

The adhesion molecule L1 regulates transendothelial migration and trafficking of dendritic cells

Luigi Maddaluno,^{1,3} Sue Ellen Verbrugge,^{1,3} Chiara Martinoli,^{2,3} Gianluca Matteoli,^{2,3} Andrea Chiavelli,^{2,3} Yiping Zeng,⁴ Elizabeth D. Williams,⁴ Maria Rescigno,^{2,3} and Ugo Cavallaro^{1,3}

¹The FIRC Institute of Molecular Oncology (IFOM), 20139 Milan, Italy

²Department of Experimental Oncology, European Institute of Oncology, 20141 Milan, Italy

³IFOM-IEO Campus, 20139 Milan, Italy

⁴Centre for Cancer Research, Monash Institute for Medical Research, Monash University, Victoria 3800, Australia

The adhesion molecule L1, which is extensively characterized in the nervous system, is also expressed in dendritic cells (DCs), but its function there has remained elusive. To address this issue, we ablated L1 expression in DCs of conditional knockout mice. L1-deficient DCs were impaired in adhesion to and transmigration through monolayers of either lymphatic or blood vessel endothelial cells, implicating L1 in transendothelial migration of DCs. In agreement with these findings, L1 was expressed in cutaneous DCs that migrated to draining lymph nodes, and its ablation reduced DC trafficking in vivo. Within the skin, L1 was found in Langerhans cells but not in dermal DCs, and L1 deficiency impaired Langerhans cell migration. Under inflammatory conditions, L1 also became expressed in vascular endothelium and enhanced transmigration of DCs, likely through L1 homophilic interactions. Our results implicate L1 in the regulation of DC trafficking and shed light on novel mechanisms underlying transendothelial migration of DCs. These observations might offer novel therapeutic perspectives for the treatment of certain immunological disorders.

CORRESPONDENCE

Ugo Cavallaro:
ugo.cavallaro@
ifom-ieo-campus.it

Abbreviations used: CAM, cell adhesion molecule; cDNA, complementary DNA; CHS, contact hypersensitivity; EC, endothelial cell; HA, hemagglutinin; HU-VEC, human umbilical vein EC; LEC, lymphatic EC; moDC, monocyte-derived DC.

L1 (also known as L1CAM or CD171) is a transmembrane glycoprotein belonging to the Ig superfamily of cell adhesion molecules (CAMs [Ig-CAMs]), which mediate calcium-independent cell–cell adhesion. The L1 gene is located on the X chromosome in human, mouse, and rat. The extracellular portion of the protein contains six Ig-like domains and five fibronectin type III repeats, followed by a transmembrane region and a cytoplasmic domain (1). L1 has long been characterized as a cell recognition molecule within the nervous system, where it is involved in neurite fasciculation, synaptogenesis, axonal growth and path finding, and cell migration. In humans, mutations in the L1 gene cause abnormal brain development, which is characterized by mental retardation and defects in the central nervous system (2). These neurological alterations were, at least in part, recapitulated in mice where the L1 gene was disrupted (3, 4).

L1-dependent cell–cell adhesion is mediated by the homophilic binding between L1 molecules

located on adjacent cells. However, L1 also engages in heterophilic interactions with different molecular partners, including other Ig-CAMs, integrins, and growth factor receptors. These interactions, together with the association of its cytoplasmic tail with a broad spectrum of intracellular partners, endow L1 with the signal-transducing properties that underlie its neural activities (1).

Besides the nervous system, L1 expression has been reported in various normal tissues, ranging from some epithelia to certain lineages of the hematopoietic system, as well as in several tumor types. In these nonneuronal tissues, however, L1 function is still poorly understood. Within the hematopoietic system, L1 has been detected in cells of myelomonocytic and lymphoid origin such as lymphocytes and DCs (5).

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DCs play a key role in the activation of specific immunity, and their trafficking to secondary lymphoid organs is crucial for this function. Indeed, upon microbial contact and stimulation by inflammatory cytokines DCs take up antigens and migrate from peripheral tissues, via the afferent lymphatics, into the T cell area of the draining lymph node where they present the antigens to T lymphocytes, thus triggering the immune response. The migration of DCs into and out of tissues depends on a cascade of discrete events including the induction of chemokines, the activation of chemokine receptors, and the regulation of adhesion molecules. In particular, transendothelial migration is of paramount importance during DC-induced immune response because DCs need to cross both the blood vessel wall, to move from the bloodstream to the peripheral tissue, and the lymphatic endothelium, to reach the lymph nodes via the lymphatic circulation (6). Based on these considerations and on the reported role of L1 in cellular motility and in intercellular recognition, we investigated the involvement of L1 in DC function and, in particular, in the transmigration of DCs across the endothelium.

To this goal, we generated conditional knockout mice in which L1 expression was ablated in the hematopoietic precursors as well as in endothelial cells (ECs). L1-deficient DCs derived from these mice were impaired in both adhesion to the endothelium and in transendothelial migration. Moreover, DC migration to afferent lymph nodes upon contact sensitization was also defective in conditional L1 knockout mice, likely also involving endothelial L1. Thus, we have provided evidence that highlights the important role of L1 in DC trafficking, which may open novel therapeutic perspectives for the treatment of immune disorders.

RESULTS

Generation of conditional L1 knockout mice and characterization of DCs

L1 has been detected in human DCs (5). To investigate whether mouse DCs also express L1, we collected lymph node cells from C57BL/6 mice and determined L1 expression in CD11c⁺ cells. Approximately 55% of DCs were found to be positive for L1 (Fig. 1 A). The analysis of DC subpopulations showed L1 expression in 45% of CD4⁺, 40% of CD8⁺, and 40% of B220⁺ DCs, whereas 85% Langerhans cells were positive for L1 (Fig. S1 A). Similar results were obtained in DCs isolated from the spleen (Fig. S1 B). The widespread expression of L1 in Langerhans cells was also confirmed in the epidermis (see fourth paragraph).

To gain insight into the role of L1 in DC function, we first undertook a genetic approach in mice. The tyrosine kinase receptor Tie2 is expressed in early precursors of hematopoietic and ECs (7). Hence, transgenic mice expressing Cre recombinase under the control of the *Tie2* gene promoter (8) were intercrossed with *L1*^{flaxed} mice carrying two floxed alleles of the *L1cam* gene (9). The genotype of the mice was determined by PCR on genomic DNA (Fig. S2, A and B). Because the *L1cam* gene maps on chromosome X (and, therefore, only one copy is present in male genome), Cre-mediated

recombination was expected to be more efficient in L1-floxed males. Hence, only Tie2-Cre-positive males carrying the floxed *L1cam* allele (referred to as *Tie2-Cre;L1*^{flaxed} mice) were used throughout the study.

The correct function of the Tie2-Cre transgene was verified by Cre immunoblotting analysis on undifferentiated bone marrow precursors (Fig. S2 C, top). These precursors showed no expression of L1 at this stage (Fig. S2 C, bottom). The ablation of L1 in Tie2⁺ hematopoietic progenitors did not cause major defects in mouse hematopoiesis, as blood cell counts for erythrocytes and the different leukocyte populations gave very similar values for both *L1*^{flaxed} and *Tie2-Cre;L1*^{flaxed} littermates (unpublished data). In addition, the loss of L1 did not affect the cellular composition of mouse lymph nodes (Fig. S2 E), including the relative amounts of DC subpopulations (Fig. S2 F). Finally, although Tie2-Cre mice have been used to target genes expressed in ECs (8) and L1 expression in the vessels has been reported under pathological conditions (10, 11), no gross vascular defects were noted in *Tie2-Cre;L1*^{flaxed} mice.

To investigate the role of L1 in DCs, bone marrow precursors were cultured in the presence of GM-CSF, a classical inducer of DC differentiation. This treatment yielded a nearly pure population of CD11c⁺ cells (Fig. 1 B), confirming their differentiation into DCs. Bone marrow-derived DCs isolated from *L1*^{flaxed} mice exhibited high levels of L1, which is detectable by both FACS and immunoblotting analysis (Fig. 1 B, top; and Fig. S2 D). In contrast, L1 was not detected in DCs derived from the bone marrow of *Tie2-Cre;L1*^{flaxed} mice (Fig. 1 B, bottom; and Fig. S2 D), indicating that Tie2 promoter-driven expression of Cre recombinase results in the ablation of L1 in this cell type.

