Brief Definitive Report

Reverse Transcriptase–dependent and -independent Phases of Infection with Mouse Mammary Tumor Virus: Implications for Superantigen Function

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Summary

Mouse mammary tumor virus (MMTV) encodes a superantigen (SAg) that promotes stable infection and virus transmission. Upon subcutaneous MMTV injection, infected B cells present SAg to SAg-reactive T cells leading to a strong local immune response in the draining lymph node (LN) that peaks after 6 d. We have used the reverse transcriptase inhibitor 3′-azido-3′-deoxythymidine (AZT) to dissect in more detail the mechanism of SAg-dependent enhancement of MMTV infection in this system. Our data show that no detectable B or T cell response to SAg occurs in AZT pretreated mice. However, if AZT treatment is delayed 1–2 d after MMTV injection, a normal SAg-dependent local immune response is observed on day 6. Quantitation of viral DNA in draining LN of these infected mice indicates that a 4,000-fold increase in the absolute numbers of infected cells occurs between days 2 and 6 despite the presence of AZT. Furthermore MMTV DNA was found preferentially in surface IgG^+ B cells of infected mice and was not detectable in SAg-reactive T cells. Collectively our data suggest that MMTV infection occurs preferentially in B cells without SAg involvement and is completed 1–2 d after virus challenge. Subsequent amplification of MMTV infection between days 2 and 6 requires SAg expression and occurs in the absence of any further requirement for reverse transcription. We therefore conclude that clonal expansion of infected B cells via cognate interaction with SAg-reactive T cells is the predominant mechanism for increasing the level of MMTV infection. Since infected B cells display a memory (surface IgG^+) phenotype, both clonal expansion and possibly longevity of the virus carrier cells may contribute to stable MMTV infection.

Superantigens (SAgs) are proteins of bacterial or viral origin that bind to MHC class II molecules and interact specifically with TCR Vβ domains (1). Mouse mammary tumor virus (MMTV) is a milk-transmitted type B retrovirus that encodes a SAg in its 3′ LTR (2, 3). After infection of mice by MMTV, T lymphocytes react with the SAg molecule expressed on the surface of infected B cells, leading to a local immune response (4, 5). This SAg-dependent immune reaction ultimately results in amplification of MMTV infection and virus transmission (5–8); however the precise sequence of events that follow MMTV infection have not been clarified. Here we use the reverse transcriptase inhibitor 3′-azido-3′-deoxythymidine (AZT) to identify an initial SAg-independent phase of MMTV penetration and integration. Subsequent amplification of infection occurs primarily (if not exclusively) via clonal expansion of the initially infected B cells due to SAg-mediated cognate interaction with T cells.

Materials and Methods

Mice and Injections. Adult BALB/c mice were purchased from HO Harlan OLAC Ltd. (Bicester, UK). *Mtv*-7^+ (Mls-1a) congenic BALB/c (BALB.D2) mice were derived from breeding pairs originally provided by H. Festenstein (London Hospital Medical College, London, UK) (9). BALB/c mice were injected with either 10^6 *Mtv*-7^+ B cells or $\sim 10^8$ MMTV (SW) virus particles in the footpad. AZT (Sigma Chemical Co., St. Louis, MO) was administered to mice in drinking water (1 mg/ml). On the first day of treatment AZT was also injected intraperitoneally at a dose of 3 mg (corresponding to a daily dose of AZT taken up through drinking water) (10).

Flow Microfluorometry and Cell Sorting. PBL were recovered from samples of heparinized blood by centrifugation on a cushion of Ficoll-Hypaque (Pharmacia LKB, Uppsala, Sweden). LN cells or PBL were labeled in a single step with direct FITC conjugates of either anti-Vβ6 (44.22.1) (11) or anti-Vβ14 (14.2) (12) mAbs together with anti-CD4-PE (GK1.5) (Becton Dickinson & Co., Mountain View, CA). B cells were detected with FITC labeled rabbit $F(ab)'_2$ anti–mouse Ig. All samples were analyzed using a FACScan® and the Lysys II program (Becton Dickinson & Co.). For sorting, LN cells were stained either with FITC-labeled anti-Vβ6 and anti-CD4-PE or with biotinylated goat anti–mouse IgG (Caltag Laboratories Inc., San Francisco, CA) followed by avidin PE plus FITC labeled rabbit $F(ab)'_2$ anti–mouse Ig. Cells were sorted to a purity of >95% using a FACStar Plus® (Becton Dickinson & Co.).

