INTEGRIN CROSSTALK IN THE UPSTREAM MIGRATION OF CD4+ T

LYMPHOCYTES

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To my parents, Jin Woo Kim and Tae Sung Ahn

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ABSTRACT

INTEGRIN CROSSTALK IN THE UPSTREAM MIGRATION OF CD4+ T LYMPHOCYTES

Hyun Ji Kim

Daniel A. Hammer

Leukocytes travel between different tissues, ranging from an inflamed site, bone marrow to lymph node after hematopoiesis for further development and maturation, to another tissue, through the vasculature. To efficiently reach the destination, circulating leukocytes in the blood stream must be able to adhere and exit the circulation. Recently, T lymphocytes have been shown to distinguish the direction of shear flow and migrate in a direction that is determined by adhesion molecules on the surface. T lymphocytes with LFA-1-ICAM-1 interaction migrate upstream (i.e. in the opposite direction of shear flow), and those with VLA-4-VCAM-1 binding migrate downstream (i.e. with the direction of flow). In the first aim, we study the crosstalk of LFA-1 and VLA-4 integrins in the upstream migration. We showed that while LFA-1 and VLA-4 mediated migration independently show very different directional response, LFA-1 and VLA-4 together potentiates the upstream migration during and post flow. The persistent direction of migration is mediated by PI3K. We uncover the intracellular signaling mechanism responsible for the upstream migration of CD4+ T lymphocytes with mice models in collaboration with Dr. Janis K. Burkhardt at Children's Hospital of Philadelphia. We found that, downstream of LFA-1, Crk and c-Cbl are key in the upstream direction. Lastly, in the third aim we investigate how the substrate mechanics affect the motility of

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CD4+ T lymphocytes under static and flow conditions. Specifically, we reported that while LFA-1-mediated migration under static conditions showed stiffness-dependent motility, the upstream migration under flow was unaffected. Together, we provide more insight to understanding the mechanism of the upstream migration.

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CHAPTER 1: Motivation

Leukocytes are an essential part of the immune system, maintaining homeostasis and responding to foreign challenges (1). To efficiently challenge foreign invaders at inflammatory sites, leukocytes such as neutrophils, as part of the innate immune system, circulate within the blood vessel to travel to sites of inflammation to enter tissues via leukocyte adhesion cascade, a multi-step process that involves the capture, rolling, adhesion and migration (2,3). Leukocytes are led by chemokines and cellular adhesion molecules expressed by endothelium for efficient transmigration to sites of inflammation. During the process, leukocytes receive and process multiple extracellular signals coordinate the behavior of leukocytes. Chemokines, selectins and adhesion molecules that mediate initial capture, rolling, firm adhesion trigger receptor-ligand mediated downstream signals for leukocytes (4,5). The multistep cascade of rolling, firm adhesion and migration is universally employed by all leukocytes to traffic to appropriate locations to transmit biological information. For example, lymphocytes are employ this same multistep method to home to immune organs, such as lymph nodes and Peyers patches to find and interact with antigen presenting cells (6,7). During egress, leukocytes bind to and migration on endothelium under hydrodynamic flow, forcing any receptor-mediated interactions to withstand the shear forces.

First reported by Valignat and co-workers, T lymphocytes, mediators of the adaptive immune response, can migrate in the direction opposite of imposed hydrodynamic flow. T lymphocytes orient and migrate upstream only on surfaces coated with Intracellular Adhesion Molecule-1 (ICAM-1), a key adhesion ligand of the integrin

known as Leukocyte Function-associated Antigen-1 (LFA-1, $\alpha_L\beta_2$) (8), which is found on many leukocytes including T cells. In contrast, Very Late Antigen-4 (VLA-4, $\alpha_4\beta_2$) integrins on T lymphocytes mediate downstream migration under flow on surfaces made with its cognate ligand, Vascular Cell Adhesion Molecule-1 (VCAM-1) (9). Our group previously published a follow-up study in which we made surfaces with different ratios of ICAM-1 and VCAM-1, and showed that only a small amount of binding between LFA-1 and ICAM-1 is required for upstream migration (9). The ability of T lymphocytes to crawl against the direction of flow is quite extraordinary, because cells must be able to not only withstand hydrodynamic flow but actively migrate against it. Aside from the obvious questions regarding the physiological relevance of upstream migration, the chemomechanical mechanisms that underlay upstream migration require further study,

Interestingly, upstream migration under flow is not limited to T cells, but also shown in marginal-zone B cells and hematopoietic stem cells (10,11). Although Valignat and co-workers originally showed that neutrophils crawl downstream on ICAM-1, the Hammer laboratory later showed that was because of the binding of Mac-1 to ICAM-1; when Mac-1 was blocked, the only available integrin to binding ICAM-1 was LFA-1, and upstream migration returned (12). T cell migrating upstream has also been observed on HUVECs and *in vivo* (13,14).

