Endogenous MMTV Proviruses Induce Susceptibility to Both Viral and Bacterial Pathogens

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Most inbred mice carry germline proviruses of the retrovirus, mouse mammary tumor virus (MMTV) (called *Mtvs*), which have multiple replication defects. A BALB/c congenic mouse strain lacking all endogenous *Mtvs* (*Mtv*-null) was resistant to MMTV oral and intraperitoneal infection and tumorigenesis compared to wild-type BALB/c mice. Infection of *Mtv*-null mice with an MMTV-related retrovirus, type B leukemogenic virus, also resulted in severely reduced viral loads and failure to induce T-cell lymphomas, indicating that resistance is not dependent on expression of a superantigen (Sag) encoded by exogenous MMTV. Resistance to MMTV in *Mtv*-null animals was not due to neutralizing antibodies. Further, *Mtv*-null mice were resistant to rapid mortality induced by intragastric inoculation of the Gram-negative bacterium, *Vibrio cholerae,* but susceptibility to *Salmonella typhimurium* was not significantly different from BALB/c mice. Susceptibility to both MMTV and *V. cholerae* was reconstituted by the presence of any one of three endogenous *Mtvs* located on different chromosomes and was associated with increased pathogen load. One of these endogenous proviruses is known to encode only Sag. Therefore, *Mtv*-encoded Sag appears to provide a unique genetic susceptibility to specific viruses and bacteria. Since human endogenous retroviruses also encode Sags, these studies have broad implications for pathogen-induced responses in mice and humans.

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Introduction

Pathogens are recognized by the immune system, which has been divided into innate and adaptive responses. Innate immunity detects pathogen-associated molecular patterns (PAMPs) on the invading organisms [1]. PAMPs interact with pattern recognition receptors, which often contain a cytosolic region called the Toll/IL-1 receptor domain [2]. One group of pattern recognition receptors, the Toll-like receptors (TLRs), is evolutionarily conserved from *Drosophila* to humans and is expressed by a variety of cell types [3]. Signaling through the Toll/IL-1 receptor domains leads to activation of inflammatory responses and initiation of adaptive immunity [4]. Another form of innate immunity (also known as intrinsic immunity) appears as non-inducible barriers to pathogen replication as exemplified by apolipoprotein B mRNA-editing enzyme catalytic polypeptides, which lead to the degradation or mutation of viral genomes [5]. Finally, adaptive immunity provides an antigen-specific response that can evolve and provide immunological memory [4].

Pathogenic organisms have developed a number of mechanisms to avoid innate, intrinsic, or adaptive immunity or to modify these responses. Retroviruses, including the betaretrovirus mouse mammary tumor virus (MMTV), represent one group of pathogens that uses cells of the immune system to establish infection [6,7]. MMTV is transmitted as exogenous virus through the milk of infected female mice to newborn pups or through endogenous proviruses (*Mtvs*) integrated in the germline [8]. Recently, we have shown that MMTV is a complex retrovirus with features similar to the human retroviruses, human endogenous retroviruses type K (HERV-K), HIV, and human T-cell leukemia virus (HTLV) [9].

In the first few weeks of life, milk-borne MMTV enters the small intestine, where it penetrates M cells of the epithelial layer to invade the underlying lymphoid tissue [10]. B lymphocytes and dendritic cells are the initial targets of MMTV infection [11]. These infected antigen-presenting cells (APCs) express virus-encoded superantigen (Sag), a type II transmembrane glycoprotein, at the cell surface in association with major histocompatibility complex (MHC) class II molecules [12]. Sag-MHC complexes on APCs interact with particular variable regions on the β chains (Vβ) of T-cell receptors (TCRs) to initiate a helper T-cell response [11]. Unlike conventional antigens, these APC interactions allow activation of 5% to 35% of naïve CD4+ T cells, which then release cytokines to induce rapid proliferation of bystander cells that are susceptible to MMTV infection [13]. Dividing lymphocytes provide a reservoir of mobile infected cells, which subsequently transmit MMTV to dividing mammary

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Abbreviations: APC, antigen-presenting cell; CFU, colony-forming unit; CTL, cytotoxic T lymphocyte; ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence-activated cell sorter; HYB-MTV, infectious hybrid proviral clone of MMTV; LD_{50}, lethal dose for 50% of infected animals; MMTV, milk-borne mouse mammary tumor virus; *Mtv*, endogenous proviral DNA copies of MMTV integrated into the germline; PBL, peripheral blood lymphocyte; Sag, superantigen; TBLV, type B leukemogenic virus; TCR, T-cell receptor; TLR, Toll-like receptor; Vβ, variable region of the beta chain of the T-cell receptor

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Synopsis

Mouse mammary tumor virus (MMTV) is a retrovirus that causes breast cancer in mice. MMTV-induced breast cancers represent a well-established model for human breast cancer research. Mice become infected by virus particles in mother's milk (exogenous MMTV), and insertion of exogenous MMTV into somatic cell DNA sporadically leads to genetic damage and cancer. Alternatively, if exogenous MMTVs infect germline cells, the viral genome becomes a stable part of the mouse genome and may be inherited from either parent (endogenous MMTV). In this report, the authors demonstrate that mice carrying endogenous MMTV in their genomes are more susceptible to disparate infectious agents, such as exogenous MMTV and the cholera-producing bacteria, *Vibrio cholerae*. However, this effect was selective since disease induced by another related bacteria, *Salmonella typhimurium*, was not affected. Genetic evidence suggests that a viral protein produced by endogenous MMTVs enhances pathogen-induced disease and death. Further, the human genome contains many human endogenous virus type Ks, which highly resemble MMTVs and also may influence disease. Therefore, this study suggests that endogenous retroviruses found in the mouse and human genomes alter the course of disease induced by multiple pathogenic organisms.

cells during puberty [14]. Later during infection, there is gradual deletion or anergy of the Sag-reactive T cells [15]. Thus, Sag plays an essential role in T-cell stimulation to establish milk-borne MMTV infection. Disruption of T-B cell interactions, which occurs in CTLA4 immunoglobulin-transgenic mice [16], or mice carrying several different knockout mutations (Foxn1, immunoglobin heavy chain, CD40L, or CD28) [17–20], reduces or abolishes the Sag-mediated immune response as well as viral spread and transmission.

Common laboratory mice carry between two and eight *Mtv* proviruses that often are defective for replication and fail to produce infectious particles [21]. However, most *Mtv*s encode functional Sags that induce intrathymic or peripheral deletion of Sag-reactive T cells [22]. These Sags are polymorphic at their C-termini, which interact only with specific TCRs [23]. Thus, endogenous *Mtv* proviruses reshape the adaptive immune system by altering the T-cell repertoire of the mouse [24] and may potentially facilitate milk-borne MMTV infection by generating partial tolerance to virally encoded proteins. Further, mice transgenic for the *sag* gene of C3H strain MMTV are protected against exogenous MMTVs encoding the same Sag specificity, leading to the hypothesis that endogenous *Mtv*s are maintained to provide protection from milk-borne MMTV infection [6].

