Distinct roles for LFA-1 affinity regulation during T cell adhesion, diapedesis and interstitial migration in lymph nodes

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ABSTRACT

During the course of homing to lymph nodes (LN s), T cells undergo a multi-step adhesion cascade that culminates in an LFA-1-dependent firm adhesion to the luminal surface of high endothelial venules (HEVs). The importance of LFA-1 affinity regulation in supporting T cell arrest on HEVs has been well established, however, its importance in the post-adhesion phase which involves intraluminal crawling and diapedesis to the extravascular space remains elusive. Here we have shown that LFA-1 affinity needs to be appropriately regulated to support these essential steps in the homing cascade. Genetically engineered T cells that were unable to properly down-regulate LFA-1 affinity underwent enhanced, chemokine-independent arrest in HEVs but showed perturbed intravascular crawling to transmigration sites and compromised diapedesis across HEVs. By contrast, the extravascular migration of T cells was insensitive to the affinity-enhancing LFA-1 mutation. These results highlight the requirement for balanced LFA-1 affinity regulation in intra- and trans-vascular, but not extra-vascular, T cell migration in LN s.
INTRODUCTION

The constant recirculation of naïve T cells through secondary lymphoid organs (SLOs) is critical for immune surveillance. A central event in this process is homing of T cells to LNs via HEVs. A current model of the homing cascade includes a sequence of at least 4 distinct steps: 1) recruitment of circulating T cells to the luminal HEV surface, involving a rolling interaction and its subsequent conversion to firm adhesion upon chemokine activation; 2) intravascular migration of luminally adherent T cells that allows the translocation from the initial attachment site to a suitable exit site; 3) transendothelial diapedesis across HEVs; and 4) random migration of T cells within the extravascular compartment in LN parenchyma. Considerable information is available on the molecular and cellular mechanisms involved in the first and last step in this homing cascade; however, little is known about the rules that control the access of luminally adherent cells to the LN parenchyma.

Integrin LFA-1 (αLβ2) is the predominant cell adhesion molecule present on T cells. LFA-1 is an α/β heterodimeric transmembrane membrane protein that contains the ligand binding inserted (I) domain at the most distal part of the extracellular portion. LFA-1 undergoes dynamic and regulated conformational changes in response to internal cues (e.g., the intracellular signaling elicited by chemokine and T cell receptors,) as well as in response to external cues (e.g., ligand densities and shear stress). A series of in vitro investigations propose a model that explains how these sequential engagements of internal and external cues regulate LFA-1 conformations in T cell interactions with ICAM-1, the major LFA-1 ligand on endothelial cells. In naïve unstimulated T cells, LFA-1 is predominantly in a default bent form containing a low-affinity (LA) I domain. Upon encountering endothelial cell-bound chemokines that trigger G-protein-coupled receptor
(GPCR) signaling, this latent form of LFA-1 is converted into a “primed” extended form possessing an intermediate-affinity (IA) I domain. In physiologically perfused microvessels, the IA LFA-1 is rapidly stabilized into a fully active extended form with a high-affinity (HA) I domain via the interaction with ICAM-1, supporting T cell arrest on ICAM-1 \(^{14}\). In T cells laterally migrating on ICAM-1 substrates \textit{in vitro}, LFA-1 affinity needs to be spatio-temporally regulated: whereas HA LFA-1 mediates adhesion at the anterior of polarized cells, the heterodimer reverts to the LA form and, thus promotes de-adhesion at the posterior end, supporting balanced cycles of adhesion and de-adhesion \(^{15,16}\).

Previous studies using LFA-1 blocking antibody \(^{17}\) and LFA-1 deficient mice \(^{18}\) have shown that lymphocyte homing to LNs is critically dependent on LFA-1. Intravital microscopy (IVM) investigations of lymphocyte behavior in LNs have revealed that inhibitors of LFA-1 \(^{19,20}\) block intravascular lymphocyte arrest on HEVs. In addition, similar loss-of-function strategies have been used to suggest that LFA-1 might be dispensable for leukocyte migration in the LN interstitial space. For example, Woolf et al. reported that \(\beta_2\) integrin-deficient T cells lacking LFA-1 exhibited only moderately impaired interstitial motilities \(^{21}\). In addition, Lämmermann et al. reported entirely integrin-independent interstitial migration of dendritic cells (DCs) \(^{22}\). However, it is still unclear how the conformational regulation of LFA-1 activation and, in particular, regulated LFA-1 de-adhesion affect T cell homing. Moreover, loss-of-function approaches that abrogate LFA-1 function are not suitable to explore the role of LFA-1 in the post-adhesion phase of the homing cascade prior to entry into the extravascular space. Thus, it is currently unknown whether LFA-1 contributes to intravascular T cell crawling or diapedesis \textit{in vivo}. To fill this gap in knowledge, we have generated a mutant mouse, in which LFA-1 de-adhesion is perturbed by a knock-in mutation \(\alpha_L\)-I306A that constitutively up-regulates ligand binding affinity of LFA-1. By studying the adhesive interactions of \(\alpha_L\)-I306A T cells with HEVs
using multiphoton-intravital microscopy (MP-IVM), here we show the importance of properly down-regulating LFA-1 affinity in promoting the intravascular crawling and diapedesis of T cells during physiologic homing to peripheral LNs.
RESULTS

Constitutive affinity upregulation of LFA-1 by a genetic perturbation of the I domain
α-helical conformational constraint residue

To perturb LFA-1 de-adhesion in vivo, we sought to use a knockin mutation that
constitutively up-regulates LFA-1 affinity. To this end, we investigated the structural
information of human LFA-1 I domain. The I domain adopts a Rossmann fold with a
metal ion-dependent adhesion site (MIDAS) at the “top” of the domain, whereas its N- and
C-terminal connections occur on the distal “bottom” face (Figure 1 A). Upon cellular
activation, the C-terminal α7 helix is induced to slide down along the side of the I domain,
thereby triggering the conformational conversion of the default LA I domain to the IA, and
then to the HA form. In the LA I domain, an invariant isoleucine (Ile-306 in the αL I
domain) located at the second half of the α7 helix, intercalates deeply into a hydrophobic
pocket, thus providing the stabilization needed for this conformation (Figure 1 A). In
this way, I306 constrains the movement of the α7 helix, thereby maintaining LFA-1 in a
default LA non-adhesive state. Indeed, the alanine substitution of I306 has been shown to
constitutively activate human LFA-1 in transfectants.

