Brief Definitive Report

The Nasal-associated Lymphoid Tissue of Adult Mice Acts as an Entry Site for the Mouse Mammary Tumor Retrovirus

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Summary

Mouse mammary tumor virus (MMTV) is a B type retrovirus transmitted to the suckling offspring through milk. MMTV crosses the intestinal barrier of neonates, initially infects the lymphoid cells of the Peyer's patches, and later spreads to all lymphoid organs and to the mammary gland. Adult mice can be infected systemically, but not by oral MMTV administration. In this study, we show that nasal administration of infected milk induces the infection of adult mice. Nasal MMTV infection shared the main features of systemic and neonatal intestinal MMTV infections: deletion of the superantigen (SAg)-reactive T cell subset from the peripheral T cell population, presence of viral DNA in lymphoid cells, and transmission of MMTV from mother to offspring. Viral DNA was restricted to the lungs and nasal-associated lymphoid tissue (NALT) 6 d after nasal infection. Furthermore, SAg-induced T cell proliferation was only detected in NALT. These results demonstrate that MMTV crosses the intact epithelium of the upper respiratory tract of adult mice and infects the lymphoid follicles associated with these structures.

Mouse mammary tumor virus (MMTV) is a B type retrovirus present in the milk of lactating infected female mice (1). Infectious MMTV, in contrast to other viruses, requires an intact immune system to establish an efficient, lifelong infection and to complete its life cycle. MMTV crosses the intestinal barrier of neonates and initially infects the lymphoid cells of the Peyer's patches (PP), later spreading to all lymphoid organs and to its final target the epithelial cells of the mammary gland (2). After footpad injection of infectious MMTV, MMTV primarily infects B cells in the draining popliteal lymph nodes (3). After integration of the MMTV genome in B cells, a protein with a superantigenic activity is produced (SAg molecule), which, in the context of MHC class II, triggers an intense proliferation of $CD4^+$ T cells expressing the appropriate T cell receptor Vβ domain, thereby providing T helper function to infected B cells that in turn proliferate. These early cognate interactions between B and T cells facilitate the subsequent spread of MMTV to the mammary gland via lymphocytes (4).

Although systemic infection has been intensively analyzed, little is known about the early steps in mucosal infection. The critical elements allowing mucosal infection by MMTV have not been elucidated. One can hypothesize that uptake and transport of MMTV across the intestinal barrier is mediated by a receptor. As adult mice are resistant to oral infection, such a receptor should be downregulated at weaning, its disappearance correlating with the onset of intestinal resistance to MMTV (5). We could exclude that the neonatal Fc receptor, which is expressed by enterocytes during the first 2 wk of life, mediates the transport of MMTV across the intestinal barrier (5). M cells are specialized epithelial cells which deliver samples of foreign material by transepithelial transport from the lumen to underlying organized lymphoid tissues within the mucosa (6, 7). It is possible that MMTV could cross the intestinal mucosa via M cells. If M cells are able to mediate transport, it is difficult to understand why infection is restricted to the neonatal period, since M cells are present both in newborns and adults. The resistance to oral MMTV infection after weaning may also reflect the postnatal maturation of the digestive functions of the gastrointestinal tract.

We hypothesized that mucosal MMTV infection is not restricted to the neonatal period, and that other lymphoid organs associated with an epithelium other than the intestinal epithelium could be the site of MMTV entry. We infected adult mice with MMTV by the nasal route to gain access to the mucosal lymphoid organs associated with the nasal cavity and the lungs. We found that MMTV, when given nasally, infects adult mice. Furthermore, we localized the initial site of viral propagation to the mucosal-associated lymphoid organs of the nasal cavity. We could detect both the proliferation of the T cell subset induced by the SAg molecule and viral DNA in the nasal associated lym-

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phoid tissue (NALT) 6 d after intranasal administration of infected milk. Viral DNA was also detected in lungs at this time, but not the SAg-reactive T cell proliferation. Altogether, these results show that MMTV can infect adult mice by a mucosal route, and that MMTV can initiate its infectious cycle in lymphoid tissues associated with the nasal cavity.

Materials and Methods

Mice. BALB/c mice were obtained from Harlan Olac (London, U.K.). MMTV (SW)-infected mice were obtained from IFFA Credo (L'Arbresle, France) and bred in our animal facility.

