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# Expression of Low Levels of Peripheral Lymph Node-Associated Vascular Addressin in Mucosal Lymphoid Tissues: Possible Relevance to the Dissemination of Passaged AKR Lymphomas

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Lymphoid tumors display a wide variety of growth patterns in vivo, from that of a solitary extralymphoid tumor, to a general involvement of all lymphoid organs. Normal lymphocytes are uniquely mobile cells continuously recirculating between blood and lymph throughout much of their life cycle. Therefore, it is reasonable to propose that disseminating malignant lymphocytes may express recirculation characteristics or homing properties consistent with that of their normal lymphoid counterparts. Trafficking of lymphocytes involves the expression and recognition of both lymphocyte homing receptors and their opposing receptors on endothelium, the vascular addressins. These cell surface elements direct the tissue-selective localization of lymphocyte subsets in vivo into organized lymphoid organs and sites of chronic inflammation where specific binding events occur between lymphocytes and the endothelium of specialized high endothelial venules (HEV). In a recent murine study of 13 lymphoma lines, we found that lymphomas that bind well to high endothelial venules, in the Stamper-Woodruff in vitro assay (an assay of lymphocyte binding to venules in frozen sections of peripheral lymph nodes or Peyer's patches), spread hematogenously to all high endothelial venule bearing lymphoid organs, whereas non-binding lymphomas did not. In some cases lymphomas that bound with a high degree of selectivity to peripheral lymph node (PLN) high endothelial venules exhibited only limited organ preference of metastasis, involving the mucosal lymphoid organs Peyer's patches (PP) in addition to the peripheral lymph nodes of adoptive recipients. Here we demonstrate that Peyer's patch high endothelial venules express a low but functional level of peripheral lymph node addressin (MECA-79) that can be recognized by lymphomas expressing the peripheral lymph node homing receptor (MEL-14 antigen). Utilization of the peripheral lymph node associated endothelial cell recognition system could explain the ability of lymphoid neoplasms, selectively expressing lymph node homing receptors, to disseminate to both peripheral lymph nodes and to organized mucosal lymphoid tissues in vivo.

**Key words: metastasis, lymphoma, homing receptor, high endothelial venule, addressin**

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Migrating lymphocytes travel between lymphoid organs and other tissues of the body by the lymph and blood. This recirculation process is facilitated by the ability of migrating lymphocytes to enter organized lymphoid tissues by specifically adhering to and migrating through the walls of post-capillary high endothelial venules (HEV) [1]. The extravasation of lymphocyte subsets into diverse lymphoid tissues exhibits remarkable specificity [2], which appears to be determined at least in part by selective mechanisms of endothelial cell recognition. In vivo and in vitro studies have revealed the existence of two functionally distinct lymphocyte-high endothelial cell recognition systems in the mouse, mediating lymphocyte interactions with HEV in mucosal lymphoid tissues (Peyer's patches) and in peripheral lymph nodes [3,4]. Lymphocyte surface molecules thought to be involved in these tissue-selective interactions between lymphocytes and high endothelial cells, referred to as "homing receptors," have been identified by monoclonal antibodies (mAbs) in mouse, rat, and human systems [4–7]. The tissue-selective interactions of lymphocytes and high endothelial cells (HEC) implicate distinct cell surface recognition elements on HEC from mucosal and peripheral lymphoid tissues. Two of these endothelial recognition elements for lymphocytes have been identified. These "vascular addressins" appear to convey tissue position or tissue specificity information to circulating lymphocytes [9,11].

In our recent studies of lymphoma progression in AKR and C3H mice, we have demonstrated that the ability of lymphoma cells to bind HEV in vitro correlates with the in vivo metastatic behavior in adoptive recipients (summarized in Table I) [8], supporting a role for normal lymphocyte homing processes in lymphoma metastasis. HEV-binding lymphomas metastasized via the blood to all lymph nodes and Peyer's patches (PP) and yielded gross enlargement of those tissues. In contrast, lymphomas that did not bind HEV in vitro, gained access to the blood, and involved the bone marrow and spleen, but nevertheless produced no significant growth in distant (non-draining) peripheral lymph nodes or Peyer's patches. One particularly intriguing feature of this study was the observation that a lymphoma (BK37) which selectively bound PLN-HEV in the in vitro assays metastasized to involve both PLN and Peyer's patches when assessed in vivo in AKR mice. This was also our observation with the 38C13 lymphoma in C3H mice. Thus in both cases there is a paradox between the highly selective PLN-HEV binding of these lymphomas in vitro, and their in vivo metastasis not only to PLN, but also to PP.

