Unique Resistance of I/LnJ Mice to a Retrovirus Is Due to Sustained Interferon γ-dependent Production of Virus-neutralizing Antibodies

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Abstract

Selection of immune escape variants impairs the ability of the immune system to sustain an efficient antiviral response and to control retroviral infections. Like other retroviruses, mouse mammary tumor virus (MMTV) is not efficiently eliminated by the immune system of susceptible mice. In contrast, MMTV-infected I/LnJ mice are capable of producing IgG2a virus-neutralizing antibodies, sustain this response throughout their life, and secrete antibody-coated virions into the milk, thereby preventing infection of their progeny. Antibodies were produced in response to several MMTV variants and were cross-reactive to them. Resistance to MMTV infection was recessive and was dependent on interferon (IFN-γ) production, because I/LnJ mice with targeted deletion of the INF-γ gene failed to produce any virus-neutralizing antibodies. These findings reveal a novel mechanism of resistance to retroviral infection that is based on a robust and sustained IFN-γ-dependent humoral immune response.

Key words: IFN-γ • virus-neutralizing antibodies • retrovirus • infection • resistance

Introduction

Exogenous mouse mammary tumor virus (MMTV)* is transmitted through the milk of infected females to suckling newborns, where it initially infects cells of the immune system (1). The MMTV genome encodes a superantigen (Sag; reference 2) that is presented by MHC class II molecules expressed on infected B cells and is recognized by TCRs expressed on cognate T cells (3, 4). Different Sags stimulate distinct subsets of T cells (each with a particular TCR Vβ chain), causing them to proliferate and allowing them to become infected with the virus (5). After the initial proliferation of T cells, steady deletion of the cognate T cells is observed in all infected mice (6). From the cells of the immune system, the virus passes to the mammary tissue and causes mammary carcinomas as a result of insertional mutagenesis (7).

Three mechanisms of resistance to MMTV infection in mice have previously been discovered. The first was mapped to the MHC locus, H2 (8). Inbred mice of b, f, q, and s H2 haplotypes do not express I-E MHC class II molecules because of mutations in either the H2-Ea or H2-Eb gene (9, 10). As I-E H2 molecules are better suited for Sag presentation, mice with the I-E-negative H2 haplotypes are relatively resistant to MMTV infection and MMTV-induced mammary tumors (11–13).

The second mechanism of resistance to MMTV was found in mice that carry endogenous proviruses, which encode Sags similar to Sag of exogenous virus. Viral Sags expressed by endogenous Mtv's cause negative selection of T cells bearing cognate Vβ elements in the thymus and in the periphery (14–16). As a result, mice exposed to an exogenous MMTV that encodes a Sag with the same specificity as the endogenous Mtv's are resistant to MMTV infection and do not develop mammary gland tumors (5, 17).

Previously we have identified a third mechanism of resistance to MMTV inherited by I/LnJ mice (18). We showed that resistance to MMTV(C3H)-induced mammary tumors in mice from this strain is due to impaired mammary gland infection and is not related to the MHC locus or presence of endogenous Mtv's (18). Here we found that production of avirulent virions by infected I/LnJ cells underlies the impaired mammary gland infection and sought to identify the mechanism involved.

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*Abbreviations used in this paper: AP, alkaline phosphatase; MMTV, mouse mammary tumor virus; MuLV, murine leukemia virus; Sag, superantigen.
Materials and Methods

**Mice.** All mice used in this study were bred and maintained at the animal facility of The Jackson Laboratory. I/LnJ, C3JK-H2\(^{a}\) H2-T18/Sn (C3.JK), and BALB/cJ mice were purchased from The Jackson Laboratory. I/LnJ mice were crossed to B6.129S7-\(Ifn^{γ-}\)/Tc (19) for 10 repetitive generations, and heterozygous N10 mice were intercrossed to generate I/LnJ mice with targeted mutation of IFN-\(γ\) (I/LnJ IFN-\(γ\) KO). C3H/HeN MMTV\(^+\) mice infected with the MMTV(C3H) virus variant were purchased from the National Cancer Institute, Frederick Cancer Research Facility, Frederick, MD. The MMTV(LA) virus variant (20) was passed on BALB/cJ mice.

**Antibodies and Flow Cytometry.** Mononuclear peripheral blood lymphocytes were stained with FITC-coupled monoclonal antibodies against \(\mathrm{V}_{\beta}2\), \(\mathrm{V}_{\beta}6\), and \(\mathrm{V}_{\beta}14\) TRC chains (BD Biosciences). Anti-CD4 antibodies coupled to PE (Life Technologies) were used in the second dimension. Leukocytes were recovered from heparinized blood samples by centrifugation through a Ficoll/Hypaque cushion. Peripheral blood lymphocytes were analyzed using a FACScan (Becton Dickinson) flow cytometer and the CELLQuest software program.

