

Lymphocyte Migration in Lymphocyte Function-associated Antigen (LFA)-1-deficient Mice

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Summary

Using lymphocyte function-associated antigen (LFA)-1^{-/-} mice, we have examined the role of LFA-1 and other integrins in the recirculation of lymphocytes. LFA-1 has a key role in migration to peripheral lymph nodes (pLNs), and influences migration into other LNs. Second, the $\alpha 4$ integrins, $\alpha 4\beta 7$ and $\alpha 4\beta 1$, have a hitherto unrecognized ability to compensate for the lack of LFA-1 in migration to pLNs. These findings are confirmed using normal mice and blocking LFA-1 and $\alpha 4$ monoclonal antibodies. Unexpectedly, vascular cell adhesion molecule (VCAM)-1, which is essential in inflammatory responses, serves as the ligand for the $\alpha 4$ integrins on pLN high endothelial venules. VCAM-1 also participates in trafficking into mesenteric LNs and Peyer's patch nodes where mucosal addressin cell adhesion molecule 1 (MAdCAM-1), the $\alpha 4\beta 7$ -specific ligand, dominates. Both $\alpha 4\beta 1$, interacting with ligand VCAM-1, and also LFA-1 participate in substantial lymphocyte recirculation through bone marrow. These observations suggest that organ-specific adhesion receptor usage in mature lymphocyte recirculation is not as rigidly adhered to as previously considered, and that the same basic sets of adhesion receptors are used in both lymphocyte homing and inflammatory responses.

Key words: adhesion • integrin • homing • bone marrow • lymphocyte

The integrin LFA-1 (CD11a/CD18), which belongs to the $\beta 2$ family of integrin receptors, is expressed by all leukocytes and has a central role in the functions of these cells. When leukocytes respond to inflammatory signals, LFA-1 acts together with other adhesion receptors to direct the cells into injured tissues (see references 1–4). Thus, the selectin adhesion receptors allow transient leukocyte contact with stimulated endothelium, causing a rolling movement which is further arrested by the action of the $\alpha 4$ integrins. This stage is followed by a signaling event that activates LFA-1 to promote firm adhesion of the leukocytes and their migration across the endothelium towards the area of injury.

In contrast, mature lymphocytes, but not myeloid cells, circulate continually through secondary lymphoid tissue

of the peripheral lymph nodes (pLNs),¹ mesenteric LNs (mLNs), and Peyer's patches (PPs), thereby increasing the opportunity for an encounter with antigen. To migrate across the specialized high endothelial venules (HEVs) of these LNs, lymphocytes have been proposed to use organ-specific sets of adhesion receptors (4, 5). Migration into mucosal tissue such as PPs is greatly dependent on the $\alpha 4$ integrin, $\alpha 4\beta 7$, which has no apparent role in homing to the pLNs (6, 7). On the other hand, homing of naive lymphocytes to pLNs has been considered to be dependent upon L-selectin (CD62L) but not to involve the $\alpha 4$ integrins (8, 9). However, parallels are emerging between the

¹Abbreviations used in this paper: CT, CellTracker™; ES, embryonic stem; HEV, high endothelial venule; HUVEC, human umbilical vein endothelial cell; ICAM, intercellular adhesion molecule; MAdCAM-1, mucosal addressin cell adhesion molecule 1; pLN, peripheral lymph node; PNAd, peripheral node addressin; PP, Peyer's patch; VCAM, vascular cell adhesion molecule.

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processes of lymphocyte recirculation and the response to inflammatory signals. LFA-1 influences migration into mucosal tissue as well as the pLNs (10), and there is now evidence that recirculating lymphocytes also undergo an activation step when they contact the HEVs. LFA-1 can be activated by signals arising from the binding of lymphocyte L-selectin to HEV ligand, GlyCAM-1 (11). HEV-expressed chemokines B lymphocyte chemoattractant (BLC) and secondary lymphoid tissue chemokine (SLC) cause adhesion of naive B cells (12) and T cells (13), respectively, and SLC has been demonstrated to activate LFA-1.

Ligands for most of the lymphocyte adhesion receptors have been identified on the HEVs. L-selectin recognizes peripheral node addressin (PNAd), a group of fucosylated and sialylated LewisX-expressing proteins on pLN HEVs (see reference 14). LFA-1 functions through recognition of its ligands intercellular adhesion molecule (ICAM)-1 or ICAM-2 (see reference 15). The $\alpha 4\beta 7$ integrin recognizes MAdCAM-1, which is expressed on HEVs in PPs and mLNs (16, 17), and VCAM-1 and fibronectin. The alternative $\alpha 4$ integrin, $\alpha 4\beta 1$, which has a major role in inflammation (18–20), also recognizes ligands VCAM-1 and fibronectin. VCAM-1 is expressed by endothelia under inflammatory conditions (21, 22) and is thought to be absent on normal LN endothelium (22, 23). Fibronectin is generally associated only with the subendothelial matrix, but there is a report that a spliced variant is expressed in rheumatoid arthritis tissue (24). Thus, the ligand binding activities of the two $\alpha 4$ integrins are distinctive, in that MAdCAM-1 is involved in $\alpha 4\beta 7$ -mediated naive lymphocyte homing to mucosal sites whereas VCAM-1 primarily serves as the ligand for $\alpha 4\beta 1$ at inflammatory sites.

Recently, two studies using LFA-1-deficient mice have confirmed many of the previous assignments of LFA-1 function, for example, in assays for T cell function such as MLR and delayed-type hypersensitivity responses (25, 26). In this study, we have used LFA-1^{-/-} mice to reexamine the role of LFA-1 in the recirculation of lymphocytes and to investigate alternative mechanisms used in its absence. We confirm an important role for LFA-1 in LN localization, identify an unexpected role for $\alpha 4$ integrins, $\alpha 4\beta 7$ and $\alpha 4\beta 1$, in pLN homing that is mediated by HEV-expressed VCAM-1, and finally demonstrate that $\alpha 4\beta 1$ /VCAM-1 together with LFA-1 is involved in migration of lymphocytes to bone marrow.

Materials and Methods

Generation of LFA-1 Mutant Mice. A 7-kb PCR product amplified from murine 129/Sv genomic DNA and containing exon 2 of the *Lfa-1* gene was subcloned into pSP72. A selection cassette containing stop codons in all three frames, an independent ribosomal entry sequence (IRES) followed by the LacZ gene with an SV40 polyadenylation signal, and a neomycin phosphotransferase gene was introduced into exon 2. Culture and transfection of GK129 embryonic stem (ES) cells was done as described previously (27). EcoRI-digested ES cell DNA was analyzed by Southern blot analysis using probe A, and homologous recombination

was confirmed using probe B (see Fig. 1 a). Blastocyst injection and breeding have been described previously (27). Mice were genotyped from EcoRI-digested tail DNA by Southern blot (data not shown) or PCR (Fig. 1 b). A multiplex PCR using primers on either side of the selection cassette and one within the neomycin resistance gene yielded fragments of 90 bp (wild-type) and 660 bp (LFA-1-deficient), respectively.

