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A developmental switch in lymphocyte homing receptor and endothelial vascular addressin expression regulates lymphocyte homing and permits CD4+CD3− cells to colonize lymph nodes

(ontogeny/MAdCAM-1/lymphocyte migration)

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ABSTRACT **In adult mice, the dominant adhesion molecules involved in homing to lymph nodes are L-selectin homing receptors on lymphocytes and the peripheral lymph node addressins on specialized high endothelial venules. Here we show that, from fetal life through the first 24 hr of life, the dominant adhesion molecules are the mucosal addressin MAdCAM-1 on lymph node high endothelial venules and its counterreceptor, the Peyer's patch homing receptor, integrin $\alpha4\beta7$ on circulating cells. Before birth, 40–70% of peripheral blood leukocytes are L-selectin-positive, while only 1–2% expresses $\alpha4\beta7$. However, the fetal lymph nodes preferentially attract $\alpha4\beta7$-expressing cells, and this can be blocked by fetal administration of anti-MAdCAM-1 antibodies. During fetal and early neonatal life, when only MAdCAM-1 is expressed on high endothelial venules, an unusual subset of CD4+CD3− cells, exclusively expressing $\alpha4\beta7$ as homing receptors, enters the lymph nodes. Beginning 24 hr after birth a developmental switch occurs, and the peripheral node addressins are upregulated on high endothelial venules in peripheral and mesenteric lymph nodes. This switch in addressin expression facilitates tissue-selective lymphocyte migration and mediates a sequential entry of different cell populations into the lymph nodes.**

Lymphocyte homing to lymph nodes (LNs) in adult mice involves defined sets of primary adhesion molecules expressed on both lymphocytes and endothelial cells. T and B lymphocytes in the bloodstream enter lymph nodes by adhering to and migrating through specialized postcapillary venules, the high endothelial venules (HEVs; refs. 1–3). The primary adhesion molecules are homing receptors on lymphocytes and vascular addressins on cognate endothelial cells (for review see ref. 4). The primary adhesion molecules are involved in initial "rolling" contacts, to be followed by secondary adhesive events required for arrest and subsequent transendothelial migration of these cells (5–7). To enter an adult peripheral LN (PLN), lymphocytes express high levels of the homing receptor L-selectin (8–11). L-selectin can permit lymphocyte or leukocyte adherence to the PLN addressins (PNAds) expressed on HEVs (12, 13). Several LN HEV ligands for L-selectin have been identified, all of which are sulfated glycoproteins (14–16). A monoclonal antibody (mAb), MECA-79, recognizes the PNAds on HEV in adult PLNs and mesenteric LNs (MLNs) and specifically blocks lymphocyte binding to HEV in PLNs and MLNs (12). It recognizes sulfated carbohydrates present on multiple PNAd species (13, 16).

The Peyer's patch (PP) lymphocyte homing receptor, integrin $\alpha4\beta7$ (17–20), mediates lymphocyte binding to HEV in PPs and also permits rolling and adhesion of lymphocytes in lamina propria venules (21). The endothelial cell counterreceptor for this molecule has been identified as the mucosal addressin, MAdCAM-1, an immunoglobulin superfamily member (22, 23). Monoclonal antibody MECA-367 recognizes MAdCAM-1 and blocks the interaction of $\alpha4\beta7$ with MAdCAM-1 and therefore binding of lymphocytes to HEV in PPs (24). In addition, MAdCAM-1 is glycosylated and, when expressed by HEV, can display carbohydrates recognized by MECA-79. MAdCAM-1 contains a single mucin domain, and this glycosylated mucin can mediate the binding of L-selectin to MAdCAM-1 *in vitro* and *in vivo* (25, 26). Although L-selectin facilitates initial interaction of circulating lymphocytes with PP HEV, $\alpha4\beta7$ is required for and, in some instances, is sufficient for PP HEV interaction *in situ* (26). In contrast, L-selectin is required for lymphocyte homing to adult PLN, where $\alpha4$ integrins normally appear dispensable (11, 19, 27). Thus, $\alpha4\beta7$ critically controls lymphocyte homing to PP and lamina propria, whereas L-selectin dominates and controls the specificity of PLN homing. The MLN is a composite mucosal and peripheral lymphoid organ. The homing of lymphocytes to MLNs involves either of the homing receptors, L-selectin and $\alpha4\beta7$, on lymphocytes and their counterreceptors on high endothelial cells.

This homing pattern of lymphocytes to lymphoid organs is observed in adult animals. When we focused on the adhesion molecules involved in lymphocyte homing to LNs of fetal and newborn mice, we observed that homing events are quite different when compared with lymphocyte homing to adult LNs. Here we demonstrate that the first lymphocyte homing to LNs in C57BL mice involves MAdCAM-1 and $\alpha4\beta7$, but not L-selectin. This changes rapidly after birth. Antibodies that block MAdCAM-1 or $\alpha4\beta7$-mediated homing *in vivo* block the colonization of LNs by these cells.