In this report we present studies that may help to explain this observation. We provide evidence that Peyer's patch-HEV express functional if low levels of PLN addressin, allowing recognition of HEV in these mucosal lymphoid organ even by lymphomas that appear to exclusively express PLN homing receptors. Furthermore, we show that BALB/c Peyer's patch-HEV express significantly less PLN addressin than Peyer's patch-HEV in AKR mice, an observation that helps explain the previous discordance between results of in vitro HEV binding assays (carried out in earlier studies on BALB/c Peyer's patches) vs in vivo metastatic behavior (in AKR mice).

## MATERIALS AND METHODS

### Mice and Tissues

Tissues: Axillary, Peyer's patches, brachial and ingunial lymph nodes were from 8–12-week-old BALB/c and AKR/J mice (Institute for Medical Research, San Jose, CA; and Jackson Labs, Bar Harbor, ME, respectively). These tissues were collected and

**TABLE I. Properties of Murine Lymphomas: *In Vitro* BALB/c HEV-Binding, *In Vivo* Tissue Localization, and MEL-14 Staining**

| Cell | *In vitro* HEV-binding[a] | | *In vivo* progression | | MEL-14 staining |
|---|---|---|---|---|---|
| | PLN | PP | PLN[b] | PP[c] | |
| Control cells | | | | | |
| Normal MLN lymphocytes | + | + | NA[d] | NA[d] | ++ |
| HEV non-binding lymphomas | | | | | |
| BW5147 | − | − | − | − | − |
| TK2 | − | − | − | − | − |
| TK5 | − | − | − | − | − |
| L1-2 | − | − | − | − | − |
| HEV binding lymphomas | | | | | |
| TK1 | +/− | +++++ | +++ | +++ | +[e] |
| TKJ43 | + | ++++ | ++ | +++ | +[e] |
| TK23 | + | ++ | ++ | ++ | ++ |
| BK37 | ++ | +/− | ++++ | + | ++++ |
| 38C13 | ++++ | + | ++++ | + | ++++ |

[a]Binding to HEV is expressed in relative terms, where the binding of normal MLN lymphocytes (as an internal standard) has been defined (+). PLN, peripheral lymph nodes; PP, Peyer's patches.
[b]Tumor-progression to peripheral lymph nodes was qualitatively determined by comparing the weight of lymph nodes from syngeneic control and lymphoma-bearing animals. Lymph nodes from mice bearing HEV non-binding lymphomas were not significantly larger than lymph nodes from control animals, suggesting little or no lymph node involvement (−). In contrast, the lymph nodes from mice bearing HEV binding lymphomas were much larger than control lymph nodes.
[c]Peyer's patches in syngeneic lymphoma-bearing animals were examined histologically for the presence of tumor. "−" indicates no detectable tumor, and "+" indicates tumor involvement.
[d]NA, not applicable.
[e]Note that these lymphomas were previously reported to be completely MEL-14 negative and Peyer's patch-HEV-specific [3]. This has been explained by the subsequent observation that the MEL-14 antigen and lymph node HEV binding are lost on lymphoma cells that are tested directly upon thawing after storage in liquid nitrogen in 10% DMSO. Frozen-thawed lymphoma cells used in those studies and in subsequent studies (where cells completely lacking in lymph node homing receptor expression were desired) acted as specificity tools. Here we have used cells taken directly from tumor of adoptive recipients, as their behavior is more relevant to the metastatic patterns observed.

frozen in TISSUE-TEK O.C.T. embedding compound (Miles Scientific, Naperville, IL). Subsequently, these tissue blocks were cryostat sectioned for both immunoperoxidase staining and in vitro HEV binding assays.