To determine the structural basis of the Ile-306 constraint in the mouse LFA-1 I
domain, we first built a structural model, since there is no crystal or solution structure
currently available. We carried out homology modeling using the closed LA conformation
of a human LFA-1 I domain crystal structure (pdb code: 1ZON) as a template. This
model showed that the conserved Ile-306 is located in a hydrophobic cavity of the closed
conformation of the mouse I domain (Figure 1 A), thus supporting the hypothesis that this
isoleucine likely helps maintain the mouse LFA-1 I domain in the LA state.
To disable the Ile-306 I domain conformational constraint in vivo, we employed standard gene targeting approaches; specifically, we mutated the conserved Ile-306 into alanine in the murine αL I domain, thereby generating a mutant Ilgal-I306A (Figure 1, B and C). We have obtained knock-in (KI) mice homozygous for the mutant αL allele Ilgal I306A/I306A, which were designated αL-I306A. αL-I306A mice did not exhibit any gross abnormalities or developmental defects except for a smaller sized peripheral LN [WT, 1.62 ± 0.11 mm; αL-I306A, 1.06 ± 0.08 mm, (diameter) P < 0.001], which contained less lymphocytes compared with WT (Table S1).

Increased binding to and perturbed migration on ICAM-1 of αL-I306A LFA-1 in vitro

To characterize the effect of αL-I306A mutation to LFA-1 on the cell surface, we initially analyzed mutant and WT lymphocyte adhesion in vitro. αL-I306A lymphocytes showed reduced cell-surface expression of LFA-1 but not other integrins (Figure S1). Despite reduced LFA-1 expression, αL-I306A lymphocytes exhibited enhanced basal activity to bind to ICAM-1, but not to VCAM-1, substrates in the presence of physiological concentrations of Mg2+ and Ca2+, which favor the default LA state of integrins9 (Figure 2 A and Figure 2S, A and B). The increased basal adhesion of αL-I306A cells to ICAM-1 was further enhanced by extracellular Mn2+-stimulation which mimics cellular stimulation, thus inducing the HA conformation9 (Figure 2 A, left). As measuring monomeric affinity of LFA-1 on the cell surface is technically difficult29, we sought to assess LFA-1 affinity status using dimeric ICAM-1-Fc. Consistent with the cell adhesion assays, the assessment of LFA-1 affinity status using soluble ICAM-1-Fc showed that αL-I306A LFA-1 adopts a constitutively active IA conformation, which can be reversibly activated to the HA conformation upon exposure to Mn2+ (Figure 2 B).
Chemokine-driven transmigration of WT and αL-I306A lymphocytes across uncoated and VCAM-1-coated Transwell inserts proved comparable, suggesting that chemokine responses and α4 integrin-mediated transmigration in αL-I306A cells are intact (Figure 2 C, right). By contrast, transmigration of αL-I306A cells across ICAM-1-coated inserts was suppressed compared with that of WT cells (Figure 2 C, left). The more ICAM-1 density on inserts increased, the more severe was the perturbation of αL-I306A T cells (Figure 2 C, left).

We next attempted to study two-dimensional (2D)-migrations of naïve WT lymphocytes, in which basal LFA-1 adhesiveness was maintained at low levels. However, these cells failed to exhibit efficient 2D-migration on either ICAM-1/CXCL12- or ICAM-1/CCL21-coated substrates (data not shown). Similar observations have been made in a previous report 21. As an alternative we therefore used IL-15-induced Central Memory-like T cells (TCM), since in these cells LFA-1 is known to display little basal adhesiveness to ICAM-1, while still readily upregulating adhesion upon chemokine stimulation 30. WT TCM robustly migrated on ICAM-1/CXCL12 (Figure 2 D and Supplementary Movie 1 A). In contrast, αL-I306A TCM showed efficient adhesion, but reduced 2D-migration, on the same substrate (Figure 2, D-G and Supplementary Movie 1B). During the migration of αL-I306A cells, the leading and trailing edges were not well coordinated. Whereas WT cells polarized in a characteristic fashion, displaying a typically hand mirror-like shape with a flattened leading edge followed by a short narrow tail, αL-I306A cells exhibited extremely extended uropods that were highly enriched in αL-integrin (Figure 2, H and I). Continuous forward movement of both the leading edge and the body of the αL-I306A cell appeared to be frustrated by the inefficient detachment of the uropod. In addition, migrating αL-I306A, but not WT, T cells left behind LFA-1-rich membrane debris on ICAM-1 substrates, suggesting that the mutant LFA-1 present in the tail was unable to detach from the ICAM-1.
substrates (Figure 2 H). The dis-coordination between the body of αL-I306A cells and their trailing edge reduced not only the migratory velocity, but also their propensity to make a constant forward movement in the same direction, resulting in ~34% reduced meandering index (Figure 2 G). Thus, the decreased migration efficiency of αL-I306A cells on and across ICAM-1 substrates appears to be related to the perturbed detachment of the uropod.