The role of L1 in DC adhesion to endothelium and transendothelial migration

To study the role of L1 in DC biology, we first asked whether this adhesion molecule is involved in the maturation of DCs. Bone marrow-derived DCs were stimulated with LPS and then the expression of classical activation markers was analyzed. The loss of L1 did not affect LPS-induced up-regulation of CD86 (Fig. S3 A), CD80, and MHC class II (not depicted), indicating that DC maturation is not influenced by L1. In addition, the level of L1 was not affected by LPS stimulation of DCs (Fig. S3 B).

Next, we investigated whether L1 is involved in the interaction of DCs with the lymphatic vessel endothelium, a key process in DC trafficking to lymphoid organs (12). To this goal, DCs derived from *L1*^{flaxed} or *Tie2-Cre;L1*^{flaxed} bone marrows were subjected to adhesion assays on monolayers of lymphatic ECs (LECs). Two mouse LEC lines were used, MELCs (13) and SV-LECs (14). In both cases, *Tie2-Cre;L1*^{flaxed} DCs exhibited a lower adhesion capacity to lymphatic endothelium as compared with DCs from control *L1*^{flaxed} mice (Fig. 2, A and B). Furthermore, L1-positive DCs spread and extended cellular protrusions upon adhesion to LECs, whereas *Tie2-Cre;L1*^{flaxed} DCs retained a round morphology

(Fig. 2 B, inset). The stronger adhesion of L1-expressing bone marrow-derived DCs was not the result of an L1-dependent regulation of $\beta 2$ integrins because no difference in $\beta 2$ expression was observed between $L1^{flox}$ and $Tie2-Cre;L1^{flox}$ DCs and the two cell populations adhered to purified ICAM-2 (a major $\beta 2$ ligand) with similar efficiency (unpublished data). The role of L1 in the interaction of DCs with the lymphatic endothelium was also assessed using DCs freshly isolated from lymph nodes. In this case, $L1^+$ and $L1^-$ DCs were separated by FACS sorting and labeled with different dyes before adhesion assays on SV-LEC monolayers. As shown in Fig. 2 C, lymph node-derived $L1^+$ DCs adhered twice more efficiently than $L1^-$ cells to the lymphatic endothelium, confirming the results obtained with bone marrow-derived DCs. $L1^-$ DCs isolated from the lymph nodes of $Tie2-Cre;L1^{flox}$ mice showed an adhesion rate to lymphatic endothelium comparable to that of $L1^-$ DCs from $L1^{flox}$ mice (unpublished data). These results supported the notion that L1 is required for DC-LEC interaction.

We next asked whether the loss of L1 also affected the migration of DCs across a lymphatic endothelial barrier. Both basal-to-apical and apical-to-basal directions were tested to mimic intra- and extravasation of DCs, respectively. By analogy to cell adhesion, the migration rate of L1-deficient DCs through a lymphatic endothelial monolayer was markedly lower than that of control cells (Fig. 3 A). L1 was required for both apical-to-basal and basal-to-apical DC transmigration (Fig. 3 A, left and middle). Moreover, because transendothelial migration of DCs also occurs across the wall of blood vessels (12), we included blood vascular ECs in our transmigration assays, using the mouse EC line 1G11 (15). As in the case of LECs, the loss of L1 resulted in the impairment of DC migration through 1G11 monolayers (Fig. 3 A, right), implicating L1 in the trafficking of DCs across both lymphatic and blood vessel walls. Very similar results were obtained when the transendothelial migration of either immature or mature DCs across lymphatic or blood vessel ECs was stimulated by the chemokines CCL3 or CCL19, respectively (Fig. S4 A). Notably, L1 deficiency by

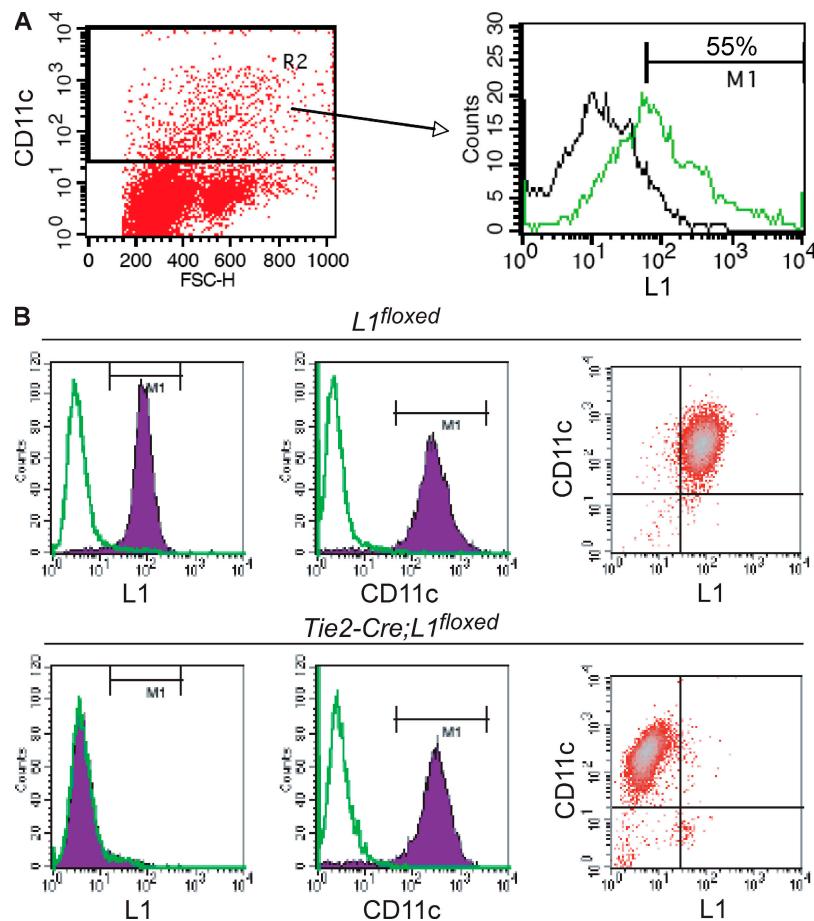


Figure 1. Ablation of L1 in DCs from *Tie2-Cre;L1^{flox}* mice. (A) CD11c-positive cells from the lymph nodes of C57BL/6 mice (left) were gated and analyzed for L1 expression (right). Background staining was determined with a control isotype-matched antibody (black line). The experiment was repeated with lymph nodes from five individual mice with similar results, and the figure refers to one representative analysis of one mouse. (B) FACS analysis of CD11c and L1 coexpression in bone marrow-derived DCs from *L1^{flox}* and *Tie2-Cre;L1^{flox}* mice. The experiment was repeated with similar results on four individual mice for each genotype, and the figure refers to one representative analysis.

itself did not affect the migratory ability of DCs, as neither the chemotactic migration toward the CCL3 or CCL19 chemokines (Fig. S4 B) nor the motility of DCs within three-dimensional collagen type I matrix (not depicted) were affected in *Tie2-Cre;L1^{flxed}* DCs. This argued against a cell autonomous effect of L1 on DC motility and further supported its specific involvement in DC–EC interactions.

The difference between L1-proficient and deficient DCs in adhesion to and migration through endothelial barriers was

confirmed with DCs obtained from at least five mice for each genotype. Moreover, each experiment was performed comparing DCs isolated from *L1^{flxed}* and *Tie2-Cre;L1^{flxed}* littermates to rule out the effect of genetic variability. Nevertheless, to validate these observations in an isogenic model, DCs derived from *L1^{flxed}* mice were transduced with the Tat-Cre fusion protein, which is known to promote the nuclear translocation of Cre recombinase (16). As a control, cells were treated either with buffer or with an inactive form of Tat-Cre