Despite its prevalence, we lack a fundamental understanding of the mechanisms behind upstream migration. My thesis will explore fundamental aspects of the upstream migration of CD4+ T lymphocytes. Specifically, we will determine key intracellular signaling molecules downstream of the two key integrins of CD4+ T lymphocytes, VLA-

4 and LFA-1. We also investigate the effect of stiffness in the upstream migration, closely recreating endothelium *in vitro*.

Aim 1: Show that CD4+ T lymphocytes remember shear flow under co-stimulation of VLA-4 and LFA-1 and contribute to persistent upstream migration during and/or post flow.

Dominguez *et al* reported that when CD4+ T lymphocytes are exposed to alternating ten minute periods where flow is turned on and turned off, only cells migration on surfaces containing a 1:1 mixture of VCAM-1 to ICAM-1 migrated in the upstream direction in the absence of flow (9). In this aim, we hypothesize that the simultaneous activation of VLA-4 and LFA-1 contribute to persistent upstream migration under flow. Using soluble factors, such as inhibitors and antibodies, we show that we can activate VLA-4 and that the crosstalk between VLA-4 and LFA-1 potentiates upstream migration.

Aim 2: Determine the mechanism of the upstream migration of CD4+ T lymphocytes and downstream signaling of LFA-1.

In collaboration with Nathan H. Roy and Janis K. Burkhardt, we propose key signaling molecules downstream of LFA-1 that are essential for upstream migration in murine CD4+ T lymphocytes. Using a mouse that endogenously expresses Cas9, CRISPR-Cas9 edit was used to delete signaling molecules from murine CD4+ T cells. In this aim, we identify several key molecules downstream of LFA-1 binding but upstream of PI-3-kinase that are required for T-cells to migrate upstream successfully.

Aim 3: Determine whether substrates with varying degrees of stiffness affect migratory behavior of CD4+ T lymphocytes.

While uncovering signaling mechanisms is key to understanding a biological phenomenon, previous *in vitro* studies on upstream migration are limited to polystyrene or PDMS spin-coated glass surfaces coated with ICAM-1. Since upstream migration is a mechanical phenomenon, it is reasonable to ask if the elasticity of a substrate could play a role in modulating upstream migration. Matrix stiffness affects many cellular processes, such as cell morphology, spreading, and migration itself (15). Thus, we hypothesized that LFA-1 is mechanosensitive and that sufficiently stiff substrates are needed to support upstream migration. Using polyacrylamide gels of different stiffnesses we illustrate that upstream migration is inhibited on very soft surfaces but the effect of softs substrates can be overcome by activating VLA-4 with soluble VCAM-1.

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CHAPTER 2: Background

Immune System

The immune system is an integral part of an organism's ability to maintain homeostasis and react to foreign, infectious materials. In mammals, the immune system consists of a variety of different cells that have differentiated from hematopoietic stem cells. Among blood cell types, lymphocytes have the ability to distinguish non-self pathogens and respond with protective effector function (1).

Circulating leukocytes emerge from hematopoietic stem cells in the bone marrow. The first step in differentiation is the emergence of myeloid or lymphoid progenitor cells (2). Further differentiation leads to a variety of leukocytes that constitute the adaptive or innate immune response. Once differentiated, leukocytes can stay in the bone marrow or travel to secondary tissues for further maturation and activation.



Figure 2.1. Hematopoiesis. Hematopoiesis is a process of hematopoietic stem cells differentiation to myeloid or lymphoid cell lineages. CLP, common lymphoid progenitor; CMP, common myeloid progenitor; GMLP, granulocyte-macrophage-lymphocyte progenitor; GMP, granulocyte-macrophage progenitor; MEP, megakaryocyte-erythrocyte progenitor; MPP, multipotent progenitor; NK, natural killer. Adapted from (2) with permissions.

Figure 2.1 illustrates hematopoietic stem cell-derived leukocyte lineages. Common myeloid progenitor cells give rise to different forms of granulocytes, such as neutrophils, monocytes, macrophages, eosinophils, basophils, mast cells, and dendritic cells. Common myeloid progenitor cell lineages are often associated with the innate immune response, where cells directly interact with pathogens based on conserved elements. Common lymphoid progenitor cells differentiate further into T and B cells, natural killer (NK) cells, and NK-T cells (3). These cells constitute the adaptive immune system and mediate further propagation with molecular specificity of long-term memory responses (4,5).