**RNase T\(_1\) Protection Assays.** RNaseT\(_1\) protection assays were performed as described previously (21), using probes specific for BALB2, BALB, and BALB14 viral transcripts (20). 40 \(\mu\)g of total RNA isolated from the lactating mammary glands and 5 \(\mu\)g of RNA isolated from milk were used.

**Production of Cytokines by Activated T Cells.** Anti-CD3 antibodies (5\(\mu\)g/ml) were attached to 6-well plates for 1.5 h at 37°C in PBS buffer. Wells were washed with PBS two times. 9 \(\times\) 10\(^6\) cells isolated from the lymph nodes pooled from three mice were added per a well and incubated for 24 h in the CO\(_2\) incubator. RNA isolated from activated cells was subjected to RNase protection analysis with Riboquant probes using the manufacturer’s protocol (BD Biosciences). 20 \(\mu\)g of splenic RNA and 5 \(\mu\)g of lymph node RNA (before and after activation) were loaded on a 6% urea/acylamide gel.

**Mammary Gland Tumorgenesis.** Mammary gland tumor incidence in MMTV(LA)-infected BALB/cJ and I/LnJ mice was monitored by weekly palpation of the animals. Tumor-bearing mice were killed and DNA isolated from a portion of each tumor was subjected to Southern blot analysis as described previously (22). All of the tumors contained new MMTV integrants, indicating that the tumors were caused by the virus (unpublished data).

**Production of Monoclonal Antibodies against MMTV Virion Proteins.** Endogenous Mtv-\(\beta\)-free male mice, a gift of Dr. Marrack (National Jewish Medical and Research Center, Denver, CO; reference 23), were immunized with 1% Triton X-100-treated MMTV(LA) virions (100 \(\mu\)g of proteins) in CFA by subcutaneous injection in two hind footpads and four locations in the back. Mice were challenged 3 wk later with the same dose of virus. Fusion of the tumors was challenged with the same amount of Mtv integrants by intravenous injection. Fusion was done according to standard protocol. Antibodies were characterized by ELISA and Western blot analysis.

**ELISA with MMTV Virion Proteins.** Either MMTV(C3H) isolated from the milk of C3H/HeN MMTV\(^+\) mice or MMTV(LA) isolated from the milk of BALB/cJ mice as described (20, 23) was resuspended in PBS with 0.2% Triton X-100 and bound to the plate at 0.5 \(\mu\)g/ml in PBS buffer overnight at 4°C. Non-specific binding was blocked by 1% bovine serum albumin for 2 h at 37°C. Secondary antibodies against different mouse immunoglobulins classes conjugated with alkaline phosphatase (AP) diluted in the ELISA buffer (PBS, 0.05% Tween 20, and 0.1% sodium azide) were used at the second step. Background obtained with the secondary antibody alone (goat anti–mouse immunoglobulins coupled to AP) was subtracted. To test for antiviral antibodies in mouse sera, serum samples were diluted in the ELISA buffer and incubated with virion proteins bound to plastic before adding the secondary antibodies.

**Western Blot Analysis.** Viral particles were isolated from BALB/cJ milk. Viral proteins were electrophoresed on 10% polyacrylamide gels under reducing conditions in the presence of 1% sodium dodecyl sulfate. The proteins were then transferred to nitrocellulose membranes, treated with different serum samples or anti-MMTV protein monoclonal antibodies at the first step and conjugated to horseradish peroxidase (Upstate Biotechnology) at the second step, and detected with Western Blot Detection Reagents (Amersham Biosciences).

**Immunization.** Preimmune sera were collected from 2-mo-old BALB/cJ and I/LnJ mice, which were then immunized with MMTV(LA) virus proteins isolated from 100 \(\mu\)l of milk in CFA by subcutaneous injection in two hind footpads and four locations in the back. Mice were challenged 3 wk later with the same dose of antigen in IFA. Serum samples were collected 10 d later and the reactivity of pre and postimmune serum samples to MMTV(LA) virion proteins was compared by ELISA. AP-labeled goat anti–mouse immunoglobulin antibodies were used in the second step (Sigma-Aldrich).

**MMTV Infection.** Biologically active MMTV virions were isolated from the milk of MMTV(LA)-infected BALB/cJ females as described previously (22, 24). Proteins from virions isolated from 100 \(\mu\)l of milk were injected intraperitoneally into 3– to 4-wk-old I/LnJ and BALB/cJ females as described previously (24).