Virus Preparation and Infection. Milk was prepared as described earlier (5). For nasal MMTV infection, adult mice (8–10-wk-old) were anesthetized by the injection of a mixture of ketasol (Dr. E. Gräub, Bern, Switzerland) and rompun (Bayer, Zurich, Switzerland). Infected milk was diluted to 1:2 in PBS and 20 μl pipetted into the nostrils of the mice.

Isolation of Lymphoid Cells. Peripheral blood was taken from the tail vein and mononuclear cells were isolated by centrifugation of heparinized samples (diluted with PBS) on a Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) cushion. Lymph nodes, spleen, and thymus were dissociated mechanically. PP were recovered as described earlier (2). The intraparenchymal lung lymphoid cells were prepared as described by Abraham et al. (8) with a few modifications. The lung vascular bed was flushed by injection of 5–10 ml chilled (4°C) heparinized PBS into the right ventricle. At this time, the color of the lungs became white. The lungs were excised, washed twice in ice-cold PBS, minced finely, and then incubated in 5 ml of RPMI 1640 with 10% FCS containing 110 and 80 U/ml, respectively, of type II and IV collagenases (CII-28 and CIV-28; Seromed, Berlin, Germany). The suspensions were gently shaken at 37°C in a 15-ml conical tube (model 2095; Falcon, Basel, Switzerland) for 75 min. After incubation, the largest lung pieces were dissociated mechanically and the suspension was left to sediment for 10 min at 4°C. The supernatants were filtered through a cell strainer (2350; Becton Dickinson, San Jose, CA) and centrifuged (4°C). The pellets were recovered in 4 ml of 40% Percoll solution (17-0891-01; Pharmacia), layered on a 4-ml cushion of 80% Percoll solution, and centrifuged for 15 min at 1,900 *g* (15°C). The cells of the interface were recovered and washed in ice-cold RPMI, 10% FCS and used for FACS® analysis or DNA preparation. For NALT isolation, the mice were killed by cervical dislocation, and the skin and excess soft tissue from the head were removed. The skull of the mouse was cut off with a large (No. 10) scalpel 5 mm behind the eyes. The lower jaw was removed and the end of the snout was cut off just behind the upper incisor tooth. For DNA sample preparation, the snout was cut along the nasal septum and the lymphoid tissues located near to the base of the nasal cavity were removed with the end of a scalpel. The tissues obtained were treated as for the minced lung preparations, i.e., collagenase digestion and the recovery of lymphoid cells after a Percoll gradient. For analysis of the lymphoid cell population present in NALT by FACS®, the snout was prepared as described above and the epithelium of the roof of the upper jaw was carefully removed under dissecting lens. The NALT, identified as two small longitudinal strips of tissue attached to the palate was dissected out and mechanically dissociated to obtain lymphoid cell suspensions.

Flow Cytometry. Lymphoid cells from different lymphoid organs or blood were labeled with a mixture of anti-CD4 (anti-L3T4, PE conjugate; CALTAG, South San Francisco, CA) and affinity purified, FITC-conjugated anti-Vβ6 (44-22-1; reference 9) or anti-Vβ14 (14.2; reference 10). All samples were analyzed using a FACScan® and the Lysys II program (Becton Dickinson). Dead cells were excluded by a combination of forward and side scatter.