Animal Husbandry. All mice were kept under specific pathogen-free conditions and in accordance with United Kingdom Home Office regulations. The mice were monitored on a six-monthly basis for a wide selection of parasites, bacteria, and fungi and were regularly found to be pathogen free. LN sections from sentinel mice belonging to litters used in the reported experiments were inspected for the presence of germinal centers by hematoxylin and eosin staining and for positive MAdCAM-1 staining in pLNs (28). These latter indicators of an inflammatory response were routinely negative for mice housed in London and Hamburg.

mAbs and Other Reagents. The following purified rat mAbs were used in this study: LFA-1 α subunit mAbs H35.89.9 (IgG2b) and H68 (IgG2a), both obtained from Dr. Michel Pierres (Centre d'Immunologie, INSERM-CNRS de Marseille-Luminy, Marseille, France); $\alpha 4$ subunit mAb PS/2 (IgG2b); anti-VCAM-1 mAb MK2.7 (IgG1); anti-MAdCAM-1 mAb MECA-367 (IgG2a), obtained from American Type Culture Collection; anti-PNAd mAb MECA-79 (IgM; obtained from Drs. Mark Singer and Steve Rosen, University of California, San Francisco, CA); MECA-325 (IgG1), obtained from Dr. A. Duijvestijn (University of Limburg, Maastricht, Netherlands); CD54 mAb YN1/1.7 (IgG2a; obtained from Dr. Fumio Takei, Terry Fox Laboratory, BC Cancer Research Centre, Vancouver, Canada); CD4, CD8, and B220 mAbs (PharMingen); and control rat IgG1 mAb PyLT-1 (IgG1; Imperial Cancer Research Fund) and control null rat IgG1 and IgG2a mAbs (PharMingen). Secondary detection antibodies were FITC-conjugated goat anti-rat Ig (Jackson Immuno-Research Laboratories) and biotinylated rabbit anti-rat Ig (Vector Laboratories). Fab fragments were prepared by papain digestion using either the ImmunoPure[®] Fab kit (Pierce Chemical Co.) or HPLC to remove any residual intact IgG (7).

Preparation of Cells for Flow Cytometry. Single cell suspensions from 8–12-wk-old mice were prepared by mincing or rubbing between glass slides with frequent rinsing with 5% FCS/RPMI or 0.2% BSA/PBS. Bone marrow cells were harvested by flushing with 5% FCS/RPMI via cut ends of tibias and femurs, followed by disaggregation and filtering through nylon gauze. Cells were washed and incubated at 5×10^6 cells/ml with specific primary mAbs, then FITC-conjugated goat anti-rat Ig (1:200; Jackson ImmunoResearch Laboratories) at 4°C for 30 min. Cells were fixed in 1% HCHO/PBS before analysis on a FACScan[™] instrument (Becton Dickinson).

In Vivo Homing Experiments Using CellTracker[™] Fluorescent Dye-labeled Lymphocytes. Lymphocytes from pLNs and mLNs of LFA-1^{+/+} and LFA-1^{-/-} mice were prepared and adjusted to 5×10^6 cells/ml in 5% FCS/RPMI for labeling as described (29). LFA-1^{+/+} lymphocytes were incubated with 0.2 mM CellTracker[™] (CT) Green 5-chloromethylfluorescein diacetate (CMFDA; Molecular Probes) and LFA-1^{-/-} lymphocytes with 5 mM CT Orange 5-chloromethyl tetramethylrhodamine (CMTMR) at 37°C for 45 min. The two cell preparations were washed and counted, then mixed together to achieve a 1:1 ratio of differentially labeled LFA-1^{+/+} and LFA-1^{-/-} lymphocytes for injection into recipient 8–12-wk-old C57BL/6 mice for a period of 1 h. A correction factor was applied to the subsequent data if the lymphocyte ratio

was not precisely 1:1. Reversing the CT dyes had no effect on results. The lymphocytes were frequently tested for activation status before and after the labeling procedure by measuring the level of L-selectin that can be enzymatically cleaved after an activating stimulus (30). In general, a total of 2×10^7 cells in a volume of 200 μ l was injected per tail vein. In experiments involving antibody blocking, the cells and mAbs were coinjected. Fab fragments were used in amounts of 250–400 μ g per mouse. Irrelevant control Fab fragments were without effect.

In Vivo Homing Experiments Using ^{51}Cr -labeled Lymphocytes. Lymphocytes obtained as above were labeled with 20 μCi ^{51}Cr /ml as described previously (29), and dead cells removed by centrifugation on a 17% Nycodenz cushion (Nycomed). 5×10^6 lymphocytes in 200 μ l were injected into the tail vein of recipient 8–12-wk-old C57BL/6 mice. mAbs were coinjected as Fab fragments and at 300 μg /200 μ l. Sample groups of four mice were used in each condition. The mice were killed after 1 h, and the distribution of radioactivity was determined for the different organs and for the residual body mass. PBLs were calculated for a blood volume of 1.5 ml.

Labeling, Injection, and Detection of Donor Cells in the Host pLNs. LN cells were labeled by dissolving 1 mg digoxigenin in 0.5 ml DMSO and incubating the lymphocytes for 15 min at a final concentration of 5×10^7 cells/ml with 60 μg digoxigenin. After washing, 0.8×10^7 cells were injected intravenously, and pLNs were removed 30 min later and frozen in liquid nitrogen. To localize the donor cells in the HEVs, two antigens were revealed simultaneously as described (31). In brief, cryostat sections were incubated with the anti-HEV mAb MECA-325, which was revealed by the alkaline phosphatase anti-alkaline phosphatase (APAAP) technique (blue [31]). The donor cells were identified by a peroxidase-conjugated antidigoxigenin antibody (brown) as described previously (31). The area of the HEVs (excluding the lumen) together with the area within 4-cell diameters surrounding the vessel was then determined (31), and the number of injected cells adhering to and within the endothelium, and within the tissue was analyzed.

Immunohistochemistry. Sections of 6 μm were cut from snap-frozen tissues of pLN, mLN, and PP and placed on silane-coated slides which were fixed in acetone for 10 min and allowed to dry. Slides were washed in standard Tris-buffered saline (TBS),

pH 7.6, and primary mAb was added for 1 h at room temperature. mAbs were used at optimal dilution: 10 μg /ml for anti-VCAM-1 mAb MK2.7 (IgG1) and IgG1 isotype control mAb PyLT-1, MECA-79 at 4 μg /ml, and MECA-367 as tissue culture supernatant. Sections were also tested with null IgG1 and IgG2a mAbs (PharMingen; data not shown). Biotinylated rabbit anti-rat Ig (1:100; Vector Laboratories) was added for 35 min. A tertiary stage was carried out using the StreptABCComplex/HRP kit following the manufacturer's instructions (K377; Dako), and the slides were incubated again for 35 min before exposure to 1 mg/ml 3,3'-diaminobenzidine.

Results

Disruption of the *Lfa-1* Gene. *Lfa-1*, the gene for the LFA-1 α subunit, was mutated in ES cells using a replacement-type targeting vector and the strategy shown in Fig. 1 a. G418-resistant colonies were identified by Southern blot analysis of EcoR1-digested ES cell genomic DNA using the probe shown in Fig. 1 a. Of 400 G418-resistant ES cell colonies, 10 correctly targeted colonies were identified. Three different homologously targeted ES cell clones were injected into C57BL/6 blastocysts, and all of them were transmitted through the germline. Southern blot analysis of mouse tail DNA enabled identification of LFA-1 wild-type (+/+), heterozygous (+/-), and gene-deleted status (-/-) (data not shown). This was confirmed by PCR analysis (Fig. 1 b).