**Virus-Neutralization Assay.** The in vitro neutralization procedure was performed at room temperature as published previously (25). Briefly, purified MMTV(LA) or MMTV(C3H) virions (virions isolated from \(\sim\)100 \(\mu\)l of milk in 20 \(\mu\)l of PBS) were incubated with 0.05 \(\mu\)l of serum from uninfected BALB/cJ mice for 30 min, and bound virions were subjected to Southern blot analysis. For the in vivo neutralization experiment, newborn BALB/cJ mice suckling on viremic BALB/cJ females were injected intraperitoneally with 50 \(\mu\)l of serum from either uninfected or MMTV(LA)-infected I/LnJ mice (diluted 1/10 in PBS) every other day for 9 d (5 injections). Mice were bled, and RNA isolated from their lactating mammary glands after the first pregnancy was subjected to RNase T\(_1\) protection analysis with MMTV-specific probes.

**Results**

The Mammary Gland Cells of I/LnJ Mice Become Infected with MMTV(LA), but Viruses Shed into Milk Are Not Infectious. In our previous studies we showed that in I/LnJ mice exposed to the exogenous MMTV(C3H), infection did not progress to the mammary glands (18). Another MMTV, MMTV(LA) originated from BALB/cJ mice, consists of three different exogenous MMTVs: BALB2, BALB, and BALB14, with \(\mathrm{V}_{\beta}2\), \(\mathrm{V}_{\beta}6\), and \(\mathrm{V}_{\beta}14\)-specific Sags, respectively (20, 26). These MMTV(LA) viruses are produced at much higher titers than are MMTV(C3H) viruses (20). To determine whether infection with MMTV(LA) progresses to the mammary glands, I/LnJ mice were fostered by BALB/cJ...
mice infected with MMTV(LA) and frequencies of Sagg-cognate T cells in the periphery were analyzed at 14 wk of age. Whereas uninfected I/LnJ mice have 9.6 ± 0.7% of CD4+/VB14+ and 6.5 ± 0.25% of CD4+/VB2+ T cells (n = 12), MMTV(LA)-infected 14 wk-old I/LnJ mice have 0.89 ± 0.6% of CD4+/VB14+ T cells and 0.64 ± 0.21% of CD4+/VB2+ T cells (n = 12). We did not analyze a subset of CD4+/VB6+ T cells affected by a third virus, MMTV(LA), present in the mixture, because I/LnJ mice inherit Mtv7 and thus, do not have CD4+/VB6+ T cells (18). Infected I/LnJ females were bred, tested for the presence of virus in their milk, and monitored for mammary tumors. Mammary glands of all fostered I/LnJ mice become infected, as they secreted all three MMTVs into their milk (Fig. 1 A, lanes 1–3). Quantitative analysis of viral RNA isolated from the milk of infected mice ruled out differences in virus titers between infected BALB/cJ and I/LnJ mice (Fig. 1 D). Thus, in contrast to MMTV(C3H), infection with a stronger MMTV(LA) virus variant progressed to the mammary glands of I/LnJ mice.

Although mammary glands of fostered I/LnJ mice became MMTV infected, they were resistant to mammary tumor development. Whereas 80% of MMTV(LA)-infected BALB/cJ females (16/20) developed mammary tumors by 1 yr of age, whereas none of 24 I/LnJ females infected with the same viruses had developed tumors even at 1.5 yr of age.

To determine whether the virus secreted by infected I/LnJ mice was passed to their offspring, we produced second-generation females and tested their milk for virus presence. Almost all animals either had eliminated the viruses (Fig. 1 B, lanes 2, 3, 4, 6, and 7) or produced them at significantly reduced titers compared with their mothers (Fig. 1 B, lanes 1 and 5). Thus, after only one passage through I/LnJ mice MMTV(LA) was severely attenuated.

To investigate whether MMTV(LA) produced by infected I/LnJ mice was modified in such a way that it was no longer infectious in susceptible mice, we examined MMTV-susceptible BALB/cJ and C3H/HeN mice that were foster-nursed by the same MMTV(LA)-infected I/LnJ females. All fostered BALB/cJ and C3H/HeN mice either did not produce virus or produced virus at much reduced titers as determined by RNase T1 analysis with MMTV(LA)-specific probes (Fig. 1 C, data shown for BALB/cJ mice only). Thus, although I/LnJ mice became infected with MMTV(LA), the viruses they produced were modified such that they lost infectivity.