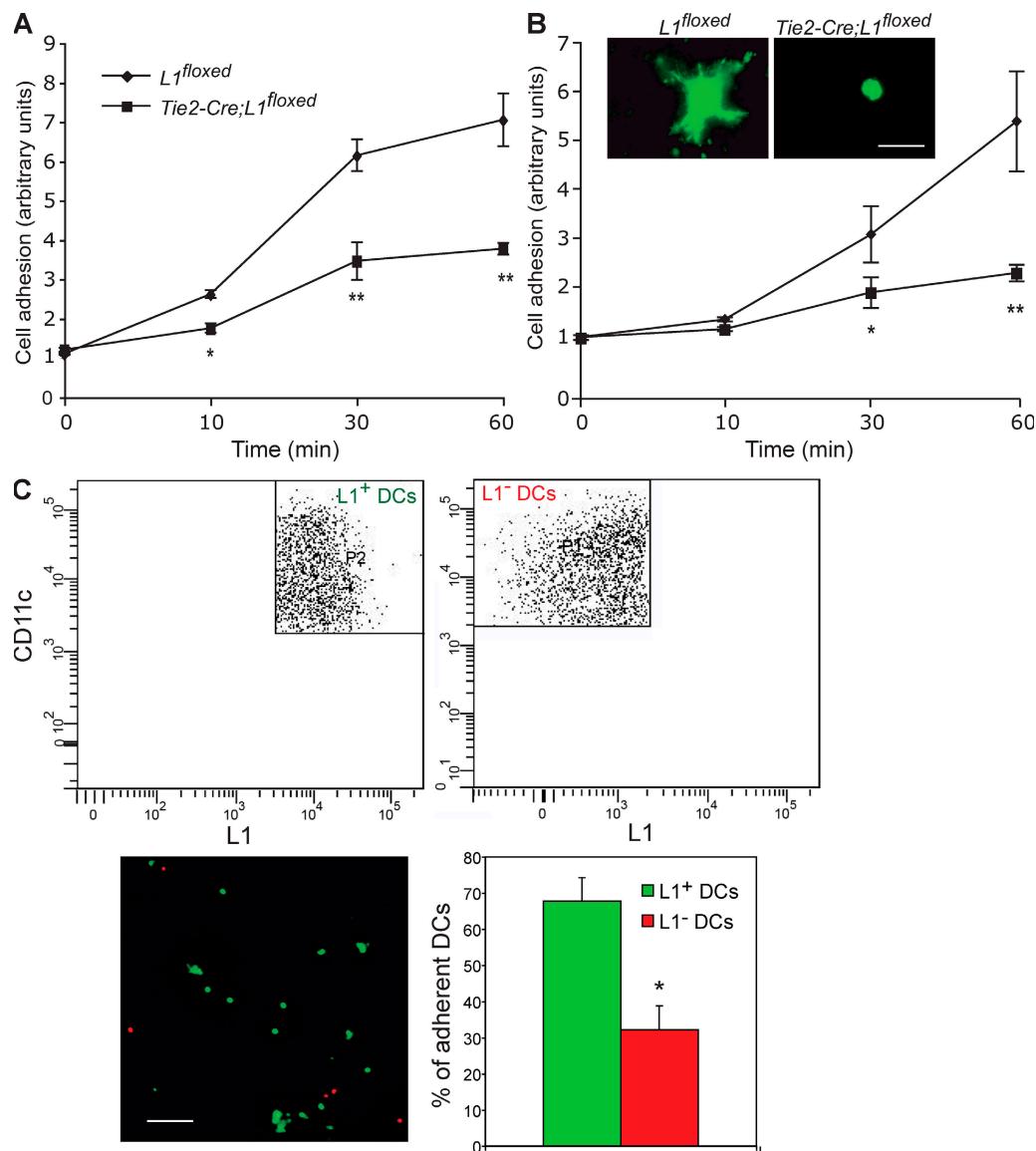


Figure 2. L1 regulates the adhesion of DCs to endothelium. (A and B) CFSE-labeled bone marrow-derived DCs from *L1^{flxed}* and *Tie2-Cre;L1^{flxed}* mice were seeded on TNF- α -stimulated MELC (A) or SV-LEC (B) monolayers and allowed to adhere for the indicated time lengths. After washing and fixation, cell adhesion was measured as described in Materials and methods. Data represent the means \pm SD of a single representative experiment performed in triplicate. The experiment was independently repeated five times, each time using DCs from different mice. The insets in B show the morphology of DCs seeded on SV-LEC monolayers. Bar, 10 μ m. *, $P < 0.05$; **, $P < 0.005$ (relative to *L1^{flxed}* DCs). (C) Mouse inguinal lymph node cells were enriched for CD11c $^{+}$ cells and then FACS sorted into CD11c $^{+}$ /L1 $^{+}$ and CD11c $^{+}$ /L1 $^{-}$ DCs (top, postsorting cell populations), which were then labeled with CFSE (green) and PKH26 (red), respectively, before adhesion assays on TNF- α -stimulated SV-LEC monolayers (bottom left, example of DC adhesion; bar, 30 μ m). Data in the bottom right represent the means \pm SD from three independent experiments, each performed with lymph nodes from three mice. *, $P < 0.05$ (relative to L1-positive DCs).

(unpublished data). The transduction of *L1*^{flaxed} DCs with Tat-Cre resulted in the almost complete ablation of L1 expression (Fig. S5 A). This, in turn, caused a dramatic decrease in both apical-to-basal and basal-to-apical transendothelial migration of DCs (Fig. S5 B), thus confirming that the loss of L1 impairs this process. Collectively, these observations point to L1 as an important player in the endothelial adhesion and transendothelial migration of DCs.

The role of L1 in DC trafficking in vivo

Our in vitro data on the role of L1 in the interaction of DCs with the endothelium might reflect an involvement of this adhesion molecule in the vascular trafficking of DCs

in vivo. To address this issue, we performed a series of FITC skin painting assays, in which FITC was applied on the skin of *L1*^{flaxed} or *Tie2-Cre;L1*^{flaxed} mice, and then the uptake of FITC by cutaneous DCs and their trafficking to draining lymph nodes was determined by FACS analysis. As shown in Fig. 3 B, the number of FITC⁺/CD11c⁺ cells in the lymph nodes of *Tie2-Cre;L1*^{flaxed} mice was markedly lower than in their *L1*^{flaxed} littermates. More than 85% of FITC⁺/CD11c⁺ DCs detected in the lymph nodes of *L1*^{flaxed} mice were positive for L1 (Fig. 3 C), confirming that this molecule is expressed in skin migratory DCs. Collectively, these results indicate that L1 is required for the trafficking of DCs in vivo.

