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ORIGINAL ARTICLE

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Expression of L-selectin and efficient binding to high endothelial venules do not modulate the dissemination potential of murine B-cell lymphoma

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Abstract The homing receptor L-selectin is essential for the migration of naive lymphocytes into peripheral lymph nodes. In contrast to naive lymphocytes, activated and memory cells down-regulate L-selectin and enter peripheral lymph nodes by an L-selectin-independent mechanism. In view of the concept that lymphomas present the malignant counterparts of normal lymphocytes at a defined stage of differentiation, it has been suggested that in contrast to lymphomas with a memory/activated cell phenotype, L-selectin is essential for dissemination of lymphomas that represent naive cells. 38C-13 is a murine B-cell lymphoma with an immature naive cell phenotype. 38C-13 cells express high levels of L-selectin and bind to lymph node high endothelial venules in an L-selectin-dependent manner. In this study we demonstrate that treatment of 38C-13 tumor-bearing mice with anti-L-selectin antibodies did not inhibit tumor dissemination to peripheral lymph nodes. Moreover, L-selectin-negative 38C-13 variant cells disseminated as efficiently as wild-type cells. Thus, in spite of its expression, L-selectin is not required and does not affect the metastatic potential of the tumor. L-selectin of the malignant cells and of normal lymphocytes appears to be functionally different. Thus, whereas antibody cross-linking of L-selectin resulted in down-modulation of the receptor in normal lymphocytes, cross-linking had no effect on L-selectin expression in 38C-13 cells, suggesting that, in spite of comparable levels of surface expression in normal and malignant cells, L-selectin may be functionally impaired in some malignant cells.

Key words Adhesion molecules · Metastasis · B-cell lymphoma

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Introduction

Lymphocytes recirculate continuously between blood and lymphoid organs. Their extravasation to tissues is a multistep process that is mediated by a set of adhesion molecules that are expressed on lymphocytes and their counterparts on endothelial cells [11]. The distinct receptors that are involved in each step determine the organ specificity of extravasation. The lymphocyte homing receptor L-selectin mediates binding of lymphocytes to high endothelial venules (HEV), specialized venules that control the extravasation of circulating lymphocytes from the blood into lymphoid organs and sites of chronic inflammation [10, 21]. L-selectin-deficient mice, as well as antibody inhibition studies, have shown that L-selectin is essential for lymphocyte migration into peripheral lymph nodes and into inflamed non-lymphoid tissues [3, 28, 38].

Since lymphoma cells often express the same adhesion molecules as their normal counterparts, it has been postulated that these adhesion molecules are involved in the dissemination of lymphoma cells to target organs. Accordingly, it has been suggested that L-selectin has an essential role in the formation of lymph node metastases by lymphoma cells. In agreement with this concept, it has been demonstrated that the capacity of murine lymphoma cells to bind in vitro to HEV strongly correlates with their capacity to form lymph node metastases in vivo, suggesting that L-selectin is critical for lymphoma dissemination and that its expression may predict the metastatic behavior of tumor cells [4, 37]. However, this idea has more recently been challenged by studies that have shown a lack of correlation between the expression of L-selectin and the metastatic potential of lymphoma cells [15, 22]. These studies demonstrated that L-selectin-negative T-cell lymphomas that do not bind to HEV readily metastasize to lymph nodes. Such lymphomas may, however, represent memory T-cells, which enter peripheral lymph nodes via the afferent lymphatic vessels [7, 8, 29]. On the contrary, lymphomas

that represent naive lymphocytes may enter peripheral lymph nodes via HEV by an L-selectin-dependent mechanism. In view of the concept that lymphomas represent the malignant counterparts of normal lymphocytes "frozen" at a certain stage of maturation/activation, it is conceivable that the same mechanisms regulating normal lymphocyte traffic are, at least to a certain extent, operating in lymphoid malignancies. If so, lymphomas with a memory/activated cell-like phenotype would be expected to reach lymphatic organs predominantly by an L-selectin-independent mechanism via afferent lymph, whereas those with a naive cell phenotype may depend on L-selectin for their hematogenous spread.