**αL-I306A LFA-1 enhances the interactions of T cells with lymph node venules**

Next, we sought to study how LFA-1 de-adhesion would affect T cell homing to LNs. We performed a competitive homing assay, in which WT and αL-I306A cells labeled with carboxyfluorescein diacetate, succinimidyl ester (CFSE) and 5-(and-6)-(((4-chloromethyl) benzoyl) amino) tetramethylrhodamine (CMTMR), respectively, were mixed and intravenously administered into C57BL/6J-Ly5.1 WT recipient mice. Organs were harvested 2 h after injection and the homing indices were determined. We found that αL-I306A T cells homed ~50% less well to the peripheral and mesenteric LNs, Peyer’s patches, small and large intestines, while mutant and WT T cells were equally represented in peripheral blood, spleen, bone marrow, and lung (Figure 3). In contrast, more αL-I306A T cells accumulated in the liver than WT cells (Figure 3).

Reduced homing of αL-I306A T cells to LNs raised the possibility that although αL-I306A cells showed enhanced adhesion to ICAM-1 under static condition *in vitro*, constitutively IA LFA-1 might confer to T cells a diminished ability to arrest on HEVs. To directly address this possibility, we employed epi-fluorescence-IVM imaging of peripheral (inguinal) LNs, investigating the adhesive interactions of WT and αL-I306A T cells with physiologically perfused LN venules. The venular tree in inguinal LNs is composed of 5 different venular branching orders: orders III to V are cortical HEVs, whereas orders I and
Il are medullary-collecting venules with flat endothelia. Only high order venules simultaneously express ligands for L-selectin, ligands for LFA-1, and CCR7-activating chemokines that trigger LFA-1 activation and support the bulk of naïve lymphocyte traffic into peripheral LNs. Thus, we focused our analysis on the ability of fluorescently tagged WT and mutant T cells to roll and arrest in order II-V HEVs of WT recipient mice.

Although both cell types displayed comparable rolling fractions (Figure 4 A), $\alpha$-I306A T cells exhibited a slower rolling velocity than did WT cells on high-order venules (Figure 4 C). Both cell types express comparable levels of L-selectin, and their rolling was primarily mediated by L-selectin, since a mAb to L-selectin abrogated all rolling interactions (data not shown). The reduction in rolling velocity of KI T cells was mediated by $\alpha$-I306A LFA-1, since LFA-1 mAb treatment reverted it to levels comparable to those of WT cells (Figure 4 C) without affecting rolling fractions (Figure 4 B). Compared with WT, $\alpha$-I306A T cells also exhibited markedly increased sticking fractions in order IV and V venules (Figure 4 D). LFA-1 mAb treatment abrogated the firm adhesion of both cell types in HEVs (data not shown). Thus, reduced homing of $\alpha$-I306A T cells to LNs is unlikely due to their diminished ability to arrest on HEVs.

$\alpha$-I306A T cells exhibit perturbed intravascular crawling and diapedesis

To investigate the mechanisms underlying the reduced homing of $\alpha$-I306 T cells in spite of enhanced sticking to HEVs, we conducted a series of MP-IVM experiments in intact popliteal LNs by injecting WT and mutant T cells into bone-marrow (BM) chimeric mice. These chimeric mice were made by irradiating and reconstituting actin promoter-driven GFP-transgenic mice with WT BM cells. In these mice, only radio-resistant stromal cells, particularly LN endothelial cells are GFP-positive. Indeed, we found that LN endothelial cells exhibited much higher GFP levels than any other cell type in BM.
chimeras, thus allowing us to discriminate individual vessels from the surrounding stromal cells (Figure 5 A and Supplementary Movie 2). Unlike IVM methods involving the use of fluorescent plasma markers that highlight only the luminal compartment of LN vessels, the utilization of GFP chimeras enabled us to directly visualize and delineate the vascular wall of HEV and to undertake a detailed examination of interactions between donor T cells and endothelial cells during the entire homing process.

For MP-IVM imaging, an equal number of WT and KI T cells were fluorescently labeled with 7-amino-4-chloromethylcoumarin (CMAC) (blue) and CMTMR (red), respectively, and adoptively transferred by i.v. injection (in some experiments the fluorescent dyes were swapped with identical results). Adhesive interactions of donor T cells with GFP+ LN venules were recorded. HEVs were morphologically and functionally identified as those GFP+ LN venules in which WT T cells could be detected (Figure 5 A, and Supplementary Movie 2). During the course of our observations (0 to ~180 min after adoptive transfer), we continually noted that ~2 times more $\alpha_{L}$-I306A cells firmly adhered to the apical sides of HEVs than WT cells (Figure 5 B). This finding is consistent with the increased sticking fraction of $\alpha_{L}$-I306A cells to high order venules observed in epi-fluorescence-IVM. To determine whether $\alpha_{L}$-I306A LFA-1 can support firm adhesion to HEVs independently of GPCR activation, WT and $\alpha_{L}$-I306A T cells were pre-treated with pertussis toxin (PTX) and adoptively transferred into GFP-chimeric mice. While PTX treatment greatly reduced the number of arresting WT T cells as expected $^{19}$, it barely affected the number of arresting KI cells (Figure 5 B). However, neither PTX-treated WT nor KI T cells that arrested in HEV underwent subsequent diapedesis, indicating that this step requires guidance by GPCR-dependent chemo-attractants (Supplementary Movie 3).

To study the motility of T cells on the apical surface of HEVs, we analyzed those T cells present on the luminal side of GFP+ vessels by delimiting a Region of Interest (ROI)
to the vessel compartment. This analysis approach confirmed that more KI cells were present in the vessel compartment than were WT cells (Figure 5, C and D). Compared with WT T cells, $\alpha_L$-I306A T cells displayed aberrant, “frustrated” movements characterized by a decreased meandering index and an increased mean three-dimensional instantaneous velocity (3D-IV) (Figure 5, C and D). This type of movement of KI T cells suggests an inefficient migration as seen in our in vitro 2D migration data (Supplementary Movie 1b), in which they fail to move forward due to an impaired release of the trailing edge from the substrate, while rapidly displacing the leading edge. This result led us to study in detail how efficiently WT and KI T cells migrate on and across HEV (Figure 5; E-K).