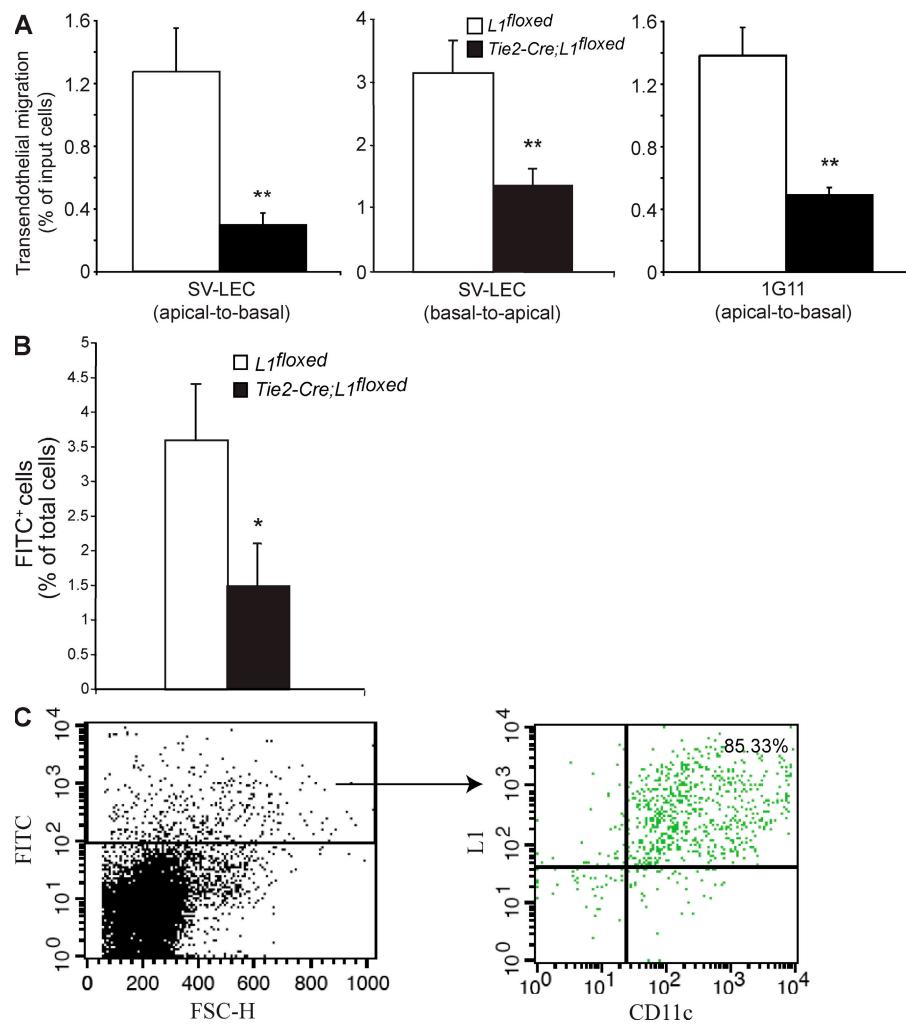


Figure 3. L1 is required for DC transendothelial migration and trafficking to lymph nodes. (A) For apical-to-basal transmigration assays, SV-LEC (left) or 1G11 cells (right) were seeded on the upper side of gelatin-coated Transwell filters and allowed to form dense monolayers. For basal-to-apical migration assays (middle), SV-LECs were cultured on the bottom side of the filters. ECs were pretreated with TNF- α before transmigration assays. CFSE-labeled bone marrow-derived DCs from *L1*^{flaxed} and *Tie2-Cre;L1*^{flaxed} mice were added to the upper chamber of Transwell inserts. After 3 h, DC transmigration was measured as described in Materials and methods. Data represent the means \pm SD of representative experiments performed in triplicate with DCs from five mice for each genotype. *, P < 0.05; **, P < 0.005 (relative to *L1*^{flaxed} DCs). (B) FITC skin painting was performed on the abdomen of *L1*^{flaxed} or *Tie2-Cre;L1*^{flaxed} mice. After 24 h, inguinal lymph nodes were excised and subjected to FACS analysis for FITC and CD11c. Data are expressed as the percentage of FITC-positive cells and represent the means \pm SD of a representative experiment (six mice per group) out of three performed. *, P < 0.05 (relative to *L1*^{flaxed} mice). (C) FITC-positive cells in inguinal lymph nodes (left) were gated and analyzed for L1 and CD11c expression (right).

The role of L1 in Langerhans cell trafficking

To investigate whether the loss of L1 affects DC-dependent immune response, we focused on skin immunity and performed contact hypersensitization assays. However, no difference was observed between *L1*^{flaxed} and *Tie2-Cre;L1*^{flaxed} mice (Fig. 4 A). Because skin immunity has been proposed to implicate dermal DCs rather than Langerhans cells (17, 18), we asked whether L1 expression is restricted to specific subtypes of cutaneous DCs. The costaining of mouse and human skin tissues for L1, CD11c, and the Langerhans cell–specific marker Langerin revealed that L1 is specifically expressed in Langerhans cells but not in dermal DC (Fig. 4 B and Fig. S6). The staining of epidermal sheets confirmed that L1 expression is a general feature of Langerhans cells (Fig. S7). The absence of L1 in dermal DCs provided a possible explanation for the unaffected contact hypersensitivity (CHS) in *Tie2-Cre;L1*^{flaxed} mice. Indeed, when fluorescent latex beads were injected into mouse derma to track the migration of dermal DCs or of infiltrating monocytes to draining lymph nodes (19), we found no difference in DC migration in the presence or absence of L1 (Fig. S4 C). These observations pointed to L1-expressing Langerhans cells as the most prominent DC

type that migrates to lymph nodes upon skin painting. This hypothesis was confirmed by a FACS analysis on the lymph nodes of *L1*^{flaxed} mice subjected to TRITC skin painting assays, which revealed that almost 100% of TRITC⁺/CD11c⁺ cells are Langerhans cells and that ~97% of TRITC⁺ cells coexpress langerin and L1 (Fig. 4 C, left). When the same analysis was performed in *Tie2-Cre;L1*^{flaxed} mice, we observed a dramatic decrease (about fourfold) in TRITC⁺ Langerhans cells in the lymph nodes (Fig. 4 C, right), which correlated with the efficiency of Cre-mediated ablation of L1 (not depicted). This is exemplified in Fig. 4 C (right), where, of the residual TRITC⁺ cells that migrated to the lymph nodes, the majority expressed L1. Previous studies using TRITC skin painting assays reported that, besides Langerhans cells, dermal DCs also migrate to the lymph nodes (18, 20), an event which was not observed under our experimental conditions. Although the reason for such a discrepancy remains unclear, it may depend on the different genetic background of the mice used in those studies (129/SV;BALB/c) as compared with ours (C57BL/6). Our data further support the notion that L1 is critical for Langerhans cell trafficking. The reduction in Langerhans cell migration was not caused by a lower

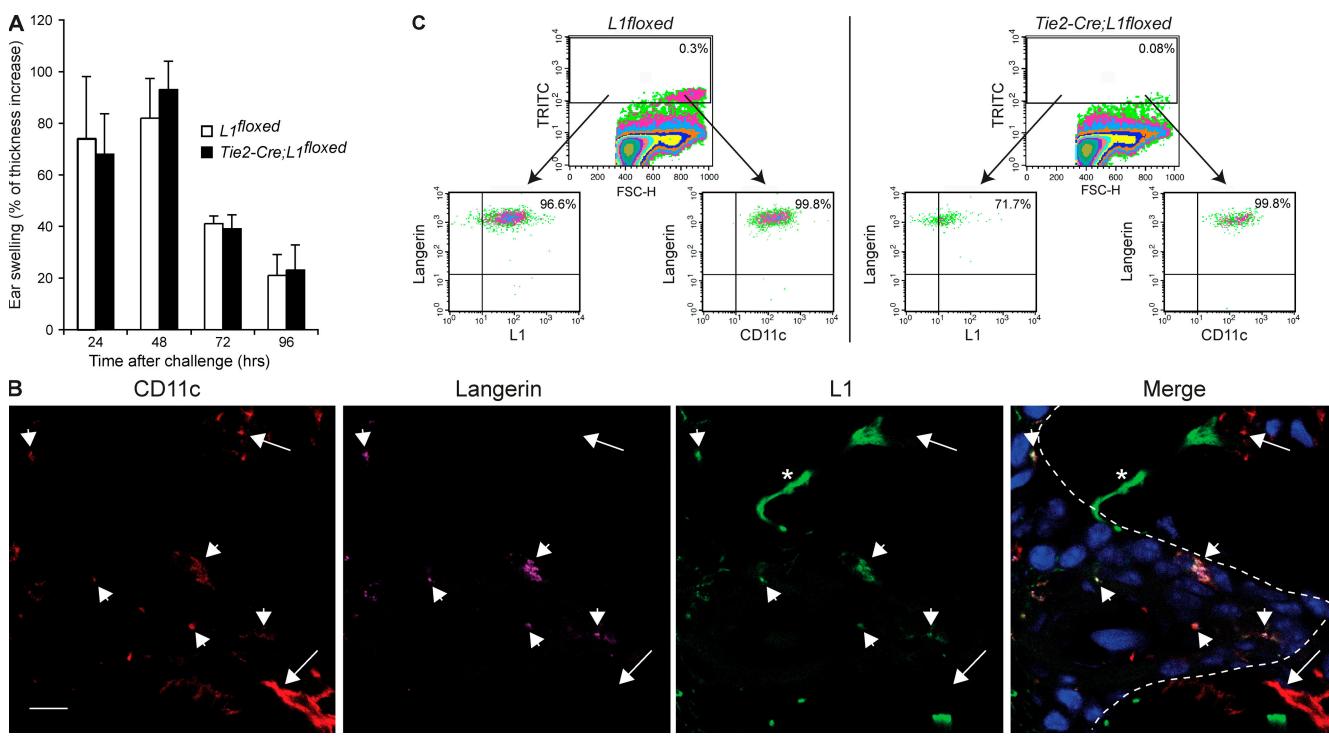


Figure 4. L1 is not required for CHS but is involved in Langerhans cell trafficking. (A) CHS was determined in *L1*^{flaxed} or *Tie2-Cre;L1*^{flaxed} mice by ear-swelling assay at different time points (six mice per genotype), as described in Material and methods. Error bars show the SD among the six individual mice of the same genotype. (B) Skin tissue sections from C57BL/6 mice were subjected to immunofluorescence triple staining for CD11c, Langerin, and L1, followed by confocal analysis. The images were taken from a single confocal plane. The dashed line indicates the boundary between epidermis (left) and dermis (right). Arrowheads indicate CD11c⁺/Langerin⁺ Langerhans cells that express L1 and arrows indicate CD11c⁺/Langerin⁻ dermal DCs that do not express L1. Asterisks indicate an L1-positive nerve that served as internal control. DAPI staining (right) was used to visualize nuclei. Bar, 10 μ m. (C) *L1*^{flaxed} (left) or *Tie2-Cre;L1*^{flaxed} (right) mice were subjected to TRITC skin painting, followed by excision of inguinal lymph nodes after 48 h and FACS analysis for CD11c, langerin, and L1 on TRITC⁺-gated cells. Four mice were analyzed individually for each genotype, giving similar results, and the figure refers to a representative analysis of one mouse per genotype.

number of Langerhans cells in the skin of *Tie2-Cre;L1^{fl/fl}* mice because we found no difference in the distribution and density of Langerhans cells and dermal DCs between *L1^{fl/fl}* and *Tie2-Cre;L1^{fl/fl}* mice (unpublished data). Overall, these results indicate that in the skin Langerhans cells are the only DC type expressing L1 and are affected by L1 deficiency in their ability to migrate in vivo. It is noteworthy, however, that the skin painting procedure induced L1 expression in skin vessels (unpublished data). Thus, vascular L1 could contribute to Langerhans cell migration, accounting to some extent for the defect observed in *Tie2-Cre;L1^{fl/fl}* mice, where L1 is also ablated in the endothelium.