MMTV also interacts with the immune system through the expression of the surface glycoprotein SU, which binds to the transferrin receptor 1 (TfR1/CD71) to mediate viral entry into host cells [25]. In addition, MMTV SU binds to TLR2 and TLR4, and this interaction is believed to trigger the secretion of proinflammatory cytokines and chemokines as well as the upregulation of the entry receptor CD71 [26]. Cytokine secretion is diminished from APCs if the cells have a double mutation in TLR2 and TLR4 or if cells are tolerized by prior exposure to lipopolysaccharide [26]. These results indicate that MMTV manipulates both the innate and adaptive immune responses through virally encoded SU and Sag.

In this study, we have developed BALB/c congenic mice that lack all endogenous *Mtv*s (BALB/*Mtv*-null). These mice are resistant to mammary tumorigenesis and leukemia induction by two different MMTV strains, as well as mortality induced by the Gram-negative pathogen, *Vibrio cholerae*. Both viral and bacterial loads are diminished in *Mtv*-null animals compared to their *Mtv*-positive littermates, and this phenotype is reversed by restoration of any one of three endogenous *Mtv*s found in BALB/c mice. Since at least one of the endogenous *Mtv*s appears to encode only superantigen, our data suggest that MMTV Sag is a negative regulator of the innate immune response to multiple pathogens.

Results

Development of a BALB/c Congenic Mouse Strain That Lacks MMTV Proviruses

To obtain *Mtv*-null mice on the BALB/c background, we mated BALB/c females (H-2^d) to males from the recently inbred PERA/Ei (H-2^k) strain that lack endogenous *Mtv*s [27]. The F1 animals were then backcrossed to PERA mice to obtain the N1 generation. Subsequently, N1 mice were screened for the absence of endogenous *Mtv*s using PCR from tail-derived genomic DNA, and *Mtv*-free progeny were then mated to BALB/c mice. Every other generation, the progeny were intercrossed to obtain *Mtv*-free animals that were then backcrossed to BALB/c. This strategy was continued for ten generations to obtain the *Mtv*-null strain (H-2^d), which has been maintained by brother-sister matings for more than 3 y.

The absence of endogenous *Mtv*s was verified by PCR analysis of mouse genomic DNA using primers specific for each of the three endogenous MMTV proviruses of BALB/c mice, *Mtv6, 8,* and *9* (Figure 1A). The lack of *Mtv*s was also confirmed by flow cytometry to detect the levels of specific T cells (Figure 1B). Deletion of specific T-cell subsets is a sensitive indicator of the expression of MMTV superantigen on the surface of antigen-presenting cells [28]. As expected, BALB/c mice had very low levels of CD4+ T cells reactive with Sags encoded by *Mtv6, 8,* and *9* proviruses (TCR variable-region beta [Vβ] 3, 5, 7, and 12). However, significant populations of these same T-cell subsets were detected in *Mtv*-null mice, consistent with the absence of the corresponding endogenous MMTV proviruses. Further, Southern blotting of genomic DNA from *Mtv*-null animals and hybridization with MMTV long terminal repeat-specific probes confirmed the absence of proviruses (unpublished data). Together, these data indicate that BALB/*Mtv*-null mice are free from endogenous *Mtv*s.

BALB/*Mtv*-Null Mice Are Resistant to MMTV-Induced T-Cell Deletion and Mammary Tumorigenesis

To determine the susceptibility of BALB/*Mtv*-null mice to MMTV infection, *Mtv*-null pups were allowed to nurse on C3H MMTV-infected mothers (C3H/HeN MMTV+ mice) (foster nursing). This method allows the natural milk-borne transmission of MMTV from mothers to offspring. As anticipated, milk-borne MMTV infection of wild-type BALB/c mice gave a 100% incidence of mammary tumors with an average latency of 5.8 ± 0.4 mo (Table 1). However, milk-borne infection of *Mtv*-null mice only gave a 10% incidence of mammary tumors (one of ten animals) with the only tumor appearing at 15 mo.

Mice were also infected by inoculation with rat XC

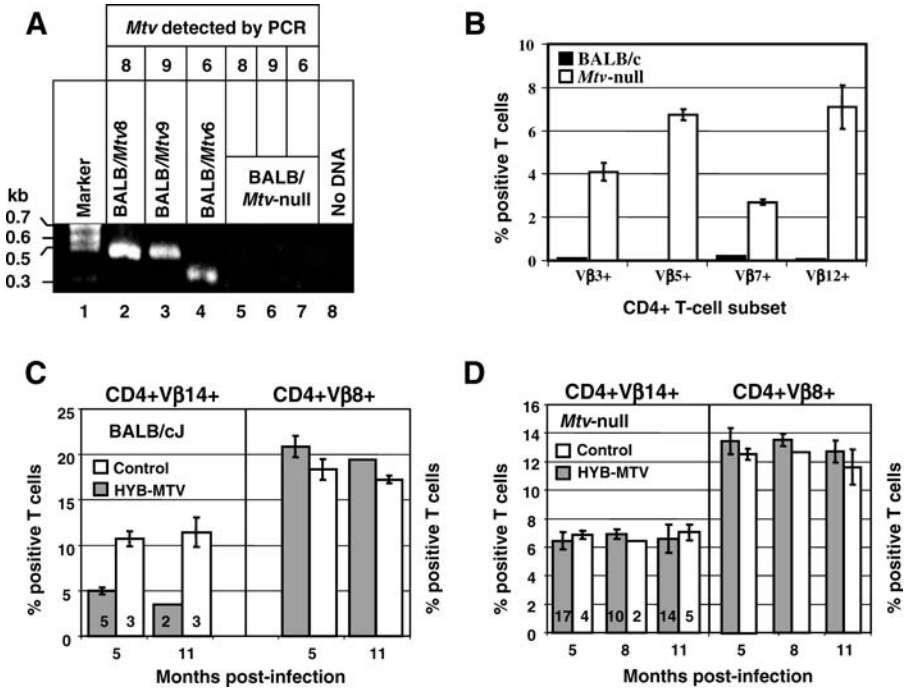

Figure 1. BALB/*Mtv*-Null Mice Lack Endogenous *Mtv*s and Have Impaired Sag-Mediated T-Cell Deletion after Exogenous MMTV Infection

(A) PCR analysis of mouse genomic DNA using endogenous *Mtv*-specific primers. Genomic DNA samples from mice with single endogenous *Mtv*s were used as positive controls for PCR amplification of individual proviruses (lanes 2–4). Genomic DNA samples from three *Mtv*-null mice were analyzed (lanes 5–7). PCR with no added DNA template was the negative control (lane 8).

