Neonatal infection with a milk-borne virus is independent of β7 integrin- and L-selectin-expressing lymphocytes

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Mouse mammary tumor virus (MMTV) is acquired by neonates through milk and first infects lymphocytes in Peyer's patches. We show here that newborn mice lacking β7 integrin or L-selectin were infected with MMTV at wild-type levels in both their lymphoid and mammary tissues. Superantigen-mediated activation and cognate T cell deletion were also unimpaired in both types of null mice. A large proportion of neonatal Peyer's patch lymphocytes in wild-type mice were β7 and β1 integrin low and both populations increased in response to MMTV infection. These results suggest that adhesion molecules other than β7 integrin or L-selectin play a role in lymphocyte homing in the gut, peripheral lymph nodes and mammary gland in response to MMTV infection.

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1 Introduction

Lymphocyte trafficking depends on the expression of specific adhesion molecules on their surface that interact with counter receptors on the target tissue. The best-studied example of this type of cell-cell interaction is found in gut-associated lymphoid tissue (GALT). Lymphocyte homing to Peyer's patches (PP) and the intraepithelial spaces of the intestine (IE) requires expression of α4β7 integrin and αEβ7 integrin, respectively [1]. Indeed, adult mice with targeted deletion of the β7 integrin gene have a greater than 90% reduction in both PP and IE lymphocytes [2]. Antibody blocking studies have also implicated L-selectin in lymphocyte homing to the PP [3] and mice that express neither β7 integrin nor L-selectin have greater than 95% reduction in GALT lymphocytes [4].

Much less is known about what governs lymphocyte migration to other mucosal-associated lymphoid tissue (MALT). Homing to vaginal epithelia in response to Chlamydia infection has implicated α4β1 integrin [5] and VCAM-1, the counter-receptor for this integrin, was found in the high endothelial venules (HEV) of human bronchial and nasal mucosa (reviewed in [6]). Although lymphocyte trafficking to mammary epithelial tissue during lactation is critical to the immunological health of both the mother and newborn, few studies have addressed the adhesion molecule composition of lymphocytes in this MALT. The ligands for α4β7 and αEβ7, MAdCAM-1 and E-cadherin, respectively, are expressed in the mammary gland and *in vitro* adherence of lymphocytes to mammary tissue can be blocked with anti-MAdCAM-1 antibodies [7]. The L-selectin ligand GlyCAM-1 is also found on mammary epithelial cells, but lacks the sulfate groups necessary for binding to L-selectin [8].

MMTV is a milk-acquired virus that depends on lymphocytes to establish infection [9, 10]. MMTV enters through M cells in the PP and mice that lack these cells are immune to infection [10]. MMTV first activates then infects B lymphocytes [11, 12], which in turn present a viral superantigen (Sag) to T cells bearing specific T cell receptor (TCR) Vβ chains (reviewed in [13]). These Sag-cognate T cells proliferate and produce cytokines that stimulate nearby B cells, resulting in the establishment of a reservoir of infection-competent and infected lymphocytes [14]. Infection then spreads to the mesenteric

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Abbreviations: MMTV: Mouse mammary tumor virus **Sag:** Superantigen **GALT:** Gut-associated lymphoid tissue **LN:** Lymph node **PP:** Peyer's patch **KO:** Null

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lymph nodes (LN) that drain the GALT and later to peripheral tissues [11].

Lymphocytes are also required for subsequent virus spread to mammary gland. The target mammary epithelial cells can only be infected with MMTV at a time when they are driven to divide, that is, during the hormonal stimulation that accompanies puberty and pregnancy. Thus, the creation of an infected lymphoid reservoir following neonatal exposure allows the virus to overcome the temporal block to infection. Both B and T cells can be infected with and transmit MMTV [15–17]. Lymphocytes infected initially in the gut and subsequently in peripheral lymphoid organs must migrate to the mammary gland to deliver MMTV to epithelial cells. Mice that lack B or Sag-responsive T cells are resistant to infection via either the neonatal route or by direct injection of virus into the mammary gland [9, 18, 19].

The aim of these studies was to determine whether β7 integrin or L-selectin were required for (a) the initial acquisition of milk-borne MMTV in the gut, and (b) the migration of MMTV-infected lymphocytes from gut to mammary gland. Despite the paucity of target lymphocytes present in the gut of β7-null mice, their lymphocytes were efficiently infected with MMTV and loss of L-selectin, either on its own or in conjunction with the lack of β7, had no effect on infection. We also found that cells with a naive phenotype increased in the PP of wild-type neonatal mice in response to MMTV infection. In the lactating mammary glands of the adhesion molecule-null mice, there were significant numbers of T and B cells. Mammary gland infection was not affected by the absence of L-selectin or β7 and both types of null mice developed mammary tumors with similar incidence and kinetics. Thus, other homing molecules may contribute to lymphocyte migration to GALT during the neonatal period when MMTV is acquired and to their subsequent homing to peripheral lymphoid tissue and mammary gland.

2 Results

2.1 Mammary gland lymphocyte populations of β7- or L-selectin-null mice

To determine whether loss of β7 integrin or L-selectin affected their migration, we analyzed the lymphocyte subsets in the lactating mammary glands of wild-type and null mice. There was variability in the isolated cell populations from different mice, as previously reported [20]; some of this variability may be due to the different background strains of the various mice (see Sect. 4). There were no significant differences in the percentage of B cells in MMTV-infected wild-type, β7- or L-selectin-null mice (Table 1), while uninfected β7-null mice had a greater percentage of B cells than wild-type mice. Additionally, the mammary tissue of the β7-null mice had a higher percentage of T cells. The B cell population has been shown by others to be composed predominantly of IgA^+ plasmablasts that originate from the GALT [21]. In all cases, the percentage of B cells was smaller than T cells, as previously observed [22].