**MMTV-infected I/LnJ Mice Produce Antibodies against MMTV.** The results of described experiments suggest that MMTV proteins required for virus infectivity were either modified or blocked by anti-virus neutralizing antibodies produced by infected I/LnJ mice. To test this hypothesis, virus was isolated from the milk of MMTV(LA)-infected I/LnJ and BALB/cJ females and subjected to an ELISA to test for the presence of immunoglobulins coating the viral particles. Virus isolated from the milk of infected I/LnJ females was coated with immunoglobulins of the IgG2a isotype, while virus isolated from infected BALB/cJ milk was not (Fig. 2 A, left and right graph).
Neutralizing Antibodies and Resistance to Retroviruses

Next we sought to determine whether anti-MMTV antibodies were present in the sera of MMTV(LA)-infected I/LnJ mice. Serum samples were collected from I/LnJ and BALB/cJ mice that were either infected with MMTV(LA) through foster nursing or were virus-free. Again we used ELISA to test for the reactivity of different sera samples to MMTV(LA) virion proteins bound to the plate. In contrast to BALB/cJ mice, sera of MMTV(LA)-infected I/LnJ mice contained antibodies reactive to the MMTV virion proteins, and these antibodies belonged to the IgG2a isotype (Fig. 2 B, top graph).

Unlike MMTV(LA)-infected I/LnJ mice, I/LnJ mice infected with MMTV(C3H) showed impaired mammary gland infection, whereas hybrid F1 mice obtained from crosses between susceptible C3H/HeN females and resistant I/LnJ males were susceptible to infection (18). However, when the F1 females were backcrossed to I/LnJ males, an N2 generation was produced in which 50% of the females were mammary gland infected, whereas the other 50% of the females showed impaired mammary gland infection (18). Furthermore, all susceptible N2 females, but not resistant N2 females, developed mammary tumors (18). When serum samples from MMTV(C3H)-infected resistant and susceptible N2 females were tested in an ELISA for their reactivity against MMTV virion proteins, it appeared that all sera from the resistant N2 females contained anti-MMTV antibodies of the IgG2a isotype (Fig. 2 B, bottom graph). In contrast, no MMTV(C3H)-infected susceptible N2 mice showed production of anti-MMTV antibodies (Fig. 2 B, bottom graph). Thus, resistance to mammary gland infection and subsequent mammary tumor development cosegregated with production of virus-neutralizing antibodies and are controlled by a single recessive gene.

Such an efficient development of anti-virus antibodies in I/LnJ mice was unexpected, as these animals were exposed to MMTV as neonates and, thus, were anticipated to be tolerant to the viral antigens. Previously we showed that newly integrated MMTV proviruses were detected in thymi of I/LnJ mice exposed to the virus as neonates suggesting that they were infected (18). Furthermore, Sag-cognate T cells were deleted from both CD4+ and CD8+ single-positive (SP) thymocytes subsets. The normal percentage of Vα14+ T cells among SP CD4+ and CD8+ T cells in thymus of uninfected I/LnJ mice is 11.0 ± 0.7 and 4.0 ± 0.4, n = 3, respectively. This percentage declined to 6.1 ± 0.9 and 1.2 ± 0.9, n = 5, respectively, in thymi of infected 14-wk-old I/LnJ mice. Thus, although MMTV infection of the thymus in I/LnJ mice results in deletion of Sag-reactive T cells, it does not lead to tolerance to other viral proteins.