We therefore describe a developmental switch in the expression of functionally significant primary adhesion molecules on lymphocytes and their HEV counterparts. The HEV switch appears to occur independently of circulating lymphocytes. The consequence of these endothelial changes in the expressed genetic program has a quantitative and functional impact on lymphocyte distribution that may well influence immune function.

EXPERIMENTAL PROCEDURES

Animals. C57BL/KA-Thy1.1 mice were maintained in the animal colony at Stanford University and kept under routine laboratory conditions. Timed matings were used for *in utero* injections of monoclonal antibodies.

Antibodies. The following antibodies were used for staining: anti L-selectin, Mel-14 (8); anti-αe, M290 (28, 29); anti-$\beta7$, M298 (30); anti-$\alpha4$, R1-2 (17); anti-$\alpha4\beta7$ heterodimer, DATK-32 (31); anti-PNAd, MECA-79 (12); anti-mucosal ad-

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Abbreviations: HEV, high endothelial venule; LN, lymph node; PLN, peripheral LN; MLN, mesenteric LN; PNAd, PLN addressin; PP, Peyer's patches.

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dressin, MECA-367 (24); anti-CD4, RM4-5 (PharMingen); anti-CD4, GK1.5 (32); anti-CD8, 53–6.7 (33); anti-CD3-ε, 145–2C11 (PharMingen); anti-γδ T-cell receptor, 3A10 (34); and anti-Vγ2 T cell receptor, UC3-10A6 (PharMingen).

***In Vitro* HEV-Binding Assay.** Lymphoma adherence to HEVs *in vitro* was assayed by the method of Butcher *et al.* (35), a modification of the assay originally described by Stamper and Woodruff (36).

Immunohistochemistry. Cryostat sections of LNs (5-μm thickness) were stained with various monoclonal antibodies as described (12).

Injections of Monoclonal Antibodies. Monoclonal antibodies [MECA-367; MEL-14; anti-CD5, 53–7.3 (33); anti-α4, PS/2 (37); and anti-β7, FIB504 (31)] were injected i.p. daily at a dose of 250 μg into pregnant mice starting between day 10 and 15 of pregnancy up until the day of birth. The presence of the injected antibodies in the newborn animals was checked by staining of either cell suspensions or sections with the second step reagents and analysis by either flow cytometry or microscope, respectively. Level of expression was compared with stainings with first and second step reagents. Experiments with injection of MECA-367, FIB30, FIB504, PS-2, and Mel-14 during pregnancy and analysis of newborn mice at day 1 were repeated three, two, two, two, and two times, respectively. Experiments with injection of MECA-367 during pregnancy and 5 days after birth were repeated three times. Results shown are representatives of the duplicate or triplicate experiment.

RESULTS

A Developmental Switch Occurs in Expression of Addressins in Mouse LN HEV During the Neonatal Period. Differential expression of addressins at various sites, such as LNs and PPs, directs the selective homing of lymphocyte subsets to these sites. LNs are formed before birth and contain lymphocytes on the day of birth. To see if the adult expression profile of addressins also regulates the early colonization of LNs, we studied addressin expression on HEV in MLN and PLN early in their development. To our surprise, neonatal LNs, taken within 24 hr after birth, contain HEV that are predominantly PNAd-negative, as defined by MECA-79 (Fig. 1*a*). However, HEV in these organs express high levels of the mucosal addressin MAdCAM-1, as shown by MECA-367 staining (Fig. 1*b*). This pattern can be observed in both PLN and MLN (data not shown).

Analysis of LNs from postnatal mice revealed that the MECA-79 epitope begins to be expressed on HEV no earlier than 24 hr after birth and is highly expressed at 2–4 days after birth (Fig. 1*c* at 4 days). Until 2 weeks after birth, both the MECA-79 epitope and MAdCAM-1 can be found on HEV in both PLN and MLN. At ≈2 weeks of age, a small subset of HEV in PLN start to exhibit a reduction in MAdCAM-1 expression, while HEV in MLN express MAdCAM-1 at high levels. During the 3rd and 4th week of postnatal life, the MAdCAM-1 expression level on PLN HEV continues to decline until it is almost completely absent at the level of sensitivity of immunohistology (Fig. 1 *h* and *j*). Occasionally, rare high endothelial cells expressing MAdCAM-1 can be observed in adult PLN.

To determine if the mucosal addressin, expressed exclusively on HEV until 24 hr after birth, is functional, we performed *in vitro* binding studies. For this assay, a mixture of two lymphoma cell lines, TK-1 and 38C13, was used. TK-1 lacks expression of L-selectin, but expresses α4β7 and can therefore bind selectively to the mucosal addressin. Lymphoma 38C13 expresses L-selectin but not α4β7 and therefore binds selectively to the PNAds. Lymph node sections from mice taken at different ages were overlayered with a mixture of TK-1 and 38C13 cells. The input ratio of TK-1 and 38C13 cells was selected in preliminary assays on adult MLN to yield a ≈1:1 ratio among cells binding HEV. Fig. 2 shows that, at 1 day after birth, HEV in PLN selectively bind the α4β7+ lymphoma (TK-1). This binding profile reverses over a period of 2–3 weeks after birth. This confirms that MAdCAM-1, expressed solely on HEV until 1 day after birth, mediates specific binding of the lymphoma TK-1 to neonatal LN HEV, and that this changes once the PNAds start to be expressed.