(B) Flow cytometry of endogenous *Mtv* Sag-reactive T-cell subsets in PBLs. PBLs from 6-mo-old BALB/c and *Mtv*-null mice were dually stained with mouse specific CD4-phycoerythrin and mouse-specific TCR Vβ3, 5, 7, or 12-FITC antibodies followed by FACS and software analysis. Three individual animals of each strain were tested, and the average proportion of T-cell subsets (± standard deviation) is shown. Flow cytometry also was performed on PBLs isolated after MMTV infection of BALB/c J (C) or *Mtv*-null (D) mice. The numbers inside the bars indicate the numbers of animals tested. At 11 mo post-infection of BALB/c animals, only two animals were tested for T-cell deletion because the other animals had died from mammary tumors. Cells were stained with mouse-specific CD4-PE and mouse-specific Vβ8 or Vβ14-FITC antibodies. The percentages of C3H MMTV Sag-reactive (CD4+Vβ14+) (left panels) and non-reactive (CD4+Vβ8+) (right panels) T cells among the PBLs isolated from mice inoculated with C3H MMTV-infected rat cells (dark bars) were compared to non-injected control mice (light bars). PE, phycoerythrin.

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fibroblasts stably expressing an infectious cloned MMTV provirus, HYB-MTV [29]. The stably transfected cells (XC/HYB-MTV) [30] were injected intraperitoneally into BALB/c and *Mtv*-null weanlings. Wild-type mice developed mammary tumors with an incidence of 90% and an average latency of 8.9 ± 2.2 mo, whereas *Mtv*-null mice had a 10% mammary tumor incidence (three of 29 animals inoculated) with an average latency of 12.7 ± 3.2 mo (Table 1). Therefore, the difference between *Mtv*-null and BALB/c animals in disease susceptibility was not influenced by the route of viral infection.

One explanation for these results is that MMTV infection is limited in *Mtv*-null mice and reduced replication of the virus leads to a decreased frequency of proviral integration into the cellular genome. MMTV is believed to induce tumors by insertional mutagenesis and activation of cellular oncogenes [31–33] and, therefore, reduced integration will limit mammary tumor incidence. Since deletion of superantigen-reactive T cells is a sensitive indicator of MMTV infection, MMTV-infected animals were analyzed for Sag-specific T-cell deletion.

As anticipated, wild-type BALB/c animals inoculated with MMTV-infected rat cells showed statistically significant (69%) deletion of C3H MMTV Sag-reactive (CD4+Vβ14+) T cells compared to uninfected BALB/c mice (Figure 1C, left panel). In contrast, *Mtv*-null mice inoculated with the same infected cells showed no detectable C3H Sag-specific T-cell deletion (Figure 1D, left panel). T-cell deletion was specific since there was no observable deletion in a T-cell subset that is non-reactive with C3H MMTV Sag protein (CD4+Vβ8+; Figure 1C and 1D, right panels). These results are consistent with the idea that MMTV replication is restricted in *Mtv*-null mice, thus reducing proviral loads and mammary tumorigenesis. Deletion of Sag-specific T cells was also not detectable in progeny of MMTV-infected *Mtv*-null mothers (unpublished data).

To determine whether there was a difference in viral loads between MMTV-inoculated BALB/c and *Mtv*-null mice, spleen RNA was obtained from multiple infected animals and subjected to semi-quantitative RT-PCR using long terminal repeat primers that were specific for C3H MMTV (Figure 2A). Exogenous MMTV RNA levels were consistently higher in BALB/c mice than in *Mtv*-null animals inoculated with HYB-MTV infected cells. Similar data were obtained using tissues from mice infected with MMTV by foster nursing (Figure 2A). Also, note that MMTV RNA was barely detectable in *Mtv*-null mammary tumors (Figure 2A, lane 18, and unpublished data), but was easily detected in the BALB/c tumors (lanes 19 and 20). These results indicate that *Mtv*-null mice have reduced viral loads after MMTV infection.

Table 1. Tumor Incidence of Mice Infected with MMTV or TBLV

Mouse Strain	Infection By:					
	Foster Nursing with C3H MMTV[a]		Intraperitoneal Inoculation with MMTV[b]		Intraperitoneal Inoculation with TBLV[c]	
	Tumor Incidence (Number of Mice with Tumors/Total)[d]	Average Latency (mo)[e]	Tumor Incidence (Number of Mice with Tumors/Total)[d]	Average Latency (mo)[e]	Tumor Incidence (Number of Mice with Tumors/Total)[f]	Average Latency (mo)[e]
BALB/c	100% (6/6)	5.8 ± 0.4	90% (9/10)[d]	8.9 ± 2.2	50% (9/18)	5.7 ± 1.5
Mtv-null	10% (1/10)	15	10% (3/29)	12.7 ± 3.2	0% (0/8)	—
BALB/cX *Mtv*-null	ND[g]	ND	86% (12/14)	7.3 ± 2.4	ND	ND
BALB/*Mtv6*	ND	ND	38% (5/13)	12.8 ± 1.8	ND	ND
BALB/*Mtv8*	ND	ND	75% (6/8)	9.1 ± 3.6	ND	ND
BALB/*Mtv9*	ND	ND	53% (8/15)	8.6 ± 2.3	ND	ND

[a]Newborn animals were infected by nursing on MMTV-infected C3H/HeN mothers.
[b]Weanling animals were infected by intraperitoneal inoculation with rat XC cells stably transfected with an infectious MMTV provirus.
[c]Weanling animals were infected by intraperitoneal injection of human Jurkat T cells stably transfected with an infectious TBLV provirus.
[d]Number of female mice with mammary tumors/total number of infected females. Mice that died before 12 mo of age from birthing problems were excluded from the study.
[e]Mean ± standard deviation.
[f]Number of animals with T-cell lymphomas/total number of infected males and females.
[g]Not done.
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BALB/*Mtv*-Null Mice Are Resistant to TBLV-Induced T-Cell Lymphomas

Sag-mediated amplification of MMTV in lymphoid cells is required for efficient viral transmission and dissemination in the mammary gland [6,14]. Therefore, resistance of *Mtv*-null mice to MMTV-induced disease could be attributed to reduced Sag function in these animals. To test this possibility, we analyzed infection by an MMTV variant, TBLV, which induces T-cell tumors rather than mammary cancers [34,35], by a Sag-independent mechanism [34]. Injection of TBLV-infected cells intraperitoneally into weanling BALB/c mice gave a 50% incidence of T-cell lymphomas with an average latency of 5.7 mo. In contrast, BALB/*Mtv*-null mice inoculated in the same manner failed to develop lymphomas within the 10-mo observation period (Table 1). Similarly, BALB/*Mtv*-null mice did not develop lymphomas after injection with a TBLV mutant that encodes only 17 amino acids of Sag, whereas this same mutant was lymphomagenic in BALB/c mice (unpublished data) [34]. Thus, both Sag-dependent and Sag-independent MMTV strains have reduced tumorigenicity in *Mtv*-null mice.

To test whether resistance of *Mtv*-null mice affects viral replication, we performed RT-PCR analyses on RNA extracted from tissues of TBLV-inoculated mice. TBLV transcripts were detected by RT-PCR in splenic RNA from 10-mo-old infected animals, and the identity of PCR products was confirmed by sequencing (Figure 2B and unpublished data). Viral loads were greatly reduced in the infected *Mtv*-null mice compared to those from infected BALB/c animals. Since viral RNA was detected only sporadically, our results suggest that *Mtv*-null mice are less susceptible to MMTV and TBLV infection. Nevertheless, viral RNA cannot be demonstrated in every organ of MMTV or TBLV-infected wild-type BALB/c mice (unpublished data), a result that might be expected from the negative regulation of MMTV transcription in most tissues except lactating mammary gland [36,37]. Together with the lack of Sag-specific T-cell deletion observed in *Mtv*-null mice infected by MMTV (Figure 1), these experiments suggest that *Mtv*-null mice restrict both TBLV and MMTV replication.