Detection of Viral DNA. Cells derived from different lymphoid organs (5×10^6) were washed in Tris-buffered saline, pH 7.4, and the DNA was phenol extracted after proteinase K treatment using a standard technique (11). DNA was ethanol-precipitated, redissolved in 100 μl of 10 mM Tris (pH 8.0)–0.1 mM EDTA, and 0.5 μl was used in PCR amplification reactions. Oligonucleotides were chosen to amplify MMTV (SW) *orf* sequences exclusively. The 5′ oligonucleotide, AGGTGGGTCACAATCAACGGC (MS10), is common to various endogenous MMTV (mtv) *orf* sequences, and the 3′ oligonucleotide, GCGACCCCCATGAGTATATTT (IM2), is specific for the SW *orf* sequence. After 5 min at 95°C, 1 min at 60°C, and 1 min at 72°C, followed by 30 cycles of 30 s at 94°C, 30 s at 60°C, and 30 s at 72°C, the reaction was terminated by an elongation of 10 min at 72°C. Specific product was detected by liquid hybridization, with 10 μl of the PCR reaction mixture being hybridized in 15 mM NaCl and 0.25 mM EDTA with 50 fmol of specific ^{32}P-labeled internal probe common to various mtv *orf* sequences MS11 (CAAGGAGGTCTAGCTCTGGCG). The conditions for denaturation and hybridization were 5 min at 98°C, 15 min at 55°C, and a rapid cooling to 4°C. 10 μl of reaction product was size separated on a 1.5% agarose gel, dried on paper (2589B; Schleicher and Schuell, Dassel, Germany), and autoradiographed at −70°C overnight (12). For controlling the quality and the quantity of the DNA samples, we amplified of the endogenous *orf* sequences present in the BALB/c genome. Oligonucleotides used were VJ77 (GATCGGATCCATGCCGCGCCTGCAGCAGA) and VJ71 (GTGTCGACCCAAACCAAGTCAGGAAACCACTTG). After 5 min at 95°C, 1 min at 60°C, and 1 min at 72°C, followed by 30 cycles of 30 s at 94°C, 30 s at 55°C, and 30 s at 72°C, the reaction was terminated by an elongation of 10 min at 72°C. The products were loaded on 1.5% agarose gel and the specific band (1,200-bp) was visualized by ethidium bromide staining.

Results

Adult Mice Can Be Infected by MMTV Via the Nasal Route. The establishment of an efficient MMTV infection is characterized by the deletion from the peripheral T cell population of the T cell subset reactive to the SAg molecule encoded by the infectious MMTV (13). Hence, efficient MMTV infection can be easily detected by the disappearance from the peripheral lymphoid organs or blood of the SAg-reactive T cells. Anesthetized adult mice were administered intranasally with PBS, or with uninfected or infected milk. Blood was taken 5 and 10 wk after nasal administration and the presence of SAg-reactive T cells in peripheral blood was measured by flow cytometry. Fig. 1 shows that adult mice delete from their peripheral blood lymphocytes the $CD4^+V\beta6^+$ T cells which are reactive to the SAg molecule encoded by MMTV (SW). This deletion was not observed in unmanipulated mice or mice inoculated intranasally with uninfected milk or PBS (Fig. 1). The deletion kinetics of the SAg-reactive T cells observed after nasal MMTV infection were slower than that observed after neonatal infection, probably reflecting a lower effi-

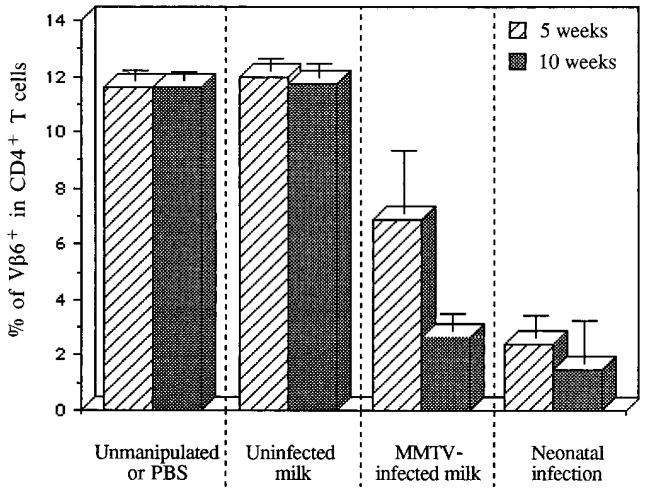

Figure 1. Clonal deletion of $CD4^+V\beta6^+$ T lymphocytes in peripheral blood of BALB/c mice infected nasally with MMTV (SW). Mice were anesthetized and received in the nostrils 20 μl of milk from infected (nine mice) or uninfected (seven mice) lactating females. As controls, seven mice received 20 μl of PBS or were only anesthetized. As positive controls, the kinetics of deletion of the SAg-reactive T cell in mice infected neonatally are shown (two mice). The indicated times are the number of weeks after nasal MMTV infection or ages of mice infected during the neonatal period.