To determine if the presence of recirculating lymphocytes is important for the induction of the PNAds on HEV, we analyzed LNs of SCID and Rag2$^{-/-}$ mice. In those LNs, we observed the same pattern of addressin expression: at birth MAdCAM-1 is primarily expressed on HEV, while only a few cells express the MECA-79 epitope. Twenty-four hours after birth, we see higher levels of the MECA-79 epitope, while MAdCAM-1 is still being expressed at high levels. At 4–5 weeks after birth, we see a predominant expression of the PNAd on HEV in PLNs (data not shown).

The First Cells That Enter the Lymph Nodes Are α4β7-Positive. The change in addressin expression on HEVs prompted us to determine which homing receptors were expressed on cells in the bloodstream. Analysis of peripheral blood leukocytes of newborn mice showed that ≈80% of the cells in the bloodstream express L-selectin, while only 2–4% are α4β7-positive (Fig. 3*A*). Since α4β7 is the cognate homing receptor for MAdCAM-1, the addressin expressed exclusively at that time on HEV, it was important to determine which homing receptors were expressed on the first cells that appear in the LNs.

We analyzed by FACS the lymphocyte subpopulations in the LNs that express α4β7. At first we evaluated CD3+ cells. On the day of birth, the majority of CD3+ cells are γδ T cells, which are CD4− and CD8−. Analysis of γδ T cells in these LNs shows that ≈98% of the cells express α4β7, as recognized by mAb Datk-32 (Fig. 3*B*). The remaining CD3+ cells are CD4+ or CD8+. On day of birth, the absolute numbers of CD3+CD4+ and CD3+CD8+ cells are very low, but they increase rapidly after birth. The first CD3+CD8+ and CD3+CD4+ that come into the LN are all α4β7-positive. At 4 days after birth, gating on CD3+CD8+ and CD3+CD4+ cells shows that most of the CD3+CD8+ and CD3+CD4+ cells that reside in the LNs express α4β7 (Fig. 3*C*).

When stained for B220 antigen with mAb 6B2, few B cells could be found (Fig. 3*B*). Approximately 60% of the B220+ cells present in the LNs were positive for α4β7, when gated for B220+ cells (Fig. 3*B*). A few Mac-1-positive cells could be found in the LNs, most of them expressing α4β7 (Fig. 3*B*).

In the process of determining the different α4β7 positive subsets that home to LNs before birth, we found an unusual population of cells, which is present in the LNs at birth at relatively high numbers. A subset of CD4+ cells that do not express CD3 constitutes ≈10-15% of the cells in the LNs at day of birth. The existence of this subset has been described (38). At birth, the majority of the CD4+ cells are CD3−, but within 1–2 days, massive numbers of CD4+CD3+ cells enter the LN, which makes the CD4+CD3− population less obvious. About 90% of the CD4+CD3− cells in LN at birth express α4β7 (Fig. 3*B*).

When γδ T-cell receptor, CD3+CD8+, and CD3+CD4+ cells, present in LNs, were analyzed over time for expression of α4, β7, and L-selectin, we observed that expression of L-selectin increases over time, while high levels of α4 expression declines (Fig. 4).

However, when we analyzed the CD4+CD3− cells in young and adult mice, we could only see a few cells expressing L-selectin, while >50% of CD4+CD3− cells maintained high levels of α4 and medium levels of β7 expression (Fig. 5). The expression of α4β7 is therefore likely to be necessary for these cells to enter LNs during late fetal and early neonatal life. Their numbers remain relatively constant in the face of massive B and CD3+ T cell immigration, and so their percent representation in LNs drops dramatically in the postnatal period.

The First Wave of Lymphocytes Homing to Lymph Nodes Can Be Blocked with mAbs Against MAdCAM-1, α4, and β7.