The percentage of $TCR\alpha\beta^+$ T cells in the lactating mammary gland was between 20% and 35% in all cases, except for $MMTV^+$ β7-null mice where it was 54.3% (Table 1). This was significantly higher than in MMTV-infected wild-type and L-selectin-null mice ($p<0.01$ for both groups). There were more $CD8^+$ than $CD4^+$ cells for all groups except the MMTV-negative β7-null mice, in

Table 1. Analysis of lymphocyte populations in lactating mammary glands

Mouse[a)]	% $B220^+$	% $TCR\alpha\beta^+$	% $CD4^+/TCR\alpha\beta^+$	% $CD8^+/TCR\alpha\beta^+$	*n*[b)]
C3H/HeN $MMTV^-$	1.9 ± 1.8[c)]	21.3 ± 3.6	24.2 ± 10.1	42.3 ± 5.9	4
C3H/HeN $MMTV^+$	4.0 ± 0.8	27.5 ± 3.8	25.7 ± 6.1	39.8 ± 6.8	4
β7 KO $MMTV^-$	13.3 ± 4.3	35.4 ± 5.2	24.1 ± 3.6	21.2 ± 5.6	4
β7 KO $MMTV^+$	3.4 ± 1.6	54.3 ± 0.4	12.4 ± 3.0	36.9 ± 7.0	4
L-sel KO $MMTV^-$	2.7	15.6	1.0	24.0	1
L-sel KO $MMTV^+$	2.8 ± 1.0	28.6 ± 5.5	4.0 ± 0.6	30.6 ± 9.9	6

a) Lymphocytes were isolated from the lactating mammary glands of MMTV-infected and uninfected wild-type mice (C3H/HeN), L-selectin (L-sel KO) and β7 integrin knock-out mice during the first week of lactation at the third pregnancy, and stained for expression of the indicated markers.
b) Number of mice analyzed.
c) Data are presented as mean ± SD.

which the percentage of $CD4^+$ cells and $CD8^+$ cells were equivalent. Similar results were seen for L-selectin-null mice, with the exception of a decrease in the percentage of $CD4^+$ T cells. The percentage of $\gamma\delta^+$ T cells was higher than is seen in non-mucosal tissue in all mice, averaging 9%, as previously reported [20] and was unaffected by virus infection (data not shown).

To determine if the absolute lymphocyte numbers were altered, immunohistochemical staining for B and T cells was performed on mammary gland sections from infected mice. The lack of either adhesion molecule had no effect on B cell numbers and caused a small increase in T cell numbers (Fig. 1). Importantly, the lack of β7 integrin or L-selectin did not result in the complete loss of either B or T cells in the lactating gland.

We next examined the expression of homing markers for GALT (α4β7 and αEβ7), other mucosal sites (α4β1) and peripheral LN (L-selectin). In wild-type mice, about 70% of the lymphocytes were αE and β1 positive, while about 40% were α4 and β7positive (Table 2). A similar percentage of lymphocytes from L-selectin-null and wild-type mice expressed α4 and β1 integrins, whereas these percentages were decreased in the β7-null mice (Table 2); this might represent the lack of cells bearing both the α4/β1 and α4/β7 integrins. There was no alteration in the L-selectin$^+$ lymphocyte population in the absence of β7, nor was there a difference in the $\beta7^+$ population in the absence of L-selectin. Surprisingly, there were a considerable percentage of αE^+ lymphocytes in β7-null mice, although this was decreased compared to wild-type and L-selectin-null mice (Table 2). There is no known integrin besides β7 that pairs with αE [23, 24]. Both wild-type and

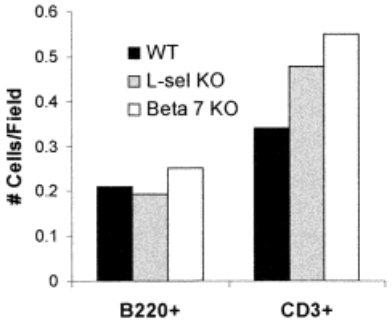

Fig. 1. Lymphocyte numbers in lactating mammary gland of MMTV(LA)-infected β7- and L-selectin-null mice. Lactating mammary glands were isolated from MMTV(LA)$^+$ wild-type, β7- and L-selectin-null mice and frozen sections were stained with anti-CD3 (T) and anti-B220 (B) antibodies. Data are presented as the average number of lymphocytes per field.

β7-null mice showed a reduction in αE^+ and $\beta1^+$ lymphocytes in response to MMTV infection (Table 2). This decrease was also seen in the T cell population, the major lymphocyte population of the mammary gland (not shown).

In summary, these data showed that the loss of β7 or L-selectin had no effect on B and T cells numbers and did not greatly alter the percentage of lymphocyte subpopulations in the lactating mammary gland. Furthermore, the lactating mammary gland lymphocytes expressed a variety of adhesion molecules important for migration.

Table 2. Integrin and L-selectin expression on mammary gland lymphocytes.