## Monoclonal Antibodies

Production and characterization of monoclonal antibodies used in this study MEL-14 and MECA-79 are described previously [7,11]. MEL-14, a rat IgG2b monoclonal antibody, recognizes the peripheral lymph node homing receptor of mouse lymphocytes and blocks it's HEV binding function in vivo as well as in vitro. The rat anti-mouse IgM monoclonal MECA-79 defines the peripheral lymph node addressin, an HEV-specific molecule in PLN; it blocks binding of lymphocytes to PLN in vivo as well as in vitro. Class-matched mAbs 30G12 [7] and OZ-42 are rat IgG2b and IgM, respectively; 30G12 recognizes mouse T200 and OZ-42 recognizes a brain-specific determinant in developing postnatal mouse cerebellum [12].

### Immunoperoxidase Staining

Immunoperoxidase staining of acetone-fixed frozen sections was performed using a two-stage system as previously described [9]. In brief, sections were incubated with 100 μg of mAb in PBS, washed, and treated with a solution of PBS containing a 1:50 dilution of the second-step horseradish peroxidase-conjugated rabbit anti-rat immunoglobulins (DAKO, Santa Barbara, CA) and 4% NMS. Detection of the second-stage antibody was achieved by exposure to diaminobenzidine 0.05% and 0.009% $H_2O_2$ in 0.05 M Tris (pH 7.6) and then enhanced by incubation in 0.5% copper sulfate in saline.

### Immunofluorescence Staining

Lymphoma cells were stained with MEL-14 monoclonal antibody and class-matched control as previously described [4]. Briefly, cells were incubated with 10 μl of 100 μg/ml mAb/$10^6$ lymphoma cells in DMEM, 4% newborn calf serum (NBCS) and incubated on ice for 20 min. The cells were then washed through a NBCS cushion, incubated with Sigma goat anti-rat IgG (St Louis, MO) 1:50 dilution in DMEM, 4% NBCS, 5% mouse sera, incubated on ice for 20 min, and then spun through NBCS. The cells were then evaluated by UV-epi-illumination microscopy for staining.

### In Vitro Lymphoma HEV Binding Assay

In assessing the blocking action of test and class-matched control mAbs, lymphoma cells or tissue sections were pretreated with antibody for 30 min at 7°C as follows: $10^7$ lymphoma cells were preincubated with 1 ml of 100 μg/ml MEL-14 or class-matched control and washed; sections were pretreated with 50 μl of 100 μg/ml of MECA-79 or class-matched control, and antibody was aspirated away before incubation with the lymphomas. Following this pretreatment, the in vitro assay was performed as described below.

The standard in vitro binding assay has been described [10]. Briefly, 1–3 × $10^6$ cells in 100 μl of medium were applied to 12 μm thick, freshly cut tissue sections (Peyer's patches or peripheral lymph nodes). After mild rotation for 30 min at 7°C, sections were fixed in 1.5% glutaraldehyde in PBS. When appropriate, an internal standard population of fluorescein isothiocyanate labeled lymphocytes was added to test populations, to allow quantitative comparisons. Cell binding to HEV was accessed microscopically under darkfield illumination and UV-epi-illumination.

### Lymphomas

The lymphomas used included BW5147 (American Type Culture Collection, Rockville, MD), an AKR T cell lymphoma; L1–2, an Abelson virus-induced C57 lymphoma; TK1, TK2, TK5, and TK23 spontaneous AKR/Cum T cell lymphomas [3]; TKJ43, an AKR/J lymphoma [3]; BK37, an AKR/Cum B cell lymphoma [3]; and 38C13 a C3H B cell lymphoma [4]. The T-cell lymphoma TKJ43 was employed as a representative mucosal binder in the binding and blocking studies as it binds mucosal HEV 2–4× better than normal mesenteric node lymphocytes (NMNL), while binding at low levels to PLN HEV. The B cell lymphoma 38C13 [12] was chosen as the peripheral specific lymphoma in this study because of its 4× better binding to peripheral lymph node HEV than NMNL (by in vivo serial passage through lymph nodes resulting in selection of a high binding, highly metastatic form) and it's well-characterized PLN HEV-specific binding behavior [4,9,11].