Figure 2. MMTV-infected I/LnJ mice produce IgG2a-specific antibodies against MMTV. (A) Viral particles isolated from the milk of I/LnJ mice fostered by BALB/cLA mothers are coated with antibodies of the IgG2a isotype. Left graph, virions were purified from the milk of MMTV(LA)-infected I/LnJ and BALB/cJ mice, bound to plastic, and analyzed for coating immunoglobulins by ELISA. The reaction was developed with goat anti-mouse isotype-specific immunoglobulins coupled to AP. Right graph, purified anti-gp52 mAbs of the IgG1 isotype were bound to plastic at 3 μg/ml followed by incubation with virions collected from milk of MMTV(LA)-infected BALB/cJ and I/LnJ mice. ELISA was developed with anti–mouse IgG2a-specific immunoglobulins coupled to AP. (B) IgG2a-specific antibodies reactive against MMTV virion proteins are present in the sera of MMTV-infected I/LnJ mice and resistant N2 mice. Top graph, the ELISA was performed with MMTV(LA) virion proteins isolated from 8-wk-old BALB/cLA mice and serum samples from age-matched uninfected or MMTV(LA)-infected I/LnJ and BALB/cJ mice and developed with either goat anti-mouse polyvalent immunoglobulins or goat anti-mouse IgG2a-specific immunoglobulins coupled to AP. Bottom graph, serum samples from MMTV(C3H)-infected resistant and susceptible N2 mice, as well as from MMTV(C3H)-infected I/LnJ, C3H/HeN, and F1 mice were tested for reactivity against MMTV virion proteins by ELISA. Anti–mouse IgG2a antibodies coupled to AP were used at the second step. Sera were diluted 10−3.
Antibodies Produced by Infected I/LnJ Mice React with Major Virion Proteins and Are Capable of Neutralizing MMTV. To determine which viral proteins are recognized by anti-MMTV antibodies, lysates of MMTV(LA) viral particles purified from the milk of BALB/cLA females were separated by denaturing PAGE, transferred to nitrocellulose, and incubated with sera from mice infected or uninfected with MMTV. Antibodies against gp52 (a-gp52), p27 (a-p27), gp36 (a-gp36), or sera (10⁻² dilution) from infected I/LnJ mice (I/LnJ MMTV⁺) or uninfected I/LnJ mice (I/LnJ⁻) were used. Blots were developed with anti-mouse polyclonal, or antimouse IgG2a-specific or IgG1-specific antibodies coupled to horseradish peroxidase. 1–6, individual mice of 30 tested. (B) Sera from MMTV-LA-infected I/LnJ mice protect susceptible mice from MMTV infection. Purified MMTV(LA) virions incubated with different sera were injected directly into the mammary glands of previously uninfected BALB/cJ mice. All successfully infected mice show deletion of Sag-cognate T cells (6, 27). The deletion of Sag-cognate T cells measured 8 wk after infection was used as indicator of virus infectivity. Uninfected control BALB/cJ mice have 9.2 ± 0.8% of peripheral CD4⁺ Vβ14⁺ T cells, n = 10. BALB/cJ mice injected with virus preincubated with infected BALB/cJ serum, uninfected BALB/cJ serum, or uninfected I/LnJ serum became infected with MMTV, showing a diminution in the percentage of CD4⁺ Vβ14⁺ to 2.4 ± 0.6%, n = 5, to 2.1 ± 0.7%, n = 5, and to 2.8 ± 1.2, n = 5, respectively. Only BALB/cJ mice injected with MMTV virions preincubated with MMTV(LA)-infected I/LnJ serum did not show deletion of CD4⁺ Vβ14⁺ (10.5 ± 0.4%, n = 5), suggesting that they were virus-free. Furthermore, the same BALB/cJ mice did not show mammary gland infection as determined by RNase T₁ protection analysis (unpublished data). We have also performed experiments with MMTV(C3H) virions preincubated with MMTV(LA)-infected I/LnJ sera and with MMTV(LA) virions preincubated with MMTV(C3H)-infected I/LnJ sera and obtained similar results (unpublished data). Thus, MMTV pretreatment with serum from infected I/LnJ mice in vitro results in virus neutralization and block of infection.

Second, in order to determine whether antibodies produced by infected I/LnJ mice were capable of neutralizing virus in vivo, newborn mice infected with MMTV were used. Two groups of newborn BALB/cJ mice suckling on viremic mothers were injected with sera from MMTV-infected or uninfected I/LnJ mice. Pubescent mice were bred and RNA isolated from their lactating mammary glands of these animals was screened for viral transcripts in an MMTV-specific RNase T₁ protection analysis (BALB2 and BALB14 probes shown).

The Kinetics of Anti-MMTV Antibody Production and a Class Switch to IgG2a in Naturally Infected I/LnJ and BALB/cJ Mice. By 8 wk of age naturally infected I/LnJ mice produce virus-specific antibodies of the IgG2a isotype (Fig. 2 B). To determine when this antibody response is
initiated and whether it is unique to I/LnJ mice, susceptible BALB/cJ mice and resistant I/LnJ mice were fostered on viremic BALB/cJ females and presence of antibodies against MMTV in their sera was analyzed by ELISA at different time points. Two different secondary antibodies were used to detect antibodies present in the sera by ELISA: anti–mouse Ig nonisotype specific and IgG2a-specific antibodies (Fig. 4). These experiments revealed that the initial production of anti-MMTV antibodies could be detected in both infected I/LnJ and BALB/cJ mice from 3 to 6 wk after birth. However, antibodies were no longer observed in BALB/cJ mice after they reached 6 to 7 wk of age. In addition, the temporal production of anti-MMTV antibodies in BALB/cJ mice did not include a specific increase in antibodies of the IgG2a isotype, as no such antibodies were detected in BALB/cJ sera. Thus, susceptible BALB/cJ mice were incapable of mounting a long-lasting antibody response against virus and did not class-switch to the IgG2a isotype. In contrast, in I/LnJ mice, after a wave of poly-isotypic early response, a steady and increasing production of anti-MMTV antibodies of the IgG2a isotype began at ~6 wk of age (Fig. 4).