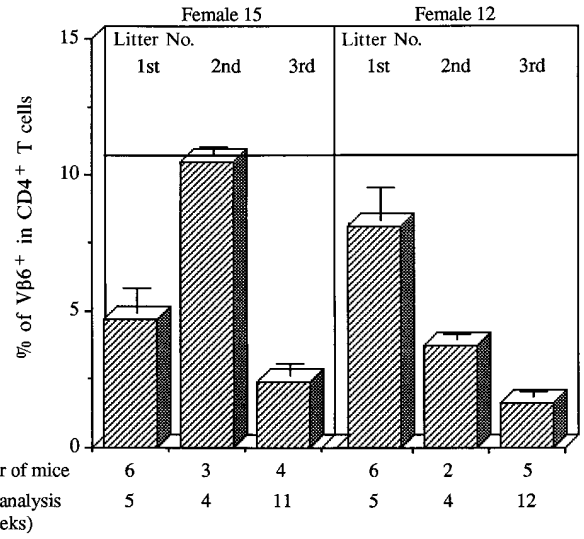

Figure 2. Female mice infected nasally by MMTV transmit the disease to their offspring. Two mice were infected by the nasal route and mated 14 wk after infection. Blood was recovered from the pups of three litters (*1st, 2nd, 3rd*) and the percentages of $CD4^+V\beta6^+$ T cells were determined by flow cytometry. The horizontal line represents percentages of $CD4^+V\beta6^+$ T cells in peripheral blood of noninfected mice.

ciency of nasal MMTV infection (Fig. 1). The same results were obtained whether or not the mice were anesthetized (data not shown). 5 mo after nasal infection, the $CD4^+V\beta6^+$ T cells were deleted from blood, spleen, cervical lymph nodes, and the nasal-associated lymphoid tissue. In comparison, the $CD4^+V\beta14^+$ T cells which are not reactive to the SAg molecule encoded by MMTV (SW) were not deleted (data not shown). These results demonstrate that MMTV given by the nasal route was able to infect adult mice.

Adult Mice Infected by the Nasal Route Transmit the Disease to their Offspring. We tested whether MMTV completes its life cycle after nasal infection of adult mice, i.e., whether nasally infected adult female mice could transmit the disease to their offspring. We mated nasally infected female mice with noninfected males. The $CD4^+V\beta6^+$ T cell deletion in the peripheral blood of the pups was determined in three litters to monitor for disease transmission. Out of the six litters tested, five showed deletion of the SAg reactive T cells (Fig. 2). These results clearly establish that adult female mice infected nasally by MMTV (SW) transmit the disease to their offspring, indicating that MMTV (SW) does complete its life cycle after adult nasal infection.

SAg-driven T Cell Proliferation Occurs in the Nasal-associated Lymphoid Tissue. Having demonstrated that adult mice can be infected via the nasal route, we next tried to identify the initial organized lymphoid organ infected by this retrovirus. We reasoned that if we could locate the site of the T cell proliferation which occurs 4–6 d after MMTV infection, we would identify the lymphoid organ(s) in which initial retroviral infection occurs. Indeed, after neonatal or systemic MMTV infection, the SAg response is only found in lymphoid tissues draining the site of MMTV infection: the popliteal lymph node after footpad infection or the PP during neonatal infection (2, 13). For this purpose, we nasally infected adult mice and killed them 6 d after infection. We then determined the percentage of SAg-reactive T cells in different lymphoid organs. We did not find any SAg-reactive T cell proliferation in the PP, spleen, cervical lymph nodes (upper or lower), mediastinal or brachial lymph nodes, in the lymphoid cell population isolated from the lungs, or in peripheral blood (data not shown).

However, we did find an increase of the percentage of SAg-reactive T cells in the lymphoid organ of the nasal cavity (Fig. 3). A dilution of 1:8 of the same pool of infected milk did not induce an SAg response in NALT after nasal infection, whereas dilutions up to 1:2,000 were still able to induce proliferation of reactive T cells in the draining lymph node after footpad injection. This is probably due to systemic infection being more efficient than nasal infection. This result demonstrates that initial MMTV infection is localized at least in the nasal-associated lymphoid tissue.