Mouse[a)]	% αE^+	% $\beta7^+$	% $\alpha4^+$	% $\beta1^+$	L-selectin	*n*[b)]
C3H/HeN $MMTV^-$	88.2 ± 4.6[c)]	38.9 ± 6.1	49.5 ± 5.5	92. ± 4.3	4.8 ± 1.7	4
C3H/HeN $MMTV^+$	68.4 ± 4.9	30.8 ± 4.4	40.6 ± 4.7	56.0 ± 6.4	3.5[d)] (1.9, 5.2)	4
β7 KO $MMTV^-$	61.4 ± 4.0	NA[e)]	31.4 ± 4.4	61.9 ± 6.1	ND[f)]	4
β7 KO $MMTV^+$	44.7 ± 25.5	NA	30.3 ± 18.7	13.5 ± 4.8	5.2[d)] (9.7, 0.7)	4
L-sel KO $MMTV^-$	85.6	ND	23.2	26.2	NA	1
L-sel KO $MMTV^+$	79.3 ± 4.3	32.5 ± 3.6	46.7 ± 5.3	76.1 ± 9.9	NA	6

a) Lymphocytes were isolated from the lactating mammary glands of the indicated mice during the first week of lactation and stained for the expression of the adhesion molecules shown.
b) Number of mice analyzed.
c) Data are presented as mean ± SD.
d) Two mice were analyzed; the % of staining cells for each is shown in parentheses.
e) Not applicable.
f) Not done

2.2 β7- and L-selectin-null mice have normal B cell responses and Sag presentation

Subcutaneous injection of MMTV initially results *in situ* B cell activation in the draining LN [12]. After B cell infection, Sag presentation to T cells causes their activation; this peaks at 96 h after virus introduction [14]. The Sag response within the draining LN is due to activation of resident lymphocytes as well as their recruitment from the circulation [25]. Because L-selectin or β7 integrin deficiency could affect the Sag response if homing to LN was disrupted, we tested whether B and T cell activation in response to exogenous virus was intact in the adhesion molecule-deficient mice.

Not surprisingly, the absence of β7 did not impair the initial B cell stimulation or Sag presentation in response to exogenously acquired MMTV (Table 3). Although the cellularity of the L-selectin-null mice LN was dramatically reduced, at 24 h after MMTV injection, L-selectin-null mice had an increase in the percentage of activated B cells ($CD69^+/B220^+$) in the draining LN (Table 3), similar to that seen in wild-type or β7-null mice. By 96 h post injection, the percentage of cognate T cells in the draining LN doubled in both wild-type and L-selectin-null mice and there was a proportional increase in cell numbers. The loss of L-selectin and β7 also had no effect on the T cell-dependent activation of B cells at 96 h (Table 3). Thus, neither β7 integrin nor L-selectin plays a role in the migration of naive lymphocytes to the LN or their activation in response to MMTV Sag.

Milk-borne MMTV infection is characterized by progressive deletion of Sag-cognate T cells that is proportional to the level of infection [26, 27]. β7- and L-selectin-deficient mice were foster-nursed on $MMTV(LA)^+$ mothers and starting at 1 month of age, the percentage of Sag-cognate T cells in peripheral blood was analyzed. In MMTV negative mice, 9-12% of peripheral T cells are $V\beta6^+/CD4^+$ (Fig. 2); this is reduced to approximately 5% by 1 month and less than 1% by 5 months in mice nursed on $MMTV(LA)^+$ mothers. Similar levels and kinetics of deletion occurred in MMTV(LA)-infected β7- and L-selectin-null mice (Fig. 2). Therefore, the β7- and L-selectin-null mice showed no impairment in the Sag-mediated deletion of cognate T cells.

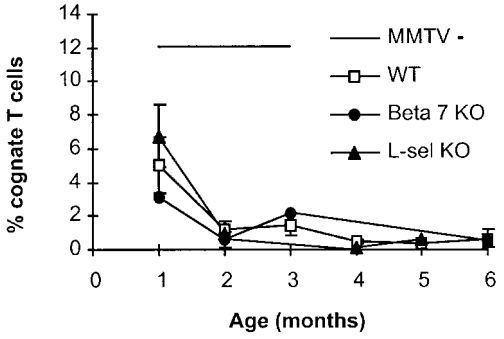

Fig. 2. Kinetics of peripheral Sag-cognate T cell deletion in β7- and L-selectin-null mice after MMTV infection. β7- and L-selectin-null mice were foster-nursed on C3H/HeN $MMTV(LA)^+$ mothers, and peripheral blood lymphocytes were stained for Vβ6 and CD4 at the indicated times. Shown is the mean ± standard deviation. Two to twelve mice per group were analyzed at each time point.

Table 3. Activation of lymphocytes in response to MMTV infection

	24 h		96 h						
	% $CD69^+/B220^+$		$CD69^+/B220^+$		% $V\beta8^+/CD4^+$		cell no. ($\times 10^{-6}$)		
Mouse[a)]	D	C	D	C	D	C	D	C	*n*[b)]
wt	26.3 ± 5.3[c)]	7.4 ± 2.4	78.0 ± 8.9	22.1 ± 2.2	43.7 ± 3.4	20.1 ± 2.8	4.9	0.3	3–5
L-sel KO	49.2 ± 21.0	7.8 ± 3.0	52.4 ± 6.5	33.7 ± 14.7	42.6 ± 6.1	23.4 ± 4.4	0.4	0.05	3–5
wt	41.5 ± 8.7	9.2 ± 2.3	77.0 ± 1.9	15.5 ± 2.6	33.5 ± 1.2	16.1 ± 1.1	ND[d)]	ND	4
β7 KO	50.7 ± 12.5	11.8 ± 2.9	83.1 ± 2.4	19.5 ± 1.8	36.7 ± 0.9	17.1 ± 1.4	ND	ND	4

a) Lymphocytes were isolated from draining (D) and non-draining control (C) LN 24 h and 96 h following injection of MMTV(FM) and stained to determine the percentages of activated B cells ($CD69^+/B220^+$), and Sag-cognate $CD4^+$ T cells ($V\beta8^+$). Total cell counts were determined for L-selectin KO mice and compared to wild-type mice.
b) Number of mice analyzed.
c) Data are presented as mean ± SD.
d) Not done.