MMTV Resistance Is Recessive and Segregates with Endogenous MMTV Proviruses

To determine the gene(s) involved in the resistance of BALB/*Mtv*-null mice to MMTV and TBLV infection, we obtained F1 hybrids between BALB/c and *Mtv*-null mice. Weanling F1 animals were injected intraperitoneally with MMTV-infected rat cells and observed for Sag-reactive T-cell deletion and development of mammary tumors. Deletion of C3H-MMTV Sag-specific (Vβ14+) T cells was detected in all MMTV-infected F1 animals similar to that observed with infected wild-type BALB/c mice (Figure 3A). Deletion was not observed in the Sag non-reactive T-cell population (Vβ8+) (Figure 3F). The injected F1 mice also developed mammary tumors with an incidence of 86% and an average latency of 7.3 ± 2.4 mo (see below; Table 1). RT-PCR was used to identify expression of C3H MMTV in these tumors. As anticipated, these tumors expressed MMTV RNA as detected by RT-PCR, and cleavage of the products with *Cla*I was consistent with the presence of C3H MMTV transcripts (unpublished data). Therefore, resistance of the *Mtv*-null mice to mammary tumors as well as Sag-specific T-cell deletion is a recessive trait.

During the establishment of the *Mtv*-null strain, we noted that the resistance to MMTV-induced mammary tumors was retained after six backcross generations. This result suggested that resistance is a multi-gene trait. To determine whether the resistance of *Mtv*-null mice segregates with any of the endogenous *Mtv* loci of BALB/c mice, we derived BALB/c congenic strains carrying single *Mtv* proviruses called BALB/*Mtv6,* BALB/*Mtv8,* and BALB/*Mtv9* (single-positive strains). The same crosses also yielded siblings that lacked all three endogenous MMTV proviruses. The presence or absence of endogenous *Mtv*s in these strains was verified by PCR analysis of genomic DNA and by flow cytometry of peripheral blood lymphocytes (PBLs) (unpublished data).

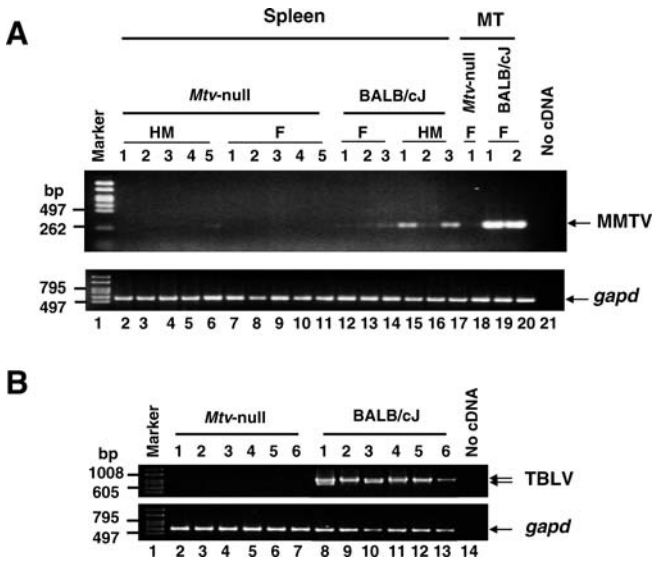

Figure 2. Exogenous MMTV RNA Loads Are Higher in the Presence of Endogenous *Mtv* Proviruses

(A) RT-PCR indicates higher levels of C3H MMTV RNA in spleens derived from infected BALB/c mice compared to those from infected *Mtv*-null mice. The numbers above each lane represent separate BALB/c or *Mtv*-null animals as indicated. Mice injected with cloned HYB-MTV-producing cells are designated HM, whereas mice infected by foster-nursing are designated F. Organs were harvested between 6–15 mo post-infection in BALB/c mice at the time of tumor development, whereas organs from *Mtv*-null mice were harvested between 10–15 mo post-infection. MT, mammary tumor

(B) RT-PCR analysis shows lower TBLV levels in *Mtv*-null versus *Mtv*-positive strains. Organs were harvested between 4–10 mo post-infection in BALB/c mice at the time of tumor development; organs from *Mtv*-negative mice were obtained at 10 mo post-infection.

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Weanling single-positive strains were inoculated intraperitoneally with MMTV-infected rat cells and observed for deletion of Sag-reactive T cells and for the development of mammary tumors. Interestingly, all three single-positive strains demonstrated equivalent C3H MMTV-specific T-cell deletion with similar kinetics (Figure 3B–3D). Deletion of CD4+Vβ14+ T cells at 9 mo post-inoculation was 38% (BALB/*Mtv6*), 48% (BALB/*Mtv8*), and 45% (BALB/*Mtv9*). As expected, BALB/*Mtv*-null siblings showed no deletion (Figure 3E). Moreover, the Sag non-reactive T-cell population (Vβ8+) was not affected (Figure 3F–3J), validating the specificity of the deletion observed.

Mice carrying single *Mtv* proviruses were also susceptible to C3H MMTV-induced mammary tumors (Table 1). Interestingly, the incidence of mammary tumors correlated with the extent of T-cell deletion. The incidence of tumors was highest in BALB/*Mtv8* mice (75%), whereas the tumor incidence was 53% and 38% in BALB/*Mtv9* and BALB/*Mtv6* mice, respectively. Three of the 29 injected *Mtv*-null mice also developed mammary tumors (~10% incidence). The tumor incidence of BALB/c mice carrying single *Mtv* proviruses was statistically higher than those lacking MMTV proviruses. Expression of C3H MMTV RNA was verified in mammary tumors using RT-PCR with virus-specific primers followed by cleavage of the products with *Cla*I (unpublished data). Because *Mtv6, 8,* and *9* are located on different mouse chromosomes, these results suggest that susceptibility to Sag-mediated deletion and mammary tumorigenesis is conferred by the presence of any of three endogenous *Mtv* proviruses rather than linked cellular genes.

BALB/*Mtv*-Null Mice Infected with MMTV Lack a Neutralizing Antibody Response

Since a single endogenous *Mtv* provirus is sufficient to allow Sag-mediated deletion and MMTV-induced mammary tumors, it is possible that BALB/c mice have immune tolerance to MMTV-encoded proteins. If this is true, then *Mtv*-null mice may have a strong neutralizing antibody response against virion antigens that limits the infection [38]. To test this idea, BALB/c and *Mtv*-null mice were infected by nursing on MMTV-infected C3H/HeN mothers and then tested at weekly intervals for MMTV-specific antibodies by an enzyme-linked immunosorbent assay (ELISA) (Figure 4A). The majority of BALB/c mice lacked virus-specific antibodies (~94% of the animals) during the first 3–5 wk of age as reported previously [39]. Similarly, antibodies directed against MMTV were absent in 70% of the *Mtv*-null animals, despite use of a more sensitive assay as compared to previous reports [39]. To test the specificity of the sporadically occurring, low-titer antibodies, Western blots of purified MMTV were incubated with sera from individual *Mtv*-negative animals infected by milk-borne MMTV (Figure 4B). No envelope (SU)-specific antibodies were detectable, although some reactivity to capsid protein was demonstrable. Since only a small percentage of the resistant animals developed antibodies and the antibodies were not directed against surface antigens, the resistance of *Mtv*-null mice to MMTV-induced mammary tumors is not due to production of neutralizing antibodies. Therefore, the mechanism of viral resistance in *Mtv*-null mice does not resemble antibody-mediated mechanism(s) reported for other mouse strains that limit MMTV infection [38,39].