Restriction of the Proviral Form of MMTV to the NALT and Lung Tissue 6 d after Nasal Infection. To clearly demonstrate that MMTV infection after nasal administration takes place in the lymphoid organ of the nasal cavity, we analyzed by a specific PCR whether viral DNA could be detected in the lymphoid cells present in the nasal cavity. We isolated DNA from various lymphoid organs after 6 d, 8 d, 15 d, or 3 mo of infection and found that viral DNA is initially detected only in NALT and lung tissue (Fig. 4). At later time points, we could find viral DNA in the peripheral lymph nodes, PP, spleen, and thymus, showing that MMTV infection spread to all lymphoid organs as seen after systemic and neonatal MMTV infection (Fig. 4).

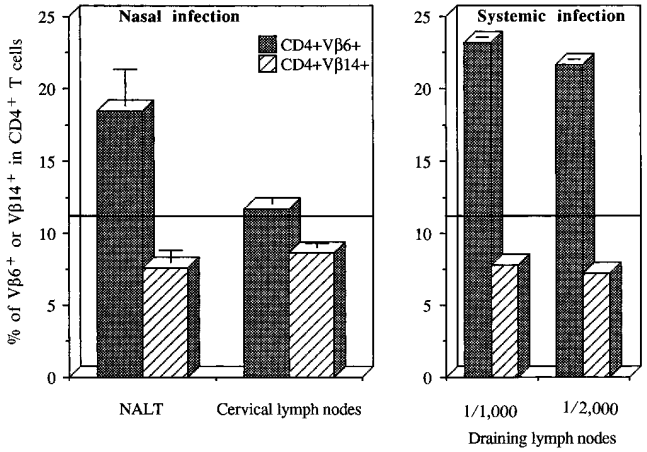

Figure 3. T cell SAg response was found in the NALT. Adult mice were infected by the nasal route (three mice) with 20 μl of infected milk diluted to 1:2 or by injection in the footpad of 20 μl of the same infected milk sample diluted to 1:1,000 or 1:2,000 (one mouse). After 5.5 d, mice were killed and the percentages of $V\beta6^+$ or $V\beta14^+$ cells among $CD4^+$ T cells in NALT and cervical lymph nodes for the nasally infected mice or in popliteal lymph nodes for the footpad-infected mice were determined by flow cytometry. The horizontal line represents percentages of $CD4^+V\beta6^+$ T cells of noninfected mice.

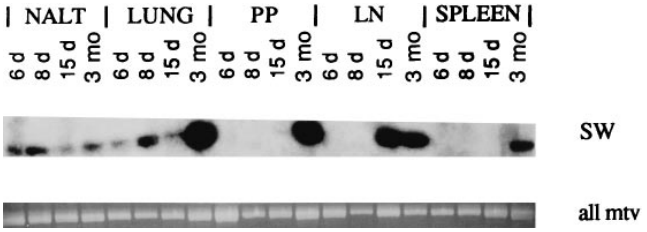

Figure 4. MMTV (SW) viral DNA in different lymphoid organs after nasal infection. Using a PCR assay, we amplified viral DNA sequences with oligonucleotides specific for MMTV (SW) SAg sequence (*top*) or common to all endogenous MMTV (mtv) SAg sequences (*bottom*). The latter PCR amplification experiments indicated that the quantities of genomic DNA taken from different lymphoid organs at the indicated times points were comparable. DNA were isolated 6 d, 8 d, 15 d, or 3 mo after nasal infection. *LN*, cervical lymph nodes.

Discussion

We report that adult mice, like neonates, are susceptible to mucosal MMTV infection. We show for the first time that MMTV crosses the epithelium of the nasal cavity and infects NALT.

After mucosal administration, whether intranasal for adult mice or intraintestinal for neonates, the course of MMTV infection is relatively similar in that the virus is first propagated in lymphoid tissue associated to the mucosal epithelium before disseminating to all lymphoid organs. At 10 d of age, both the SAg response and the viral DNA are restricted to the PP of neonates, however, the viral DNA can be detected in all lymphoid organs later (2). In adult mice, the detection of the SAg response and viral DNA in the NALT 6 d after nasal infection demonstrates that after crossing of the epithelium, MMTV infects this mucosal lymphoid structure. The detection of viral DNA in cervical lymph nodes, PP, thymus, and spleen and systemic SAg-reactive T cell deletion shows that MMTV infection spread to other lymphoid organs. Furthermore, nasal administration of MMTV-infected milk induces an efficient MMTV infection, since the disease is transmitted to the offspring.