## RESULTS AND DISCUSSION

### Homing Receptor Dependent In Vitro Lymphoma Binding

The two selected lymphomas were tested for their relative HEV binding capacities on both PLN and PP of BALB/c mice. The C3H B cell lymphoma 38C13 was characterized by MEL-14 staining (Table I), in vitro binding to HEV in Peyer's patches vs. PLN (Fig. 1A), and inhibition of in vitro binding by the mAbs antibodies MEL-14 and MECA-79 (Figs. 1B, 3) but not by MECA-367, a monoclonal antibody that blocks lymphocyte binding to PP HEV by recognition of the mucosal addressin (data not shown). The results are consistent with 38C13 binding almost exclusively via the PLN HEV recognition system. This high degree of specificity allowed us to explore the binding capacities of both BALB/c and AKR Peyer's patch-HEV to bind cells via lymph node homing receptor mechanisms. TK/J43 binds mucosal HEV in Peyer's patch by a mucosal specific interaction as shown by its mucosal vs. peripheral HEV binding preference (Fig. 1A), low MEL-14 antigen expression (Table I), and the inability of MEL-14 and MECA-79 to block the mucosal Peyer's patch HEV binding (Fig. 3). Thus, TKJ43 acts as a negative specificity control for the events that are mediated by peripheral addressin binding interactions.

### Expression of Peripheral Lymph Node Addressin in Peyer's Patches

Consistent metastatic involvement of Peyer's patches even by the PLN-HEV-specific lymphoma BK37 prompted us to examine the Peyer's patches of AKR mice for expression of the PLN-associated vascular addressin defined by the mAb MECA-79. This mAb preferentially stains PLN HEV and blocks lymphocyte interactions with PLN HEV both in vitro and in vivo [11]. Our working hypothesis was that the expression of this antigen in AKR and BALB/c mice might differ, with AKR mice exhibiting a breakdown in the tissue-preferential expression of the MECA-79 antigen, thus facilitating the binding of peripheral specific lymphomas in Peyer's patches. Such divergent expression would potentially explain the observed metastatic behavior of the lymphomas.

In BALB/c mice MECA-79 stains peripheral lymph node HEC (Fig. 2) from their lumenal surface to the basal lamina. By contrast, on Peyer's patch HEC, staining in most instances is restricted to the basal lamina. On a small subset of Peyer's patch HEV low level lumenal surface staining is observed [11]. Staining of normal AKR tissue with MECA-79 is illustrated in (Fig. 2). While tissue-selective expression of the antigen defined by MECA-79 was apparent in this mouse strain (i.e., MECA-79 preferentially stained peripheral lymph node-HEV), this antigen also appeared to be expressed at moderate levels in the Peyer's patches. As in BALB/c mice, MECA-79 intensely stained the HEV of AKR peripheral lymph nodes, while providing a weaker signal on Peyer's patch-HEV. However, unlike BALB/c mice, where few HEV lumens were seen to stain (as described previously) [11], the lumens of many of the Peyer's patch-HEV in AKR mice were stained by this MAb (see Fig. 2). These observations suggested that the antigen defined by MECA-79 could account for a low level of lymphoma localization in AKR Peyer's patches.

We used the Stamper-Woodruff in vitro assay [10] to assess the effects of MECA-79 on tissue-selective HEV binding by testing the PLN-specific lymphoma 38C13 and by mucosal HEV binding lymphoma TKJ-43 (Fig. 3). In BALB/c mice, we have previously reported that MECA-79 and MEL-14 inhibit 38C13 lymphoma binding to PLN HEV [11] (see Fig. 1B). Here we demonstrate that these mAbs also inhibit