2.3 Peripheral lymphoid infection occurs in β7 integrin- and L-selectin-deficient mice

Although these results showed that both the β7 integrin- and L-selectin-deficient mice were MMTV infected, deletion of cognate T cells occurs when very low levels of Sag are expressed [28] and thus, may not be reflective of differences in infection levels [29]. Exogenous MMTV sequences can be found in the LN, spleen, and thymus of infected mice. L-selectin is required for lymphocyte homing to non-mucosal (LN) and mucosal (PP) tissue, especially during inflammatory responses [30–34]. Thus, it was possible that infection of the lymphoid compartment, specifically peripheral LN, would be defective in L-selectin-null mice.

DNA isolated from the spleens and LN of infected mice was subjected to semi-quantitative PCR analysis, using primers specific for newly integrated exogenous MMTV and as a control, endogenous MMTV. The level of newly integrated MMTV(LA) in the spleens of L-selectin- and β7 integrin-null mice was similar to that seen in wild-type mice (Fig. 3). This was expected, because L-selectin does not play a role in the lymphocyte migration to the spleen [30–34] and β7 mediates homing to GALT but not peripheral lymphoid organs [24, 35]. However, there was no difference in peripheral LN infection in the L-selectin-null mice, indicating that virus spread occurred despite the paucity of lymphocytes at these sites (Fig. 3).

2.4 Mammary gland infection is intact in β7- and L-selectin-null mice

The ultimate target of MMTV is the mammary gland. Because β7 and L-selectin are important for lymphocyte homing within the mucosal and peripheral immune systems, respectively, and because a large percentage of the lymphocytes isolated from this tissue expressed β7 integrin (Table 2), we tested whether the mammary glands of null mice were infected. Null and wild-type mice foster-nursed on MMTV(LA) or MMTV(C3H)$^+$ mothers were force-bred and RNA isolated from milk at the first, second and third pregnancies was subjected to virus-specific RNase protection analysis to measure mammary gland infection. Although milk virus levels were somewhat variable, both β7- and L-selectin-null mice were infected since they produced wild-type amounts of virus RNA at all parities (parity 2 shown) (Fig. 4). Both MMTV, MMTV(C3H) with a relatively weak Sag, and MMTV(LA), with a strong Sag, infected the adhesion molecule-deficient mice as well as their wild-type counterparts. Thus, neither β7 nor L-selectin was required for initial lymphocyte migration to the mammary gland or for the lymphocyte-dependent spread of virus that occurs during pregnancy.

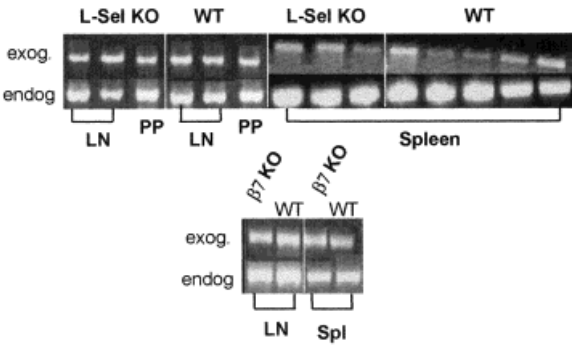

Fig. 3. MMTV(LA) infection in peripheral lymphoid tissue in β7- and L-selectin-null mice. Total DNA extracted from the peripheral LN, spleen (Spl), and PP was subjected to PCR under semiquantitative linear conditions using MMTV(LA)-specific primers (exog.), and primers which amplify all endogenous MMTV (endog.) as a control. The upper panel shows the data from L-selectin-null (L-sel KO) and wild-type (WT) mice, and the lower panel, β7-null and wild-type mice.

MMTV infection results in mammary adenocarcinomas, with a tumor incidence as high as 100% by the age of 1 year [36]. The kinetics and incidence of mammary tumorigenesis is proportional to the level of virus infection [29, 36]. As a final readout of MMTV infection, the adhesion molecule-deficient mice were examined for tumor incidence. The mean time to 50% tumor incidence was similar for all the mice (wild type, 280 days; β7-null, 250 days; L-selectin-null, 260 days) and 100% of the mice developed mammary tumors. That both null mice developed tumors with similar kinetics and incidence as wild-type mice is further indication that their mammary tissue was infected to the same level.