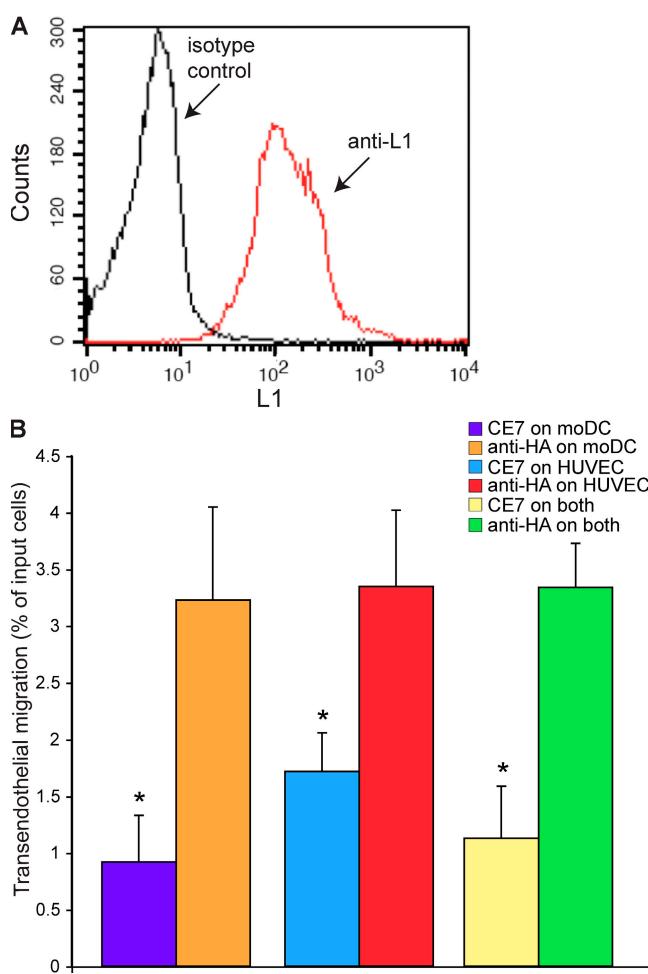


Figure 5. L1 function is required for transendothelial migration of human DCs. (A) The expression of L1 in human moDCs was assessed by FACS analysis. (B) CFSE-labeled moDCs were subjected to transmigration assays through HUVEC monolayers (see Materials and methods) for 2 h. moDCs, HUVECs, or both cell types were pretreated with 30 μ g/ml of anti-L1 CE7 monoclonal antibody or a control isotype-matched anti-hemagglutinin (HA) antibody before transmigration assays. Data represent the means \pm SD of a representative experiment performed in triplicate. The experiment was independently repeated three times. *, P < 0.005 (relative to cells treated with anti-HA antibody).

The role of L1 in the transendothelial migration of human DC

Given the difference between human and murine immune systems, we asked whether L1 is also involved in the transendothelial migration of human DCs. To this goal, we used human monocyte-derived DCs (moDCs), which express moderate levels of L1 (Fig. 5 A; reference 5). The maturation of human moDCs was not accompanied by changes in L1 levels (not depicted), confirming our observations on mouse bone marrow-derived DCs (Fig. S3 B). To evaluate the role of L1 in transendothelial migration, CFSE-labeled moDCs were pretreated with CE7, a monoclonal antibody that has been previously shown to neutralize L1 function (21), and then allowed to cross a monolayer of TNF- α -activated human umbilical vein ECs (HUVECs). The inactivation of L1 in moDCs with CE7 resulted in a dramatic reduction of the transendothelial migration as compared with moDCs treated with an irrelevant antibody (Fig. 5 B). Given the expression of L1 in activated ECs (10) as well as in TNF- α -treated HUVECs (see Fig. 7 A), we also assessed the contribution of vascular L1 to DC transendothelial migration by pretreating HUVECs with CE7 before transmigration assays. The inactivation of endothelial L1 caused a reduction in the transmigratory activity of moDCs (Fig. 5 B). Finally, when L1 was neutralized in both DCs and HUVECs, no additive effect was observed as compared with the inactivation in the individual cell types (Fig. 5 B). Notably, CE7 had no effect on chemokine-induced migration of moDCs (not depicted), which is in line with the results on L1-deficient mouse DCs (Fig. S4 B). Thus, L1 function is required for the trafficking of human DCs through an endothelial barrier.

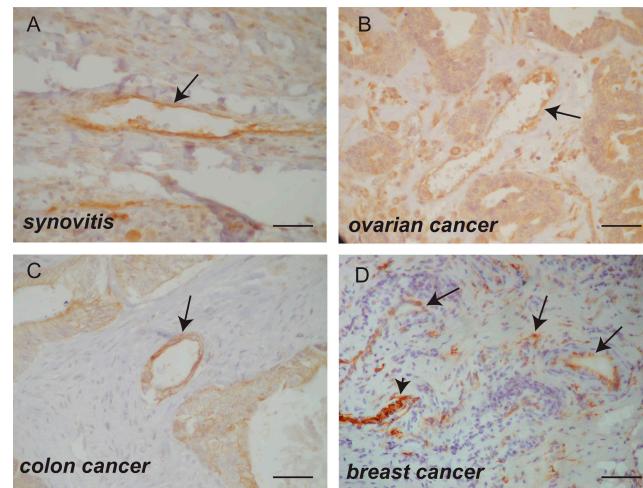


Figure 6. L1 expression in pathological vessels. Tissue sections from human synovitis, ovarian, colon, or breast carcinoma were subjected to immunohistochemical staining for L1. Arrows indicate L1 staining associated with the vessel wall, whereas the arrowhead in D indicates an L1-positive nerve that served as the internal control. The staining was performed on sections from at least four independent patients for each disease. Bars, 50 μ m.

L1 homophilic binding in DC–endothelium interaction

The results on the transmigration of moDCs through HUVEC monolayers appeared to implicate the homophilic interaction of L1 on DC surface with L1 expressed on ECs. To test this hypothesis, we first determined whether ECs express L1. Immunofluorescence and immunoblotting experiments revealed the presence of L1 in several primary cell populations isolated from the endothelium of lymphatic and blood vessels derived from different human and murine organs, as well as in established EC lines (Fig. S8, A and B). We also investigated the endothelial expression of L1 *in vivo* by immunohistochemistry. L1 was absent from normal quiescent vasculature (not depicted), but it was detected on the vessels associated to pathological conditions such as neoplastic or inflammatory diseases (Fig. 6), confirming and extending previous observations (10, 22). Because this suggested that the expression of L1 is regulated by tumor- or inflammatory cell-derived fac-

tors, we treated ECs with inflammatory cytokines, followed by the assay for L1 expression. Although LPS, IL-1 β , and IL-3 had no effect on L1 levels in HUVEC and 1G11 cells (not depicted), a marked up-regulation was induced by TNF- α , as observed both by FACS (Fig. 7, A and B) and by quantitative RT-PCR (Fig. 7 C), the latter implying a regulation at the messenger RNA level. To verify whether TNF- α induced the expression of vascular L1 also *in vivo*, the cytokine was injected subcutaneously into mice, followed by costaining of skin sections for PECAM-1 and L1. Although no L1 was detected in the vessels of control mice (Fig. 7, D–F), high levels of L1 were found in PECAM-1-positive endothelium of TNF- α -treated mice (Fig. 7, G–I), confirming this inflammatory cytokine as a strong inducer of L1 expression in the vasculature. Costaining for PECAM-1 and the lymphatic vessel-specific marker LYVE-1 (23) revealed that TNF- α -induced expression of L1 occurred in both blood and lymphatic