Whereas ~81% of arrested WT T cells completed both steps of HEV transmigration in a 60-min recording, only ~52% of KI T cells did so (Figure 5 E). While tracing the migratory paths of individual T cells that had successfully transmigrated, we noted that arrested T cells underwent distinct sequential steps while emigrating from HEVs: an initial crawling step identified by a migratory path on the apical surface of HEVs from the initial arresting point to an exit site; and the TEM step, which encompassed the movement of T cells that had reached an exit site across the HEV (Figure 5 F, and Supplementary Movie 2). To better characterize these steps of T-cell transmigration, we focused the WT and KI T cells that had successfully transmigrated across HEVs, while excluding those that had not transmigrated. We found that $\alpha_L$-I306A T cells crawled more slowly and shorter distances to exit sites than did WT cells (Figure 5, G, H, J and K). During the TEM step, $\alpha_L$-I306A T cells took ~6 times longer to transverse the endothelial layer and reach the LN parenchyma than did WT cells (Figure 5, I). Representative migration profiles show that compared with a WT T cell (Figure 5J), a KI T cell crawled for a shorter time period with a slower velocity, potentially scanning less surface areas of HEV, and then took longer to
complete transmigration (Figure 5 K). These findings suggest that the perturbed cell migration during both crawling on and transmigrating across HEVs was responsible for the lower overall homing efficacy of KI T cells.

**Impact of perturbed crawling and diapedesis on T cell homing to LNs**

In the conventional homing assays that we had performed initially (Figure 3), labeled cells were considered to have "homed" when they could be recovered from recipient tissues irrespective whether they reside in the vascular or in the interstitial compartment. To selectively assess the capacity of T cells to complete the entire homing cascade including TEM, we administered LFA-1 blocking mAb M17/4 after allowing donor cells to home to LNs for 1 h. Lymphoid organs were harvested 1 hr after mAb injection and the homing indices were determined. This delayed LFA-1 mAb treatment not only blocked further accumulation of new circulating T cells to HEVs, but also detached those T cells that were already adherent on the luminal surface of HEV but had not undergone diapedesis. Although the delayed LFA-1 mAb treatment did not affect the homing indices to the spleen, bone marrow, and peripheral blood (Figure 6), it decreased the homing indices to the peripheral and mesenteric LNs by ~50% (Figure 6). These results strongly suggest, but may not conclude definitively, that the perturbed intravascular crawling and diapedesis steps observed in KI T cells at the HEV microenvironment have an impact on T cell homing and extravasation to LNs at a systemic level.

**αL-I306A T cells do not display aberrant interstitial motility**

Next we studied WT and KI T cell migration in the LN interstitial microenvironment, which contains fibroblastic reticular cells (FRCs) and DCs expressing detectable levels of ICAM-1, albeit low levels as compared with HEVs (Figure S3). To examine T cell interstitial motility with MP-IVM, we used non-irradiated WT mice as recipients of
differentially labeled WT and KI T cells. Both WT and KI T cells crawled rapidly, displaying random walk-like behaviors (Supplementary Movie 4) with comparable motility parameters, including mean 3D-IV and meandering indices (Figure 7, A and B). We observed small, but statistically significant, decrease in motility coefficients of KI T cells (Figure 7 C), which was associated with increased turning angles (Figure 7 D). These results suggest that LFA-1 is minimally engaged in ligand binding during the rise in interstitial T cell motility, even when the integrin was in a constitutive IA state. To confirm this hypothesis, we acutely blocked LFA-1 by administering mAb M17/4 after allowing adoptively transferred T cells to enter the LN interstitium (i.e., 24 h after adoptive T cell transfer). Administered M17/4 (unlabeled) bound to T cells in the LN interstitium, as it competed for LFA-1 with a fluorescently labeled M17/4 when the labeled mAb was applied ex vivo (Figure 7E) However, this anti-LFA-1 treatment had no effect on mean 3D-IV, meandering indices, or the motility coefficients of WT and KI T cells in the LN interstitium (Figure 7, F-H), thereby indicating that neither an enhanced (intermediate) ligand binding activity nor complete functional inhibition of LFA-1 has a major impact on interstitial T cell motility in the steady state. We observed similar results in additional experiments in which WT and αL-I306A cells were alternately labeled (data not shown).

These results support the possibility that the conversion of the primed LFA-1 to the HA conformation that occurs during intravascular arrest is apparently absent from the LN interstitial space. A potential absence of shear stress in the LN interstitium might explain the mechanism that prevents such conversion to the HA conformation. Alternatively, as the LFA-1 ligand expression levels potentially affect LFA-1 activation, we studied the spatial and differential distribution of ICAM-1 and ICAM-2 in the vessel and interstitial compartments. Immunofluorescence histology revealed that while ICAM-2 was highly colocalized with PNAd (HEVs), it barely colocalized with ERTR-7+ (FRCs) or CD11c+ (DCs) (Figure S3E), confirming a previous report that ICAM-2 is expressed predominantly in
endothelial cells and only at low levels on platelets and some leukocyte subsets. By contrast, ICAM-1 was broadly expressed in the T-cell zone, where it partly co-localized with PNAd, ERTR-7, and CD11c (Figure S3; A and C). To assess the density of ICAM-1, we compared the mean fluorescence intensity (MFI) of ICAM-1 in HEVs, FRCs, and DCs. Using quantitative image analysis with a Photoshop software application, we found that the ICAM-1 MFI values in HEVs were significantly higher than those in FRCs and DCs (Figure S3; B and D). Therefore, relatively low ICAM-1 and ICAM-2 densities in the interstitial space might prevent ligand engagement of LFA-1.
DISCUSSION