Mtv-Null Mice Have an Immune Response to MMTV-Infected, but Not Uninfected, BALB/c Lymphoma Cells

The resistance of *Mtv*-null mice to MMTV infection may result from a cell-mediated immune response to the virus. To test this idea, weanling BALB/c and *Mtv*-null animals were inoculated intraperitoneally with 5×10^6 A20 cells (a BALB/c B-cell lymphoma cell line) [40]. A second set of mice was injected with the same number of A20 cells stably transfected with an infectious MMTV provirus, HYB-MTV. Injection of A20 cells gave approximately the same percentage of solid, peritoneal tumors in both BALB/c and *Mtv*-null mice (25% [3/12] with an average latency of 7.5 ± 1.5 wk versus 29% [4/14] with an average latency of 6.8 ± 2.3 wk). In contrast, 38% (5/13) of BALB/c (average latency 5.2 ± 0.5 wk), but none of the *Mtv*-null mice developed tumors when injected with MMTV-transfected A20 cells. Therefore, these results suggest that *Mtv*-null mice can direct a specific immune response against C3H MMTV-infected cells.

Resistance of BALB/*Mtv*-Null Mice to *V. cholerae* Correlates with Absence of Endogenous *Mtv*s

Since the resistance of *Mtv*-null mice to TBLV and MMTV infection may be due to an immune response, we tested whether these mice may also have an altered disease susceptibility to other pathogens. BALB/c and *Mtv*-null mice were inoculated intragastrically with *V. cholerae* strain O395,

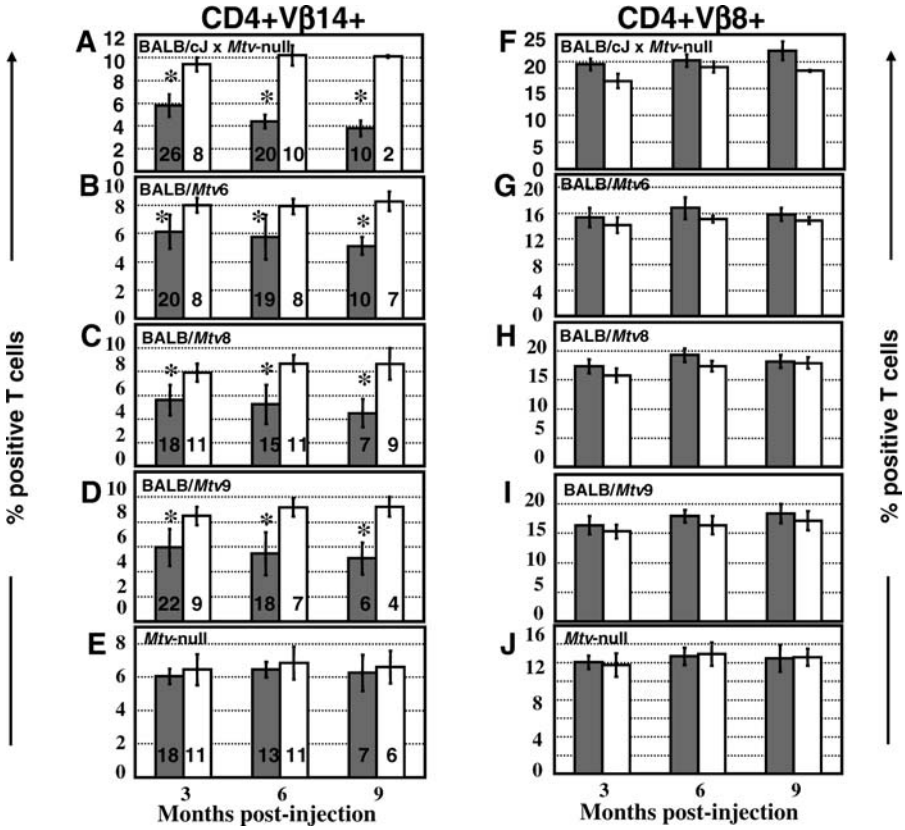

Figure 3. BALB/c X *Mtv*-Null F1 Hybrids and Mice Carrying Single *Mtv* Proviruses Exhibit C3H MMTV Sag-Specific T-Cell Deletion

PBLs isolated from MMTV-infected (dark bars) and control non-infected (light bars) mice at different times were dually stained with mouse-specific CD4-PE and Vβ8 or Vβ14-FITC antibodies followed by FACS analysis. The percentages of C3H Sag-reactive CD4+Vβ14+ (A–E) and non-reactive CD4+Vβ8+ (F–J) T cells obtained from BALB/c X *Mtv*-null F1 (A and F), BALB/*Mtv6* (B and G), BALB/*Mtv8* (C and H), BALB/*Mtv9* (D and I), and BALB/*Mtv*-null (E and J) are depicted. Numbers inside the bars represent numbers of animals tested. The average percentages of T cells in the MMTV-infected *Mtv*-positive strains (including standard deviation) were significantly different from non-infected controls of the same strains by the two-tailed Student's *t* test ($p < 0.05$) (asterisks). In contrast, infected *Mtv*-null animals failed to demonstrate T-cell deletion. PE, phycoerythrin.

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which expresses cholera toxin [41]. Mid-log phase bacterial suspensions containing 10^5, 10^6, or 10^7 colony-forming units (CFUs) were fed to 5- to 6-d-old pups, and mortality was recorded over a 48-h period. As anticipated, the average lethal dose (LD_{50}) of *V. cholerae* in BALB/c mice was 1.6×10^5 CFUs (Table 2). In contrast, the average LD_{50} of *V. cholerae* in *Mtv*-null animals was ~16-fold higher (2.3×10^6 CFUs) ($p < 0.05$). These results indicate that *Mtv*-null mice are more resistant than BALB/c mice to two different pathogens, MMTV and *V. cholerae*. The difference in disease resistance of the two mouse strains to *V. cholerae* can be observed within 24 h.

To evaluate whether the increased resistance to *V. cholerae* of *Mtv*-null mice was linked to the absence of endogenous *Mtv*s, the disease susceptibility of mouse strains carrying single *Mtv* proviruses on the BALB/c background was evaluated after intragastric inoculation of pups. The average LD_{50} values of the BALB/*Mtv6,* BALB/*Mtv8,* and BALB/*Mtv9* strains were 2.2×10^5, 1.2×10^5, and 2×10^5 CFUs, respectively, which were similar to that of the parental BALB/c strain (Table 2). Statistical analysis revealed that the LD_{50} values for *V. cholerae* in *Mtv*-null mice were significantly higher ($p < 0.05$) than those of BALB/c mice or mice carrying single *Mtv*s. Similar experiments were performed using intragastric inoculation of adult mice with *Salmonella typhimurium;* however, no statistical difference in mortality was observed between *Mtv*-null and BALB/c mice (unpublished data).