Absence of LFA-1 Expression and Function In Vitro. To establish that the capacity for LFA-1 synthesis had been completely ablated, leukocyte populations were examined for LFA-1 expression and function. Thymocytes from LFA-1^{-/-} mice were compared with LFA-1^{+/+} littermates and shown to be devoid of LFA-1 expression (Fig. 1 c). The lack of LFA-1 surface expression was also demonstrated for leukocytes from other lymphoid tissue, including pLN, mLN, PP, spleen, bone marrow, and blood (data not shown). To test for absence of LFA-1 function, control

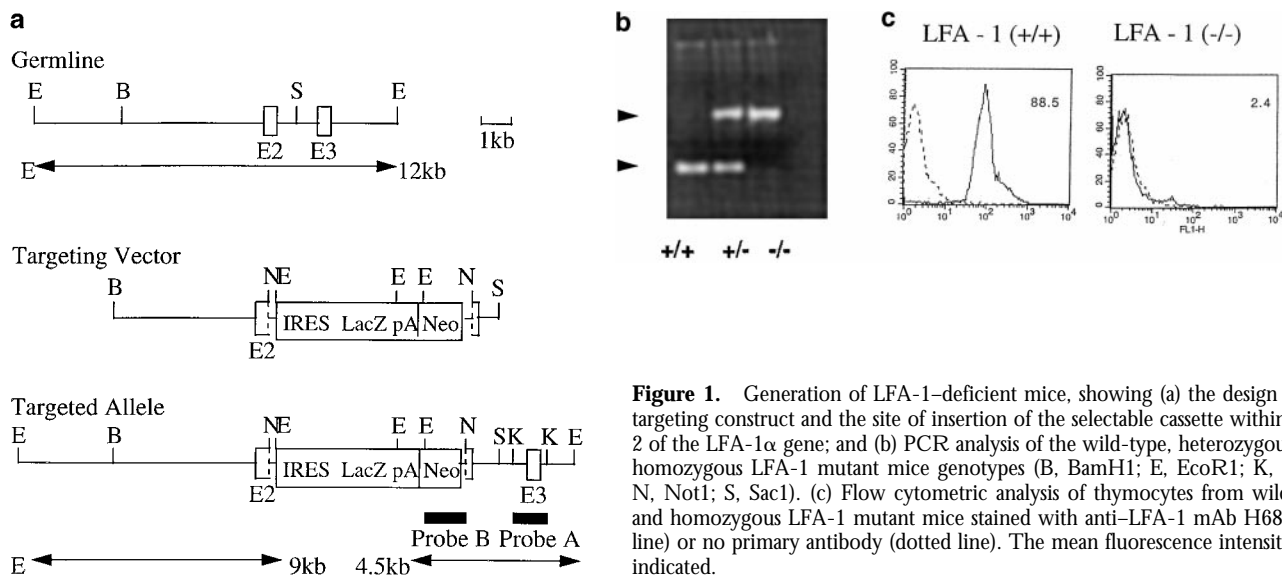


Figure 1. Generation of LFA-1-deficient mice, showing (a) the design of the targeting construct and the site of insertion of the selectable cassette within exon 2 of the LFA-1 α gene; and (b) PCR analysis of the wild-type, heterozygous, and homozygous LFA-1 mutant mice genotypes (B, BamH1; E, EcoR1; K, Kpn1; N, Not1; S, Sac1). (c) Flow cytometric analysis of thymocytes from wild-type and homozygous LFA-1 mutant mice stained with anti-LFA-1 mAb H68 (solid line) or no primary antibody (dotted line). The mean fluorescence intensities are indicated.

phorbol ester-treated LFA-1^{+/+} thymocytes were adhered to murine ICAM-1-transfected COS-7 cells, and this adhesion could be blocked with anti-LFA-1 mAb H68 (data not shown). In contrast, no adhesion was evident with LFA-1^{-/-} thymocytes. Therefore, testing for both expression and function of the LFA-1 receptor provided further proof that the LFA-1^{-/-} mice were totally deficient in this $\beta 2$ integrin.

Status of Secondary Lymphoid Tissue. Analysis of LFA-1^{-/-} compared with LFA-1^{+/+} mice revealed an increase in spleen size and a decrease in size of the pLNs, as observed previously (25). For 30-g male mice ($n = 29$), the LFA-1^{-/-} spleens were 1.7 times larger than those of LFA-1^{+/+} mice (182.0 ± 56.4 compared with 107.8 ± 30.0 mg). For 25-g female mice ($n = 37$), the same comparison yielded a 1.2-fold increase in weight (137.6 ± 33.0 compared with 118.5 ± 42.3 mg). Second, the pLNs from LFA-1^{-/-} mice were smaller than those from LFA-1^{+/+} mice, with an average decreased lymphocyte number for LFA-1^{-/-} mice of $\sim 30\%$ that of the LFA-1^{+/+} littermates ($3.54 \pm 0.74 \times 10^6$ compared with $12.15 \pm 2.90 \times 10^6$; $n = 9$).

To discover whether alteration had occurred in lymphocyte numbers in general or in a particular subtype, we next analyzed CD4 and CD8 T cell subsets and used mAb B220 to detect B cells. In the pLNs, substantial loss in numbers of CD4 and CD8 T cells as well as a deficiency in B cells was observed (Fig. 2). Furthermore, there was no difference in naive versus memory phenotype in LFA-1^{-/-} and LFA-1^{+/+} mice as indicated by expression levels of L-selectin, CD44, and CD45RB antigens (data not shown). In the other LNs, wild-type and LFA-1-deficient mice were similar in terms of lymphocyte numbers and subsets, as was expected from the fact that these LNs were comparable in size between the two types of mice. In spleen, there was a significant increase in CD4 T cells and CD8 cells (Fig. 2).

Lymphocyte Homing in LFA-1-deficient Mice. To gain further information about the diminished cellularity of the

pLNs and to investigate trafficking capabilities of LFA-1-deficient lymphocytes, short-term migration studies were performed. The approach taken was to label LFA-1^{-/-} and LFA-1^{+/+} lymphocytes with two distinguishable CT orange and green fluorescent dyes and inject equivalent numbers into the tail vein of C57BL/6 recipients. This allowed direct comparison of the homing activity of the lymphocytes within each LN setting (29). An example of the methodology is illustrated in Fig. 3 a. When the pLN lymphocytes were examined for proportions of CT orange to green after 1 h of homing, it was observed that $\sim 13\%$ of LFA-1^{-/-} cells compared with LFA-1^{+/+} cells had migrated into the pLNs (Fig. 3 a). We then examined the relative ability of LFA-1^{-/-} cells to gain entry into other secondary lymphoid tissues (Fig. 3 b). When compared with LFA-1^{+/+}, the LFA-1^{-/-} cells were most poorly represented in pLNs (0.21 ± 0.01), followed by mLNs (0.51 ± 0.02) and PPs (0.68 ± 0.03). In contrast, there was a marked increase in the LFA-1^{-/-} cells in the spleen (1.29 ± 0.04) which is likely due to redistribution of lymphocytes from LN to spleen. These findings on the effect of LFA-1 absence on the trafficking of lymphocytes were confirmed using the technique of tracking ⁵¹Cr-labeled lymphocytes to compare homing in LFA-1^{-/-} and LFA-1^{+/+} mice (data not shown). When CD4, CD8, and B220⁺ lymphocyte subsets were analyzed separately, the ratio of migrated to injected lymphocytes was identical among the subsets whether derived from wild-type or LFA-1^{-/-} mice, indicating that the lack of LFA-1 caused equal difficulty for all types of lymphocytes to gain entry into the LNs (data not shown).