LFA-1 conformation is thought to be spatially and temporally regulated, playing an important role in the promotion of T cell migration\(^{15,16}\). While T cells migrated on ICAM-1 substrates, LA LFA-1 was found to selectively localize at the trailing edge, where it was associated with myosin heavy chain IIA, which would provide the necessary force to retract the uropod\(^ {16}\). To investigate the regulated conformational activation of LFA-1, and in particular LFA-1 de-adhesion in T cell homing to LNs, we utilized \(\alpha_L\)-I306A knock-in mice. The \(\alpha_L\)-I306A LFA-1 adopts the constitutively active IA conformation that supports high-avidity cell adhesion to ICAM-1 substrates in an \textit{in vitro} adhesion assay. As previously proposed\(^ {35}\), IA LFA-1 establishes the initial contact, which is subsequently transformed into a stable high-avidity cell adhesion. One should take into consideration that V-bottom adhesion assays utilize shear stress via centrifugal force to remove unbound cells\(^ {36}\). The shear stress generated by this technique might help the conversion of the IA \(\alpha_L\)-I306A LFA-1 to the HA conformation, thereby supporting high-avidity cell adhesion in this \textit{in vitro} setting. While \(\alpha_L\)-I306A KI cells exhibited constitutively enhanced adhesion to ICAM-1 substrates, they showed perturbed transmigration across ICAM-1-coated chambers and 2D-migration on ICAM-1 substrates. Our \textit{in vitro} observations are consistent with a previous study involving another strain of knock-in mice (Lfa-1\(^{d/d}\)) expressing constitutively active LFA-1 via a deletion of the membrane-proximal \(\alpha_L\) cytoplasmic region\(^ {37}\). Perturbation of either the I domain constraint in \(\alpha_L\)-I306A mice or the cytoplasmic \(\alpha/\beta\) association in Lfa-1\(^{d/d}\) mice resulted in aberrantly activated ligand binding by LFA-1 and suppressed cell migration on ICAM-1. Importantly, by leaving the cytoplasmic domains intact, the I domain mutation in \(\alpha_L\)-I306A mice rules out the possibility that the altered interactions with cytoskeletal and cytoplasmic signaling...
molecules are induced by the partial deletion of cytoplasmic tails, a potential caveat associated with the use of Lfa-1\textsuperscript{d/d} cells \textsuperscript{37}.

Our epi-fluorescence-IVM investigations showed that while $\alpha_L$-I306A LFA-1 did not affect the rolling fraction, it decreased rolling velocity, suggesting that IA $\alpha_L$-I306A LFA-1 can stabilize rolling interactions once they are initiated by L-selectin. LFA-1 has been shown to help stabilize selectin-mediated rolling of neutrophils along inflamed mesenteric venules \textit{in vivo} \textsuperscript{38} but LFA-1 does not appear to contribute to physiological lymphocyte rolling in HEV \textsuperscript{19}, presumably because WT LFA-1 on naïve T cells adopts a predominantly LA conformation \textsuperscript{13}. The present data demonstrates that LFA-1 in the IA conformation can stabilize L-selectin-mediated T cell rolling along HEVs, suggesting that the conformational regulation of LFA-1 differs in lymphocyte and myeloid leukocytes. Consistent with this interpretation, Green et al. suggested that intermediate-affinity LFA-1 cooperates with selectins in reducing the rolling velocity of neutrophils \textsuperscript{39}. Stabilization of $\alpha_L$-I306A T cell rolling, which occurred in high-order LN venules, apparently also promoted the subsequent firm adhesion of rolling T cells. This can account for the increased sticking fraction observed preferentially in high-ordered venules, although LFA-1 ligands are broadly expressed throughout the venule tree \textsuperscript{31}.

Our MP-IVM investigations in GFP chimeric mice enabled, for the first time, a detailed examination of T cell interactions with HEV during the post-adhesion phase of homing to LNs. We identified a sequence of three distinct steps that require the fine-tuned regulation of LFA-1 to enable T cell entry into LNs: 1) firm arrest; 2) intravascular crawling to a TEM site; and 3) TEM across HEV into the extravascular space. Monocytes \textsuperscript{40} and neutrophils \textsuperscript{41,42} have been previously shown to undergo $\beta_2$ integrin-mediated intravascular crawling on inflamed vessels to the extravasation sites. However, to our knowledge, this is the first demonstration that T cells crawl along the luminal surface of microvessels to
undergo transmigration at TEM sites. While αL-I306A T cells showed increased firm arrest to HEV, their capacity for intravascular crawling and TEM was severely compromised. This disturbed migration likely stems from the inability of adherent αL-I306A T cells to efficiently detach the uropod, thereby locking them in place in the intravascular compartment. Thus, T cells must appropriately down-regulate LFA-1 affinity to efficiently crawl within and across HEVs. Our in vivo observations are in good agreement with recent in vitro findings by Shulman et al.\(^5\) who showed that human T cells must be able to regulate LFA-1 to migrate on and across a monolayer.\(^5\)

It has been suggested that lymphocytes preferentially leave HEV through discrete exit sites (termed “hot spots”).\(^43\) The inability to crawl within HEV likely limited the ability of αL-I306A T cells to reach these “hot spots”. Indeed, modifying a traditional homing assay by employing a delayed LFA-1 mAb treatment, we were able to distinguish between donor cells that had successfully accessed the LN parenchyma from those that remained arrested in the intravascular compartment. These experiments indicate that the perturbed intravascular crawling and diapedesis of αL-I306A T cells reduced their capacity to home into the LN parenchyma by as much as 60%.