To determine whether the difference in susceptibility to *V. cholerae* was due to differences in cholera-toxin–induced fluid accumulation or due to altered bacterial growth, additional BALB/c and *Mtv*-null pups were inoculated with several different doses of bacteria. Our data revealed that there was little difference in relative fluid accumulation in the guts of the two strains (unpublished data), whereas growth in the small intestines of *Mtv*-null strain animals was statistically lower than that in wild-type BALB/c mice after inoculation with three different doses of *V. cholerae* (Figure 5).

Discussion

BALB/c congenic mice that lack all endogenous *Mtv*s were resistant to mammary tumors induced by MMTV, lymphomas induced by TBLV, and mortality due to *V. cholerae*. In each case, pathogen infection could be demonstrated. However, the viral load was reduced in MMTV-infected *Mtv*-null mice as demonstrated by negligible amounts of Sag-specific T-cell deletion and by reduced levels of MMTV or TBLV RNA in infected tissues. Relative resistance to MMTV was not affected by the method of inoculation. *V. cholerae* infection of *Mtv*-null

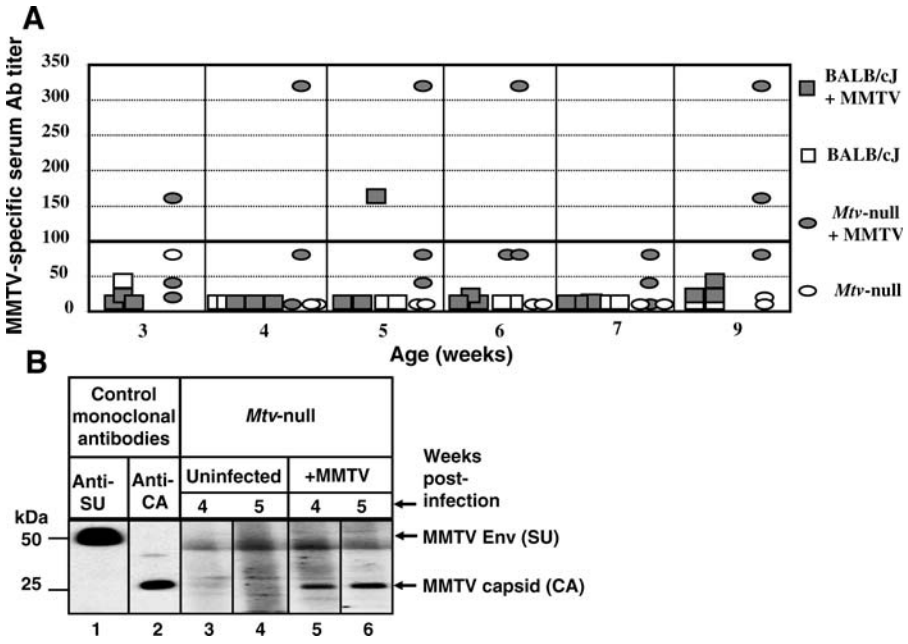

Figure 4. BALB/*Mtv*-Null Mice Infected by Milk-Borne MMTV Lack Virus-Specific Neutralizing Antibodies

(A) Both BALB/c and *Mtv*-null animals sporadically have low levels of MMTV-specific antibodies. Sera from milk-borne C3H MMTV-infected (gray symbols) or age-matched non-infected (white symbols) BALB/c (squares) and *Mtv*-null (circles) mice were analyzed weekly by ELISA to detect virion-specific antibodies. Each symbol represents an individual mouse. The serum antibody titer was calculated as the reciprocal of the highest serum dilution that reacted with MMTV virions. Since one non-infected animal showed a titer of 1:80, values above 1:100 (the heavy line) were considered positive.

(B) Sporadic, low-titer antibodies in *Mtv*-null animals have reactivity to MMTV capsid antigen. Proteins from purified MMTV particles were separated on SDS-containing polyacrylamide gels, subjected to Western blotting, and incubated with a 20-fold dilution of serum from two MMTV-infected *Mtv*-null mice with the highest antibody titers (1:320) at 4–5 wk of age. MMTV capsid antigen (CA) or surface envelope (SU)-specific monoclonal antibodies were used as positive controls. Sera from two age-matched non-infected BALB/*Mtv*-null mice were used as negative controls.

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animals also led to reduced bacterial loads compared to those observed in BALB/c mice.

Several mechanisms of resistance to MMTV infection have previously been described. A number of these mechanisms involves disruption of Sag-induced interactions of B and T cells, which are required for amplification of the number of milk-borne MMTV-infected cells and transmission of virus to the mammary gland [6,7,18]. Transgenic mice expressing high levels of C3H MMTV Sag delete their Sag-reactive T cells and are refractory to milk-borne C3H MMTV infection [6]. A similar phenotype has been reported in Vβ8.2-transgenic mice, which cannot be infected by SW MMTV whose Sag does not recognize Vβ8.2+ T cells [7]. B-cell-deficient mice are resistant to milk-borne MMTV infection as demonstrated by their failure to delete Sag-reactive T cells and the absence of proviral integrations in spleen and mammary glands [18]. Further, mice with inefficient presentation of MMTV Sag due to MHC class II mutations are resistant to viral infection [42]. Knockout mice deficient in cell surface molecules necessary for T-B cell interactions (CD28/CTLA4-B7 or CD40/CD40L) also demonstrate defects in milk-borne MMTV dissemination and transmission [16,19,20]. Thus, the resistance of *Mtv*-null mice to MMTV-induced tumorigenesis might be attributed to lack of Sag-mediated viral amplification in the lymphoid compartment.

Table 2. Resistance of *Mtv*-Null Mice to Disease Induced by *V. cholerae* Is Abolished by the Presence of a Single *Mtv* Provirus

Mouse Strain	Number Animals Tested	LD_{50} at 48 h Post-Infection (Standard Deviation)	*p*-Value[a]
BALB/c	31	1.6×10^5 ($\pm 8 \times 10^4$)	.03
BALB/*Mtv*-null	42	2.3×10^6 ($\pm 2 \times 10^6$)	—
BALB/*Mtv6*	38	2.2×10^5 ($\pm 1 \times 10^5$)	.03
BALB/*Mtv8*	31	1.2×10^5 ($\pm 4 \times 10^4$)	.03
BALB/*Mtv9*	41	2.0×10^5 ($\pm 9 \times 10^4$)	.02

[a]Single-factor ANOVA followed by Tukey's post-hoc tests were performed using SPSS 12.0. The *p*-values were obtained from pair-wise comparisons of each mouse strain with the BALB/*Mtv*-null strain.