38C-13 is a carcinogen-induced murine B-cell lymphoma with an immature B-cell phenotype [5, 6]. 38C-13 cells express high levels of L-selectin and bind efficiently to HEV in an L-selectin-dependent manner [17]. It was therefore expected that 38C-13 cells transmigrate into peripheral lymph nodes through HEV by an L-selectin-dependent mechanism. In this report we demonstrate that L-selectin is dispensable for dissemination of 38C-13 to lymph nodes, suggesting that lymphomas with a naive cell phenotype metastasize to lymph nodes by an L-selectin-independent mechanism, hence resembling in this regard memory/activated cell-like lymphomas.

Materials and methods

Mice

C3H/eB mice were obtained from the animal facility of Tel-Aviv University and used at 8–10 weeks of age. All procedures with animals were carried out in accordance with institutionally approved protocols.

Antibodies

The hybridoma cell line MEL-14, secreting rat anti murine L-selectin [17], was obtained from the American Type Culture Collection (Rockville, Md.) and grown as ascites. The monoclonal antibody (mAb) was purified from ascites fluid by protein G-Sepharose (Pharmacia Biotech, Uppsala, Sweden). The hybridoma cell line 2B6 secretes a rat mAb that is specific for an idiotypic determinant of the 38C-13 IgM [30].

Cells

38C-13 cells [5,6] and their L-selectin-negative variants were maintained in RPMI-1640 medium supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 50 μM 2-mercaptoethanol. L-selectin-negative variants of 38C-13 were selected as previously described [19]. Briefly, cells were fluorescein-labeled with MEL-14 mAb. Cells expressing no detectable L-selectin were then separated by sorting for 0.5% of the most negative cells, using a fluorescent activated cell sorter (Becton Dickinson, Mountain View, Calif.). Sorted cells were allowed to expand in culture, and the selection process was repeated three more times, until most of the selected cells were negative for L-selectin. At this stage, cells were cloned by limiting dilution.

Tumor inoculation and MEL-14 treatment

Mice were inoculated i.p. with 1×10^4 38C-13 cells or s.c. with 1×10^5 38C-13. One hour later mice were injected i.p. or i.v. with 0.2 mg MEL-14 or with 0.2 mg rat IgG (Sigma, St. Louis, Mo.). Antibody injection was repeated every three days thereafter, until termination of the experiment (day 18).

Detection of lymph node invading tumor cells

Inguinal, brachial and axillary lymph nodes were removed, and cell suspensions were prepared. Cells were incubated for 24 h at 1×10^5 cells/well in flat-bottom 96-well microplates. They were then pulsed for 20 h with 1 μCi/well of [^{3}H]TdR and harvested. [^{3}H]TdR uptake was assessed by liquid scintillation counting. Under these conditions, presence of 38C-13 cells in lymph nodes was manifested by massive cell proliferation (high rates of thymidine uptake) compared to low proliferation rates in normal lymph nodes. Results were expressed in cpm (mean ± SD).

Invasion of lymph nodes by 38C-13 tumor cells was also demonstrated by fluorescence staining of lymph node cells with the 2B6 anti-idiotype mAb, which is specific for the IgM of 38C-13.

Flow cytometry

Cells were incubated for 10 min at 4 °C with 60 μg aggregated human IgG (Sigma, St. Louis, Mo.) to block Fc receptors. Primary antibodies were then added for an incubation of 30 min at 4 °C. Cells were then washed and incubated for 30 min at 4 °C with secondary FITC-conjugated mouse anti-rat IgG (Jackson ImmunoResearch, West Grove, Pa.). Cells were washed again, fixed with 1% paraformaldehyde and analyzed on a FACSort flow cytometer (Becton Dickinson, Mountain View, Calif.).

ELISA

ELISA was used to determine the amount of 38C-13 idiotype present in serum of tumor-bearing mice. The assay was performed as described [30]. Briefly, 96-well ELISA plates were coated for 18 h at 4 °C with 10 μg/ml of purified anti-idiotype mAb. Plates were then washed, blocked with 1% BSA for 1 h at 37 °C and washed again. Serial dilutions of test sera or purified idiotype were then added to plates for 18 h at 4 °C. Plates were washed, and horseradish-peroxidase-conjugated donkey anti-mouse IgM (Jackson ImmunResearch, West Grove, Pa.) was added for 1 h at room temperature. Following washes, plates were developed for 15 min with 1,2-orthophenylenediamine. The reaction was terminated with 1 M H_2SO_4 and absorbance at 490 nm was measured by ELISA reader.