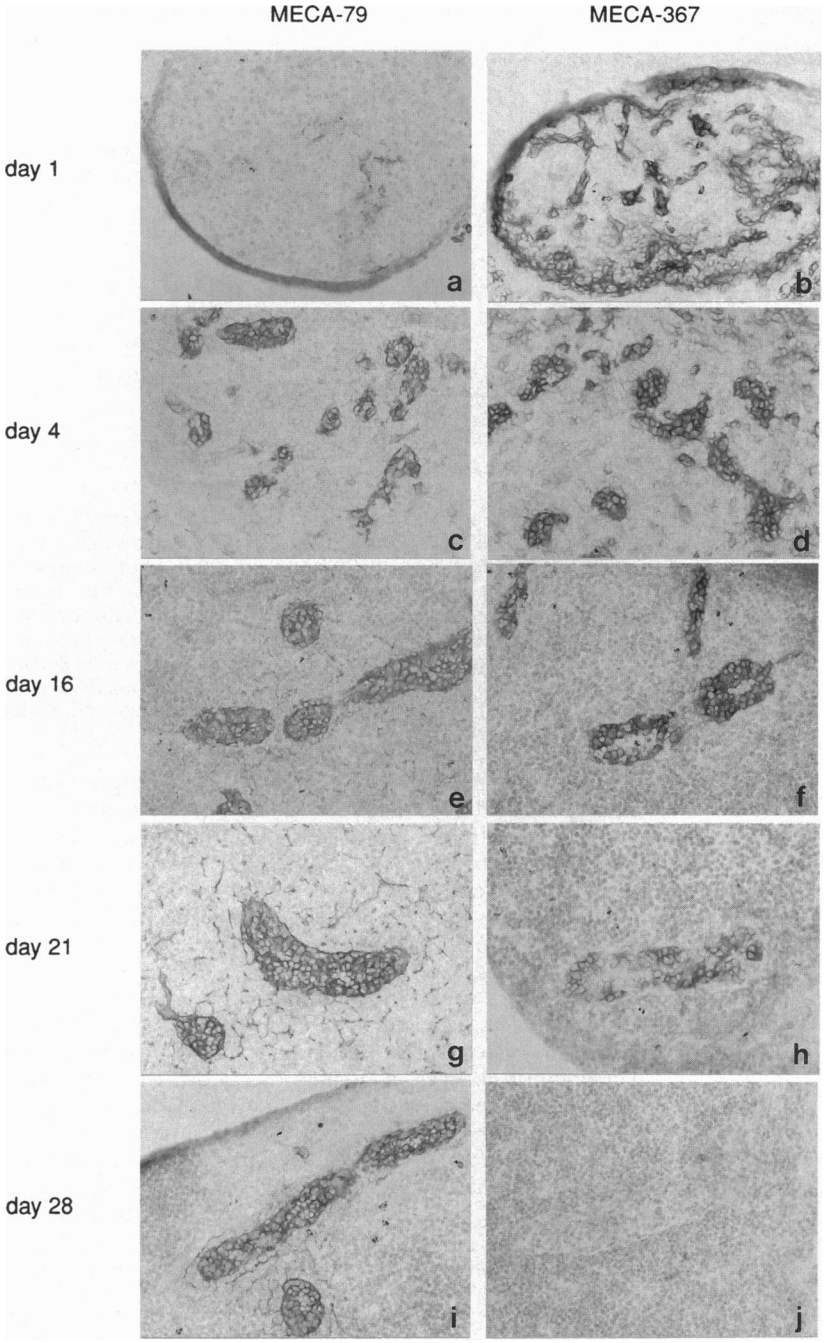

FIG. 1. PLNs, taken at 1, 4, 16, 21, and 28 days after birth, stained with MECA-79 recognizing the PNAds (*a*, *c*, *e*, *g*, and *i*) and MECA-367 recognizing the mucosal addressin (*b*, *d*, *f*, *h*, and *j*). At 1 day after birth, predominant expression of only the mucosal addressin can be observed, while at 4 and 16 days after birth, PNAds and mucosal addressins can be observed on HEV in PLN. At 16 days after birth, however, HEVs can be found that only partially express the mucosal addressin. During the third and fourth week the mucosal addressin disappears from HEVs in PLN.

If $\alpha4\beta7$ (but not L-selectin) homing receptors expressed on lymphocytes and MAdCAM-1 on endothelial cells is indeed involved in the first lymphocyte homing to LNs, then mAbs to these coreceptors should inhibit the early entry of T cells and CD4+CD3− cells into LN. To test this hypothesis, blocking mAbs were administered to pregnant mothers beginning on gestational day 10, as described in *Experimental Procedures*, and the total number and/or the relative representation of the different subsets of lymphocytes in the LNs were analyzed after birth. Injection of the anti-mucosal addressin mAb MECA-367 on days 10, 13, 15, and 17 of embryonic life almost completely blocked the recruitment of CD3+ cells ($\gamma\delta$ T cells) into LNs, while partially inhibiting the accumulation of CD4+CD3− cells (Fig. 6). When mice from the same mother were analyzed on the second day after birth, there were still fewer CD3+ cells in LNs of the MECA-367-treated animals compared with control animals (Fig. 6). The MAdCAM-1 exclusive entry mechanism is lost by day 5 (see below).