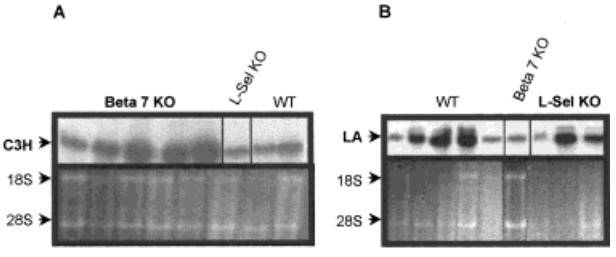

Fig. 4. Mammary gland infection in β7 and L-selectin-null mice. RNA was isolated from the milk of MMTV$^+$ β7 KO, L-selectin-null (L-sel KO), and wild-type (WT) mice at the second pregnancy and subjected to RNase protection assay using a probe specific for MMTV(C3H) (A), and MMTV(LA) (B). The upper panel shows the RNase protection and the lower panel is a formaldehyde gel of the same RNA to indicate the integrity of the RNA. The 18S and 28S ribosomal bands are indicated.

2.5 Mice deficient in both β7- and L-selectin-mediated homing show wild-type MMTV infection

Mice that lack both β7 integrin and L-selectin have a 99% reduction in both the size and cellularity of their PP [4]. To determine whether MMTV infection in the β7-or L-selectin-null mice was due to a compensatory effect of the remaining adhesion molecule, we treated β7-null mice foster-nursed on MMTV(LA)$^+$ mothers with an antibody that blocks L-selectin (MEL-14) from birth to weaning and then examined them for MMTV infection. The lack of both β7 and L-selectin had no effect on Sag-mediated deletion of peripheral T cells (Fig. 5A), since the percentage of Vβ6$^+$ T cells was reduced to approximately 1% in both the MEL-14- and PBS-treated β7-null mice. Moreover, virus spread was not affected, since the level of newly acquired exogenous provirus was the same in the spleens of MEL-14- and PBS-treated β7-null mice (Fig. 5B). Similar levels of newly acquired exogenous provirus were also seen in the mammary gland tissue of both PBS- and MEL-14-injected β7-null mice (data not shown). Thus, neither β7 integrin- nor L-selectin-bearing lymphocytes are required for milk-borne MMTV infection.

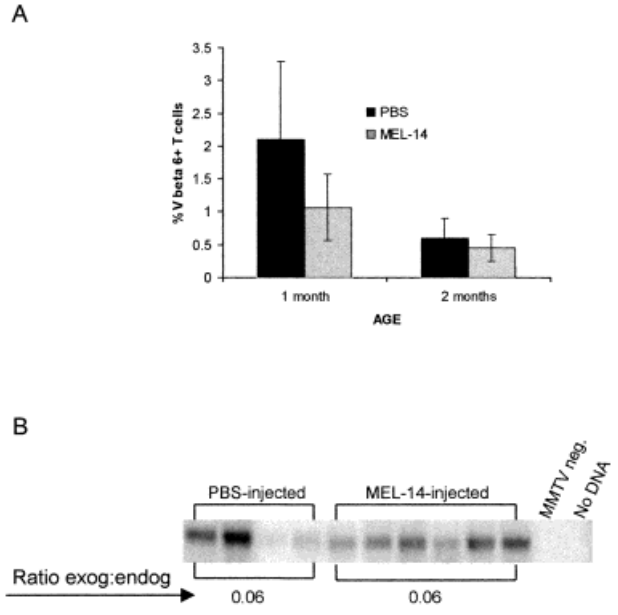

Fig. 5. MMTV infection in β7-null mice treated with anti-L-selectin. β7-null mice were foster-nursed on MMTV(LA)$^+$ mothers and treated from birth to day 30 with mAb MEL-14 or PBS. MMTV infection was determined by Sag-mediated cognate T cell deletion (A), and peripheral lymphoid infection (B). (A) Mice were bled at the indicated times and peripheral blood lymphocytes were stained for Vβ6 and CD4. (B) Splenic DNA (1 μg) was subjected to semiquantitative PCR as in Fig. 2. The PCR products were blotted to nitrocellulose, and reacted with a ^{32}P-labeled LTR-specific probe. This panel represents the MMTV(LA) exogenous products for four individual PBS-injected β7-null mice (lanes 1-4), six individual MEL-14-injected β7-null mice (lanes 5-10), and one MMTV-negative β7-null mouse (lane 11).

2.6 Naive lymphocytes are increased in neonatal PP following MMTV infection

That loss of either β7 integrin or L-selectin had no affect on infection was surprising since together these molecules account for most lymphocyte migration into PP, the tissue that serves as the entry point for MMTV. Therefore, we examined the lymphocyte population of neonatal PP in response to MMTV infection. Four-day old pups from MMTV-negative mothers were either foster-nursed on MMTV(LA)-infected mothers for 6 days, or left with their MMTV-negative mothers. At 10 days, the mice were killed, lymphocytes were isolated from their PP and examined by FACS for adhesion and activation molecule expression. Because the PP of β7-null mice were greatly diminished in size and cellularity, even following MMTV exposure, similar analyses could not be performed on these mice. Therefore, hematoxylin/eosin-stained sections of the small intestines of foster-nursed β7-null mice were examined.

The PP of infected wild-type neonates were three to four times larger than controls and both the B220$^+$ and CD4$^+$ lymphocyte populations were increased (Table 4). The PP of neonatal β7-null mice nursed on MMTV$^+$ mothers were also increased in size relative to uninfected controls, although they could only be detected microscopically and there were many fewer cells than in wild-type infected neonates (not shown). There were also significant increases in the percentage of naive lymphocytes (L-selectin^{high}-β7^{low}, L-selectin^{high}-LFA$^+$, and L-selectin^{high}-CD44^{low}) in both the CD4 and B220 subsets (L-selectin^{high}-CD4$^+$, β7^{low}-CD4$^+$, L-selectin^{high}-B220$^+$ and β7^{low}-B220$^+$), even in the presence of Sag-mediated activation. These results suggest that the increase in lymphocyte percentages could be due to homing of naive CD4$^+$ and B220$^+$ cells to PP. Indeed, the migration of CFSE-labeled neonatal splenic lymphocytes to the PP was found to be significantly higher in MMTV-infected pups (data not shown). It is also possible that MMTV infection results in the alteration of cell surface molecule expression or the proliferation of cells *in situ* in the PP.