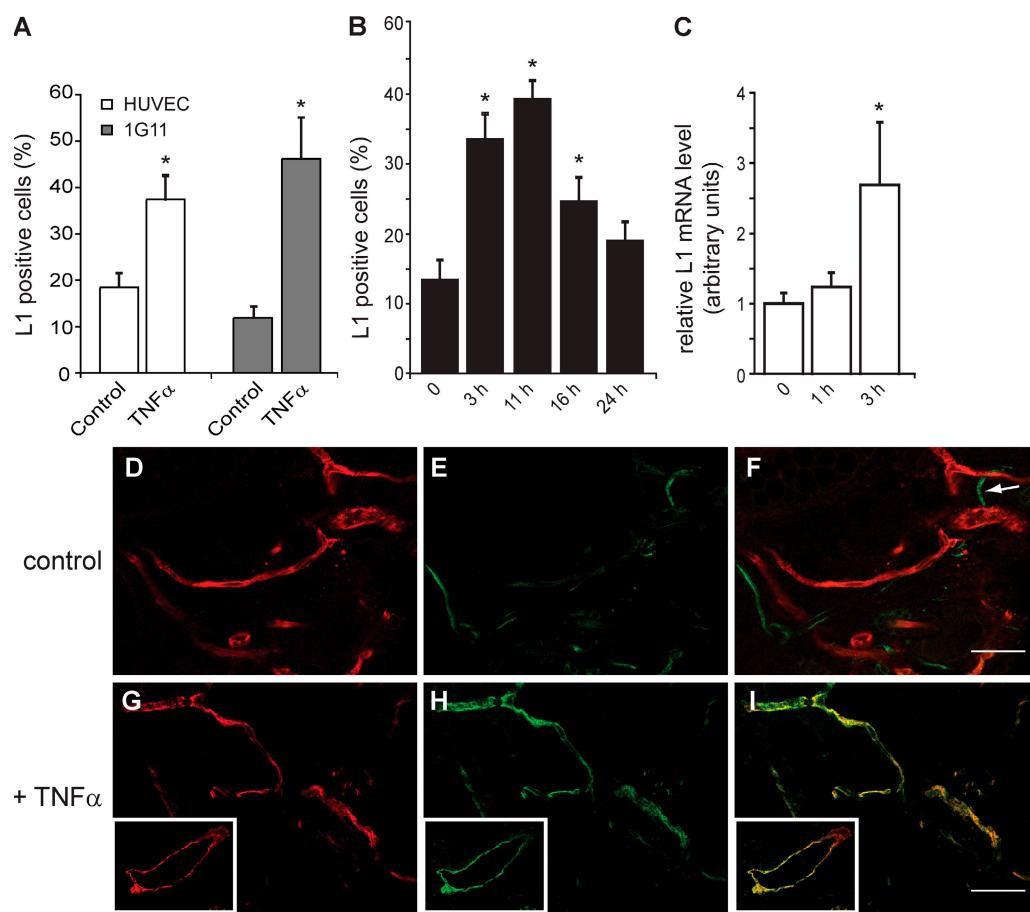


Figure 7. TNF- α induces L1 expression in endothelium. (A) HUVEC or 1G11 cells were starved of serum and endothelial growth factors and then treated with 20 ng/ml TNF- α for 3 h, followed by FACS analysis for L1 expression. (B) HUVEC were treated with 20 ng/ml TNF- α for the indicated time lengths, followed by FACS analysis for L1 expression. The data refer to the percentage of L1-positive cells in a representative experiment. Each experiment was repeated three times with similar results. (C) HUVECs were treated with 20 ng/ml TNF- α for the indicated time lengths before isolation of RNA and quantitative RT-PCR analysis for L1 expression. Data represent the means \pm SEM of three experiments performed. *, P < 0.05 (relative to untreated cells). (D–I) C57BL/6 mice (three mice per group) were subjected to subcutaneous injection of 100 μ l of either vehicle (D–F) or 40 ng/ml TNF- α (G–I) and sacrificed after 16 h. Skin fragments from the injection sites were fixed and costained for PECAM-1 (red) and L1 (green) before confocal analysis. Insets show a blood vessel cross section with the ECs positive for both PECAM-1 and L1. The arrow in F indicates an L1-positive nerve that served as an internal control. Bars, 40 μ m.

endothelium (Fig. S8 C). These findings provided the rationale for testing whether L1 homophilic interactions accounted for the adhesion of DCs to the endothelium. Indeed, L1-expressing DCs, both from murine bone marrow and from human monocytes, adhered to gelatin supplemented with the extracellular portion of L1 much more efficiently than to L1-free gelatin (Fig. S9 A). In contrast, L1-deficient DCs failed to adhere to L1-containing substrates (Fig. S9 A). In an attempt to mimic the inflammation-associated induction of endothelial L1 and to recapitulate L1 homophilic binding during DC–endothelium interaction, 1G11 ECs were transduced with L1 complementary DNA (cDNA) or with an empty vector (Fig. S9 B) and then used for transendothelial migration assays with *L1^{flaxed}* or *Tie2-Cre;L1^{flaxed}* DCs. Notably, control *L1^{flaxed}* DCs exhibited a significantly higher rate of transmigration across L1-expressing 1G11 monolayers than mock-transduced cells. In contrast, the forced expression of L1 in 1G11 cells had no effect on the transendothelial migration of *Tie2-Cre;L1^{flaxed}* DCs (Fig. S9 C). Collectively, these findings support the notion that the interaction between DCs and ECs implicates the homophilic binding of L1 molecules expressed on the two cell types.

DISCUSSION

The contribution of L1 to various developmental processes in the nervous system has long been known. Recent studies have also implicated L1 in the aggressiveness of different tumor types of nonneuronal origin such as colon cancer (24), melanoma (25), and ovarian carcinoma (21, 26). However, the functional role of L1 in normal tissues outside the nervous system has remained elusive. With regard to DCs, previous work has shown the expression of L1 in this cell type (5) and its contribution to DC-dependent activation of T cells (27). In the present study, we addressed the role of L1 in DCs in greater detail by combining a genetic approach in mice with antibody-mediated neutralization in human DCs. Our results highlighted a novel function of L1 in promoting DC trafficking both *in vitro* and *in vivo*. In particular, L1 is involved in the adhesion of DCs to the endothelium and in their transmigration through endothelial barriers. Interestingly, L1 mediates both apical-to-basal and basal-to-apical transendothelial migration of DCs, suggesting an involvement in both extra- and intravasation, respectively. However, the L1-dependent migration of DCs was not a universal characteristic of skin DCs, and only L1-deficient Langerhans cells were affected in their migratory properties *in vivo*. The functional significance of this property remains to be elucidated.

L1-expressing DCs were able to cross an L1-negative endothelium (Fig. S9 C), likely implicating heterophilic interactions with different partners on EC surface. However, the forced expression of L1 in ECs results in a marked enhancement of DC transmigration (Fig. S9 C), which is consistent with the hypothesis that, during inflammation, the induction of L1 expression in vessels (e.g., triggered by TNF- α) potentiates the transendothelial trafficking of DCs. Although the

heterophilic binding partners of L1 involved in DC–EC interactions remain elusive, members of the integrin family are suitable candidates. Indeed, the cross talk between integrins and Ig-like CAMs, such as members of the ICAM and JAM families, is a key step during leukocyte transmigration (28). In support of this hypothesis, not only has L1 been reported to interact with integrins in other experimental systems (10, 29, 30) but L1 homophilic binding upon cell–cell adhesion has been shown to promote integrin recruitment and activation (31). In this context, L1-mediated stimulation of the integrin $\alpha_v\beta_3$ favors the interaction of melanoma with ECs, a process that precedes melanoma cell intravasation (32). The inflammation-associated induction of L1 expression in ECs is intriguing. Despite the fact that L1-positive vessels in inflammatory lesions have been reported (10) and confirmed by our immunohistochemical analysis, L1 has not been investigated as part of the adhesion molecule repertoire that is induced by inflammatory stimuli in the endothelium (28). Our study provides the first evidence that L1 is indeed a transcriptional target of an inflammatory cytokine, such as TNF- α , in ECs. *In vivo*, L1 is not expressed on ECs under steady-state conditions but it is up-regulated after TNF- α treatment, supporting the notion that this event is part of the inflammatory reaction rather than a phenomenon restricted to cultured ECs. Based on our results on human ECs where the neutralization of L1 causes a reduction in DC transmigration, it is conceivable that inflammation-induced vascular L1 serves the function of enhancing the transendothelial trafficking of DCs. In this context, the *Tie2-Cre* transgene is also expressed in the endothelium (8), implying that the induction of vascular L1 under inflammatory conditions would not occur in *Tie2-Cre;L1^{flaxed}* mice. The possibility that endothelial L1 contributes to DC trafficking presents an attractive hypothesis that deserves further investigation. Moreover, although our data suggest that L1 on DCs establishes heterophilic interactions with EC surface molecules (see beginning of paragraph), it remains to be established whether endothelial L1 also binds to different molecules on the surface of DCs. This would implicate a complex network of L1-mediated interactions in DC transendothelial migration during inflammation.