Injection of mAbs that bind to $\alpha4$ or $\beta7$ also inhibited population of LN by $\gamma\delta$ T cells (Table 1). The representation of CD4+ cells, which are mostly CD3−, was reduced to a lesser extent than the CD3+, which are mostly $\gamma\delta$ T cells. When the total numbers of CD4+ cells that had entered the LNs were analyzed, there was a 75% inhibition of CD4+ cells using a mAb against $\alpha4$, while a mAb against $\beta7$ only reduced the recruitment of CD4+ cells by 25% (Table 1). The number of CD8+ cells in LNs was also reduced, suggesting that $\alpha4$, $\beta7$, and MAdCAM-1 are the principal adhesion molecules involved in the first wave of lymphocytes homing to the LNs in these mice.

We observed that, at birth, LN HEVs predominantly express MAdCAM-1, with very little or no PNAd. An increase in expression of the PNAds is observed within 24–48 hr after birth. To test if there is any involvement of L-selectin and its ligand in the colonization of LNs, the anti-L-selectin mAb Mel-14 was injected into pregnant mothers to provide the mAb during fetal life. Analysis of the LNs of newborn mice show

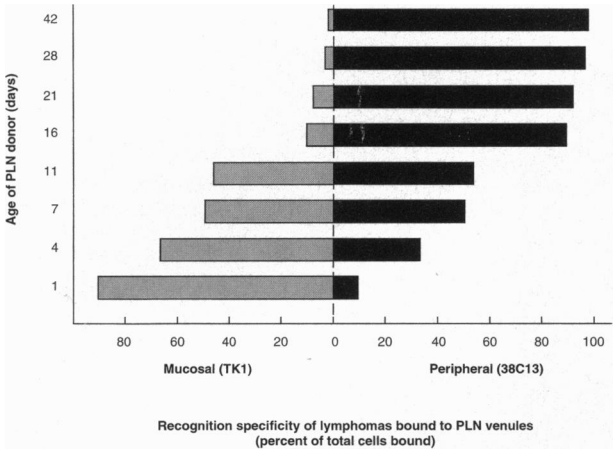

FIG. 2. *In vitro* binding of tissue-selective lymphomas to HEVs in PLNs taken at various time points after birth. A mixture of TK-1 and 38C13 lymphomas was used at a ratio of 6:1, which gave a binding ratio to HEVs in adult MLN of 1:1. TK-1 (mucosal lymphoma) preferentially bind to the mucosal addressin, while 38C13 (peripheral lymphoma) preferentially bind to the PNAds. At 1 day after birth, predominant binding of the mucosal lymphoma to HEV in PLNs can be observed, which shifts over time to predominant binding of the peripheral lymphoma at 3–4 weeks after birth. Results are shown as percent mucosal or peripheral lymphomas bound per total cells bound.

that the entrance of lymphocytes into LNs could be inhibited only slightly by treatment with anti-L-selectin mAb (Table 1). If anything, this treatment led to a relative increase in the unusual CD4+CD3− population. This supports the hypothesis that the first wave of lymphocyte homing to LNs does not involve L-selectin and the PNAds.

As noted above, a global inhibition of lymphocyte recruitment by anti-MAdCAM-1 mAb alone is unlikely, as the dramatic blocking effect of anti-MAdCAM-1 mAb was limited

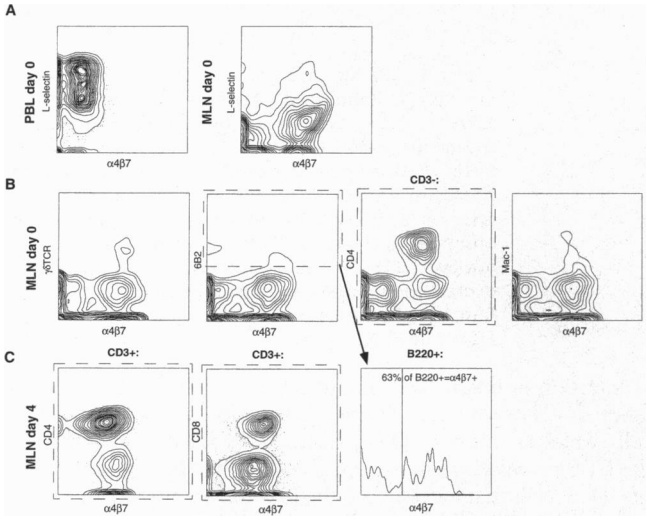

FIG. 3. (*A*) Expression of α4β7, as recognized by mAb DATK-32, and L-selectin, as recognized by Mel-14, on leukocytes in peripheral blood and MLNs of newborn mice (day of birth). (*B* and *C*) Expression of α4β7 on different leukocyte subsets in MLNs at day of birth (*B*) or 4 days after birth (*C*). Single cell suspensions were stained with mAbs Mel-14, DATK-32, 3A10, 6B2, GK1.5, 145-2C11, M1/70, and 53-6.7 recognizing L-selectin, α4β7, γδ T-cell receptor, B220, CD4, CD3, Mac-1, and CD8, respectively. In the case of CD4+CD3−, CD4+CD3+, and CD8+CD3+ cells, α4β7 expression was analyzed by gating on CD3− (for CD4+CD3−) and CD3+ (for CD4+CD3+ and CD8+CD3+) cells. The expression of α4β7 on B220+ cells was further analyzed by gating on B220+ cells, as implicated by the boxed area in the figure.