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Two lines of evidence argue against a defect in Sag-mediated amplification in *Mtv*-null animals. First, these mice are resistant to TBLV-induced lymphomas (Table 1). TBLV encodes a truncated Sag protein that lacks the C-terminal domain necessary for interaction with the Vβ region of the TCR and does not induce Sag-mediated deletion after infection, yet induces T-cell lymphomas in susceptible BALB/c mice [34,43]. Second, *Mtv*-null animals are resistant to tumorigenesis by the TBLV *sag*-frameshift virus (unpublished data). Published results indicate TBLV variants engineered with a double-frameshift mutation in the truncated *sag* gene induced lymphomas with the same efficiency as wild-type TBLV [34]. Failure of TBLV or TBLV *sag* frameshift viruses to induce tumors in *Mtv*-null mice suggests that resistance is linked to a host immune response at a step upstream of the Sag response, but common to both MMTV and TBLV infections.

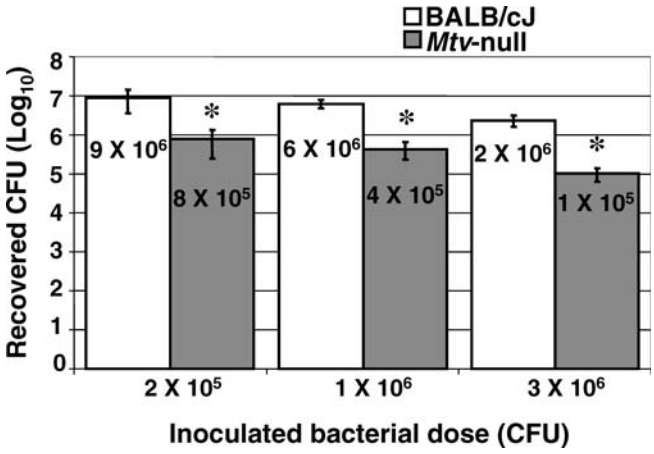

Figure 5. Bacterial Load Is Decreased in *Mtv*-Null Mice

Three different doses of *V. cholerae* were given to 5- to 6-d-old pups (three to four animals/dose). After 24 h, the mice were sacrificed, and the small intestines were assayed for bacterial growth. The means (numbers shown inside bars) and standard deviations for the recovered CFUs of each set of animals are indicated. Two-tailed Student's *t* tests were used for pair-wise comparisons of BALB/c and *Mtv*-null animals infected at each dose; *p*-values of <0.05 are shown by an asterisk.

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Production of a neutralizing antibody response has been shown to prevent induction of MMTV-induced mammary tumors [38,39]. The I/LnJ mouse strain, which contains endogenous *Mtv*s, produces IgG2a MMTV-specific neutralizing antibodies that block viral transmission from lymphoid to mammary gland cells. Unlike the MMTV-susceptible BALB/c strain, production of antibodies in this strain is sustained and dependent on interferon γ [39]. Experiments in BALB.D2 mice, which lack endogenous *Mtv*s, have also shown that a neutralizing antibody response does not dramatically interfere with viral amplification in lymphoid cells, but limited infection of the mammary gland [38]. In contrast, the BALB/*Mtv*-null mice show only low level, sporadic infection of lymphoid organs, and no transmission is demonstrable to progeny. I/LnJ and BALB.D2 mice both showed a significant MMTV Sag response following infection [38,39], whereas BALB/*Mtv*-null animals did not. The experiments in BALB.D2 mice appeared to involve incomplete backcrossing between the BALB.D2 and 38CH strains, which may account for some of the differences with our results. Further, the BALB.D2 results were obtained with SW MMTV, which is known to induce a strong Sag response in adult mice, but causes mammary tumors at a low incidence [38]. Therefore, *Mtv*-null mice appear to demonstrate a unique genetic mechanism of resistance to MMTV.

To our knowledge, our data represent the first report that a retroviral susceptibility mechanism may extend to bacteria. Previous reports from the Benjamin lab indicated that endogenous *Mtv7* Sag-mediated deletion of TCR Vβ6+ cells renders mice susceptible to polyomavirus-induced tumors [27,44]. This observation resulted from the absence of a TCR Vβ6+ population of polyoma-specific cytotoxic T lymphocytes (CTLs). In contrast, our studies show that any one of three BALB/c endogenous MMTVs with different Sag specificities was necessary and sufficient to confer susceptibility to completely disparate pathogens. Since the resistance to both MMTV and *V. cholerae* is observed in the very early stages of primary infection (within 24 h for *V. cholerae*), it is unlikely that Sag-mediated removal of CTLs is the primary reason for susceptibility to infection in the presence of endogenous MMTVs. Our results also raise interesting questions regarding the evolution and maintenance of endogenous *Mtv*s in mouse populations. The dynamic compromise for mutual survival of both MMTV and the mouse will be influenced by complex interactions between the MMTV-modulated murine immune system and numerous environmental pathogens.

Although the exact process leading to MMTV disease susceptibility in BALB/*Mtv*-null animals remains to be determined, several clues are available. An enhanced immune response to MMTV-encoded proteins was observed in *Mtv*-null mice, consistent with proposals that there is immunological intolerance to viral antigens in the absence of endogenous proviruses [38]. However, *Mtv*-null mice were resistant to both MMTV and *V. cholerae,* which share few, if any, antigenic determinants, and virus infection could be detected in *Mtv*-null organs, even as long as 15 mo post-infection. Further, susceptibility of mice to MMTV and *V. cholerae* was reconstituted by any one of three endogenous *Mtv* proviruses, *Mtv6, Mtv8,* or *Mtv9.* One of these proviruses, *Mtv6,* lacks >6 kb of sequence encoding most of *gag* and *env,* as well as the entire *pol* gene [45], thus eliminating most of the immunodominant CTL epitopes [46]. These results suggest that (i) an MMTV-encoded gene product confers susceptibility to two disparate pathogens, and (ii) the viral product is not encoded by the *gag, pol,* or *env* genes.

The *Mtv6* provirus is believed to encode only a single mRNA, which has been functionally shown to encode Sag [47], and our data are consistent with these observations (unpublished data). Since *Mtv6* confers susceptibility to *V. cholerae* very early after infection, we suggest that MMTV Sag acts as a negative regulator of innate or intrinsic immunity. Failure to suppress innate immunity would explain early effects on bacterial and viral loads in *Mtv*-null animals, as well as their heightened ability to use the adaptive CTL response to reject C3H MMTV-expressing tumor cells. A common feature of the innate immune response to both *V. cholerae* and MMTV is the ability to signal through TLR4 [26,48,49]. Lipopolysaccharide of Gram-negative organisms, such as *V. cholerae,* as well as the MMTV envelope protein, SU, both bind to TLR4 to stimulate an immune response [26,48]. Although bacterial superantigens of Gram-positive bacteria appear to increase TLR4 mRNA and protein levels [50], our preliminary experiments have detected no differences in the surface TLR4 expression of BALB/c and BALB/*Mtv*-null macrophages. In support of these results, the LD_{50} values of BALB/c and *Mtv*-null mice for *V. cholerae* O395 treated with formalin (which preserves lipopolysaccharide, but inactivates infectivity) are not significantly different, and no differences in susceptibility to *S. typhimurium* were noted (unpublished data). Thus, Sag or an unknown MMTV-encoded product may manipulate novel aspects of innate or intrinsic immunity. Our studies suggest that many mouse models of human disease may be affected by the presence of endogenous MMTV proviruses. Furthermore, the ability of the related HERV-K proviruses to encode superantigens raises the possibility that human retroviruses alter the innate or intrinsic immune responses to pathogens.