LFA-1^{-/-} Lymphocytes Show Limited Adhesion to LN HEVs. The above findings showed that LFA-1^{-/-} cells migrated less effectively, particularly to the pLNs, within the 1-h period but did not reveal the stage at which the lack of LFA-1 had its effect. To discover where LFA-1 deficiency caused difficulty, we turned to histochemistry and examined pLN tissue sections from mice injected with

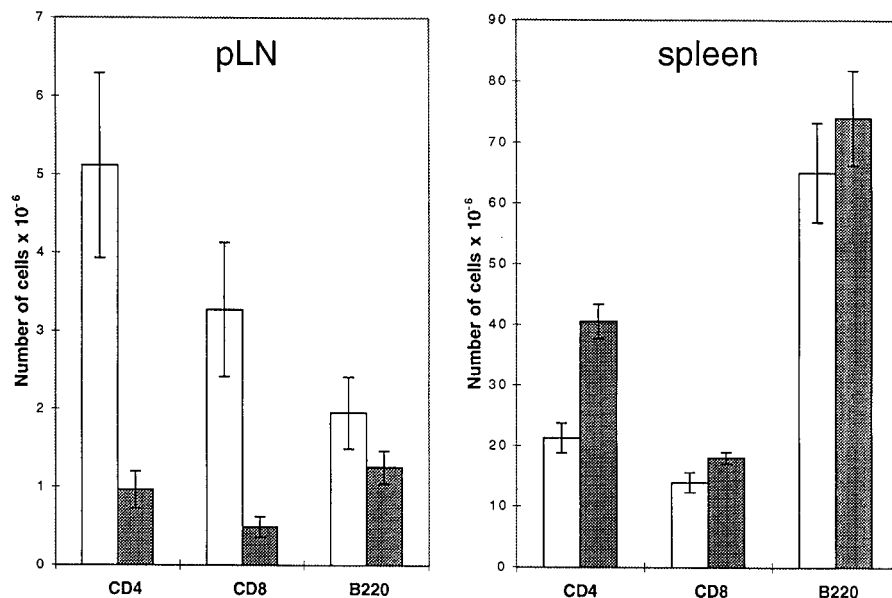


Figure 2. A comparison of lymphocyte numbers in the pLNs and spleens of wild-type and LFA-1-deficient mutant mice. In the pLNs, decreased numbers of CD4 ($P < 0.001$), CD8 ($P < 0.001$) T cells and B cells are present in LFA-1^{-/-} compared with wild-type mice. The spleens of these mice show increased numbers of CD4 ($P < 0.0001$) and CD8 ($P < 0.02$) cells. White bars, LFA-1^{+/+}; gray bars, LFA-1^{-/-}. Statistical analysis was performed using the *t* test.

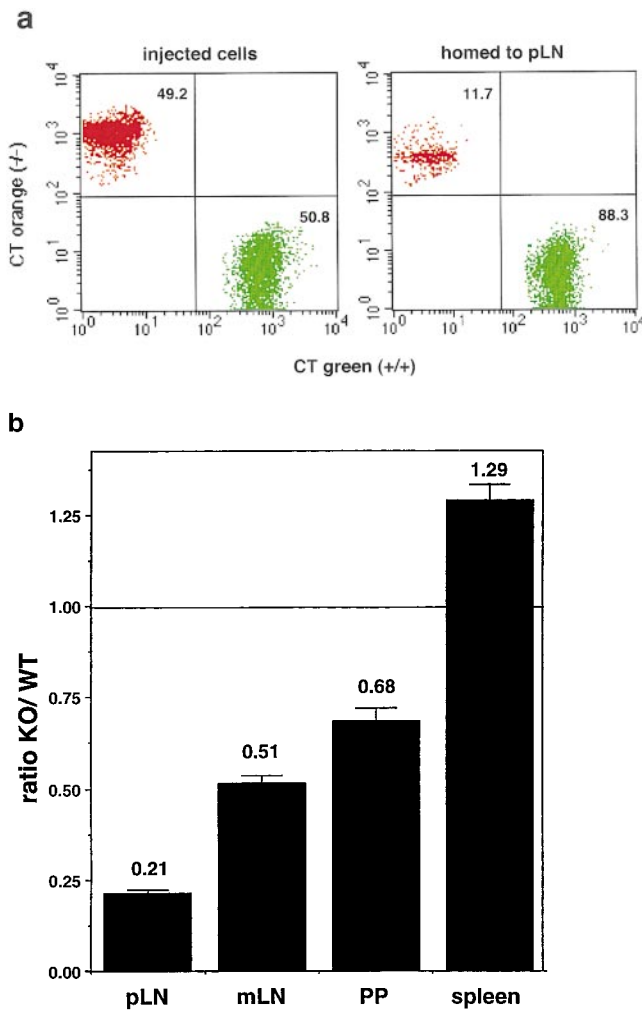


Figure 3. Differential rates of homing of CT-labeled lymphocytes from wild-type and LFA-1^{-/-} mice. (a) Two-color FACS[®] analysis of the cell mixture of LFA-1^{-/-} and LFA-1^{+/+} cells injected intravenously into a wild-type C57BL/6 mouse (left) and the fluorescent cells recovered from the pLNs after a 1-h homing period (right). For details, see Materials and Methods. (b) Homing experiments show that within 1 h, LFA-1^{-/-} lymphocytes migrate into pLNs, mLNs, and PPs with 21, 51, and 68% of the efficiency of LFA-1^{+/+} lymphocytes, respectively. Conversely, LFA-1-deficient lymphocytes occur in larger numbers in the spleen than do wild-type cells (ratio of 1.29). These data are averaged from 10–12 experiments. In all cases, the mean ratios are significantly different from 1.0 ($P < 0.05$) as assessed by *t* test.

LFA-1^{-/-} and LFA-1^{+/+} lymphocytes for 30 min. As shown in Fig. 4, the LFA-1-deficient lymphocytes bound less well to HEVs than the LFA-1-expressing lymphocytes. The total numbers of lymphocytes at the HEV level and surrounding 4-cell diameters were 285 ± 70 cells/mm² for LFA-1^{+/+} (Fig. 4, a and b) and 85 ± 10 cells/mm² for LFA-1^{-/-} lymphocytes (Fig. 4, c and d) ($n = 3$). These data suggest that loss of LFA-1 expression reduces lymphocyte adherence to and transmigration across HEVs by 70%, thereby causing substantially diminished recruitment into pLNs. When the cells were divided between those adhering to and within the HEVs and those found within 4-cell diameters beyond HEVs, the following proportions were observed: for LFA-1^{+/+} lymphocytes, 73 ± 7 and $27 \pm 2\%$; for LFA-1^{-/-}

lymphocytes, 78 ± 1 and $22 \pm 1\%$, respectively. These data suggest that the major block is at the level of the HEVs. If the deficiency had been acting selectively at the level of the transmigration step, a larger accumulation of lymphocytes at the HEV level would have been expected.

A General Role for $\alpha 4$ Integrins and LFA-1 in LN Homing. Although LFA-1 was obviously playing a critical role at the HEV level, a proportion of lymphocytes remained capable of migrating into lymphoid tissue (Fig. 3 b, and Fig. 4). Of other adhesion receptors that might substitute for LFA-1, the $\alpha 4$ integrins were attractive candidates, as they were active in migration in other situations. In the next series of experiments, the migratory behavior of CT-labeled LFA-1^{-/-} and LFA-1^{+/+} cells was compared in host animals simultaneously injected with Fab fragments of $\alpha 4$ mAb or $\alpha 4\beta 7$ -specific mAb. The $\alpha 4$ mAb completely prevented the migration of the residual numbers of LFA-1^{-/-} lymphocytes homing to pLNs, mLNs, and PPs (Fig. 5). In addition, the $\alpha 4\beta 7$ -specific mAb blocked lymphocyte entry into pLNs to $\sim 22\%$ and completely prevented migration into mLNs and PPs. There was no significant effect of $\alpha 4$ or $\alpha 4\beta 7$ mAbs on migration into spleen.