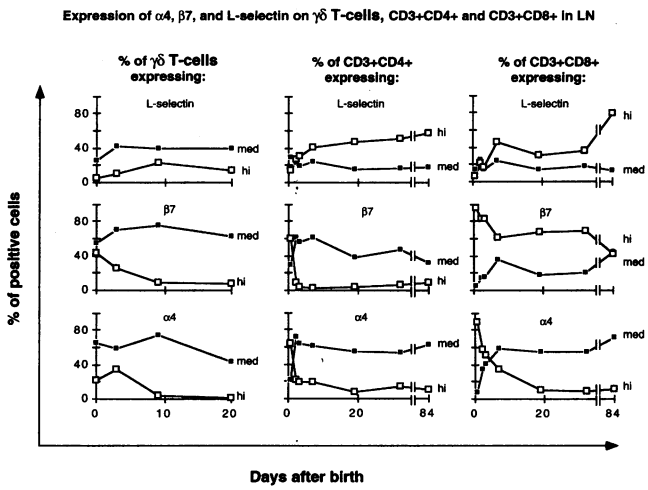

FIG. 4. Percentage γδ T cells in the MLNs of mice at 0, 3, 9, and 20 days after birth and percentage of CD3+CD4+ and CD3+CD8+ cells in MLN at 0, 2, 3, 7, 19, 28, and 84 days after birth expressing L-selectin, β7, or α4. The data were gated to include the obvious population. L-selectin medium (med) cells were gated between 2 and 17 units of intensity, as was β7 (med); α4 (med) cells were gated between 2 and 8 units. Single cell suspensions of MLNs were stained with mAb Mel-14, M298, and R1-2, recognizing L-selectin, β7, and α4, respectively, and analyzed by fluorescence-activated cell sorter (FACS; Becton Dickinson).

to the prenatal period of exclusive MAdCAM-1 expression; in animals injected with MECA-367 *in utero* and neonatally until 5 days after birth, analysis of cells present in LNs reveals that by day 5 after birth, CD4+, CD8+, CD3+, γδ T-cell receptor+, B220+, and Mac-1+ cells have entered the LNs in abundance, in spite of the presence of circulating anti-MAdCAM-1 mAb (data not shown). The total number of all cell populations, except Mac-1+ cells, is decreased.

In the above cases, we could not exclude other effects of mAb treatment, such as alterations in lymphocyte maturation, that might effect representation of lymphocyte subsets independently of changes in homing. To address homing more directly, we injected mature lymphocytes in the presence of blocking mAb. Embryos were derived by caesarean section on the day of expected birth. Congenic lymphocytes were injected i.v. in the presence of FIB30 (anti-β7 mAb), DATK-32 (anti-α4β7 mAb), or PS-2 (anti-α4 mAb). Three hours later, the

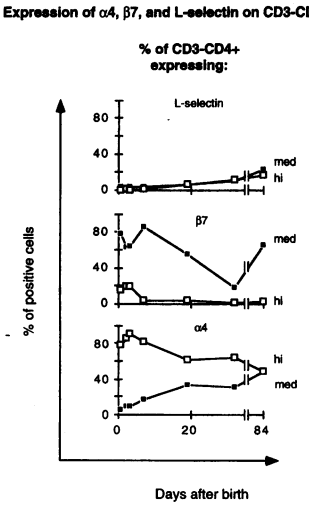

FIG. 5. Expression of L-selectin, α4, and β7, as recognized by Mel-14, R1-2, and M298, respectively, on CD3−CD4+ cells in MLN at 0, 2, 3, 7, 19, 28, and 84 days after birth. The same gates as in Fig. 4 were used.

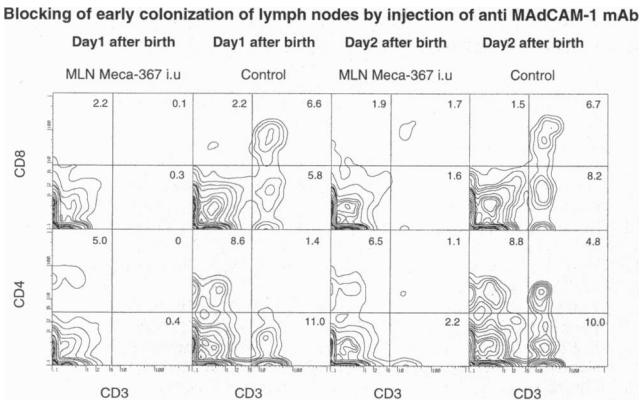

FIG. 6. Effect of injections of mAb MECA-367 *in utero* on populations of lymphocytes in LNs. Pregnant mothers were treated from day 10 of gestation until birth with blocking mAb MECA-367, recognizing MAdCAM-1. Lymph nodes were analyzed 24 hr and 48 hr after birth (day 1 and day 2, respectively). Numbers represent the percentage of cells of the total population that falls within the gates as marked in the figure.