Results

Anti-L-selectin mAb does not inhibit lymph node dissemination of 38C-13

Idiotypes of the surface Ig of B-cell lymphomas are tumor specific-antigens that can be used to detect tumor cells in different organs [18, 26, 30]. Hence, dissemination of 38C-13 in tumor-inoculated mice was demonstrated by staining with anti-idiotype mAb. When 38C-13 cells were inoculated into mice, a progressive increase in the percentage of tumor cells was found in peripheral blood and in different organs [30]. By day 18 following tumor inoculation, lymph nodes were heavily infiltrated by 38C-13 cells (shown in Fig. 7).

To assess the effect of anti-L-selectin on 38C-13 lymph node dissemination, 38C-13 inoculated mice were injected with 0.2 mg MEL-14 on the day of tumor inoculation and every 3 days thereafter. Mice were killed on day 18, when lymph nodes were heavily loaded with tumor, and the effect of antibody treatment was assessed by thymidine uptake determination in lymph node cell cultures. Cell proliferation, rather than staining for idiotype, was used for this purpose, because the MEL-14 antibody bound to tumor cells in MEL-14-treated mice and thereby complicated staining with anti-idiotype antibodies. Infiltration of lymph nodes by 38C-13 cells is manifested by massive cell proliferation compared to low proliferation rates in normal lymph nodes. A variety of experimental designs were tested in which tumor was inoculated i.p. or s.c. and antibodies were administered i.p. or i.v. Only distant lymph nodes were examined. Local lymph nodes draining the site of tumor cell injection were not collected. Thus, when tumor cells were injected s.c. in one side of the animal, only lymph nodes from the opposite non-injected side were examined. Representative results are shown in Fig. 1. It was demonstrated that anti-L-selectin mAb has no effect on dissemination of 38C-13 to lymph nodes, suggesting that extravasation through HEV by an L-selectin-dependent mechanism is not an essential prerequisite for 38C-13 dissemination to lymph nodes.

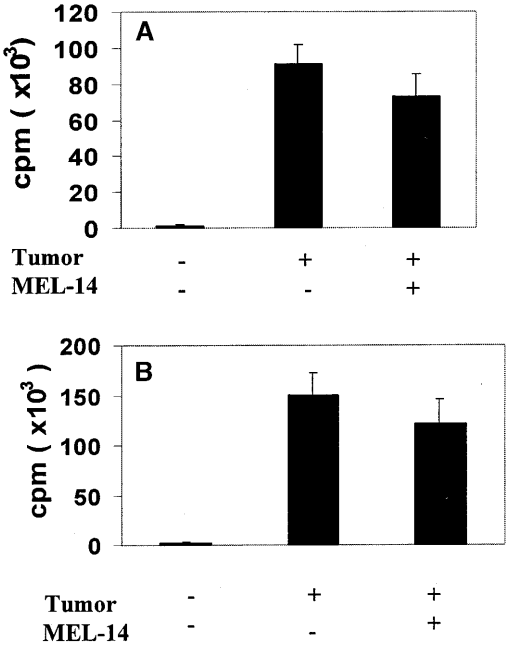

Fig. 1A, B Lymph node dissemination of 38C-13 is not inhibited by anti-L-selectin mAb. Mice inoculated s.c. **(A)** or i.p. **(B)** with 38C-13 cells were injected with 0.2 mg MEL-14 mAb or with control IgG on the day of tumor inoculation and every 3 days thereafter. Lymph nodes were removed on day 18 post tumor inoculation and cell suspensions were tested for thymidine uptake

Anti-L-selectin mAb has no effect on tumor load

One possible explanation for the finding that similar levels of tumor cells were present in lymph nodes of antibody-treated and untreated mice is that MEL-14 mAb stimulated proliferation of 38C-13 cells in antibody-treated animals. L-selectin is involved in intracellular signaling. Ligation of L-selectin has been reported to activate several signal transduction pathways [9, 27, 32, 33]. Although the physiological significance of the L-selectin-transduced signals remains to be elucidated, such signals may accelerate tumor cell growth. It is therefore possible that while MEL-14 did inhibit HEV-mediated homing to lymph nodes, a few evading tumor cells reached the lymph nodes and were subsequently induced to undergo accelerated proliferation by MEL-14, hence reaching high tumor cell numbers. If this were the case, the overall tumor burden in tumor-bearing animals would be increased. Since 38C-13 cells secrete low amounts of IgM, mice bearing 38C-13 accumulate in their serum significant levels of the idiotype, which can be detected by ELISA and serve as an indicator of tumor burden [30]. Figure 2 shows that idiotype levels in serum of MEL-14-treated and untreated tumor-bearing mice were similar (mean concentration of 600 ng/ml in treated mice and 580 ng/ml in untreated mice), indicating that the antibody did not accelerate tumor growth.