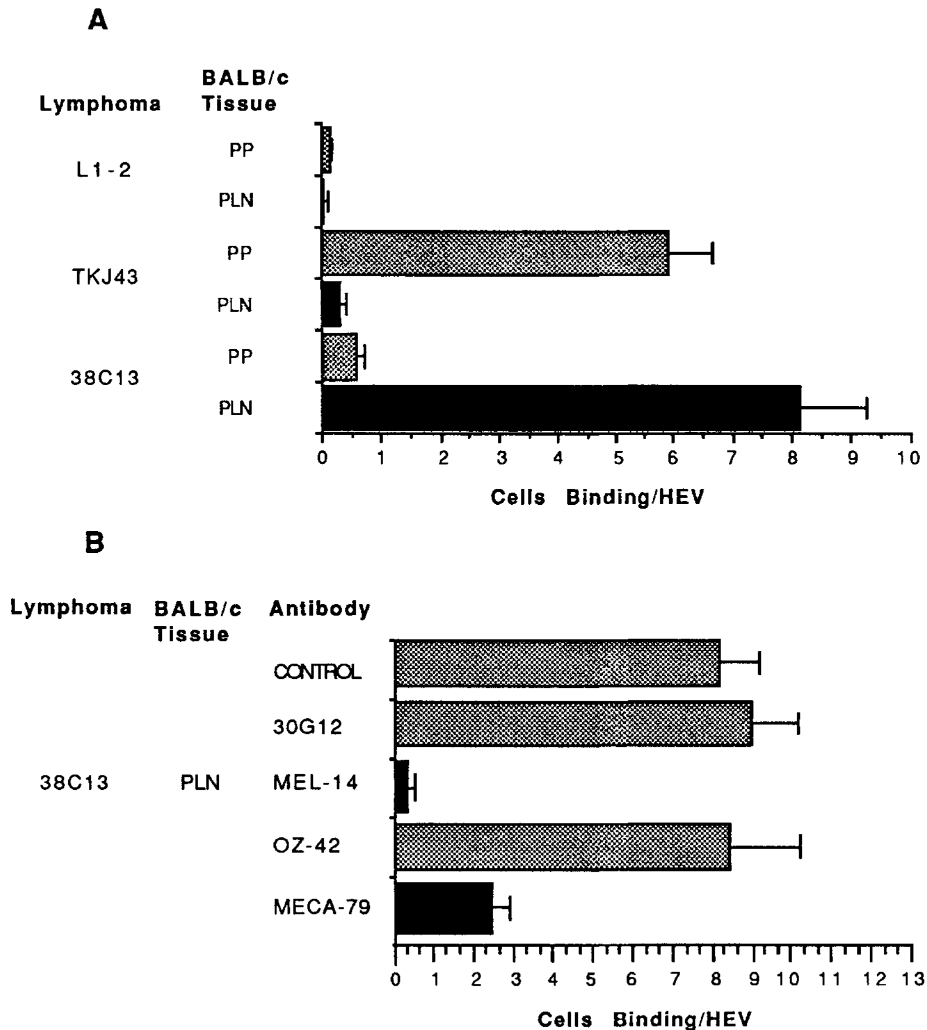


Fig. 1. **A:** HEV binding properties of selected murine lymphomas. On BALB/c peripheral lymph nodes (PLN) and Peyer's patches (PP), the lymphoma L1-2 fails to bind HEV. The lymphomas TKJ43 and 38C13 selectively bind PP and PLN, respectively. **B:** Blocking effects of MEL-14 and MECA-79 on 38C13 binding to BALB/c PLN HEV in the in vitro HEV binding assay. MEL-14 and MECA-79 effectively block 38C13 binding on PLN HEV. The class matched monoclonal antibodies 30G12 and OZ-42 have no effect on binding.

the low but significant binding of 38C13 to BALB/c PP HEV. In contrast, MECA-367 had no effect on 38C13 binding to either PLN or PP. The binding of TKJ-43 to PP HEV of BALB/c mice was not influenced by either MEL-14 or MECA-79. Normal AKR PP HEV were found to support significantly higher levels of 38C13 binding, and this binding also involved the peripheral binding specificity as demonstrated by MEL-14 and MECA 79 blocking (Fig. 3). Interestingly, the binding of TKJ-43 to AKR PP HEV was partially blocked by these monoclonal antibodies, a feature that is consistent with the low level expression of MEL-14 by this lymphoma. The results indicate that Peyer's