These results were duplicated in a ⁵¹Cr lymphocyte labeling experiment in which $\alpha 4$ mAb abolished all entry into the pLNs, mLNs, and PPs of LFA-1^{-/-} lymphocytes (data not shown). The $\alpha 4\beta 7$ mAb blocked entry into mLNs and PPs and inhibited entry into pLNs to $\sim 35\%$ (data not shown). It was possible that the $\alpha 4$ -dependent LFA-1^{-/-} cells migrating into pLNs were a specific subset of lymphocytes expanded in the LFA-1-deficient environment. However, phenotyping of these cells using a third fluorescent tag of Tricolor-conjugated anti-rat Ig showed the migrated LFA-1^{-/-} and LFA-1^{+/+} lymphocytes to have identical phenotypic profiles with regard to their levels of $\alpha 4$ and $\alpha 4\beta 7$ integrins and L-selectin (data not shown).

The suggestion that $\alpha 4$ integrins, $\alpha 4\beta 7$ acting together with $\alpha 4\beta 1$, have a role in migration to the pLNs has not previously been recognized. To further confirm the findings and to gain information about tissues other than secondary LNs, ⁵¹Cr-labeled lymphocytes from normal BALB/c mice were coinjected with 300 μ g of Fab fragments from either $\alpha 4$ mAb, LFA-1 mAb, or both into host BALB/c mice. The findings with the single mAbs were as reported previously (7, 10; Fig. 6), but the combination of $\alpha 4$ and LFA-1 mAbs totally prevented migration into pLNs as well as mLNs and PPs (Fig. 6). There was also an appreciable decrease in migration into intestinal tissue and into the “body” (see below). This mAb blockade provoked an increase in circulating blood cells as well as an increase in migration into the spleen. Put together, these observations provide evidence that the $\alpha 4$ integrins operating together with LFA-1 have an essential role in migration to pLNs, as found previously for other secondary LNs.

Ligands for $\alpha 4$ Integrins on LN HEVs. The identification of the ligand(s) recognized by the $\alpha 4$ integrins, particularly in pLNs, was next explored. Using a MAdCAM-1-specific mAb, homing to both PPs and mLNs was substantially prevented, as described previously (16, 17; Fig. 5). However,

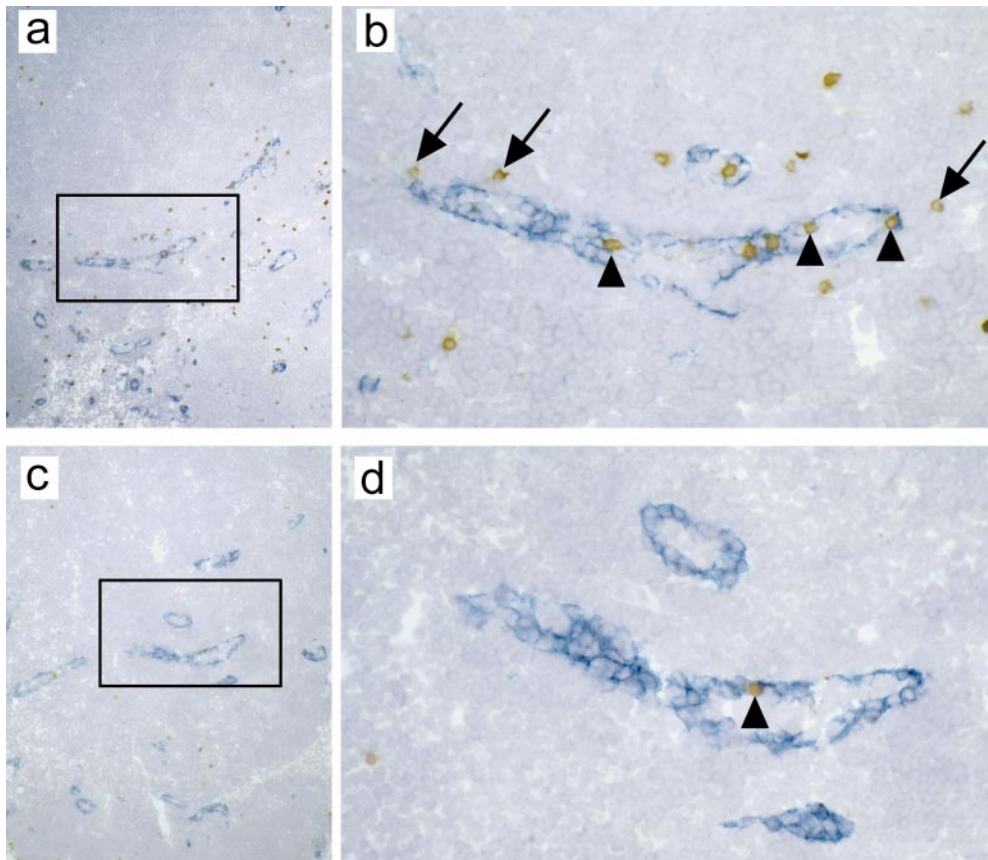


Figure 4. Tissue sections of pLNs showing the distribution of injected digoxigenin-labeled lymphocytes from LFA-1^{+/+} or LFA-1^{-/-} animals in relation to HEVs. (a) Overview of a C57BL/6 pLN 30 min after injection of LFA-1^{+/+} lymphocytes showing HEVs labeled with mAb MECA-325 (blue) and injected cells (brown). (b) Higher magnification shows injected LFA-1^{+/+} cells (brown) within and around the HEVs (blue). They are either adhered to or within the endothelium (black arrowheads), or are already within the tissue (black arrows). (c) Overview of a pLN after injection of LFA-1^{-/-} lymphocytes. (d) Higher magnification shows only one injected LFA-1^{-/-} cell can be seen within the HEV (black arrowhead), and the number of migrated lymphocytes within the node is markedly reduced. Original magnifications: a and c, $\times 60$; b and d, $\times 250$.

the mAb had no significant effect on migration of LFA-1^{-/-} lymphocytes into pLNs and spleen. VCAM-1 is another ligand recognized by both $\alpha 4\beta 1$ and $\alpha 4\beta 7$ integrins (32, 33), and in the present experiments, the anti-VCAM-1 mAb MK2.7 completely eliminated homing of LFA-1^{-/-} lymphocytes to pLNs and also substantially blocked entry into both mLNs and PPs although not into spleen (Fig. 5).

Histochemical Evidence for Expression of VCAM-1 on LN Endothelium. The foregoing experiments showed that VCAM-1 has a role in the trafficking into LNs, suggesting that this ligand is indeed expressed on the HEVs. To further address this question, immunohistochemical staining was performed using fresh frozen tissue sections of pLNs, mLNs, and PPs from LFA-1^{-/-} and LFA-1^{+/+} mice and normal C57BL/6 mice. In pLNs, anti-VCAM-1 mAb MK2.7 (IgG1) was identified to label the same HEVs as PNAd-specific mAb MECA-79 (Fig. 7, a and b). Of interest was the observation that the MAdCAM-1 mAb MECA-367 was negative, as was an IgG1 isotype control mAb, PyLT-1 (Fig. 7, c and d). In mLNs where both MAdCAM-1 and PNAd are chiefly coexpressed on HEVs (34), VCAM-1 is also present, whereas the isotype control mAb was negative (Fig. 7, e-h). In a preliminary quantitative study of mLNs, complete overlap was observed in HEV staining of VCAM-1 with one other HEV ligand, MAdCAM-1 (data not shown). The level of VCAM-1 expression was comparable on HEVs from all LNs and from LFA-1^{+/+}, LFA-1^{-/-}, and C57BL/6 mice (data not shown). Therefore the absence of LFA-1 did not in-

duce a compensatory increase in expression of VCAM-1.