Expression of surface L-selectin is not down-modulated in 38C-13 cells

Another possible explanation to the failure of MEL-14 to block dissemination was that the antibody did not bind efficiently to 38C-13 cells in vivo. To test this possibility, tumor cells from MEL-14 treated mice were stained with MEL-14, followed by FITC-anti-rat IgG, or with the second antibody alone. Cells were obtained

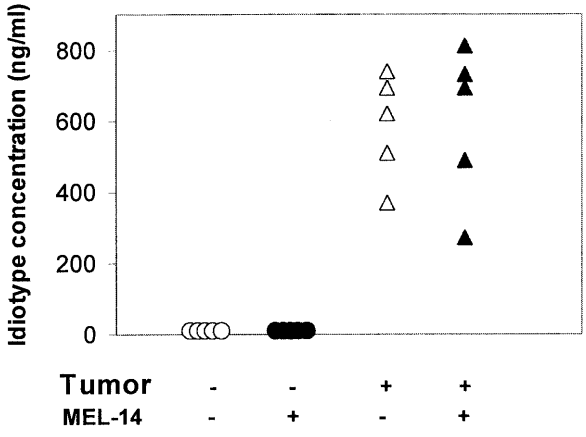

Fig. 2 Anti-L-selectin mAb has no effect on tumor load. Sera were collected from MEL-14-treated and untreated 38C-13-tumor-bearing mice on day 16 post tumor inoculation. Concentration of 38C-13 idiotype in the sera was determined by ELISA

from the primary tumor and from peripheral lymph nodes 18 days after tumor inoculation. At this time point, the primary tumor is large and the lymph nodes are heavily infiltrated by tumor, consisting predominantly of 38C-13 tumor cells (see Fig. 7). As shown in Fig. 3, tumor cells from primary tumor and from invaded lymph nodes of MEL-14 treated mice were stained by fluoresceinated second antibody alone, indicating that the injected mAb was bound in vivo to the tumor cell surface. Staining with MEL-14 followed by second antibody did not modify (in primary tumor) or only slightly enhanced (in lymph nodes) the staining pattern compared to staining with second antibody alone. These results suggest that all L-selectin molecules on tumor cells of the primary site, and the majority of L-selectin molecules on lymph node-invading cells, bound the injected antibody.

The results presented in Fig. 3 were surprising. It has been reported that in vitro antibody treatment, as well as in vivo administration of MEL-14, induced almost complete down-regulation of L-selectin on both T- and B-cells due to shedding [8, 28, 31, 35]. Therefore, the finding that expression of L-selectin on 38C-13 cells was not down-regulated following injection of MEL-14, and that the antibody was detectable on the tumor cell surface, was unexpected. In our experiments, injection of MEL-14 to tumor-free control mice mediated drastic peripheral lymph node depletion, while not affecting spleen cellularity. The remaining lymphocytes in depleted lymph nodes, as well as the spleen lymphocytes,