The aberrant motility profiles of αL-I306A cells in the vessel compartment have also been characterized. αL-I306A cells showed lower meandering indices, but higher 3D-IV, than did WT cells. In addition, αL-I306A cells tended to change direction more frequently (i.e., higher turning angles). Consistent with these data, we observed that many αL-I306A cells in the vessel compartment displayed unproductive jerky oscillations, as if frustrated by their inability to detach from endothelial cells. These oscillatory movements appear to reflect an active (albeit largely thwarted) migratory apparatus (i.e., chemotactic sensing and lamellipodia formation), as is reflected by the increased mean 3D-IV. Similarly, during 2D-migration on ICAM-1/CXCL12 substrates in vitro, αL-I306A cells displayed
disturbed migratory movements that appeared to result from the inability of aberrantly activated LFA-1 to support efficient tail detachment. Jerky migratory behaviors have also been seen in the disturbed TEM of human lymphocytes aberrantly activated by a small-molecule agonist for LFA-1. Alternatively, aberrantly regulated signaling through constitutively, or irreversibly, active αL-ι306A LFA-1 might perturb appropriate LFA-1 redistribution on the membrane, thereby suppressing efficient cell migration, as regulated redistribution of IA LFA-1 at the leading edge and HA in the mid-body have been proposed to be important for facilitating T cell migration.

Since its first implementation, MP-IVM has revealed the unexpectedly motile nature of naïve T cells in the LN interstitial compartment. However, the impact of LFA-1 activation status in this physiologic mode of migration has remained elusive. Woolf et al. observed minimal engagement of LFA-1 on naive T cells migrating in LNs, suggesting that the LFA-1 activation cascade precipitating the conversion to enhanced affinity might be inactive. However it had been unclear whether a shift in LFA-1 to the IA affinity form could impact on interstitial T cell motility. We show here that IA αL-ι306A LFA-1 exhibited enhanced firm adhesion to HEVs under shear stress in the absence of GPCR signaling. However, the expression of IA LFA-1 was apparently insufficient to engage its ligand in the extravascular space, although one could assume that such engagement would result in detectable changes in interstitial T cell motility. We observed mild changes in the motility coefficient and turning angle of KI T cells when compared to WT T cells. These parameters were unaffected after LFA-1 inhibition in vivo, thereby suggesting that these changes were independent of LFA-1 engagement. Although we did not observe an enrichment of activated T cells in LNs of KI mice, compared to those of WT mice (not shown), it is difficult to experimentally exclude the possibility that WT and KI T cell subsets that gained access to the LN interstitium might not be identical. These results suggest that
the LN interstitial space lacks essential external cues that must convert the IA LFA-1 to the HA form. It has been based on earlier studies of intravascular lymphocyte-endothelial interactions\textsuperscript{11,14} that chemokine-induced IA LFA-1 is converted to the HA form by interactions with its ligand combined with fluid shear stress. Since the interstitial milieu is proposed to lack shear stress\textsuperscript{21}, \(\alpha_L\)-I306A LFA-1 might remain in the IA conformation, which is apparently insufficient to engage in a strong adhesive interaction with ICAM-1. However, it is possible that in the LN parenchyma, cells continuously slide each other under a high-cell density microenvironment, potentially producing a physical friction that could influence LFA-1 conformations. Alternatively, as shown in our quantitative fluorescent histology data of the LN HEV and interstitium (Figure S3), lower levels of ICAM-1 and ICAM-2 expression in the LN parenchyma, compared to HEVs might prevent the conversion of IA LFA-1 on KI T cells to an HA form.

In summary, our data demonstrate that the appropriate balance of LFA-1 affinity is critical for T cell homing to LNs. Whereas LFA-1 affinity upregulation is required for T cell arrest on the luminal surface of HEVs, LFA-1 affinity needs to be properly down-regulated to facilitate intraluminal crawling and diapedesis. Perturbed de-adhesion limits the ability of T cells to crawl within and diapede across HEVs, thereby interfering with T cell homing to LNs. In contrast to the interactions with HEVs, LFA-1 engagement is dispensable for T cell migration in the interstitial space presumably due to the absence of shear and/or reduced LFA-1 ligand expression, thereby failing to convert the IA LFA-1 to the ligand competent HA form.
METHODS (ADDITIONAL METHODS ON SUPPORTING INFORMATION)

MP-IVM to study T cell interactions with HEV. In order to visualize the endothelial layer of LN vessels, α-actin GFP mice were irradiated with 1,300 rad (divided by two doses of radiation, interspaced by 6 hours) and reconstituted by intravenous injection of bone-marrow cells from WT C57BL/6J mice. Resulting GFP-chimeric mice were used as recipient, 5-6 weeks after hematopoietic reconstitution. GFP-chimeric mice were prepared for MP-IVM imaging as described. WT and KI T cells (1 X 10⁷), fluorescently labeled with CMAC and CMTMR, were adoptively transferred to the GFP-chimeric mice preparation. In some experiments, fluorescent dyes CMAC and 5-chloromethylfluorescein diacetate (CMFDA) were switched. In other experiments, donor T cells were pre-treated with 100 ng/ml pertussis toxin (PTX) for 1h at 37°C prior to injection. The kinetics of intravascular behaviors of donor T cells were evaluated by MP-IVM imaging on the HEV from time 0 to 180 min after injection. Volocity software (Improvision), was used for semi-automated tracking of cell motility in three dimensions inside vessels. These regions were defined by the delimitation of a Region Of Interest (ROI) containing GFP+ vessels and both T cell types. Parameters of cell motility were determined as previously described. Analysis of migratory paths of donor T cells arresting to HEVs and the quantification of cell numbers within HEVs were performed manually and offline by playback of digital video files. Donor cells crawling on the apical surface of HEVs and those transmigrating HEVs at TEM sites were visually distinguished. The measurements of TEM were performed with ImageJ software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/, 1997-2008) using the Manual Tracking plugin.