In spite of L1's role in DC transendothelial migration *in vitro* and trafficking *in vivo*, contact hypersensitization was not affected in mice with L1-deficient DCs. This might be accounted for by the residual fraction of DCs that migrated to the draining lymph nodes in *Tie2-Cre;L1^{flaxed}* mice, which would have been sufficient to induce specific immunity. Another explanation (not mutually exclusive with the previous one) relies on the fact that contact hypersensitization assays reflect the induction of skin immunity, which is mediated by Langerhans cells and dermal DCs. Recent studies have specifically implicated dermal DCs in contact hypersensitization (18), whereas Langerhans cells would not be involved in this process (17). Collectively with our observation that in mouse skin L1 is expressed in Langerhans cells but not in dermal DCs, this likely accounts for the normal contact hypersensitization response of *Tie2-Cre;L1^{flaxed}* mice. Future studies

should address the impact of L1 deficiency in different types of immune response that involve DCs in compartments other than the skin because the role of L1 in T cell activation has also been reported (27).

Our observation that L1 is found in specific subpopulations of DCs (e.g., Langerhans cells, bone marrow DCs, and moDCs but not dermal DCs or 45% of lymph node DCs) raises the hypothesis that the microenvironment is involved in the modulation of L1 expression in DCs in a tissue-specific manner. Along this line, we have previously reported that intestinal epithelium plays a pivotal role in determining the phenotype of DCs (33). Hence, the regulation of L1 expression might be part of the “education” of DCs by the local environment, which would enable DCs to carry out specialized functions that are required to deal with tissue-associated challenges.

Although the biological significance of L1 expression on cancer-associated vasculature remains elusive, it is tempting to speculate that endothelial L1 in tumors triggers the trafficking of DCs in the absence of an overt inflammation, resulting in the migration to lymph nodes of immature nonimmunogenic DCs (19). These cells could present tumor antigenic peptides in a tolerogenic fashion, thus contributing to tumor immunoevasion.

The impairment of DC trafficking upon loss of L1 might have important clinical implications. Other mouse models have revealed a role of L1 in the immune system, although not directly involving DCs. In L1-deficient mice, the architecture of the white pulp border in the spleen was disrupted (34), and L1 was implicated in the tissue remodelling of lymph nodes that occurs during the immune response (35). With regard to L1 function in humans, mutations in the L1 gene cause various neurological disorders that are grouped under the name L1 syndrome (2). This phenotype is largely recapitulated in L1 knockout mice (4, 36–38). Although patients carrying L1 mutations are thoroughly examined for brain development and functions, no information is available on their immune system. Our findings raise the possibility that L1 syndrome is associated with a defective DC trafficking and provide the rationale for investigating the impact of L1 mutations on the patients’ immune response. Besides the possible benefit for the clinical management of L1 syndrome patients, such an approach might also contribute to assign the DC-regulatory function of L1 to specific domains and/or residues of the protein. Indeed, numerous syndrome-associated L1 mutations have been described, which are distributed across all domains (2). Hence, the analysis of DC function in patients carrying different L1 mutations would help to determine the relative contribution of individual L1 domains to DC trafficking.

Our study also points to L1 as a potential therapeutic target to modulate DC function, a notion which is supported by the blockade of transendothelial migration of human DCs upon inactivation of L1. In this context, the design of L1-targeting strategies *in vivo* would benefit both from preclinical studies where L1-neutralizing antibodies showed therapeutic efficacy in tumor-bearing mice (21, 39) and from the use of L1 antibodies for imaging purposes in cancer patients (40).

These studies strengthen the rationale for assessing the inhibition of L1 as a strategy to repress DC trafficking in certain immunological disorders.

MATERIALS AND METHODS

Mice

L1^{fl/fl} mice (9) were provided by M. Schachner and F. Morellini (University of Hamburg, Hamburg, Germany). *Tie2-Cre* transgenic mice (8) were provided by E. Dejana (Milan, Italy). All mouse strains were backcrossed into the C57BL/6 background for eight or more generations. To obtain *Tie2-Cre;L1^{fl/fl}* mice, *L1^{fl/fl}* females were crossed with *Tie2-Cre* males. Genomic DNA of the offspring was isolated from tail biopsies and the genotype was determined by PCR (supplemental Materials and methods). All experiments were performed in accordance with the guidelines established in the Principles of Laboratory Animal Care (directive 86/609/EEC) and approved by the Italian Ministry of Health.

Antibodies

The following antibodies were used: hamster anti–mouse CD11c (clone HL3; BD); rat anti–mouse PECAM-1 (clone MEC13.3; BD); rat anti–Langerin (clone 929F3; Dendritics); rabbit anti–human L1 ectodomain (from M. Schachner, Hamburg, Germany) and rabbit anti–human L1 cytoplasmic tail pcyt-L1 (from V. Lemmon, Miami, FL; reference 41); mouse anti–human L1 (clone CE7; from K. Blaser, Davos, Switzerland; reference 42); and rat anti–L1 (clones I4.2 and S10.33) generated against mouse L1 (but cross reacting with human L1; unpublished data) and characterized in our laboratory.

Cells

DCs. Bone marrow–derived immature DCs were generated from single cell suspensions of marrow from femurs of 8–10-wk-old *L1^{fl/fl}* or *Tie2-Cre;L1^{fl/fl}* male mice. After 10–11 d of culture in GM-CSF–containing DC medium (43, 44), the homogeneity of DCs was evaluated by FACS analysis with anti–CD11c (BD). No differences were observed between *L1^{fl/fl}* or *Tie2-Cre;L1^{fl/fl}* bone marrow precursors in the proliferation rate or in the yield of CD11c-expressing DCs during GM-CSF–induced differentiation (unpublished data).

Human moDCs were obtained from healthy volunteers as described previously (45). After 5–7 d of culture, cells were analyzed for DC markers and used for functional assays.

ECs. The mouse blood vessel EC line 1G11, isolated from the lung, was provided by A. Vecchi (Milan, Italy) and cultured as previously described (15). The mouse LEC lines MELC (46) and SV-LEC (14) were provided by A. Vecchi (Milan, Italy) and J.S. Alexander (Shreveport, LA), respectively, and were cultured as previously described. Primary LECs from human prostate were isolated and cultured as previously described (47) and used between passages 3 and 6. HUVECs (PromoCell) were cultured in MCDDB 131 medium (Invitrogen) supplemented with 2 mM L-glutamine, 20% FBS, 50 µg/ml heparin, and 50 µg/ml EC growth supplement (Sigma-Aldrich). 1G11 cells, MELCs, SV-LECs, and HUVECs were seeded on 0.1–1% gelatin. Prostate LECs were cultured on plates coated with 10 µg/ml fibronectin (Sigma-Aldrich). Where specified, ECs were treated with 20 ng/ml TNF-α (PeproTech) for the indicated time points.

Immunohistochemistry

Immunohistochemistry on formalin-fixed paraffin-embedded tissue sections was performed as described previously (26) using the polyclonal antibody pcyt-L1. Staining of sections was visualized with the ABC horseradish peroxidase kit (Vector Laboratories) and DAB peroxidase substrate (Sigma-Aldrich). For morphological analysis, tissues were counterstained with hematoxylin.