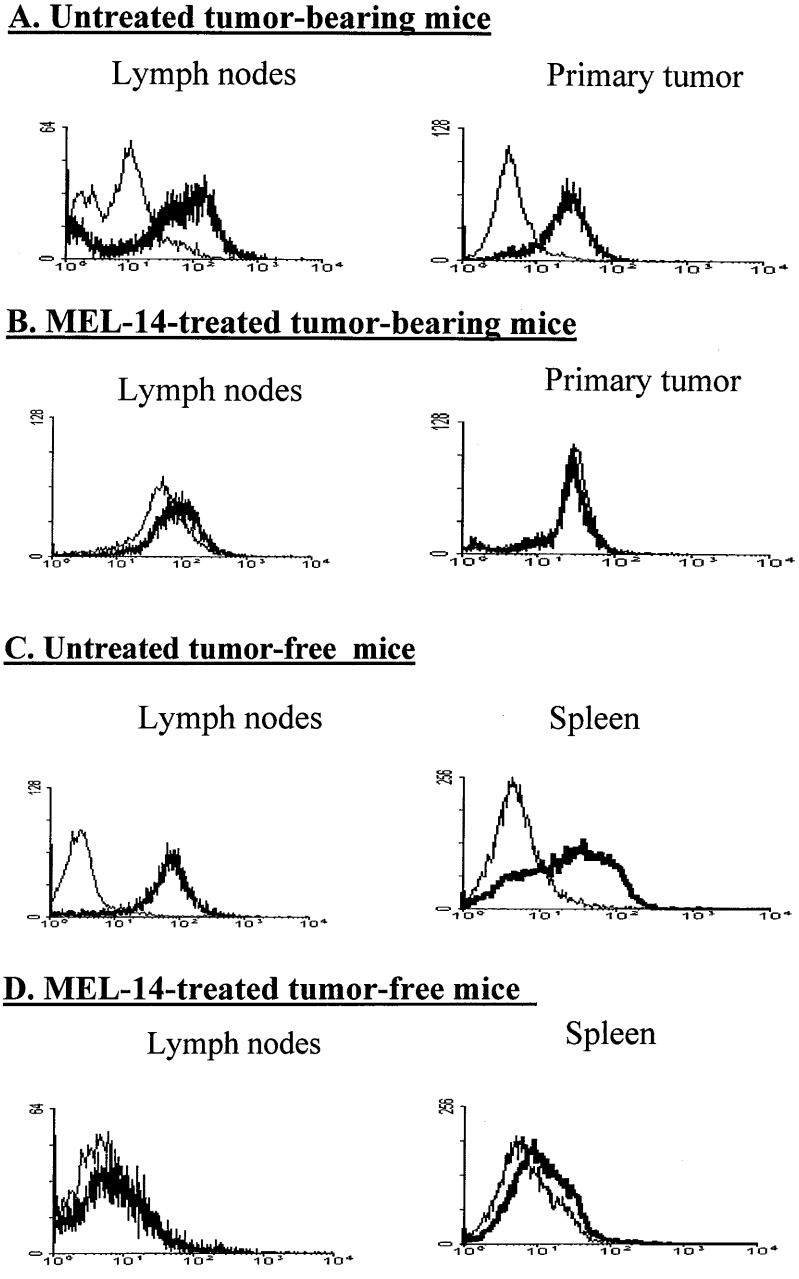

Fig. 3A–D Surface L-selectin on 38C-13 cells is not down-modulated following antibody binding. Mice inoculated i.p. with 38C-13 (**A, B**) and normal mice (**C, D**), were injected with 0.2 mg MEL-14 (**B, D**) or with rat IgG (**A, C**) on the day of tumor inoculation and every 3 days thereafter. Lymph node cells, spleen cells and tumor cells from peritoneal ascites were stained on day 18 with second antibody (anti-rat IgG) alone (*thin line*) or with MEL-14 plus second antibody (*bold line*)

revealed significant down-modulation of L-selectin expression (Fig. 3), confirming previous reports and revealing that 38C-13 cells differ from normal lymphocytes in the ability to shed L-selectin following antibody binding.

Since rapid down-regulation of L-selectin is mediated as well by lymphocyte activators such as PMA [23, 25], we determined the effect of PMA on 38C-13 cells. As shown in Fig. 4, L-selectin expression was significantly down-modulated in 38C-13 cells following 1 h treatment with PMA. These results indicate that the failure of 38C-13 cells to down-modulate L-selectin following antibody cross-linking is due to impaired signaling by L-selectin rather than a defect in the proteolytic cleavage pathway, since a non-specific stimulus mediated down regulation of L-selectin in 38C-13 cells.