Statistical analysis. Unless otherwise stated, data are expressed as the mean values ± s.e.m. Two-tailed Student’s t test was used for statistical analyses unless otherwise indicated. Statistical significance was defined as either P < 0.05*, 0.01**, or 0.001***.
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AUTHOR CONTRIBUTIONS

E.J.P. and A.P designed, performed, and analyzed all experiments unless stated otherwise; Y.I. established positive ES cell clones and discussed the data; A.G. and G.C. performed epi-fluorescent IVM experiments; C.V.C. designed, performed, and analyzed in vitro imaging; C.V.C., U.H.v.A., and M.S. designed experiments and secured funding; and E.J.P., A.P, C.V.C., U.H.v.A., and M.S. discussed the data and wrote the manuscript.
References


FIGURE LEGENDS

Figure 1. Generation of αL-I306A mice. (A) Structure of the mouse αL I domain built by homology modeling using a crystal structure of the human αL I domain (1ZON) as a template. The Mg\(^{2+}\) ion at the ligand binding site MIDAS is shown with a green sphere and the side chain of I306 is in red. The amino (N) and the carboxyl (C) termini are labeled. Note that I306 is located in the hydrophobic pocket underneath the C-terminal helix. (B) Targeted insertion to the Itgal locus of the floxed ACN cassette and the mutated exon 9 (9*) that contains αL-I306A. The targeting vector (1), wild-type Itgal locus (2), the targeted Itgal allele containing floxed ACN cassette (3), and the mutated Itgal (I306A) allele (4) are shown. Exons are shown as filled boxes. Long arm (LA) and short arm (SA) of homology, as well as the diphtheria toxin (DT) are shown. The floxed ACN cassette is deleted in chimeric male mice during spermatogenesis, leaving one loxP site (4). An engineered EcoRI site (E*) was designed to identify the targeted allele by Southern blot analysis. N, Ncol; E, EcoRI; A, AvrII; Sm, Smal; S, Spel. The thick black line indicates the probe used to screen for homologous recombinations. (C) Genotyping and confirmation of deleted ACN cassette by PCR. Genomic DNA isolated from tails was used for PCR analyses. PCR bands are shown for wild-type (WT/WT, 300 bp), heterozygote (KI/WT, 300 and 390 bp), and homozygote (KI/KI, 390 bp) samples.

Figure 2. Adhesive interactions of WT and αL-I306A (KI) cells with ICAM-1 in vitro. (A) Cell adhesion of splenocytes to ICAM-1 and VCAM-1 substrates studied using a V-bottom-well plate. Cell suspensions were added to the plates and immediately centrifuged at 200 X g for 15 min to remove unbound cells. (B) Binding of soluble ICAM-1-Fc to WT and KI splenocytes. Bound ICAM-1-Fc was detected with indirect immuno-fluorescent cytometry using FITC-anti-human IgG (Fc-specific) antibody (C) Transmigration of WT and
KI splenocytes towards a CCL21 gradient through mock-, ICAM-1-coated, and VCAM-1-coated permeable inserts was examined using a modified Boyden chamber assay with a Transwell system™. (A-C) Data are expressed as the mean ± s.e.m. of triplicates from 4 independent experiments. (D-I) 2D migration of T lymphocytes on ICAM-1. (D) 2D tracking of WT and αL-I306A (KI) TCM cells migrating ICAM-1/CXCL12 substrates. Each track (red) represents migratory paths of individual WT (n=20) and KI (n=20) over a 25-min period. Mean displacement (E), mean velocity (F), and meandering index (G) of laterally migrating TCM cells on ICAM-1/CXCL12 substrates were obtained from analysis of live-cell imaging. (E-G) Data are expressed as mean values ± s.e.m. (H) Representative confocal images of T lymphocytes migrating on ICAM-1/CXCL12 stained for actin (Alexa-488) and αL integrin (Cy3) are shown. White bars, 10 μm. (I) The polarization index of T cells from randomly selected fields. Thick horizontal bars indicate mean values. (A-C, E-F, and I) Data are expressed as the mean values ± s.e.m. Two-tailed Student’s t test was used for statistical analyses. Statistical significance was defined as either P < 0.05*, 0.01**, or 0.001***.

Figure 3. In vivo homing of T lymphocytes. Equal numbers of differentially labeled cells were mixed and injected into C57BL/6J-CD45.1+ congenic recipient mice. The number of homed donor cells and homing indices were determined 2 h after injection. Data are expressed as the mean values ± s.e.m. Two-tailed Student’s t test was used for statistical analyses. Statistical significance was defined as either P < 0.05*, 0.01**, or 0.001*** versus SP. Abbreviations: SP, spleen; PBL, peripheral blood lymphocytes; PLN, peripheral lymph node; MLN, mesenteric lymph node; PP, Peyer’s patch; SI, small intestine; LI, large intestine; BM, bone marrow; LIV, liver; LUN, lung.
Figure 4. Adhesive interactions of T cells with lymph node vessels studied by epi-fluorescent IVM on inguinal LNs. (A & B) Rolling fractions. WT and αL-I306A T cells showed comparable rolling fractions in the absence (A) or presence (B) of LFA1 blocking antibody M17/4. (C) Rolling velocity on high order venules. αL-I306A T cells rolled on high order LN venules than WT. After LFA1 blockade by M17/4 antibody, both cell types showed comparable rolling velocity. (D) Sticking fractions. KI cells The fraction of sticking cells was increased for αL-I306A T exclusively on IV and V order venules. Data are expressed as the mean values ± s.e.m. of 3 independent experiments. Two-tailed Student’s t test was used for statistical analyses. Statistical significance was defined as either P < 0.05* or 0.001***.