To confirm that VCAM-1 is expressed on the luminal surface of the endothelium and therefore accessible to recirculating lymphocytes, C57BL/6 mice were intravenously injected for 10 min with rat anti-VCAM-1 mAb MK2.7 or anti-HEV mAb MECA-325. On tissue sections stained with anti-rat Ig, both the pLN and mLN HEVs scored positive to a similar degree for both the anti-VCAM-1 and the anti-HEV mAbs (data not shown). This experiment provided a second type of histochemical proof that VCAM-1 is lumenally expressed on the HEVs of the LNs.

A Role for $\alpha 4$ Integrin-mediated Migration into Bone Marrow. In the migration experiments with ⁵¹Cr-labeled lymphocytes, significantly fewer lymphocytes distributed into the mouse carcass after treatment with a combination of LFA-1 and $\alpha 4$ mAbs (see Fig. 6). To discover the identity of the relevant tissue compartment, individual body parts of muscle, heart, kidney, thymus, and bone were isolated and counted separately. The target tissue with diminished lymphocyte migration was determined to be the limb bones, indicating that lymphocyte recirculation was occurring in the bone marrow (data not shown). Within 1 h, a single femur recruited $\sim 1\%$ of ⁵¹Cr-labeled lymphocytes, suggesting that the total bone marrow compartment attracted lymphocytes at a rate comparable to the 5–10% lymphocyte entry into the combined LNs. Which lymphocytes were migrating into bone marrow was tested by using CT-labeled lymphocytes and subsequent staining with subset-specific mAbs de-

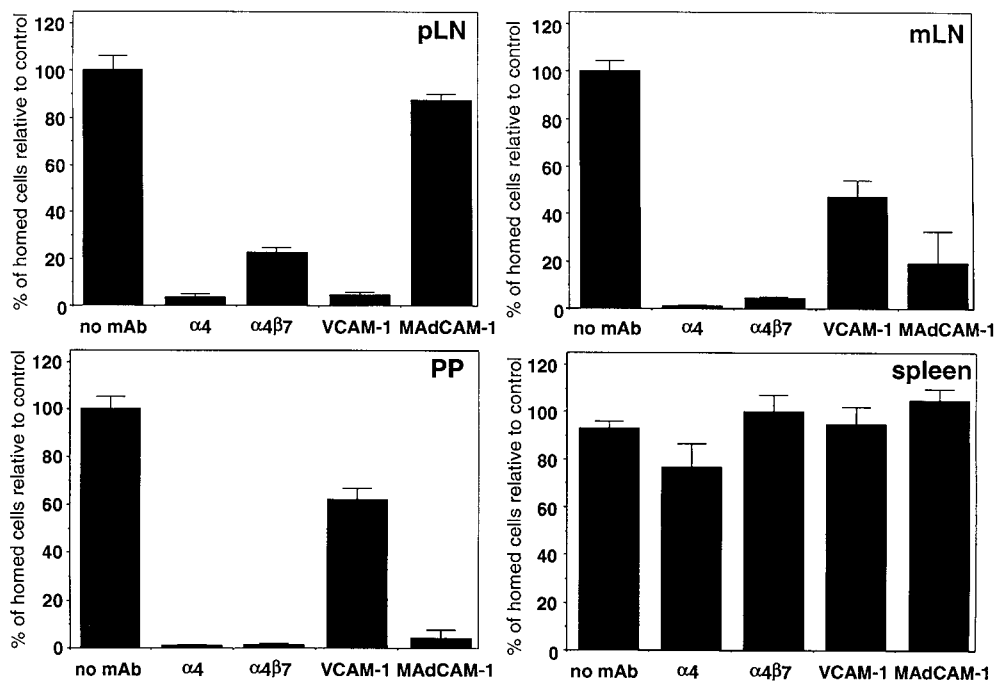


Figure 5. The effect of various mAbs on the homing of LFA-1-deficient lymphocytes to pLNs, mLNs, PPs, and spleen. In each case, the relative ratio of LFA-1^{-/-} to LFA-1^{+/+} migration (0.21, 0.51, 0.68, and 1.29, respectively; see Fig. 3 b) is set at 100% in order to assess the further reduction in migration caused by specific antibodies. Anti- $\alpha 4$ integrin (PS/2) blocked the residual ability of LFA-1^{-/-} lymphocytes to enter all three LNs, but did not significantly affect their entry into spleen. Anti- $\alpha 4\beta 7$ mAb DATK32 mirrored the effects of $\alpha 4$ mAb on mLNs and PPs, but reduced pLN migration to ~25%. Anti-VCAM-1 mAb (MK2.7) eliminated the migration of LFA-1^{-/-} cells into pLNs and partially limited their entry into mLNs and PPs. Conversely, anti-MAdCAM-1 mAb (MECA-367) blocked the homing of LFA-1^{-/-} cells into PPs, and showed no inhibition of lymphocyte entry into pLNs or spleen. Four or five animals were used per experimental group ($n = 2$ or 3; bars, \pm SD).

tected with Tricolor-conjugated anti-rat Ig. The ratio of migrated to injected CD4/CD8/B220 subsets was ~0.5/1.0/2.0. This advantage for B cell and disadvantage for CD4 T cells were independent of LFA-1 expression status.

LFA-1^{+/+} and LFA-1^{-/-} cells did not significantly differ in their trafficking to bone marrow (ratio of LFA-1^{-/-} to LFA-1^{+/+} was 1.1; data not shown), suggesting that the migration was not solely reliant on LFA-1. However, when LFA-1^{-/-}

lymphocytes were coinjected with $\alpha 4$ mAb, the trafficking to bone marrow was completely abolished (Fig. 8). These results suggest that migration into bone marrow can be accomplished either by LFA-1 or in its absence by an $\alpha 4$ integrin. As the $\alpha 4\beta 7$ mAb had a partial effect on migration, whereas $\alpha 4$ mAb blocked migration completely, we conclude that in bone marrow, in contrast to the migration into LNs, $\alpha 4\beta 1$ dominates $\alpha 4\beta 7$. Finally, the VCAM-1 mAb, but not MAd-