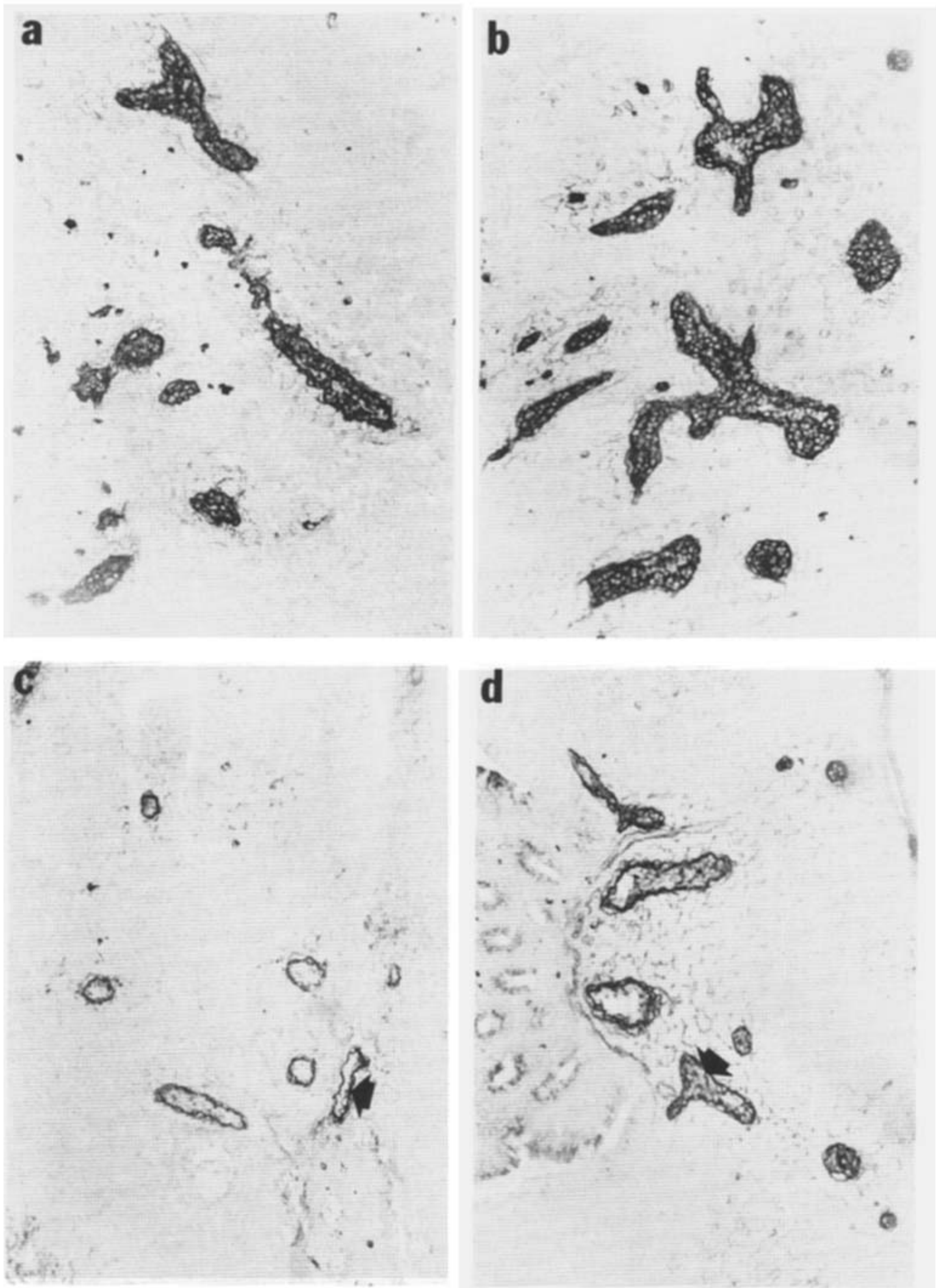


Fig. 2. Expression of the peripheral lymph node addressin on BALB/c (**a**) and AKR (**b**) PLN and BALB/c (**c**) and AKR (**d**) PP HEV. On both BALB/c and AKR PLN (a,b), MECA-79 intensely stains the HEV, from their lumenal surface to the basal lamina. By contrast on PP (c,d) MECA-79 staining of HEV is general restricted to the ablumenal aspect of the venules in BALB/c mice (arrow) (c), but is present in the lumenal surface (arrow) and on the ablumenal aspect of venules in AKR mice (d).