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PCR Analysis of MMTV Infection. At the indicated timepoints after virus injection the local (popliteal and paraaortic) LN were removed and DNA was isolated by digestion in 10 mM Tris-HCl, pH 8, 100 mM NaCl, 25 mM EDTA, pH 8, 0.5% SDS plus Proteinase K (100 μg/ml) at 52°C, overnight. After purification the DNA equivalent of 10^5 cells (~0.5 μg of DNA) was used per PCR reaction. The oligonucleotides VJ84-VJ71 were used to amplify all *Mtvs* endogenous to BALB/c mice (i.e., *Mtv-6, 8, 9*) as previously described (4). The oligo RM53 (CTAGGAACAGGAATGAACTTC) together with VJ71 (4) was used to amplify specifically MMTV (SW)/*Mtv*-7 DNA. This PCR assay was performed and analyzed as for the quantitative assay described before (5). Quantitative PCR analysis of MMTV (SW) infection normalized to endogenous *Mtv* loci has been described in detail before (5). Signal quantification was performed with a Phosphorimager SF (Molecular Dynamics Inc., Sunnyvale, CA) using MD ImageQuant software for data evaluation. The MMTV (SW) DNA content was calculated relative to the signal derived from the *Mtv*-7 locus as described previously.

Results and Discussion

We have previously identified MMTV (SW) as an infectious homologue of the endogenous provirus *Mtv*-7, which encodes a SAg that primarily reacts with Vβ6⁺ T cells (13). Infection with MMTV (SW) leads to a SAg-dependent immune response, which amplifies viral infection (5). As shown in Fig. 1 *a* a detectable response of SAg-reactive Vβ6⁺ CD4⁺ T cells was first observed in the draining LN of BALB/c mice on day 3 after MMTV (SW) injection into the footpad. This T cell response was maximal between day 4 and 6 and preceded the B cell response by ~1 d (Fig. 1 *a*) (4). To correlate these kinetic data directly with viral infection we used a PCR-based assay specific for MMTV (SW) DNA sequences located in the LTRs (4). This sensitive assay indicated that newly synthesized viral DNA was first detectable at day 2 after injection and increased thereafter (Fig. 2 *a*). Furthermore, a quantitative PCR assay (5) showed maximal MMTV (SW) infection at day 5 and 6 (data not shown). Thus the degree of MMTV infection coincides temporally with the SAg-induced immune response.

Since there are potentially multiple ways in which MMTV infection could be amplified and therefore different levels at which the MMTV SAg could act (14) we first assessed whether a SAg-induced immune response can occur in the absence of reverse transcription. To this end we injected MMTV (SW) into the footpad of AZT pretreated BALB/c mice and analyzed the draining LN 6 d later. As shown in Table 1 a specific increase of SAg-reactive Vβ6⁺CD4⁺ T cells and a corresponding B cell increase was observed in the local LN of control mice upon exposure to MMTV (SW). These responses were not seen when MMTV (SW) was injected into AZT pretreated mice (Table 1). Analysis of the viral DNA present in these LN indicated a very weak signal from MMTV (SW) injected,