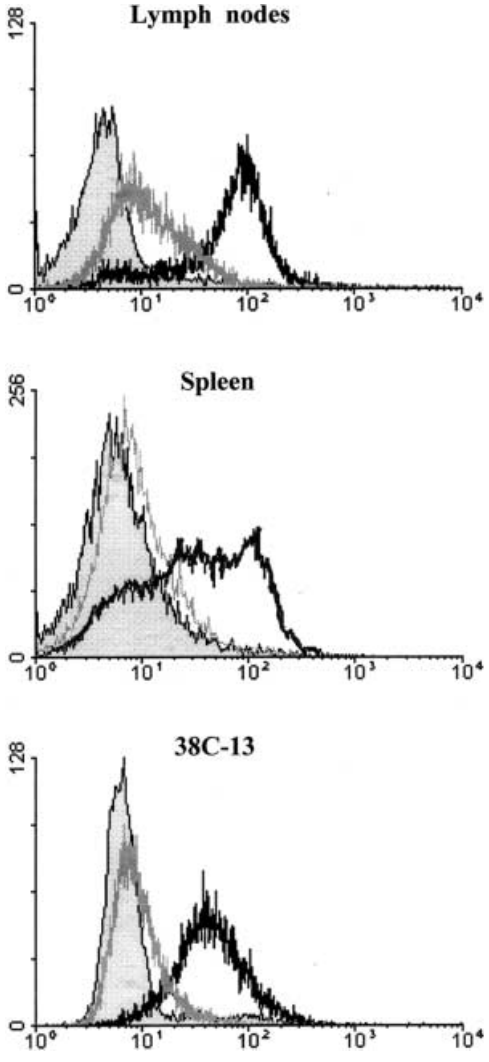

Fig. 4 Down-modulation of L-selectin following stimulation of 38C-13 cells with PMA. 38C-13 cells, lymph node cells and spleen cells were incubated for 1 h at 37 °C with (*grey line*) or without (*bold line*) 25 ng/ml of PMA. Cells were then washed and stained with MEL-14 mAb. Staining with control antibody is depicted by the filled histogram

L-selectin-negative 38C-13 cells disseminate to lymph nodes

Since injection of MEL-14 to tumor-bearing mice did not result in complete antibody saturation on 38C-13 cells (Fig. 3), it could be argued that the failure of MEL-14 to block extravasation of 38C-13 to lymph nodes resulted from insufficient antibody binding, which enabled some unbound L-selectin molecules to function. Therefore, to confirm that L-selectin is not essential for dissemination of 38C-13 to peripheral lymph nodes, we isolated L-selectin-negative variants of 38C-13 cells. These variant cells, which stably lack expression of L-selectin, were inoculated i.p. or s.c., and their dissemination to lymph nodes was determined by the cell proliferation assay. Fig. 5 demonstrates that L-selectin-negative cells disseminated as effectively as L-selectin-positive 38C-13 cells. We verified that tumor cells that invaded lymph nodes retained their L-selectin-negative phenotype and hence could not represent cells that reverted in vivo to express L-selectin (Fig. 6). In addition, we followed the kinetics of tumor infiltration into lymph nodes by staining lymph node cells with anti-idiotype antibodies and determining the percentage of stained cells at different times after tumor inoculation. Fig. 7 shows a similar infiltration pattern for wild-type and variant cells, indicating that kinetics of tumor infiltration, as well as the final tumor load, were similar for wild-type and L-selectin-negative tumor cells. These findings indicate that in spite of its expression in wild-type 38C-13 tumor cells, L-selectin is not required for lymph node metastasis.

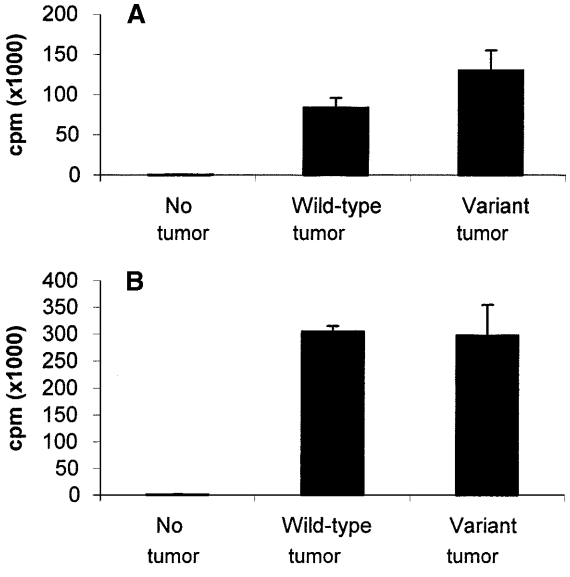

Fig. 5A, B Variant (L-selectin-negative) 38C-13 cells disseminate to lymph nodes as efficiently as wild-type (L-selectin-positive) cells. Mice were inoculated s.c. **(A)** or i.p. **(B)** with either wild-type or variant 38C-13 cells. Lymph nodes were collected on day 18 post tumor inoculation and cell suspensions were tested for thymidine uptake