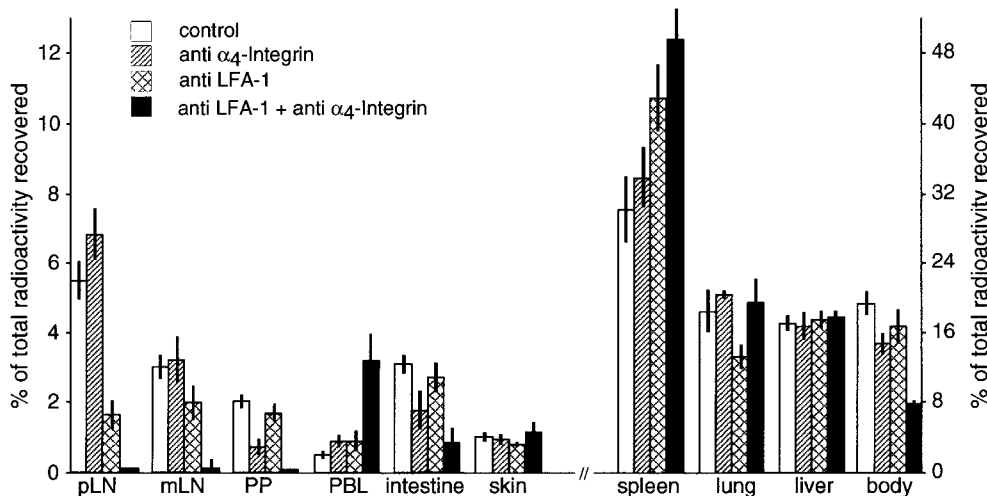


Figure 6. The effect of anti- $\alpha 4$ and LFA-1 mAbs on the migration of ⁵¹Cr-labeled BALB/c lymphocytes within 1 h after tail vein injection into host BALB/c mice. Distribution of radioactivity in various organs when lymphocytes were coinjected with anti- $\alpha 4$ mAb PS/2 (hatched bars), anti-LFA-1 mAb H35.89.9 (cross-hatched bars), anti-LFA-1 and $\alpha 4$ mAbs together (black bars), and control (white bars). The $\alpha 4$ mAb inhibited entry of lymphocytes into PPs and intestine. The LFA-1 mAb inhibited entry of lymphocytes into pLNs and redistribution to the spleen. The mAb combination inhibited entry of lymphocytes into pLNs, mLNs, PPs, intestine, and the "body" and increased redistribution into spleen. Note the increased numerical scale for the organs shown on the right. Four animals were used per experimental group ($n = 2$; bars, \pm SD).

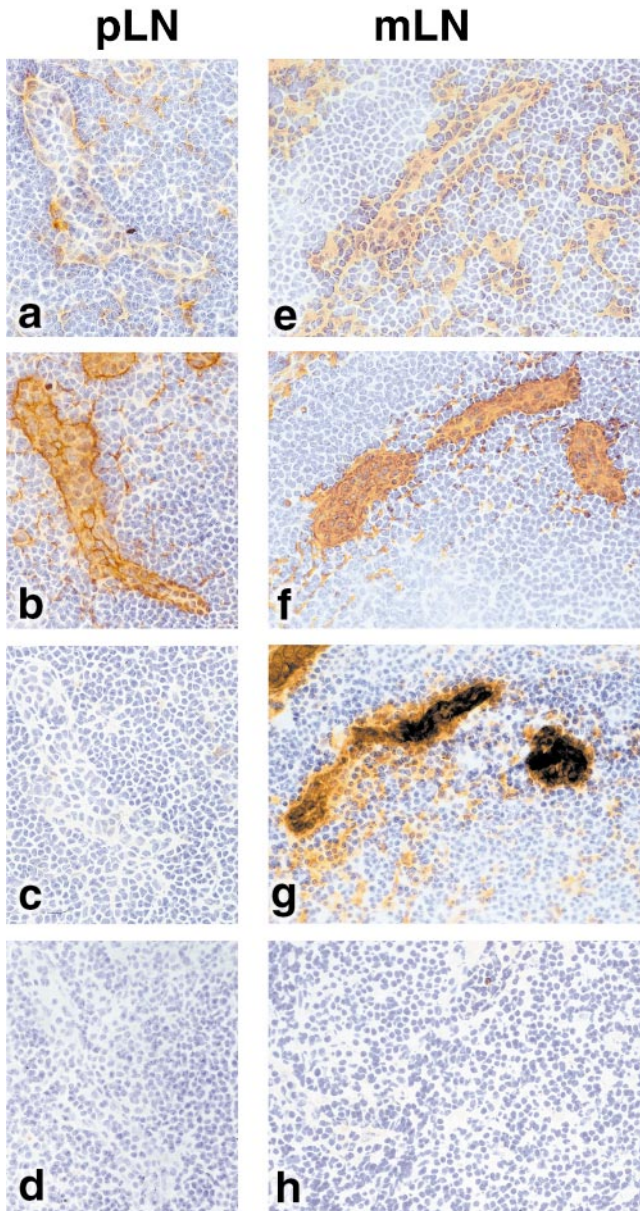


Figure 7. Histochemical demonstration of the presence of $\alpha 4$ integrin ligands in LFA-1^{+/+} pLNs (a–d) and mLNs (e–h). The panels show staining of identical tissue sections of HEVs in the pLNs with mAbs specific for (a) VCAM-1, (b) PNAd, (c) MAdCAM-1, and (d) an isotype-matched IgG1 control mAb and similarly, in the mLNs for (e) VCAM-1, (f) PNAd, (g) MAdCAM-1, and (h) IgG1 control mAb. Original magnification: a–h, $\times 150$.

CAM-1 mAb, had an inhibitory effect. Considered together, these results demonstrate that migration of recirculating lymphocytes into bone marrow can be mediated by either LFA-1 or the $\alpha 4$ integrins, and, as in pLNs, that VCAM-1 serves as the chief ligand for the latter receptors.

Discussion

This study shows that LFA-1 has a key role in migration to the pLNs, other LNs, and bone marrow but not into the

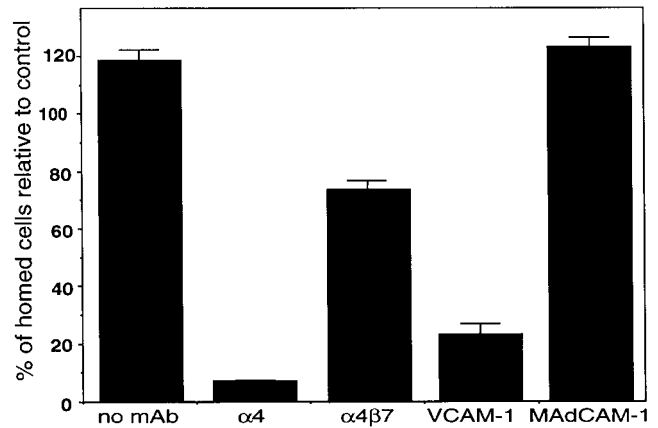


Figure 8. The effect of various mAbs on the relative entry of LFA-1^{-/-} to LFA-1^{+/+} lymphocytes into bone marrow. In the absence of antibody, the relative ratio is 1.1. Coinjection of $\alpha 4$ mAb or VCAM-1 mAb completely inhibited the migration of LFA-1^{-/-} cells, whereas $\alpha 4\beta 7$ mAb has a partial effect and MAdCAM-1 mAb is without effect.

spleen. Also revealed is a hitherto unrecognized ability of the $\alpha 4$ integrins, $\alpha 4\beta 7$ and $\alpha 4\beta 1$, to compensate for the lack of LFA-1 in lymphocyte trafficking to pLNs and other lymphoid tissues, including bone marrow. In general, these findings highlight common features between lymphocyte homing and the response to inflammatory stimuli and extend the validity of the multistep model of adhesion and transmigration.

The LFA-1^{-/-} mouse described in this report has, as its key phenotypic characteristic, pLNs that contain $\sim 30\%$ normal lymphocyte numbers, as also noted previously (25). The decreased trafficking of LFA-1^{-/-} lymphocytes to the pLNs to $\sim 20\text{--}30\%$ of wild-type lymphocytes suggested that LFA-1^{-/-} lymphocytes, irrespective of which subset, had difficulty gaining entry to the LNs. This was confirmed by microscopic studies showing that circulating LFA-1^{-/-} lymphocytes bound poorly to HEVs. Migration into mLNs and PPs was also depressed, but LN cell counts were normal, suggesting compensatory measures were in operation in these tissues but not in pLNs. The decrease in migration of lymphocytes to pLNs is consistent with previous work using function-blocking LFA-1 mAbs (10) and has recently been reported in another study using LFA-1-deficient mice (35). Thus, the general importance of LFA-1 in mature lymphocyte trafficking to secondary lymphoid tissue is confirmed.