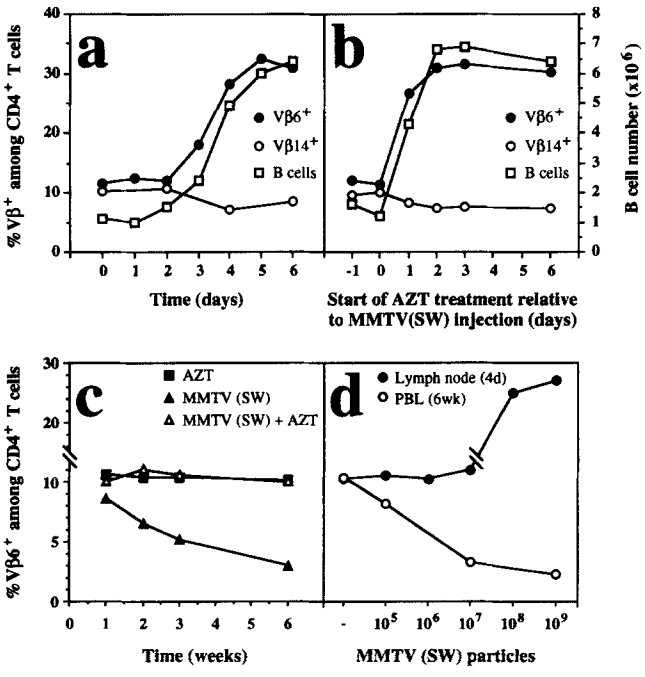

Figure 1. Kinetics of the MMTV (SW) induced SAg response and its dependence upon reverse transcriptase activity. (*a*) Kinetics of local B and T cell responses to SAg after injection of ~10^8 MMTV (SW) particles. (*b*) Local B and T cell responses to MMTV (SW) SAg (measured at day 6) in mice treated with AZT at the indicated time points. (*c*) Effect of AZT on Vβ6⁺ CD4⁺ T cell deletion in peripheral blood induced by MMTV (SW). (*d*) Dose response analysis of MMTV (SW) SAg induced local stimulation (4 d) and peripheral deletion (6 wk) of Vβ6⁺ CD4⁺ T cells.

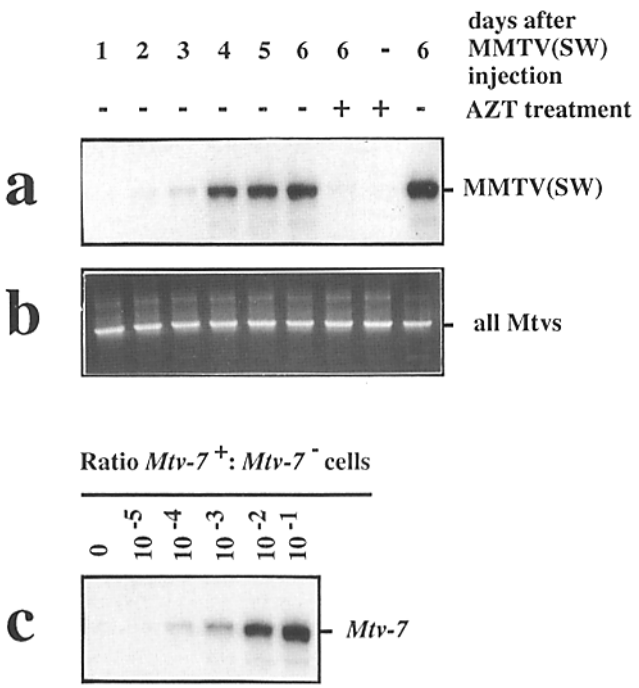

Figure 2. Effect of AZT on MMTV (SW) infection. PCR was performed with MMTV (SW)/*Mtv*-7–specific (*a*) or total *Mtv*-specific (*b*) primers using DNA purified from the draining LN taken at the indicated time points after virus was injected into normal or AZT-treated mice. (*c*) Mixing experiment to illustrate the sensitivity of the MMTV (SW)/*Mtv*-7–specific PCR. Lymphocytes from *Mtv*-7⁻ BALB/c (containing *Mtv 6, 8,* and *9*) and *Mtv*-7⁺ congenic BALB.D2 (*Mtv 6, 7, 8,* and *9*) mice were mixed in various ratios before DNA purification.