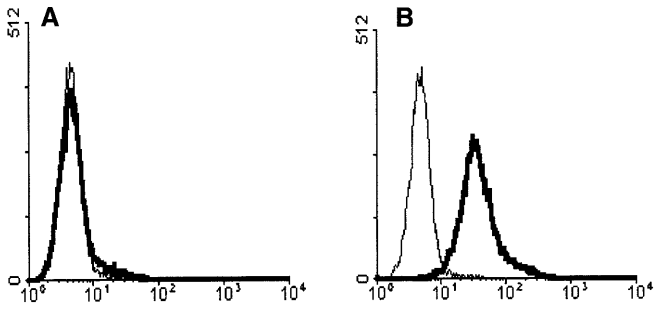

Fig. 6A, B Variant 38C-13 cells invading lymph nodes retain their L-selectin-negative phenotype. Mice were inoculated with L-selectin-negative **(A)** or with L-selectin-positive **(B)** 38C-13 cells. Lymph node cells harvested on day 18 were cultured until a homogeneous culture of 38C-13 cells was obtained. At this stage, cultured cells were stained with MEL-14 mAb (*bold line*) or with control antibody (*thin line*)

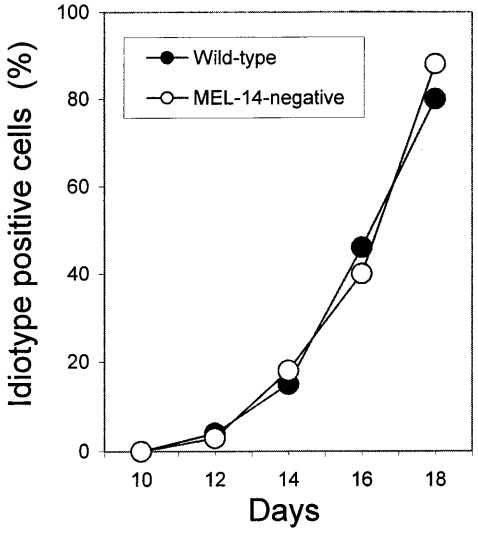

Fig. 7 The kinetics of lymph node metastasis is similar for variant and wild-type 38C-13 cells. L-selectin-negative and L-selectin-positive 38C-13 cells were inoculated into mice. At different time points post tumor inoculation, lymph node cells were stained with anti-idiotype mAb and the percentage of tumor cells in lymph nodes was analyzed

Discussion

The findings of the present study indicate that lymphoma cells with a peripheral node-homing and naive cell phenotype metastasize to lymph nodes by an L-selectin-independent mechanism. It has been suggested that the homing properties of lymphoma cells are related to their differentiation/activation phenotype. For instance, lymph node metastasis of the murine LB lymphoma, which does not express L-selectin, was inhibited by anti-CD44 antibodies and by hyaluronidase [40, 41]. It was therefore proposed that similarly to their normal counterparts, the memory cell-like LB lymphoma cells transmigrate into lymph nodes via afferent lymph in a CD44-dependent manner. Likewise, NQ22 lymphoma cells, which are L-selectin-negative and CD44-negative, do not bind to HEV and have a phenotype characteristic of poorly differentiated thymocytes, which use the $\alpha4\beta7$ integrin for spreading [15, 16]. It has been reported that in vivo administration of MEL-14 did not inhibit peripheral lymph node metastasis in TK-1 tumor-bearing mice [22]. However, since TK-1 tumor cells express undetectable to low levels of L-selectin and bind poorly to peripheral lymph node HEV in vitro [4, 37], they may resemble the above-mentioned lymphomas and hence use an L-selectin-independent lymph node extravasation mechanism. 38C-13 cells represent an immature naive B-cell phenotype [5, 6]. They bind to HEV and express high levels of L-selectin [17]. In fact, they were used as the immunogen for production of the MEL-14 mAb [17]. It was therefore suggested that, similarly to normal recirculating naive B-cells, 38C-13 cells transmigrate to lymph nodes through HEV by an L-selectin-dependent mechanism. It has been recently reported that, in contrast to normal lymphocytes, 38C-13 cells do not enter lymph nodes in significant numbers after i.v. injection [22]. As demonstrated, most of the i.v. injected 38C-13 cells were trapped in lung and liver. Accordingly, only small numbers of 38C-13 cells that remained in the circulation could reach the lymphoid organs. However, it was expected that those 38C-13 cells that reach the vascular bed of lymphoid organs extravasate by an L-selectin-dependent mechanism and grow there to fully developed metastases. Our demonstration that anti-L-selectin antibodies did not block lymph node dissemination of 38C-13 and that L-selectin-negative 38C-13 cells developed lymph node metastases as effectively as wild type 38C-13 cells indicates that L-selectin is not essential for lymph node homing of 38C-13. Thus, L-selectin-expressing lymphoma cells, that bind in vitro to lymph node HEV in an L-selectin-dependent manner, may home to peripheral lymph nodes in an L-selectin-independent mechanism.