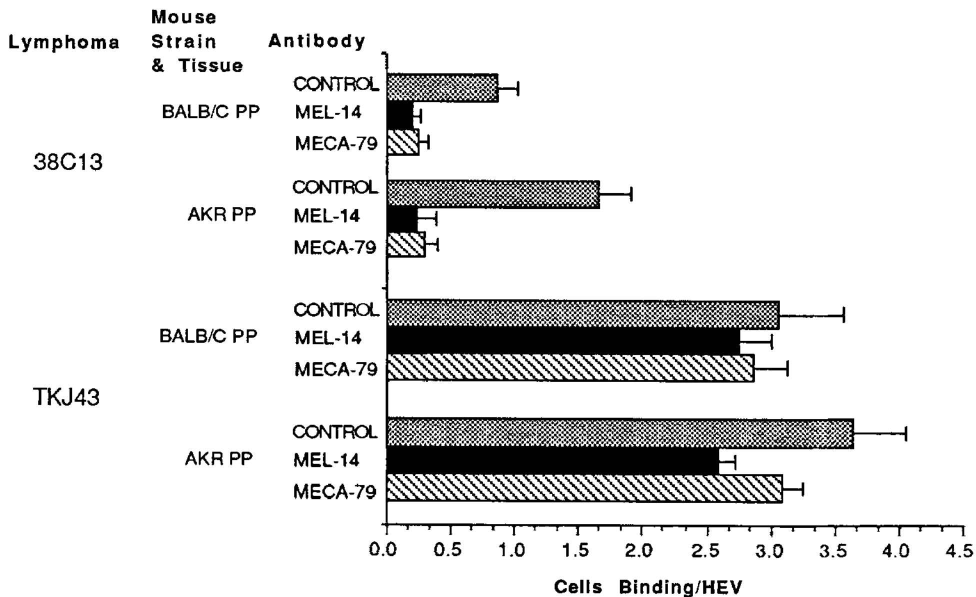


Fig. 3. Involvement of PLN homing receptors (MEL-14 antigen) and of PLN addressin (MECA-79 antigen) in lymphoma binding to Peyer's patch-HEV in BALB/c and AKR mice. 38C13 cells (a variant selected for very high peripheral node metastasis) bind significantly to Peyer's patch-HEV in both strains, but approximately 2× better to AKR than BALB/c vessels $P < 0.05$ by the Wilcoxon sign rank test. This binding is inhibited by MEL-14 and MECA-79 $P < 0.0001$ also by the Wilcoxon sign rank test. MECA-367, a monoclonal antibody that blocks lymphocyte binding to PP HEV by recognition of the mucosal addressin, had no effect on 38C13 binding to either PLN or PP (data not shown). TKJ43 binds primarily via the mucosal homing receptor-addressin mechanisms, and its binding was minimally reduced by MEL-14 on AKR Peyer's patch-HEV, while MECA-79 had no detectable effect. Neither MEL-14 nor MECA-79 blocked TKJ43 binding to BALB/c Peyer's patch-HEV. Class-matched antibody controls for MEL-14 and MECA-79 were employed as well (data not shown) and had no effect on binding. Medium only controls are depicted.

patch-HEV in both strains can bind lymphomas via mechanisms involving the PLN addressin and PLN homing receptor, but that Peyer's patch-HEV express significantly more PLN addressin in AKR than in BALB/c mice. Intravital studies of the interaction of circulating lymphoma cells with HEV in situ are currently being carried out to determine directly the role of lumenal PLN addressin in PP localization of tumor cells in the animal.

In conclusion, the expression of low levels of PLN addressin in Peyer's patch-HEV, especially in AKR mice, may in part explain the spread of PLN-specific lymphomas to these mucosal lymphoid organs. The observations further support the proposal that normal lymphocyte homing processes are involved in the progression of lymphoid malignancies [8].

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