Table 1. *The MMTV (SW)-induced SAg Response Is Dependent on Reverse Transcription*

AZT	Stimulation	n	Percent CD4 T cells expressing		B cells
			Vβ6	Vβ14	
					× 10^6
−	−	6	11.7 ± 0.6	10.6 ± 0.8	1.1 ± 0.5
+	−	6	12.0 ± 0.7	10.1 ± 0.6	1.0 ± 0.7
−	MMTV (SW)	3	29.8 ± 4.4	8.0 ± 1.2	6.6 ± 4.5
+	MMTV (SW)	3	12.6 ± 0.7	10.0 ± 0.8	1.4 ± 0.2
−	*Mtv*-7 (Mls-1)	3	33.9 ± 1.1	6.5 ± 0.5	6.3 ± 2.2
+	*Mtv*-7 (Mls-1)	3	33.0 ± 1.8	7.3 ± 0.2	5.4 ± 1.1

BALB/c mice were treated with AZT starting 1 d before injection with either ~10^8 MMTV(SW) particles or with 10^6 B cells from *Mtv*-7 congenic BALB.D2 mice. The immune response in the local (popliteal and paraaortic) lymph nodes was assessed 6 d after injection. Data are expressed as mean ± SD of the indicated number of mice.

AZT-treated compared with nontreated mice (Fig. 2 *a*) confirming that MMTV reverse transcriptase was efficiently inhibited by AZT. These results indicate that a detectable local immune response induced by the MMTV (SW) SAg is strictly dependent upon reverse transcription.

More definitive evidence that reverse transcription is a prerequisite for MMTV (SW) SAg expression was obtained by monitoring the kinetics of clonal deletion of Vβ6$^+$CD4$^+$ T cells in AZT-treated infected mice. We have shown previously that the local immune response to MMTV (SW) is followed by a progressive deletion of SAg-reactive Vβ6$^+$ CD4$^+$ T cells in the peripheral blood (13). As shown in Fig. 1 *c* this deletion does not occur in infected mice treated with AZT over a 6-wk period. Direct comparison of MMTV (SW) titration curves for local expansion (measured at 4 d) and peripheral deletion (measured at 6 wk) of Vβ6$^+$CD4$^+$ T cells after local infection indicates that the clonal deletion assay is ~1,000-fold more sensitive, since 10^5 virus particles induce detectable deletion whereas 10^8 particles are required for significant expansion (Fig. 1 *d*). Based on these data we conclude that functional SAg is unlikely to be present in the mature virion before reverse transcription or to be expressed using the RNA genome, or a portion thereof (15), as a template (16). Rather it appears that SAg expression occurs de novo after integration of the MMTV genome into the host genome. Thus, viral entry and integration most likely occur independently of SAg activity.

To rule out a nonspecific effect of AZT on the local immune response induced by MMTV SAg we injected B cells from an *Mtv*-7$^+$ mouse strain (BALB/D2) (9) into the footpad of congenic *Mtv*-7$^-$ recipients (BALB/c). This induced a local response of Vβ6$^+$ CD4$^+$ T cells comparable in magnitude to that seen upon MMTV (SW) injection (13, 17) (Table 1). However, since *Mtv*-7 is an endogenous provirus, expression of its SAg does not require reverse transcription. As shown in Table 1 the Vβ6$^+$CD4$^+$ T cell response to *Mtv*-7$^+$ cells was not affected by AZT pretreatment, indicating that the local immune responses to MMTV SAgs are not susceptible to nonspecific effects of AZT.

After virus integration and expression of functional SAg molecules, at least two mechanisms could account for the SAg-dependent amplification of infection occurring during the local immune response to MMTV. Initial infection of a few B cells (presumably without SAg involvement) followed by de novo expression of SAg and T cell proliferation could lead to activation of bystander B cells, for example via cytokine release. Preferential infection of these bystander cells (18–20) would then lead to amplification of MMTV infection. An alternative (but not mutually exclusive) mechanism would be that infected B cells are induced to clonally expand via cognate interaction with SAg-reactive T cells. As can be seen from Fig. 1 *b*, AZT exposure begun 1 d before or at the same time as MMTV (SW) injection prevented the local immune response, which was assessed in the LN 6 d later. However, if AZT treatment is delayed 1–2 d after MMTV (SW) injection, an immune response is observed that is comparable in magnitude to the control response without AZT treatment. These results indicate that MMTV reverse transcriptase activity is no longer required for the induction of an optimal SAg response 1–2 d after virus injection. Since at this time point there are no significant T or B cell responses (Fig. 1 *a*), infection of activated bystander B cells is unlikely to represent an important mechanism for further stimulation and/or maintainence of the SAg response. This conclusion is further strengthened by quantitative analysis of the degree of MMTV (SW) infection in these LN (Fig. 3). No MMTV (SW) DNA was detected in the local LN on day 6 when AZT treatment was begun 1 d before or at the time of MMTV (SW) injection. However, the levels of MMTV (SW) infection were indistinguishable in control and treated animals when AZT was given 2 d or more after virus injection (Fig. 3). These data strongly suggest that the SAg-dependent amplification