Table 4. Lymphocyte populations in neonatal PP

Marker	MMTV^{-} [a]	MMTV(LA)$^{+}$ [a]	*p*	n [b]
% B220	54.1 ± 5.2 [c]	62.6 ± 5.9	<0.05	5
% CD4	20.0 ± 0.2	36.0 ± 3.7	<0.01	4
% L-selectin^{high}-B220^{+}	4.7 ± 0.5	10.9 ± 1.0	<0.01	6
% L-selectin^{high}-CD4^{+}	22.3 ± 3.0	26.8 ± 2.8	<0.05	6
% $\beta7^{low}$-B220^{+}	34.1 ± 3.8	41.3 ± 4.5	<0.05	6
% $\beta7^{low}$-CD4^{+}	64.2 ± 7.0	70.8 ± 6.0	<0.05	6
% $\beta1^{low}$-B220^{+}	87.0 ± 4.5	94.5 ± 3.5	<0.05	5
% $\beta1^{low}$-CD4^{+}	72.9 ± 3.2	80.1 ± 4.1	<0.05	5
% L-selectin^{high}-$\beta7^{low}$	12.2 ± 0.8	19.0 ± 2.0	<0.01	3
% L-selectin^{high}-LFA^{+}	21.0 ± 1.8	27.1 ± 2.5	<0.05	3
% L-selectin^{high}-$\beta1^{low}$	20.7 ± 2.2	35.1 ± 3.4	<0.01	3
% L-selectin^{high}-CD44^{low}	22.1 ± 2.0	36.7 ± 2.3	<0.01	3
V$\beta6^{+}$/CD4^{+}	10.3 ± 1.2	19.7 ± 1.8	<0.01	4
V$\beta10^{+}$/CD4^{+}	8.5 ± 0.8	6.9 ± 0.9	NS	3

a) MMTV-negative pups were foster-nursed on MMTV(LA)$^{+}$ mothers [MMTV(LA)$^{+}$] from days 6 to 10 or left with their uninfected mothers (MMTV^{-}). PP lymphocytes were isolated at day 10 and stained for the indicated markers.
b) Number of mice analyzed.
c) Data are presented as mean ± SD.

3 Discussion

Lymphocyte recruitment into lymphoid organs and mucosal tissue occurs in part through the interaction of homing receptors with corresponding tissue-specific addressins. One well-characterized interaction is the binding of the $\alpha4\beta7$ integrin to MAdCAM-1, which has been shown in several experimental systems to be critical for lymphocytes homing to PP. Because $\beta7$ integrin is required for trafficking to this organ when paired with $\alpha4$ integrin and to the intraepithelial spaces of the small intestine when paired with αE integrin, we determined whether $\beta7^{+}$ lymphocytes were required for infection by a neonatally, gut-acquired milk-borne virus, MMTV. Surprisingly, we found that mice lacking $\beta7$ integrin were infected with MMTV, by all criteria to the same extent as wild-type mice.

MMTV represents the prototypical retrovirus acquired through gut mucosa from milk. There must be both B cells for the initial infection and responding T cells for the superantigen-dependent amplification present in the PP for MMTV infection. Although mice lacking $\beta7$ had dramatically reduced PP cellularity, even as neonates, and few naive lymphocytes enter this tissue in the absence of this molecule [37, 38], wild-type infection levels were established. This may have occurred for several reasons. First, although the severe reduction in size prevented more than gross examination of the PP in $\beta7$-null mice, there were lymphocytes that could serve as targets for viral infection. Additionally, we found that PP from neonatal wild-type mice have a defined $\beta7^{-}$-CD44^{low} population that is almost absent from adult PP (not shown), raising the possibility that homing to neonatal PP is less dependent on the $\beta7$ integrin molecule than it is in adults. Finally, several studies showed that $\beta7$ and L-selectin act in concert in the migration of lymphocytes to the PP [2, 4, 37]. Lymphocytes isolated from the PP during the first days of MMTV infection expressed low levels of $\beta7$ and $\beta1$ integrins and high levels of L-selectin. Thus, wild-type infection in $\beta7$-null mice may have occurred through lymphocytes that migrated into this tissue via expression of L-selectin. However, we also found that $\beta7$-null mice that lacked L-selectin via mAb blockade showed wild-type MMTV-infection. It is possible that lymphocytes migrated into the PP through the expression of some other adhesion molecule. For example, P-selectin has been shown to mediate migration of lymphocytes into the PP in the absence of $\beta7$ and L-selectin, although it does not appear to recruit naive lymphocytes [37].

Virus spread may have occurred in the null mice predominantly at sites other than PP, since MMTV can establish infection when introduced peripherally as well as through milk, albeit less efficiently. It is possible that the few B cells remaining in the PP of the null mice were infected and migrated to other sites such as peripheral LN. However, virus spread in L-selectin-null mice was not impaired in spite of the paucity of lymphocytes in peripheral LN, indicating that migration to these extra-mucosal sites via this molecule is not required. Moreover, the kinetics of superantigen-mediated deletion and virus infection, both of which are proportional to the extent of initial virus infection [28, 29] was not affected by the loss of β7 integrin molecule or L-selectin.