INF-γ Is Required for Anti-virus Antibody Production in I/LnJ Mice. Neonatally MMTV-infected I/LnJ mice produce anti-virus neutralizing antibodies of the IgG2a isotype and sustain this production throughout their lifetime. Class switching to the IgG2a isotype is induced primarily by IFN-γ (28). IFN-γ is a pleiotropic cytokine that plays a central role in promoting innate and adaptive mechanisms of host defense (29).

To demonstrate that IFN-γ is involved in resistance of I/LnJ mice to retroviral infection, mice with targeted mutation if IFN-γ (30) were backcrossed to I/LnJ mice for 10 generations. To investigate whether IFN-γ KO I/LnJ mice are susceptible to MMTV infection, they were fostered by viremic females along with their heterozygous littermates, and kinetics of antivirus antibody production was studied. All animals became MMTV infected since they demonstrated deletion of peripheral Sgo-cognate CD4+/Vβ14+ T cells. MMTV-LA-infected 8-wk-old IFN-γ KO/KO, KO/+ and +/+ mice had 0.8 ± 0.3% (n = 5), 0.87 ± 0.4% (n = 8), and 0.8 ± 0.5% (n = 4) of CD4+/Vβ14+ T cells, respectively, whereas uninfected mice of the same genotypes had 9.5 ± 0.7% (n = 5), 9.3 ± 0.5% (n = 5), and 9.7 ± 0.2% (n = 6) of CD4+/Vβ14+ T cells, respectively. Furthermore, all animals contained newly integrated MMTV proviruses within the lymphoid system and the virus load did not differ between mice of three different genotypes (Fig. 5 A). These data suggest that IFN-γ is not required for MMTV infection and its absence does not increase the virus load.

To determine whether IFN-γ was necessary for anti-MMTV antibodies production, infected IFN-γ−/− deficient and IFN-γ−/− sufficient I/LnJ mice were bled and their sera were tested for reactivity against MMTV in ELISA. None of MMTV-infected IFN-γ−/− deficient mice produced anti-MMTV antibodies, whereas initial polyvalent response followed by the class switch to IgG2a isotype was apparent in IFN-γ−/− sufficient mice (Fig. 5 B).

To investigate whether failure to produce anti-virus antibodies by IFN-γ−/− deficient mice results in successful
virus transmission, we fostered susceptible BALB/cJ mice by MMTV-infected IFN-γ/H9253–deficient and IFN-γ/H9253–sufficient I/LnJ mice and analyzed the frequencies of Sag-cognate T cells in the periphery of 8-wk-old mice (Fig. 5 C). All BALB/cJ mice fostered on IFN-γ/H9253–sufficient I/LnJ mice did not become MMTV infected and had normal frequencies of Sag-cognate T cells. In contrast, BALB/cJ mice fostered by IFN-γ/H9253–deficient I/LnJ mice became MMTV infected, as they showed deletion of Sag-cognate T cells (Fig. 5 C). Importantly, the steady state level of IFN-γ/H9253 in the sera of I/LnJ mice was no different from that in the sera of C3H/HeN and BALB/cJ mice and was below the detectable 30 pg/ml. Similarly, the level of IFN-γ produced by T cells in response to anti-CD3 antibodies was similar in susceptible and resistant mice (Fig. 6). Thus, antiretroviral humoral immune response in I/LnJ mice is determined by the IFN-γ produced specifically in response to viral infection.

Antivirus Antibodies of the IgG2a Isotype Are a Specialized Response to Retroviral Infection.

MMTV infection in I/LnJ mice results in production of antivirus antibodies of the IgG2a isotype (Fig. 2). It is possible that all antibody responses in I/LnJ mice were skewed toward the IgG2a isotype or it is also possible that this resulted from the activation of a specific pathway in response to retroviral infection. Immunization of I/LnJ mice with ovalbumin resulted in production of antibodies of multiple isotypes and did not differ from a response in BALB/cJ mice (unpublished data). Moreover, when both I/LnJ and control BALB/cJ mice were immunized with MMTV virion pro-
Uninfected control BALB/cJ mice had completely neutralized the virus in in vitro experiment. Incubated with sera from immunized BALB/cJ mice contains neutralizing antibodies, because MMTV virions preincubated with sera from resistant I/LnJ mice were incapable of producing viruses (Fig. 7). Thus, the immune response in I/LnJ produced antivirus antibodies of only the IgG2a isotype, whereas 7/7 and 2/7 infected adult I/LnJ mice showed production of any anti-MMTV antibodies, while 5/5 of immunized BALB/cJ mice produced anti-MMTV antibodies of both the IgG2a and IgG1 isotype. In contrast, 0/10 infected adult BALB/cJ mice showed production of any anti-MMTV antibodies, while 5/5 of immunized BALB/cJ mice produced anti-MMTV antibodies of both the IgG2a and IgG1 isotype.