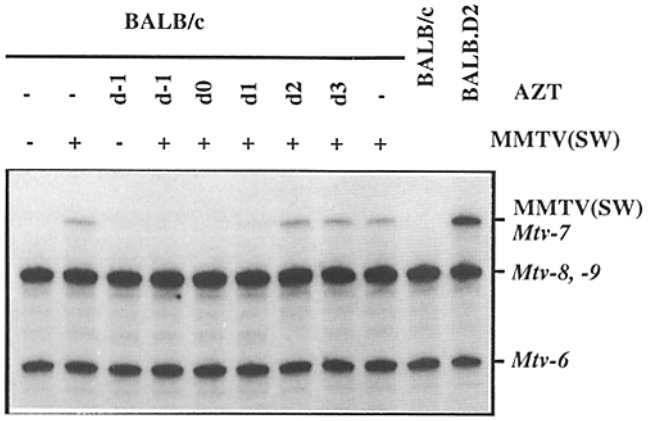

Figure 3. Time course of MMTV (SW) infection in the presence of AZT. Quantitative PCR analysis of MMTV (SW) DNA content (relative to endogenous *Mtv* loci) on day 6 in the draining LN of infected BALB/c mice treated with AZT at the indicated time points.

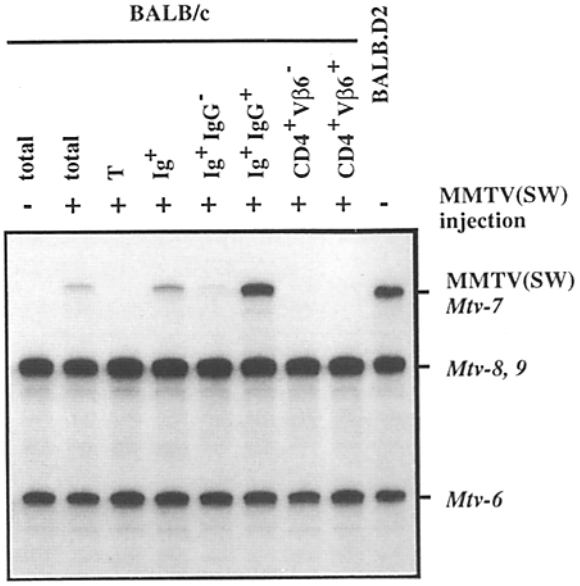

Figure 4. Phenotype of MMTV (SW) infected cells. At day 6 after virus injection LN cells were sorted into subsets according to the indicated phenotypes. The anti-IgG antibody detects ~8% surface IgG$^+$ cells among total Ig$^+$ LN B cells in control mice. At day 6 after virus injection ~12% of surface Ig$^+$ cells were found to be surface IgG$^+$. DNA isolation and the quantitative PCR assay was performed as described (5).

of MMTV (SW) infection observed after day 2 did not require reverse transcriptase activity and was therefore limited to previously infected cells.

Characterization of MMTV (SW) infected cells in LN at day 6 after virus injection confirmed our previous observation that at this stage viral DNA was exclusively detected in B and not T cells (5). More detailed analyses indicated that MMTV (SW) carriers are phenotypically surface IgG$^+$ (Fig. 4) and large, activated B cells (5). Besides this B cell subset, only SAg-reactive T cells were found to proliferate in the local LN (4, 5). However, since purified Vβ6$^+$CD4$^+$ T cells were not found to be detectably infected (Fig. 4), we would conclude that B cells (or a subset thereof) represent the primary target for MMTV infection.