FACS

FACS analysis was performed on bone marrow–derived DCs, lymph node–derived cells, and ECs. In brief, cells were resuspended in RPMI medium

containing 1% normal mouse serum and then incubated with the specific fluorophore-conjugated antibody. Cells were then analyzed by a FACSCalibur apparatus (BD). For FACS analysis on HUVEC, cells were incubated with rabbit anti-human L1 ectodomain antibody followed by Alexa Fluor 488-conjugated anti-rabbit antibody (Invitrogen).

Quantitative RT-PCR analysis

Total RNA was isolated by extraction with TRIzol (Invitrogen), and 1 μ g was reverse transcribed with random hexamers (High Capacity cDNA Archive kit; Applied Biosystems) according to the manufacturer's instructions. 5 ng cDNA was amplified in triplicate in a reaction volume of 15 μ l using TaqMan Gene Expression Assay ID Hs00240928_m1 (Applied Biosystems), which is designed for the detection of human L1 cDNA, and an ABI/Prism 7900 HT thermocycler (Applied Biosystems). Preparations of RNA template without reverse transcription were used as negative controls. For each sample, the expression level of L1 was normalized to GAPDH using the comparative threshold cycle method as previously described (48).

Adhesion assays

LECs (SV-LEC and MELC) were grown as monolayers on gelatin-coated 96-well plates and stimulated with 20 ng/ml TNF- α for 16 h before DC adhesion assays. DCs were labeled with 5 μ M CFSE, and 10^5 labeled cells per well were added and incubated at 37°C. At the indicated time points, cells were washed and fluorescence was measured using a fluorimeter (Multilabel Counter; Wallac 1420; Perkin Elmer). After subtraction of background cell binding (assessed using BSA-coated wells), cell adhesion was calculated as follows: adhesion = BF/TF \times C/A, where BF is bound fluorescence, TF is total initial fluorescence, C is the number of cells per well (10^5), and A is the well area (32 mm 2). In some experiments, DCs were purified from cell suspensions from mouse lymph nodes using CD11c MACS MicroBeads (Miltenyi Biotec) according to the manufacturer's instructions and then FACS sorted into L1-positive and L1-negative DCs using Alexa Fluor 647-conjugated anti-L1 antibody S10.33 and PE-conjugated anti-CD11c (BD). The two DC populations were then labeled with CFSE and PKH26, respectively, before adhesion assays on SV-LEC monolayers. Cell adhesion was determined by counting green and red cells.

Where specified, CFSE-labeled DCs were seeded on 96-well plates precoated with 60 μ g/ml of the extracellular portion of mouse (mL1-Fc) or human (hL1-ECD) L1 in 1% gelatin. The construct for mL1-Fc (49) was a gift from M. Schachner, and the expression vector encoding histine-tagged hL1-ECD was provided by S. Silletti (University of California, San Diego, La Jolla, CA). Both recombinant proteins were expressed in 293 cells and purified from the conditioned medium by protein G (for mL1-Fc) or nickel affinity chromatography (for hL1-ECD).

Transendothelial migration assays

MELCs, SV-LECs, HUVECs, and 1G11 cells were grown as monolayers on gelatin- or fibronectin-coated Transwell inserts with a 5- μ m pore (Costar; Corning) as described previously (50). ECs were stimulated with 20 ng/ml TNF- α for 16 h before DC transmigration assays. CFSE-labeled DCs (10^5 cells) were seeded onto the endothelial monolayers and incubated for different time lengths. In some experiments, MELCs and SV-LECs were grown on the lower side of the filter to determine basal-to-apical transmigration of DCs. Transwell inserts were thoroughly washed with PBS, fixed in PFA, and mounted onto microscope slides (Menzel-Gläser). Images of CFSE-labeled DCs were obtained with a microscope (Biosystems BX-71; Olympus). The number of CFSE-labeled DCs that crossed the filter was determined by counting the fluorescent cells.

The migration of CFSE-labeled human moDCs across HUVEC barriers was determined after preincubating DCs, HUVECs, or both with 30 μ g/ml CE7, a monoclonal antibody with L1 blocking function properties. As a control, an isotype-matched anti-HA antibody was used at the same concentration. Cell transmigration was determined as described in the previous paragraph.

Skin painting assay

Mice were painted on the shaved abdomen with 0.2 ml of either 0.5% tetramethylrhodamine-5-(and-6)-isothiocyanate (5(6) (TRITC; Invitrogen) or 0.5% FITC (Sigma-Aldrich) in a 1:1 acetone/dibutylphthalate (vol/vol) mixture. Inguinal lymph nodes were excised from treated mice after 24 or 48 h and disaggregated as described in the supplemental Materials and methods. Cell suspensions from FITC-painted mice were costained with PE-conjugated anti-mouse CD11c (BD) and Alexa Fluor 647-conjugated anti-mouse L1 clone S10.33 followed by FACS analysis. Cell suspensions from TRITC-painted mice, after staining with APC-conjugated anti-mouse CD11c or with Alexa Fluor 647-conjugated anti-mouse L1 clone S10.33, were fixed and permeabilized, followed by costaining with Alexa Fluor 488-conjugated monoclonal antibody 929F3 anti-langerin (Dendritics), which recognizes the intracellular conformation of the protein (51), before FACS analysis.

CHS assays

CHS was induced and determined as previously described (52). In brief, the hapten 4-ethoxymethylene-2-phenyl-2-oxazoline-5-one (Oxazolone [OXA]; Sigma-Aldrich) was freshly prepared before CHS assays. For sensitization, mice were painted once (day 0) on the shaved abdominal skin with 100 μ l of 3% OXA in 4:1 acetone/olive oil (vol/vol) solution. 5 d later (day +5), mice were challenged by the application of 10 μ l OXA (1%) on each side of the right ear, whereas the left ear received the vehicle alone. CHS response was determined by measuring the thickness of the antigen-painted ear compared with that of the vehicle-treated contralateral ear by a micrometer (Mitutoyo) at 24–96 h after challenge. The results were expressed as percentage of thickness increase calculated over vehicle-treated contralateral ear.

Staining of mouse endothelium

100 μ l of 40 ng/ml of mouse TNF- α or the same volume of PBS were injected subcutaneously in the inferior abdominal region of 6-wk-old C57BL/6 mice. 16 h after the injection, mice were sacrificed and the skin around the area of injection was removed, embedded in Tissue-Tek OCT (Sakura), and snap frozen in liquid nitrogen. 5- μ m frozen sections were obtained using a cryostat (CM 199; Leica) and air dried overnight. Sections were fixed in cold methanol and subjected to immunofluorescence staining using rat anti-PECAM-1 followed by Cy3-conjugated secondary antibody (Jackson Immuno-Research Laboratories). Tissue was fixed again, blocked with excess rat IgG, and then incubated with Alexa Fluor 488-conjugated rat anti-mouse L1 (clone S10.33). The tissue was then mounted onto microscope slides and images were obtained as described for cell immunofluorescence.

DC staining in mouse skin

Methanol-fixed frozen sections of C57BL/6 mouse skin were stained overnight at 4°C with rat anti-mouse Langerin and hamster anti-mouse CD11c. The day after, sections were incubated with an Alexa Fluor 647-conjugated goat anti-rat antibody (Invitrogen) and with a Cy3-conjugated goat anti-hamster antibody (Jackson ImmunoResearch Laboratories), followed by another fixation step in cold methanol. After an additional blocking step with rat IgG, sections were then incubated with Alexa Fluor 488-conjugated rat anti-L1 antibody (clone S10.33) for 2 h at room temperature. Stained tissues were then analyzed by confocal microscopy (TCS-SP2-AOBS; Leica).

Online supplemental material

The supplemental Materials and methods describes the experimental procedures used for the experiments illustrated in supplemental figures. Fig. S1 shows the phenotypic analysis of L1-expressing DCs in mouse lymph nodes and spleen. Fig. S2 illustrates the characterization of *L1*^{flaxed} and *Tie2-Cre;L1*^{flaxed} mice. Fig. S3 shows the maturation of DCs in response to LPS. Fig. S4 shows the migratory response of DCs to chemokines and to the injection of FITC-labeled beads. Fig. S5 shows the reduced transendothelial migration of *L1*^{flaxed} DCs treated with Tat-Cre. Fig. S6 and Fig. S7 show the specific expression of L1 in Langerhans cells. Fig. S8 shows the expression of

L1 in different EC types. Fig. S9 shows the role of homophilic L1–L1 interactions in the adhesion of DCs to ECs. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20081211/DC1>.

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