In our studies, LFA-1^{-/-} lymphocytes were able to gain entry into the pLNs, albeit at a lower level, suggesting involvement of further receptors. We here demonstrate these additional receptors to be the $\alpha 4$ integrins. Skewed receptor usage towards increased expression of $\alpha 4$ integrins in LFA-1^{-/-} mice as a possible cause of experimental bias was excluded (data not shown). The fact that the presence of mAbs to both LFA-1 and $\alpha 4$ integrins caused complete blockade of lymphocyte entry into normal pLNs and other LNs strongly implies that $\alpha 4$ has a critical role in migration of normal lymphocytes into pLNs. That this role for $\alpha 4$ integrin has not previously been observed might be because LFA-1 has a larger role than $\alpha 4$ integrin in adherence to

pLN HEVs than HEVs of other organs. This is in good agreement with intravital microscopy studies which show LFA-1 and L-selectin are the essential codependent pLN-specific adhesion pair for lymphocyte adherence to pLN vessels (36). Put together, the data suggest that $\alpha 4$ integrins have a less prominent role in adherence to pLN HEVs, but potentially a larger role in the transmigration step.

Our data also suggest unexpectedly that $\alpha 4\beta 7$ acts as the major $\alpha 4$ receptor in conjunction with $\alpha 4\beta 1$ in lymphocyte recirculation into pLNs. There has previously been no evidence to link $\alpha 4\beta 7$ with pLN migration, although its dominant role in trafficking into mucosal tissue of the mLNs and PPs is well documented (3, 17) and is confirmed here. The integrin $\alpha 4\beta 1$ has usually been associated with inflammatory responses (18–20) rather than with lymphocyte recirculation. However, as these two $\alpha 4$ integrins might be acting either in synergy or in sequence, the extent of the contribution of $\alpha 4\beta 1$ must await a potent murine CD29 blocking mAb. The results imply that the succession of adhesive events in lymphocyte recirculation is similar to that in inflammatory responses, and that the $\alpha 4$ integrins in cooperation with L-selectin and LFA-1 have a more specific and necessary role than previously perceived.

The data presented here identify VCAM-1 and not MAdCAM-1 as ligand on pLN HEVs for $\alpha 4$ integrins in spite of $\alpha 4\beta 7$ involvement. The result was unexpected, as $\alpha 4\beta 1$ has been identified as the major VCAM-1 binding integrin in inflammatory responses (37) in studies using transfectant (38) and cell lines (17). VCAM-1 also made a significant contribution to trafficking into mLNs and, to a lesser extent, PPs. This cooperative activity with MAdCAM-1 was also unexpected, as it has previously been considered that entry into these LNs was regulated only by MAdCAM-1. The findings presented here suggest that potential roles for $\alpha 4\beta 7$ in addition to $\alpha 4\beta 1$ should be sought in other circumstances where VCAM-1 is the major ligand.

One explanation for having overlooked the importance of VCAM-1 as an HEV ligand is its reported absence from normal lymphoid tissue (22, 23, 39), with the exception of a report on its expression on rat HEVs (40). In the present study, expression occurred at comparable levels on HEVs in pLNs, mLNs, and PP LNs and without obvious difference between LFA-1^{-/-} mice, their wild-type littermates, C57BL/6, or BALB/c mice. Such broad and constant presence of VCAM-1 implies it is constitutively expressed rather than as a consequence of an inflammatory signal or a compensatory mechanism in LFA-1^{-/-} mice.

The involvement of VCAM-1 raises the issue as to whether lymphocytes can use this ligand for migration across HEVs into the LN as well as adhesion to the luminal surface of HEVs. In this study, the lack of LFA-1 decreased migration across HEVs by ~25% only, suggesting involvement of other receptor/ligand pair(s) (data not shown). For HUVECs, VCAM-1 is reported to be restricted to an apical location and therefore available for adhesion but not for transendothelial migration (41). However, monocytes can transmigrate HUVECs, making use of $\alpha 4$ /VCAM-1 independently of $\beta 2$ integrins (42). In mice, two major forms of

VCAM-1 have been identified, the common seven-domain form and also a three-domain glycosylphosphatidylinositol (GPI)-linked splice variant which is induced by inflammation (43, 44). It is of interest that in polarized epithelial cells, the GPI-linked VCAM-1 was found apically, whereas the seven-domain VCAM-1 was localized to the basolateral surface, where it could theoretically serve as a ligand for transmigration (45). The issue of where VCAM-1 is expressed, particularly in murine HEVs, warrants further investigation.

Migration of recirculating lymphocytes to bone marrow, a primary lymphoid tissue, has been noted in the past (46). Bone marrow serves as a site of hematopoiesis and for B cell maturation in the mammal. It can also act as a reservoir for a primary immune response in the absence of a spleen and presence of L-selectin mAb (47). In this study, we find an unexpectedly high degree of mature lymphocyte recirculation in which B cells dominate into bone marrow. We suggest here that bone marrow, but not the thymus (data not shown), should be considered as a major part of the lymphocyte recirculation network.

Migration to bone marrow is restricted by adhesion mechanisms. Both human (48) and murine (49) hematopoietic progenitors make use of $\alpha 4$ integrin to lodge in the bone marrow. The $\alpha 4$ integrins are also reported to be necessary for correct lymphocyte development (50). We show that normal lymphocyte recirculation through the bone marrow is regulated by LFA-1 and the $\alpha 4$ integrins and, in contrast to the LNs, $\alpha 4\beta 1$ substantially dominates $\alpha 4\beta 7$. In this context, it is again VCAM-1 and not MAdCAM-1 which acts as ligand for the $\alpha 4$ integrins. VCAM-1 is expressed constitutively by bone marrow sinusoidal vasculature (51, 52), and as progenitor cells have been demonstrated to roll on bone marrow endothelial VCAM-1 (48), it can be speculated that other lymphocytes might behave similarly. However VCAM-1 is also expressed constitutively by stromal reticular cells (53, 54), and it is possible that lymphocytes may be retarded not only at the level of the endothelium but also within the bone marrow matrix. Factors produced by the stromal cells might be beneficial for the maintenance of the recirculating lymphocytes. For example, the interaction with VCAM-1 might suppress lymphocyte apoptosis (55). On the other hand, the incoming cells might contribute to the regulation of hematopoiesis.

In summary, the observations presented here extend and validate the concept of a multistage adhesion response requiring selectins and integrins. Rather than exclusive LN-specific use of receptors such as LFA-1 and L-selectin in trafficking to pLNs or $\alpha 4\beta 7$ to mucosal sites, we have presented evidence that LFA-1 and the $\alpha 4$ integrins can operate in migration of unstimulated lymphocytes to the pLNs as well as other secondary LNs and bone marrow. These general features, including the use of VCAM-1 as an $\alpha 4$ integrin ligand, resemble the response to an inflammatory signal. Our findings suggest a unifying hypothesis for migration of lymphocytes across HEVs into secondary lymphoid tissue and also establish further parallels between the activities of adhesion receptors in the “homing” context and the mechanisms used in the more sporadic responses to tissue injury and inflammation.

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