The absolute magnitude of the increase in MMTV (SW)–infected B cells in the draining LN under our experimental conditions can be estimated by assuming an average of four viral integrations per infected B cell. (This estimate is based on our unpublished analysis of 20 B cell hybridomas derived by fusion of day 6 infected LN). On day 2 after infection, the MMTV (SW) DNA signal is roughly equivalent to an artificial mixture of one *Mtv*-7$^+$ (diploid) LN cell/10^4 *Mtv*-7$^-$ cells (see Fig. 2, *a* and *c*). Since these draining LN contain 5×10^6 cells, this corresponds to ~250 infected cells. By day 6 the relative MMTV (SW) DNA content is 20% of the endogenous *Mtv*-7$^+$ control (Fig. 3), corresponding to ~10^6 infected cells in a draining LN containing 10×10^6 total cells. This level of clonal expansion (~4,000-fold) would require at least 12 B cell divisions in 4 d or a doubling time of ~8 h. Even shorter doubling times (6–7 h) have been reported for germinal center B cells (21). The fact that infected B cells are surface IgG$^+$ further suggests that they may constitute a pool of long-lived virus carriers (22, 23). Therefore, besides clonal expansion, longevity of infected B cells may ensure virus survival in the host and ultimately contribute to the infection of mammary tissue.

It is important to emphasize that the conclusions reached herein pertaining to the mechanism of SAg-mediated amplification of MMTV infection in lymphocytes are based on a somewhat artificial model in which adult mice are infected subcutaneously. It will therefore be important to assess the effects of SAg-mediated immune interactions during natural infection by milk-borne virus via the neonate gut mucosa. Despite this caveat, it seems very likely that the SAg-induced clonal expansion of infected B cells seen in adult mice is also important during natural infection since SAg-reactive T cells are required for MMTV transmission (5–8).

Finally the model system described here may prove useful in a more general context as a screening procedure for the efficiency of novel antiretroviral drugs. In this regard monitoring the SAg-dependent local immune response of Vβ6$^+$ CD4$^+$ T cells by flow microfluorometry represents a rapid, sensitive, quantitative, and reproducible assay that depends strictly upon MMTV reverse transcriptase activity in vivo. Importantly, immunosuppressive properties of compounds under study can be dissociated from their true antiviral effects by parallel assessment of responses to infectious and endogenous MMTV SAgs. Such a system should complement other methods of screening antiretroviral drugs in vivo, for example the SCID-human mouse model (24).