MLN were analyzed for donor-derived cells using the Ly5.2 marker. Anti-$\alpha 4$ mAb blocked lymphocyte entry by 40%, while anti-$\beta 7$ and anti-$\alpha 4\beta 7$ blocked lymphocyte entry by 59–60% (data not shown).

DISCUSSION

Lymphocytes in LNs of mice at birth are mainly $\gamma\delta$ T cells and CD4+CD3− cells. These distinct populations are rapidly diluted within the first days of postnatal life by the entry of massive numbers of CD3+CD4+ and CD3+CD8+ T cells and B cells. The adhesive systems involved in the entry of the first wave of lymphocytes to LNs, which we shall call the fetal program, are distinct from adhesion mechanisms involved in lymphocyte homing to LNs of older mice, the postnatal program. MAdCAM-1 expressed on HEV and its ligand $\alpha 4\beta 7$ on lymphocytes are critically involved in the homing of lymphocytes to LNs before and within 24–48 hr after birth. After 24–48 hr, other adhesion molecules, like L-selectin on lymphocytes and the PNAd on HEV, start to be expressed while the involvement of MAdCAM-1 starts to decline in PLNs. The switch from the fetal program to the postnatal program is made rapidly after birth. Expression of $\alpha 4\beta 7$, but not L-selectin, on CD4+CD3− cells suggests that entrance of those cells into LNs requires the fetal HEV program. The switch from the fetal HEV program to the postnatal program does not seem to depend on the presence of circulating lymphocytes in the bloodstream, since the induction of the MECA-79 epitope and the reduction in MAdCAM-1 expression on HEV in PLN of lymphocytopenic SCID and Rag2$^{-/-}$ mice takes place in an identical pattern, as seen in control mice. Our data shows that expression of $\alpha 4\beta 7$ on CD4+CD3− and $\gamma\delta$ T cells and MAdCAM-1 on HEV in LNs provides a mechanism for selective recruitment of those cells into the LNs before birth and before L-selectin$^+$ $\alpha 4\beta 7^-$ cells can enter. We speculate that the cells entering through an $\alpha 4\beta 7$-MAdCAM-1 dependent mechanism could play a role in creating the right microenvironment for the correct topography of LN subregions made up by the entry of the second wave of CD4+CD3+, CD8+CD3+, and more mature B cells.

Table 1. Blocking of LN colonization by injection of mAbs recognizing homing receptors

		Inhibition, %			
mAb treatment	Antigen	CD3 cells	CD4 cells	CD8 cells	$\gamma\delta$ T cells
PS-2	$\alpha 4$	86	75	44	83
FIB504	$\beta 7$	66	26	73	71
Mel-14	L-selectin	6	−97	33	13

Lymphocytes in LNs of newborn mice treated *in utero* with mAbs binding to homing receptors. Pregnant mothers were treated from day 15 of gestation until birth with blocking mAb Mel-14, FIB504, or PS-2, recognizing L-selectin, $\beta 7$, or $\alpha 4$, respectively. The MLNs were analyzed within 24 hr after birth. The numbers represent the percentage of inhibition of total number of cells that enter the MLNs of treated animals compared to control mice. mAbs PS-2 and FIB504 were able to block lymphocyte homing to LNs *in vivo*. mAb MEL-14 causes an increase in CD4+CD3− cells (inhibition, −97%), while other populations are only slightly inhibited.

The window in which MAdCAM-1 and $\alpha 4\beta 7$ are the main adhesion molecules involved in lymphocyte homing to LNs is very short: injections of mAb MECA-367 block lymphocyte entrance into LNs dramatically until 2 days after birth, but allowed colonization when analyzed 5 days after birth. From the *in vitro* binding studies shown in Fig. 2 and the above described reduced entry of lymphocytes *in vivo* in the presence of MECA-367, it is clear that MAdCAM-1 is still functional as an adhesion molecule in LNs after day 2 and until day 11–16. The fact that blood lymphocytes can enter LNs in the presence of mAb that block MAdCAM-1 sometime between 2 and 5 days after birth implies that cells can use both $\alpha 4\beta 7$ and L-selectin to enter LNs but that they do not necessarily need both. Thus the differential expression of homing receptors may allow different lymphocyte cell populations to enter LNs. The fact that accumulation of B220+ cells is reduced by administration of MECA-367 suggests that the colonization of LNs by B220+ cells also depends, at least in part, on $\alpha 4\beta 7$ and MAdCAM-1. It is interesting that the relative and absolute number of L-selectin+ Mac-1+ cells in the LNs goes up in MECA-367-treated mice, suggesting that these relatively depleted LNs can signal their increased entry; in controls, although Mac-1+ cells are present in large numbers in the bloodstream, they are excluded from entry into the LNs.