Surprisingly, although L-selectin-deficient mice have impaired lymphocyte migration to LN during some inflammatory responses [32, 33], its absence did not affect the response of T or B cells to Sag (Table 4), although the increase in specific Vβ-bearing T cells in response to subcutaneous injection of MMTV is primarily due to selective migration rather than proliferation [25]. This raises the possibility that Sag-induced homing of lymphocytes to peripheral LN does not require L-selectin. It has recently been suggested that L-selectin-negative T cells participate in inflammatory responses [39].

After it infects lymphocytes, MMTV traffics from gut to the mammary gland (which like gut is an MALT) via infected lymphocytes [9]. Infection of mammary tissue first occurs during puberty when the epithelial cells divide under the influence of hormones such as estrogen. Pregnancy greatly increases virus load in this tissue, most likely because of lactogenic hormone-stimulated cell division. Thus, our finding that not only does the lymphoid compartment of β7 and L-selectin-null mice become infected, but the mammary tissue as well, indicates that these adhesion molecules are not required for homing to the mammary gland during puberty or pregnancy. Indeed, there were increases in T cell numbers and percentages in the mammary gland of the β7-null mice (Fig. 1 and Table 1). Because these mice have almost no lymphocytes in their GALT, lymphocyte percentages in other tissues may show compensatory increases, similar to that observed in the periphery and spleens of L-selectin-null mice [30, 31]. Although the loss of β7-bearing lymphocytes did not cause the loss of B or T cells in mammary tissue, there may be other molecules that play compensatory roles in homing in the null mice. Additional experiments using different null mice or antibody blocking can address this issue.

Although little is known about what governs homing of lymphocytes to mammary tissue, both α4β7 and αEβ7 have been strongly implicated in this process, especially for T cells. MAdCAM-1 has been found in the epithelial cells of lactating mammary gland [7, 40] and its expression has been correlated spatially with the presence of $\alpha4\beta7^+$ T cells in this tissue [22]. Similarly, E-cadherin, the addressin for αEβ7, is expressed on the basolateral surfaces of many epithelial cells, including mammary tissue [41, 42]. A recent report showed that 60% of the $\beta7^+$ T cells in the intraepithelial spaces of the pregnant mouse mammary gland were $\alpha E\beta7^+$, while $\alpha4\beta7^+$ T lymphocytes were present in the subepithelial layer [22]. Efficient transfer of MMTV from lymphocytes to dividing mammary epithelial cells would be predicted to occur from cells that migrate into the epithelial layer, such as the αEβ7-bearing T cells. One interesting observation made here was the high expression of αE integrin on β7 lymphocytes, indicating that this integrin may exist on the surface of cells either as a homodimer or paired with a different β chain. Thus, it is possible that αE-expressing lymphocytes can be retained in the mammary gland in the absence of the β7 chain and thereby deliver virus to the epithelial cells.

What other molecules might be important for the mammary gland homing of lymphocytes? GlyCAM-1 is expressed here but lacks the sulfate-modified carbohydrate required for L-selectin interactions [8]. The other L-selectin ligand, PNad is not found in the mammary gland, and L-selectin expression was not detected on mammary lymphocytes using immunohistochemistry [22]. We also found that only a small percentage of lymphocytes isolated from mammary tissue expressed this adhesion molecule, in comparison to the large percentage that expressed the α4, αE, β7 or β1 integrins (Table 2). Recently, Finke and colleagues [43] found that MMTV-infected lymphocytes in adults that received subcutaneous injection of virus expressed α4β1 and that blocking this integrin affected migration of activated lymphocytes to many tissues including virgin mammary gland. However, others have found that VCAM-1, the ligand for this pair, is not found in lactating mammary tissue when virus is acquired [22] and it is not clear that MMTV infection in peripheral LN recapitulates what happens in GALT. The role of α4 or β1 cannot be tested using gene targeting, since deletion of either results in early embryonic lethality [44]. An alternate approach we are using is the administration of blocking mAb to neonates, which also allows multiple adhesion molecules to be blocked simultaneously.

Other factors, such as cytokines, chemoattractants, and hormones, may work in concert with adhesion molecules to determine the trafficking of lymphocytes. Recently, a novel mucosal epithelial chemokine (MEC) that is expressed in several mucosal epithelial tissues, includ-

ing mammary gland, has been identified [45, 46]. Experiments are underway to determine whether lymphocytes bearing CCR3 and CCR10, the receptors for MEC, are more highly represented in lactating mammary tissue.

A number of other retroviruses, including feline immunodeficiency virus (FIV) [47], HIV [48–50] and HTLV-1 [51, 52] are found in milk. Moreover, the major route of maternal transmission of both HIV and HTLV-1 to infants is thought to be through nursing [48–52]. Our studies of a mouse retrovirus that utilizes the same route of infection as these pathogenic human viruses provides a framework with which to understand both neonatal homing to the gut and subsequent spread of virus to lactating mammary gland.