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References

1. Marrack, P., G. Winslow, Y. Choi, M. Scherer, A. Pullen, J. White, and J.W. Kappler. 1993. The bacterial and mouse mammary tumor virus superantigens: two different families of proteins with the same functions. *Immunol. Rev.* 131:79.
2. Choi, Y., J.W. Kappler, and P. Marrack. 1991. A superantigen encoded in the open reading frame of the 3′ long terminal repeat of mouse mammary tumor virus. *Nature (Lond.).* 350:203.
3. Acha-Orbea, H., A.N. Shakhov, L. Scarpellino, E. Kolb, V. Müller, A. Vessaz-Shaw, R. Fuchs, K. Blöchlinger, P. Rollini, J. Billote, et al. 1991. Clonal deletion of Vβ14 positive T cells in mammary tumor virus transgenic mice. *Nature (Lond.).* 350:207.
4. Held, W., A.N. Shakhov, S. Izui, G.A. Waanders, L. Scarpellino, H.R. MacDonald, and H. Acha-Orbea. 1993. Superantigen-reactive CD4$^+$ T cells are required to stimulate B cells after infection with mouse mammary tumor virus. *J. Exp. Med.* 177:359.
5. Held, W., G.A. Waanders, A.N. Shakhov, L. Scarpellino, H. Acha-Orbea, and H.R. MacDonald. 1993. Superantigen-induced immune stimulation amplifies mouse mammary tumor virus infection and allows virus transmission. *Cell.* 74:529.
6. Golovkina, T.V., A. Chervonsky, J.P. Dudley, and S.R. Ross. 1992. Transgenic mouse mammary tumor virus superantigen expression prevents viral infection. *Cell.* 69:637.
7. Beutner, U., E. Kraus, D. Kitamura, K. Rajewsky, and B.T. Huber. 1994. B cells are essential for murine mammary tumor virus transmission, but not for presentation of endogenous superantigens. *J. Exp. Med.* 179:1457.
8. Pucillo, C., R. Cepeda, and R.J. Hodes. 1993. Expression of a MHC class II transgene determines both superantigenicity and susceptibility to mammary tumor virus infection. *J. Exp. Med.* 178:1441.
9. Festenstein, H., and L. Berumen. 1983. BALB.D2-Mls^a—A new congenic mouse strain. *Transplantation (Baltimore).* 37:322.
10. Kaneshima, H., C.-C. Shih, R. Namikawa, L. Rabin, H. Outzen, S.G. Machado, and J.M. McCune. 1991. Human immunodeficiency virus infection of human lymph nodes in the SCID-hu mouse. *Proc. Natl. Acad. Sci. USA.* 88:4523.
11. Payne, J., B.T. Huber, N.A. Cannon, R. Schneider, M.W. Schilham, H. Acha-Orbea, H.R. MacDonald, and H. Hengartner. 1988. Two monoclonal rat antibodies with specificity for the β-chain variable region Vβ6 of the murine T cell receptor. *Proc. Natl. Acad. Sci. USA.* 85:7695.
12. Liao, N.-S., J. Maltzman, and D.H. Raulet. 1989. Positive selection determines T cell receptor Vβ14 gene usage by CD8$^+$ T cells. *J. Exp. Med.* 170:135.
13. Held, W., A.N. Shakhov, G. Waanders, L. Scarpellino, R. Luethy, J.-P. Kraehenbuhl, H.R. MacDonald, and H. Acha-Orbea. 1992. An exogenous mouse mammary tumor virus with properties of Mls-1^a (*Mtv-7*). *J. Exp. Med.* 175:1623.
14. Coffin, J.M. 1992. Superantigen and endogenous retroviruses: a confluence of puzzles. *Science (Wash. DC).* 255:411.
15. Dickson, C., and G. Peters. 1981. Protein-coding potential of mouse mammary tumor virus genome RNA as examined by in vitro translation. *J. Virol.* 37:36.
16. Gallis, B.M., R.N. Eisenmann, and H. Diggelmann. 1976. Synthesis of the precursor to avian RNA tumor virus internal structural proteins early after infection. *Virology.* 74:302.
17. Waanders, G.A., A.N. Shakhov, W. Held, O. Karapetian, H. Acha-Orbea, and H.R. MacDonald. 1993. Peripheral T cell activation and deletion induced by transfer of lymphocyte subsets expressing endogenous or exogenous mouse mammary tumor virus. *J. Exp. Med.* 177:1359.
18. Roe, T.Y., T.C. Reynolds, G. Yu, and P. Brown. 1993. Integration of murine leukemia virus DNA depends on mitosis. *EMBO (Eur. Mol. Biol. Organ.) J.* 12:2099.
19. Miller, D.G., M.A. Adam, and A.D. Miller. 1990. Gene transfer by retrovirus vectors occurs only in cells that are actively replicating at the time of infection. *Mol. Cell. Biol.* 10:4239.
20. Springett, G.M., R.C. Moen, S. Anderson, R.M. Blaese, and F.W. Anderson. 1989. Infection efficiency of T lymphocytes with amphotropic retroviral vectors is cell cycle dependent. *J. Virol.* 63:3865.
21. Liu, Y.C., J. Zhang, P.J.L. Lane, E.Y.-T. Chan, and I.C.M. MacLennan. 1991. Sites of specific B cell activation in primary and secondary responses to T cell-dependent and T cell-independent antigens. *Eur. J. Immunol.* 21:2951.
22. Black, S.J., W. Van der Loo, M.R. Loken, and L.A. Herzenberg. 1978. Expression of IgD by murine lymphocytes. Loss of surface IgD indicates maturation of memory B cells. *J. Exp. Med.* 147:984.
23. Yuan, D., E.S. Vitteta, and J.R. Kettman. 1977. Cell surface immunoglobulin XX. Antibody responsiveness of subpopulations of B lymphocytes bearing different isotypes. *J. Exp. Med.* 145:1421.
24. McCune, J.M., R. Namikawa, C.-C. Shih, L. Rabin, and H. Kaneshima. 1989. Suppression of HIV infection in AZT-treated SCID-hu mice. *Science (Wash. DC).* 247:564.