The homing receptors that are used by 38C-13 cells for lymph node extravasation are as yet unknown. Our preliminary studies demonstrated that anti-CD44 and anti-LFA-1 antibodies did not inhibit lymph node metastasis of 38C-13. Further studies with CD44-negative and LFA-1-negative 38C-13 variant cells are underway in order to determine whether CD44 or LFA-1 are involved in lymph node spreading of 38C-13. It should be mentioned that the integrins $\alpha4\beta1$ and $\alpha4\beta7$ are not expressed in 38C-13 cells [20, 36] and hence cannot be involved in 38C-13 metastasis. However, although 38C-13 cells do not express the $\beta1$ and the $\beta7$ integrin subunits, they do express membrane-bound $\alpha4$ integrin subunits [20, 36]. It is therefore possible that an $\alpha4$ polypeptide complexed to a different β subunit is involved in dissemination.

It is not clear whether L-selectin is involved to some extent in 38C-13 lymph node extravasation under normal conditions and is replaced by other adhesion

molecules upon its blockade, or whether L-selectin is not involved at all in lymph node dissemination. If the latter is the case, it is not clear why cells that bind in vitro to lymph node HEV by an L-selectin-dependent mechanism do not utilize the same homing receptor for lymph node extravasation in vivo. One possible answer to this question may be inferred from our antibody-blocking studies. Hence, we demonstrated that in contrast to normal lymphocytes, binding of anti-L-selectin to 38C-13 cells did not result in L-selectin down-regulation. Expression of L-selectin can be rapidly down-modulated by regulated proteolysis at a membrane-proximal site [13, 24]. L-selectin cross-linking by mAb has been shown to down-modulate L-selectin surface levels [8, 28, 31, 35]. Similarly, lymphocyte activation by a variety of stimuli induces L-selectin shedding from the cell surface [12, 23, 25, 33, 34]. Moreover, transendothelial migration of leukocytes involves down-regulation of L-selectin [1, 2, 14]. Although there is a debate whether shedding of L-selectin is required for migration of neutrophils across endothelium [2, 39], it appears that L-selectin shedding is a prerequisite for lymphocyte transendothelial migration [1, 2]. Our demonstration that L-selectin is not down-regulated in 38C-13 cells, following antibody cross-linking, suggests that L-selectin may not be shed after the binding of 38C-13 cells to HEV. Hence, in spite of efficient in vitro binding to lymph node HEV, 38C-13 may not migrate across the endothelium due to impaired modulation of the adhesion receptor. Some malignant cells may express L-selectin that is not shed. Thus, it has been reported that L-selectin-positive chronic lymphocytic leukemia (CLL) cells bound to HEV at levels corresponding to the amount of L-selectin expressed on their surface, whereas L-selectin-negative CLL cells did not bind to HEV [14]. However, among L-selectin-positive CLL cells, some did not shed the homing receptor following exposure to PMA, while others shed it in a time course that was slower than in normal lymphocytes, suggesting that the signaling pathway for shedding may be less active in some malignant cells [14]. It therefore appears that, in spite of the correlation between expression of L-selectin and the capacity of malignant cells to bind to HEV, high expression of the homing receptor may not generally predict the capacity of the cells to disseminate into lymph nodes. Dissemination of malignant lymphocytes to HEV-bearing organs by an L-selectin-dependent mechanism requires the expression of a fully active adhesion molecule. L-selectin-expressing malignant lymphocytes, in which the signaling pathways for shedding or for other functional requirements are not fully active, may not disseminate effectively or may use alternative adhesion molecules for dissemination.

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