After immunization with MMTV proteins in CFA, even susceptible BALB/cJ mice were capable of producing virus-neutralizing antibodies, because MMTV virions preincubated with sera from immunized BALB/cJ mice completely neutralized the virus in vitro experiment. Uninfected control BALB/cJ mice had 9.2 ± 0.8%, n = 10 of CD4+VB14+ T cells. BALB/cJ mice injected with MMTV virions preincubated with sera from either I/LnJ or BALB/cJ mice immunized with MMTV(LA) virion proteins did not show depletion of CD4+VB14+ T cells (9.6 ± 0.37% for 5 I/LnJ sera and 9.6 ± 0.7 for 5 BALB/cJ sera), suggesting that they were virus-free. In contrast, sera from MMTV(LA)-infected BALB/cJ mice did not neutralize the virus (2.79 ± 1% of CD4+VB14+ T cells, n = 5). As expected, control sera from infected I/LnJ mice completely blocked infection (9.8 ± 0.4% of CD4+VB14+ T cells, n = 5). Thus, immunization with MMTV virion proteins in adjuvant, but not a natural infection, stimulates production of virus-neutralizing antibodies in susceptible BALB/cJ mice. In contrast, resistant I/LnJ mice have a unique ability to produce neutralizing antibodies upon infection without exogenous adjuvant.

**Discussion**

Humoral immune responses play an important role in antiviral defense. Antiviral antibodies prevent the spread of viral infections through two main mechanisms: (a) block of interactions of viral surface proteins (Env) with their receptors; (b) facilitation of virus uptake into phagocytic cells through interaction with the Fc receptors or through the complement pathway. Although for many viral infections antibody production is a key to clearance (31), retroviruses such as HIV are able to escape humoral immune responses despite the fact that anti-HIV antibodies are being produced (32). Retroviral infections persist and involve rapid mutations of genes that encode antigenic proteins, leading to selection of immune escape variants. In order for a humoral immune response against a retrovirus to be capable of blocking virus transmission, it must be robust and sustained. I/LnJ mice are unique in meeting these requirements: they efficiently generate anti-MMTV neutralizing antibodies, they are able to sustain this response throughout their lifespan (Fig. 4) and they produce antibodies against multiple viral proteins that cross-react with different MMTV variants (Fig. 3).

Our interest was drawn to I/LnJ mice because of their remarkable resistance to MMTV-induced mammary tumors (11, 18) and we searched for the mechanism underlying this resistance. We found that MMTV-infected I/LnJ mice produced antibodies against the virus that were able to (a) neutralize MMTV in vitro; (b) abort MMTV infection when injected in vivo (Fig. 3 C); and (c) coat virions secreted into milk (Fig. 2 A), thereby preventing further virus transmission. Antibodies were produced in response to four MMTV variants: MMTV(C3H), BALB2, BALB14, and BALBLA, and were cross-reactive to them. It is possible that the antibodies recognize protein determinants that viruses cannot change without losing infectivity.

The unique features of the I/LnJ response to MMTV are that such a response is elicited and maintained. We (Fig. 4) and others (33–35) have found that mice susceptible to MMTV infection and MMTV-induced tumors are capable of producing anti-MMTV antibodies. However, the kinetics of this response in I/LnJ is distinct from that in susceptible mice. The initial production of anti-MMTV antibodies in infected BALB/cJ mice started at 3 wk after birth and was terminated at 6 to 7 wk (Fig. 4). In addition, this transient production of anti-MMTV antibodies in BALB/cJ mice did not include a specific increase in antibodies of the IgG2a isotype. In contrast, I/LnJ mice demonstrated a steadily increasing response that was detectable at 4 wk after birth and class switched to the IgG2a isotype at ~7 wk (Fig. 4). This means that both MMTV-susceptible and MMTV-resistant mice did not recognize retroviral antigens as “self” when they were infected as newborns (Fig.
4). However, over time the immune system of susceptible BALB/cj mice became tolerant to these viral antigens and no longer recognizes them as foreign (Fig. 4). In contrast, in I/LnJ mice, antibody production is sustained throughout their life (Fig. 4). From these data we concluded that the immune system of I/LnJ mice is always aware of retroviral infection.

