussed previously (33). Since IgG2a is an important component of the early antibody response to MMTV as well as many other viruses (10), it was interesting to study the neutralizing activity of serum from IFN-$\gamma R^{0/0}$ and IFN-$\gamma R^{+/0}$ mice at various times after MMTV infection. A similar neutralizing activity was observed for sera taken on day 8 after infection, probably due to the presence of a virus-specific IgM response. A moderate decrease in neutralization was observed later in IFN-$\gamma R^{0/0}$ mice. However, this decrease was no longer apparent on day 200 after infection. These results show that IFN-γ contributes to the early virus-specific antibody response during MMTV infection but is not required to maintain the response in the long term. They contrast with data from Schijns et al. showing more drastic effects of disruption of the IFN-γ receptor on the neutralizing antibody response to pseudorabies virus (47).

In conclusion, our data show that IFN-α/β and IFN-γ systems do not seem to play an essential antiviral role during MMTV infection in vivo. Furthermore, the lack of a functional IFN-γ receptor was observed to affect the cytokine balance during the immune response to MMTV, with an increased production of IL-4 mRNA compatible with a skewing of the T-cell response toward a Th2 phenotype. Finally, IFN-$\gamma R^{0/0}$ mice mounted an antibody response with a perturbed isotype pattern and exhibited transiently lower virus-specific antibody titers and neutralizing activity in serum than did control animals.

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