Apart from MAdCAM-1, we also observed VCAM-1 expression on HEV using immunohistology. Although the observed expression level of VCAM-1 was lower than MAdCAM-1, VCAM-1 might also contribute to the entrance of lymphocytes into the LNs. The ligands for VCAM-1 on blood cells include $\alpha 4\beta 7$ and $\alpha 4\beta 1$ integrins (39, 40). Such a role for VCAM-1 would most likely be secondary to the role of MAdCAM-1, since administration of anti-MAdCAM-1 mAb before birth could almost completely block the entry of the first wave of lymphocytes into LNs. However, VCAM-1 could play a role in the entrance of CD4+CD3− cells, since we could never obtain a complete blockade of those cells accumulating in the LNs using mAbs to MAdCAM-1, $\alpha 4$, or $\beta 7$.

During fetal life, L-selectin-expressing cells are abundant in the bloodstream, while $\alpha 4\beta 7$+ cells constitute only 1–2% of total PBLs (data not shown). However, analysis of MLN reveals that most cells present in the LN are $\alpha 4\beta 7$+, suggesting that the fetal program is developed to selectively attract $\alpha 4\beta 7$+ cells, present at low numbers in the bloodstream. Analysis of PBLs before birth reveals that most cells are Mac-1+ and a smaller population is B220+, while CD4+CD3+ and CD8+CD3+ cells are almost absent (data not shown). Observations with fluorescein isothiocyanate injections into the thymus of newborn mice suggest that a few cells are emigrating from the thymus until 2 days after birth and that massive efflux from the thymus can be observed in 3-day-old animals (ref. 41; R.E.M., unpublished observations), and these cells are predominantly L-selectin-positive (R.E.M., unpublished observations). In concert with our results are the observations that many T cells enter the spleen of newborn mice between days 2.5 and 3.5 after birth, displacing B cells already present, from the T cells in the periarteriolar lymphocyte sheath (42, 43). The splenic T cell area is competent for T cell entry at birth, as injected adult T cells home to and displace B cells from these sites (42). Thus, a switch in release of spleen-homing T cells or in adhesion molecules on T cells/spleen entry sites is also likely to have occurred. This suggests that the entrance of cells into the

LNs is a process with different phases, each phase allowing a new subpopulation of cells to enter the LNs. The first phase is regulated by the $\alpha 4\beta 7$ homing receptor expression on cells in the bloodstream and MAdCAM-1 on HEV. The second phase starts when the fetal program switches to the adult program and HEV are beginning to express ligands for L-selectin. L-selectin-expressing cells can now enter the LNs. The third phase begins when mature CD4+CD3+ and CD8+CD3+ cells begin emigrating from the thymus in high numbers.

We have demonstrated that homing events to LNs around birth are different from lymphocyte homing to LNs in mature animals. Differences in the immune system of fetal and neonatal versus adult animals have been described for other systems; hemoglobin produced at fetal stages of human development differs from adult hemoglobin produced around birth (44–48), stem cells derived from fetal liver can give rise to different subsets of T cells when compared with stem cells isolated from adult bone marrow (49), the Vγ and Vδ genes used by $\gamma\delta$ T cells that leave the fetal thymus are different from those that leave the adult thymus (50–52), and B-cell progenitors isolated from fetal liver give rise to a different subset of B cells than B-cell progenitors isolated from adult bone marrow (53). In none of those reported cases is it clear what regulates the developmental switch, but they all show that the timing of the switch reflects at least in part inherent programming of these blood cells. Our earlier studies, in which LNs were transplanted to different local environments, also indicate that the switch from mucosal addressin expression to PNAd expression in PLNs is at least in part due to congenital programming of the gene expression potential of these endothelial cells or the underlying stromal cells (54). However, the widespread synchronic nature of this vascular addressin switch in all LNs might also derive from stimulation of HEV cells (or their precursors) by circulating inducers. It is intriguing that the targeted mutation of one cytokine—lymphotoxin (LTα)—results in the failure of development of LNs (55, 56). Perhaps it is LT that directly or indirectly regulates the fetal and/or postnatal HEV addressin programs.

In summary, during late fetal life and at birth, LN HEVs unexpectedly express high levels of MAdCAM-1, while the PNAds cannot be detected. *In vivo* blocking antibodies confirm the role of $\alpha 4$, $\beta 7$, and MAdCAM-1 in the first wave of lymphocyte homing to LNs. The expression of lymphocyte homing receptors and their vascular addressin ligands on high endothelial cells seem to be developmentally regulated. The switch from the fetal HEV program to the postnatal HEV program does not require the presence of recirculating lymphocytes. These changes in lymphocyte homing likely result from a complex, multisite generation and movement of functionally distinct lymphocyte subsets. It will be of interest to find out what genes regulate these developmental switches, and what the nature is of each of the cell subsets whose movement is programmed by these switches.

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