Figure 5. MP-IVM investigations on T cell interactions with HEV. (A) A representative image of adoptively transferred WT T cells (blue) and αL-I306A T cells (red) interacting with popliteal lymph node vessels (green) of GFP chimeric recipient mice. (B) Gαi-independent arresting of KI cells. Firm adhesion of αL-I306A T cells to HEV endothelial cells was independent of Gαi signals while WT T cell adhesion was reduced by pertussis toxin (PTX) treatment. (C & D) Motility parameters of T cells in the LN vessel compartment. Meandering index (C) and 3D-IV (D) are shown. (E) Overall transendothelial migration efficacy of arrested cells. Each migratory path of arrested WT and KI T cell on HEV was traced and analyzed. The line in the box-plot indicates the median, the box-part represents the inter-quartile range, the whiskers depict the 5th and 95th percentiles, and the crosses represent the mean of 3 independent experiments. (F) A graphic representation of the crawling- and TEM-steps during adhesive interactions of T cells with HEVs. (G & H) Velocity (G) and traveling distance (H) during the crawling step. αL-I306A T cells migrated slower and shorter distance on HEVs than did WT T cells. (I)
Time required for completing the TEM step. (J & K) Representative migration velocity profiles of WT (J) and KI (K) T cells during the crawling- and TEM-steps. (B-D, and G) Data are expressed as the mean values ± s.e.m. of 3 independent experiments. Two-tailed Student’s t test was used for statistical analyses. Statistical significance was defined as either P < 0.05* or 0.001***. (C, D, H, and I) A representative result from 3 independent experiments is shown. (C, D, and I) Thick horizontal bars indicate mean values.

**Figure 6. Impact of the delayed LFA-1 inhibition on in vivo T cell homing.** Equal numbers of fluorescently labeled WT and KI T cells were mixed and intravenously injected into C57BL/6J-CD45.1+ congenic mice. One hour after cell injection, the mice were injected intravenously with vehicle or 100 μg of anti-LFA-1 mAb M17/4. One hour later, selected organs were harvested and the homing index (KI/WT) was determined. Homing index in LNs was compared with and without delayed M17/4 treatment. Data are expressed as the mean values ± s.e.m. of at least 4 independent experiments. Two-tailed Student’s t test was used for statistical analyses. Statistical significance was defined as either P < 0.05*, 0.01**, or 0.001*** versus SP. P < 0.05# or 0.001### in groups with and without mAb (M17/4) treatment. SP, spleen; PBL, peripheral blood lymphocytes; BM, bone marrow; PLN, peripheral lymph node; MLN, mesenteric lymph node.

**Figure 7. MP-IVM investigations on T cell interstitial motilities.** Motility parameters were measured 18-24 hours after adoptive transfer of WT and αL-I306A T cells in C57BL/6 mice. (A) Mean 3D instantaneous velocity (3D-IV) (B) Meandering index (C) Motility coefficient (D) Tuning angles (E) FACS histograms showing a staining of PLN T cells with FITC-labeled anti-LFA-1 mAb M17/4. T cells were isolated from either mice that had been
administered with unlabeled M17/4 (middle panel) or those that had been mock-treated (top panel). Please note that prior in vivo administration of unlabeled M17/4 completed for LFA-1 with FITC-M17/4. (F-G) Motility parameters after LFA-1 inhibition with M17/4. M17/4 treatment affected neither meandering index (F), mean 3DIV (G), nor motility coefficient (H) of WT and KI cells. (A-H) A representative result from 3 independent experiments is shown. (C and H) Data are expressed as the mean values ± s.e.m. Data are expressed as mean values ± s.e.m. (B, F, and G). The line in the box-plot indicates the mean values, and the top and the bottom of the box represent the maximum and minimum values, respectively. The data are from 3 independent experiments. Two-tailed Student’s t test was used for statistical analyses. Statistical significance was defined as either P < 0.05* or 0.001***.
Figure 2

A. ICAM-1 and VCAM-1 binding to cells with different divalent cations (Ca^{2+}/Mg^{2+} and Mn^{2+}). Bars represent the percentage of bound cells in WT and KI samples. Asterisks indicate statistical significance: *P < 0.05, **P < 0.01, ***P < 0.001.

B. ICAM-1 binding (MFI) in WT and KI samples with different divalent cations. Bars show the mean fluorescence intensity of ICAM-1 binding. Asterisks indicate statistical significance: *P < 0.05, **P < 0.01, ***P < 0.001.

C. Migration of cells in response to different concentrations of CCL21 and ICAM-1 or VCAM-1. Bars represent the percentage of migrated cells in WT and KI samples. Asterisks indicate statistical significance: *P < 0.05, **P < 0.01, ***P < 0.001.

D. Representative images showing the migration of WT and KI cells in response to a chemoattractant. Dendrites are color-coded to indicate direction and concentration.

E. Displacement of cells in WT and KI samples. Bars represent the average displacement measured in micrometers. Asterisks indicate statistical significance: **P < 0.01, ***P < 0.001.

F. Velocity of cell movement in WT and KI samples. Bars represent the average velocity measured in micrometers per second. Asterisks indicate statistical significance: ***P < 0.001.

G. Meandering index of cell movement in WT and KI samples. Bars represent the average meandering index. Asterisks indicate statistical significance: ***P < 0.001.

H. Representative images showing the polarization of WT and KI cells. Scale bars represent 50 μm.

I. Polarization index of WT and KI cells. Scatter plot shows the distribution of polarization indices. Asterisk indicates statistical significance: ***P < 0.001.
Figure 3

Homing index (KI / WT)

- SP
- PBL
- BM
- LIV
- LUN
- PLN
- MLN
- PP
- SI
- LI

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*
Figure 6

Homing index (KI / WT)

- SP
- PBL
- BM
- PLN
- MLN

M17/4
Distinct roles for LFA-1 affinity regulation during T cell adhesion, diapedesis, and interstitial migration in lymph nodes

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