Materials and Methods

Mice. BALB/cJ (H-2^d, *Mtv6, 8,* and *9*-positive) and PERA/Ei (H-2^k, *Mtv*-free) [51] mice were obtained from The Jackson Laboratory (http://www.jax.org). Mice were bred and maintained in the Animal Resources Center at the University of Texas, Austin, Texas, United States. BALB/*Mtv*-null mice (H-2^d) lacking endogenous *Mtv*s were selected from a BALB/cJ X PERA/Ei cross followed by more than ten intercross and backcross generations; these animals have >99.9% BALB/c background. BALB/*Mtv6,* BALB/*Mtv8,* and BALB/*Mtv9* mice were derived by crosses between BALB/cJ and BALB/*Mtv*-null animals and screened by PCR and FACS analysis of PBLs for the presence of the appropriate single *Mtv* proviruses. Mice were tested periodically to ensure the absence of common bacterial and viral pathogens.

Infections. Animals infected by the milk-borne route were nursed on MMTV-positive C3H/HeN mothers. In other experiments, weanling 4- to 5-wk-old BALB/c, BALB/*Mtv*-null, or congenic mice containing single *Mtv*s were injected intraperitoneally with 2×10^7 XC/HYB-MTV or Jurkat/HYB-TBLV cells. The XC- transfected cells were cultured in 10 μM dexamethasone (Sigma, http://www.sigmaaldrich.com) for 18 h prior to injection. All injected females were bred continuously to stimulate lactogenic hormones. For determination of LD_{50} values, single colonies of *V. cholerae* O395 were grown in overnight cultures, diluted 100-fold, and then cultured to an $A_{650} = 0.5$ ($\sim 5 \times 10^8$ CFUs). Appropriate dilutions of bacteria in 50 μl of phosphate-buffered saline (PBS) containing 0.02% (w/v) Evan's blue dye were given intragastrically to 5- to 6-d-old pups, which were monitored for 48 h as previously described [52,53]. The doses of inoculated bacteria were confirmed by plating onto L agar containing 67 μg per ml streptomycin. LD_{50} values were calculated by the method of Reed and Muench [54]. In some cases, the small intestines were recovered at 24 h post-inoculation and homogenized in 1 ml of PBS prior to determination of bacterial counts on streptomycin-containing L agar. To determine the LD_{50} values for *S. typhimurium* SL1344, an isolated colony was cultured overnight at 37 °C, diluted 100-fold, and then grown to an A_{600} of 0.7 ($\sim 7 \times 10^8$ CFUs). Appropriate bacterial dilutions were prepared in PBS containing 5% sucrose and inoculated intragastrically using a gavage needle into 4- to 6-wk-old mice that had been starved for 18–20 h. Morbidity/mortality was determined during a 14-d observation period.

Cells. XC rat fibroblast cells were stably transfected with the hybrid MMTV provirus, pHYB-MTV [29] (referred to as XC/HYB-MTV). The human Jurkat T-cell line was stably transfected with the hybrid TBLV provirus (pHYB-TBLV) (referred to as Jurkat/HYB-TBLV) [34]. Culture conditions have been described [55].

Flow cytometry. Mice were bled from the retroorbital sinus at regular intervals, and PBLs were isolated and stained as previously described [56]. Fluorescein- or phycoerythrin-labeled antibodies were obtained from BD Biosciences Pharmingen (http://www.bdbiosciences.com). Cells were analyzed on a FACSCalibur using CELLQuest software (BD Biosciences).

PCR and RT-PCR analysis. Tail DNA for PCR analysis was extracted as described by The Jackson Laboratory and used for PCR with specific primers (Table S1). Initial PCR assays were performed using PCR Supermix (GIBCO-BRL, Invitrogen, http://www.invitrogen.com), but later assays used JumpStart REDAccu-Taq™ (Sigma) according to the manufacturer's instructions. Total RNA was extracted from tissues as described [56], and 20 μg was treated with 3 U of amplification grade DNase I (Invitrogen) at 37 °C for 30 min. The DNase was inactivated, and the RNA was reverse transcribed using 400 U of Moloney murine leukemia virus reverse transcriptase (RT) (Invitrogen) according to the manufacturer using poly-dT_{17} as primer. Reactions without RT verified the lack of DNA contamination. Five μl of the reactions were amplified in a 50-μl volume containing 50 mM Tris-HCl (pH 9.1), 16 mM ammonium sulfate, 3.5 mM $MgCl_2$, 150 μg/ml bovine serum albumin, 0.3 mM deoxynucleotide triphosphates, 100 ng of each primer, and 2.5 U of KlenTaq polymerase (AB Peptides, http://www.abpeps.com). 30 or 40 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 1 min in a PTC-100 Programmable Thermal Controller (MJ Research, Bio-Rad, http://www.bio-rad.com) were followed by a 5-min extension at 72 °C. Primers for these reactions are listed (Table S1). 40 cycles were used to maximize the detection of any RNA expression in *Mtv*-null animals. Samples were analyzed by electrophoresis on 2% agarose gels. In some cases, the products were purified using a QIAquick gel extraction kit (Qiagen, http://www1.qiagen.com) and sequenced. Restriction fragment length polymorphisms were used to differentiate between RT-PCR products amplified from exogenous C3H MMTV and endogenous *Mtv*s. Products were purified through Micro Bio-Spin P-30 columns (Bio-Rad Laboratories) and incubated with *Cla*I for 2 h at 37 °C after addition of a plasmid digestion control in each reaction. Major fragments were isolated and confirmed by sequencing.

ELISA and Western blotting. MMTV virion proteins were used in ELISA to detect MMTV-specific antibodies as previously described [39], except that a higher concentration of purified virus (10 μg/ml) was used for coating of the wells to increase sensitivity. Serum samples were obtained by retroorbital bleeds. Western blots were performed according to Mustafa et al. [30].

Statistical analysis. Pair-wise comparisons were performed by the two-tailed Student's *t* test. Statistics for MMTV-induced tumors were performed using the LIFETEST procedure in SAS8.2 to compute non-parametric estimates of the survivor function by the product-limit method (also known as the Kaplan-Meier method). The homogeneity of survival functions across groups was tested using log-rank and Wilcoxon tests. *V. cholerae* infections of different groups of mice were analyzed by single-factor ANOVA followed by Tukey's post-hoc test using SPSS 12.0; *p*-values of <0.05 were considered significant.

Supporting Information

Table S1. Primers Used for PCRs and RT-PCRs

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Author contributions. SB, SMP, and JPD conceived and designed the experiments. SB and MML performed the experiments. SB, MML, SMP, and JPD analyzed the data. JPD wrote the paper.

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Competing interests. The authors have declared that no competing interests exist.

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