4 Materials and methods

4.1 Mice

Mice were housed in the animal facility of the University of Pennsylvania (Philadelphia, PA) or at the Academia Nacional de Medicina (Buenos Aires, Argentina) in accordance with federal and institutional guidelines. C3H/HeN mice were purchased from the National Institutes of Health (Frederick Cancer Research Facility, Frederick, MD), L-selectin-null mice from The Jackson Laboratories (Bar Harbor, ME) [53]. β7-null mice were previously described [2]. Because presentation of the MMTV Sag is more efficient in MHC class II I-E$^+$ mice [54, 55] the adhesion molecule-deficient mice (C57BL/6 background) were backcrossed to C3H/HeN mice for two to three generations or C3H/HeN×C57BL/6 F1 mice were intercrossed to generate animals heterozygous or homozygous for the H-2^k haplotype. The mice were typed for the expression of adhesion molecules and H-2 haplotype by surface staining of peripheral blood mononuclear cells.

4.2 Viruses

Mice were foster-nursed on MMTV(C3H)- or MMTV(LA)-infected C3H/HeN mothers. For footpad injections, MMTV(FM) [57] virus was diluted in sterile PBS and injected into the right hind footpad of 1–2-month-old mice. After 24 and 96 h the draining (right) and non-draining (left) popliteal LN were harvested, and the cells were analyzed by FACS.

4.3 Antibodies and flow cytometry

The following mAb were used: FITC-conjugated anti-Vβ6 (RR4-7), FITC-conjugated anti-Vβ10 (B21.5), FITC-conjugated anti-Vβ14 (14.2), PE-conjugated anti-CD4 (H129.19), FITC-conjugated anti-CD4 (GK1.5), PE-conjugated anti-CD8a (53–6.7), FITC- and PE-conjugated anti-CD45R/B220 (RA3 6B2), PE-conjugated anti-CD69 (H1.2F3), biotin-conjugated anti-αE (2E7), PE-conjugated anti-CD49d/integrin α4 chain (R1-2), FITC-conjugated anti-β7 (M293), FITC-conjugated anti-CD29/integrin β1 chain (Ha2/5), PE-conjugated anti-CD62L/L-selectin (MEL-14), FITC-conjugated anti-H-2K^k, biotin-conjugated anti-I-Ab, streptavidin-PE (PharMingen, San Diego, CA), FITC-conjugated anti-CD8a (CT-CD8a) (Caltag, Burlingame, CA) and Alexafluor$_{488}$-conjugated goat anti-rat IgG 1:200 (Molecular Probes, Eugene, OR). Cells were acquired on a FACScan cytometer (Becton Dickinson, Mountain view, CA) and analyzed using CellQuest software (Becton Dickinson Immunocytometry Systems).

4.4 Antibody blocking

Supernatants were purified according to standard procedures from the MEL-14-secreting hybridoma (ATCC, Manassas, VA). MMTV-negative β7-null mice were fostered on MMTV(LA)$^+$ mothers and injected intraperitoneally with MEL-14 mAb or PBS every other day for 30 days as follows: week 1, 25 μg MEL-14; week 2, 50 μg; week 3, 200 μg; week 4, 400 μg. One week of treatment with MEL-14 (*i.e.* three injections) was sufficient to completely block surface L-selectin as determined by FACS (data not shown). At day 30, the mice were weaned. The mice were bled at 1 and 2 months of age to determine Sag-mediated cognate T cell deletion. At the first pregnancy (approximately 2.5 months of age), female mice were killed and MMTV levels in milk, lactating mammary gland, and spleen were analyzed.

4.5 Lymphocyte isolation from lactating mammary gland

Mammary tissue (excluding inguinal and axillary LN) (day 1–7 of lactation) was excised, minced in Krebs/Ringer bicarbonate solution with 1 mg/ml collagenase III (Worthington Biochemical Corp., Lakewood, NJ), and incubated at 37°C, 5% CO_2 for 30 min. The tissue was passed through a 10-ml syringe to disperse clumps, mixed with an equal volume of Krebs/Ringer Bicarbonate, and passed through a wire mesh. The cells were spun for 5 min at 1,500 rpm and the washed pellet was resuspended in HBSS and layered over Ficoll-Paque (Amersham Pharmacia Biotech, Piscataway, NJ). Lymphocytes were recovered from the Ficoll/HBSS interface and used for FACS analysis.

4.6 Immunohistochemistry

Sections, 5 μm thick, from lactating mammary glands of MMTV(LA)$^+$ wild-type, β7- and L-selectin-null mice (two mice of each genotype) were stained with hamster anti-mouse CD3, biotinylated goat anti-hamster IgG and streptavidin-conjugated Texas Red (T cells) or rat anti-mouse B220 and Alexafluor$_{488}$-conjugated goat anti-rat IgG (B cells). The stained sections were coded and then viewed

under a Nikon fluorescence microscope. The lymphocytes were counted under 20× magnification by a code-blinded observer. An average of 221 fields per section and 20 sections per mouse were counted.

4.7 RNase T_1 protection assay

RNase T_1 protection assays were performed as previously described using radiolabeled probes specific for MMTV-C3H or MMTV-LA [58]. Five micrograms of milk RNA or 40 μg of lactating mammary gland RNA were used.

4.8 Semiquantitative DNA PCR

Total genomic DNA isolated from the peripheral LN (axillary and inguinal), PP and spleens of wild-type and adhesion molecule-null mice was amplified by PCR with virus-specific primers, as previously described [9]. Where indicated, PCR products were Southern-blotted with a radiolabeled LTR-specific probe.

4.9 Statistics

Student's *t*-test was performed to determine statistical significance in the percentages of lymphocytes and lymphocyte subsets. A *p* value of less than 0.05 was considered to be significant.

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