6 d after infection we could detect viral DNA in NALT and in lung tissue, but the T cell proliferation characteristically induced by the SAg 4–6 d after MMTV infection was not found in the lungs. This might be due to the procedure used to isolate lymphoid cells from the lungs since we took all the lung tissue and isolated the total pool of lymphoid cells from it. It is therefore possible that the T cell proliferation only occurred in one or two lymphoid aggregates and consequently was undetectable in the total pool of isolated lymphoid cells. It is also possible that in the lungs, the cellular environment did not provide appropriate conditions for the development of an SAg reaction (14). A very early migration of infected lymphoid cells from the NALT to the lung could also account for the presence of viral DNA in the lungs 6 d after nasal infection. Whatever the explanation, we showed that NALT is one of the initial site(s) of viral spreading, since we could detect the SAg response and viral DNA at this site. Furthermore, the lungs could be either an entry site for MMTV or an early site of migration of infected cells derived from the NALT.

Adult mucosal MMTV infection seem to be less efficient than the systemic infection. Indeed the number of MMTV particles needed to induce an SAg response in NALT is 250-fold higher than the number necessary to induce the SAg response in the popliteal lymph node after footpad injection. Similarly, we could observe that the quantity of MMTV particles necessary to induce the deletion of the SAg-reactive T cells from the peripheral blood after nasal infection is also higher than the number necessary after systemic infection (data not shown). The high quantity of MMTV particles required for the successful infection of the mucosae explains the relative resistance of adult mice to the oral infection. Indeed, the acid conditions and digestive enzyme secretions of the adult gastrointestinal tract are likely to inactivate the virus and consequently prevent PP infection. In support of this assumption, we could detect, 6 d after direct injection of infected milk in adult intestine, the appearance of viral DNA in the PP (data not shown). The large viral load necessary to infect mice by a mucosal surface is probably not restricted to the adult period of life. Indeed, neonates receive a tremendous load of viral particles throughout the nursing period (3).

Although we show that MMTV can cross the epithelium of the nasal cavity, we have not elucidated how this process occurs. Although many mechanisms can be postulated to explain how a retrovirus can cross an epithelium to infect the associated lymphoid structure, we favor two of them. The first mechanism implicates M cell transport of MMTV across the epithelium, since the epithelium of PP and NALT contains M cells (6). This mechanism would allow direct access of MMTV to the lymphoid organ. In the second

mechanism, it might be possible that in the intestine, nasal, or lung cavities, MMTV infects or is taken up by dendritic cells associated with the epithelium (15, 16). The dendritic cells infected or loaded with MMTV might then migrate to draining lymph nodes and transmit the infection. These postulated mechanisms of MMTV infection are not mutually exclusive and might be proven by the study of the kinetics of nasal infection of adult mice.

Another very important point to discuss in relation to this study is the similarity between the putative modes of mucosal infection of HIV-1 and MMTV. Epidemiologic studies have revealed that worldwide, most HIV-1 infections are acquired by mucosal exposure (17). In adults, sexual transmission of HIV-1 can occur through unprotected vaginal or anal intercourse (17). Oral infection is well documented in neonates, who can acquire the virus by breast feeding (18, 19). An epidemiologic study reported that unprotected receptive oral intercourse in adults should also be viewed as high risk behavior for HIV-1 transmission (20). Furthermore, studies of oral transmission of the simian immunodeficiency virus have shown that adult macaques after nontraumatic oral exposure to cell-free simian immunodeficiency virus became infected and developed AIDS (21). These findings suggest that the oral cavity is susceptible to retroviral infections. In humans, the pharynx is guarded by the Waldeyer's ring consisting of the tonsils and adenoid lymphoid organs (22). Although mice do not have tonsils, their functional equivalent is the nasal-associated lymphoid tissue (23). In this study, we observed that MMTV can infect lymphoid cells of NALT, which suggests that, as for MMTV, HIV-1 might also infect the lymphoid cells of the Waldeyer's ring. This assumption is corroborated by the description of the presence of HIV-1 replication in the nasopharyngeal tonsil or adenoid of an infected individual (24).

In conclusion, this new adult model of mucosal MMTV infection is a powerful tool to study the mechanisms of mucosal retroviral infection and to test candidate vaccines. We are currently using this model to investigate the efficiency of a systemic antibody response directed against MMTV to prevent the early steps of mucosal infection.

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