Infection per se must be a key to production of antiviral antibodies of exclusively IgG2a isotype in I/LnJ mice, as the production of antibodies of different isotypes against viral proteins were seen in both susceptible and resistant mice when viral proteins were introduced with an adjuvant (Fig. 7). Pathogens (including viruses) are capable of activating APCs through their pathogen-associated molecular patterns (PAMPs), which are recognized by innate immune pattern recognition receptors (PRRs; references 36 and 37). Unique molecular features of viruses, such as double-stranded (ds) RNA, can serve as PAMP. Indeed, dsRNA was recently found to be recognized by Toll-like receptor (TLR)3 (38). Recently TLRs and their adaptor proteins are linked to IFN pathways and in some cases were actually induced by IFNs during viral infection (39, 40). In the case of retroviruses it remains unclear what receptors are activated, but I/LnJ mice clearly demonstrate that infection leads to an efficient immune response, implying that MMTV may activate innate immunity.

I/LnJ mice with targeted mutation of IFN-γ were unable to produce any antibodies against the virus (Fig. 5), implicating IFN-γ in the mechanism of resistance inherited by I/LnJ mice. Although we found no evidence of increased IFN-γ production by activated I/LnJ T cells or increased IFN-γ background levels in the sera of I/LnJ mice (Fig. 6), it is plausible that increased IFN-γ production might occur in response to stimuli other than T cell receptor ligation or by other cell types.

IFN-γ is mostly produced by NK cells and certain subpopulations of T cells and activates expression of IFN-γ-responsive genes whose products are engaged in the immune response (41). Other cell types, however, may be also involved in IFN-γ production. Our preliminary data showed that cells of I/LnJ bone marrow origin are capable of conveying the ability to produce anti-MMTV neutralizing antibodies by susceptible mice (unpublished data).

The precise details of the resistance mechanism in I/LnJ mice are yet to be elucidated. Previously we showed that it is controlled by a single gene, virus infectivity controller or Vic (18). Our preliminary mapping data position the gene on Chromosome 17 (not MHC-linked; unpublished data). Two alleles of the Vic gene of I/LnJ origin are required for antibody production, whereas one allele of the gene from susceptible mice is needed to suppress antibody production. The fact that resistance is recessive suggests that some type of negative regulatory activity is abolished or reduced in I/LnJ mice, allowing these mice to sustain an immune response against retrovirus. For example, it is possible that a negative feed back regulation of IFN-γ signaling is affected, leading to normal (not excessive) but sustained IFN-γ production. One well-known negative regulator of IFN-γ is the cytokine inducible SH2-containing protein 1 (CISH1), which is induced in response to IFN-γ stimulation (42). However, we did not find any differences between resistant I/LnJ mice and susceptible mice in the coding sequences of Cish1 gene or in up-regulation of Cish1 mRNA in response to IFN-γ (unpublished data). Initial recognition of MMTV as “nonself” takes place in other strains as well as in I/LnJ mice, but only I/LnJ mice continue to recognize MMTV antigens as “nonself.” Thus, susceptible mice become tolerant to MMTV, while resistant I/LnJ mice maintain antiviral immune response, a property for which IFN-γ may also be responsible. One would anticipate that these mice must be more prone to autoimmunity than other strains. This, however, is not the case, as no reports of autoimmunity in I/LnJ mice have been published, and we have not made any such observations in our colony. Moreover, I/LnJ mice as well as other mouse strains have endogenous Mtv (18), but only I/LnJ mice produce anti-viral antibodies when infected with exogenous MMTV. Thus, the mechanism that allows I/LnJ mice to sustain the antiviral response is absolutely dependent on infection with retrovirus.

Another known example of resistance to retroviral infection is resistance to Friend murine leukemia virus (MuLV) conferred by a dominant allele of the Rfv3 gene. The production of virus-neutralizing antibodies of the IgG2a isotype against Friend MuLV underlies this resistance and results in complete virus clearance (43). Although function of the Rfv3 gene also remains unknown, the mechanism of action of its encoded product is clearly different from that of the Vic gene. The resistant allele of the Rfv3 gene is dominant, since only one copy is required for the production of anti-F-MuLV neutralizing antibodies (43). In addition, the Rfv3 gene was mapped to Chromosome 15 (44, 45), whereas the Vic gene has been mapped to Chromosome 17.

Thus, we have discovered a novel mechanism of resistance to retroviral infection that results in an efficient antiviral antibody response and blocks virus transmission. Both neutralizing anti-virus antibodies and cytotoxic lymphocytes directed against viral proteins can be detected in humans infected with HIV (46). However, the level at which they control HIV infection is insufficient to prevent the disease from progressing, and virus variants that escape immune recognition are often found in HIV-infected individuals (47). If we knew a way to make the immune response against HIV and other human retroviruses robust and sustained, we would be better able to treat diseases caused by them.

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