Innate immunity distinguishes infectious foreign molecules from those of the host and relay appropriate signals to the adaptive immune system. At an early stage of infection, contact with pathogens with the aid of chemokines triggers responses among innate immune cells (6). Phagocytic cells like macrophages and monocytes engulf microbial antigens and release cytokines to further recruit other immune cells. Eosinophils, mast cells and basophils release cytotoxic granules against specific parasites and bacteria (7). Neutrophils employ several methods to engulf pathogens and they kill these pathogens by releasing reactive oxygen species. Neutrophils also produce enzymes to aid tissue repair, and produce neutrophil extracellular traps (NETs), a mesh of extracellular fibers composed of chromatins, as means of immune-modulatory response (8). Antigen presenting cells (APCs), particularly dendritic cells (DCs), phagocytose, partially degrade pathogen materials and subsequently present antigens on their surface to activate T cells (9). APCs activate the adaptive immune cells by presenting endocytosed antigen-specific peptide sequence along with major histocompatibility complex (MHC)

onto their cell surfaces (10–12). Adaptive immune cells presented with peptide-MHC (pMHC) are now activated to implement their immune functions against the target (13). Such functions include causing apoptosis of infected host cells, producing antibodies and marking pathogens for destruction, or suppress hyperactivated or prolonged inflammatory responses (13–15).

Development and classification of T lymphocytes

A key cell type in the adaptive immune system is the T lymphocyte. Once differentiated from HSCs, thymic precursors leave the bone marrow and migrate to the thymus to undergo selection processes and become mature naïve T lymphocytes. The selection establishes a proper immune checkpoint, an ability to correctly rearrange $\alpha\beta$ heterodimeric T-cell receptor (TCR) to recognize pMHC with appropriate affinity, while being able to distinguish from self-peptide MHC (1,16).

Immature thymocytes lacking the expression of CD4 and CD8, also known as double-negative (DN) cells, are selected and further differentiate in the thymus (17). T cells that pass both positive and negative selection have appropriate affinity for antigens and are able to distinguish host and non-host antigens (18,19). Cells that pass both positive selection processes are functionally competent, mature T cells and are released to the circulation (17,18,20).

Depending on the coreceptors T cells express, their classification and immune functions differ. CD4+ T cells, or 'helper cells' (Th), are involved in activating and overseeing other immune responses such as antibody production by B cells. CD8+ T cells, or cytotoxic T cells (Tc), induce apoptosis of infected host cells (Fig. 2.2). Other

subsets of T cells include immune suppressor cells like regulatory T cells (Treg), characterized by the expression of CD25 and CD4, which engage in suppressing immune responses to prevent hyperactivation and autoimmunity (14). Tissue-resident lymphocytes are also produced where these leukocytes do not circulate, but instead reside in specific tissues or organs and localize to specific niches that are not readily accessible to recirculating cells (15). Differentiation and development of CD4+ and CD8+ T lymphocytes are crucial to immunological homeostasis as T cells communicate and direct other immune cells to react to inflammation at timely manner (Figure 2.2).



Figure 2.2. Simplified schematic representation of CD4+ and CD8+ T lymphocytes. Activated CD4+ T cells further activate B cells and CD8+ T cells. Activated CD8+ T cells recognize infected host cells and induce apoptosis. Adapted from Openstax Biology with permissions (Access for free at https://openstax.org/books/biology/pages/42-2-adaptive-immune-response).

The TCR recognize peptide antigens presented in a complex with class I or II MHC to guide T cells to distinguish host cells and to become activated (13). These interactions are facilitated by the interaction of CD4 and CD8 on T helper cells and cytotoxic T cells, respectively. MHC molecules are cell surface glycoproteins on APCs that present peptide fragments to adaptive immune cells (12). APCs present foreign peptides to CD4 cells via MHC class II molecules, whereas they present antigens made by the host cell to CD8 cells via MHC class I molecules. In such case, the exogenous antigen is a product of pathogens that have been released from infected host cells or endocytosed and proteolytically processed by phagocytic leukocytes (1,13).

Naïve T cells are activated when their TCRs engage cognate pMHC complexes on the surface of APCs. Additional participation of co-stimulatory receptors, such as CD28, can augment full activation of T cells (21). Activated T cells will then proliferate into a clone of T cells specific to the antigen. Th1 cells produce cytokines, such as interferongamma (IFNγ), that primarily induce cell-mediated cytokine responses. Th2 cells produce other ILs to stimulate antibody production (1). Some activated T cells remain in the lymph nodes and become central memory cells, which are often characterized by removal of CD45RA and presence of CD45RO surface molecules. CD45RO+ CD4+ T cells retain the information of the pathogen for the long term. In case the host is infected with the same strain of pathogen, memory T cells can quickly expand and trigger downstream proliferation and activation of other immune cell types, shortening the response time by a significant amount (22–25).

Leukocyte Adhesion Cascade (LAC)

Circulating leukocytes in the bloodstream arrive to sites of inflammation by a multi-step process known as the leukocyte adhesion cascade (LAC). LAC is a step-wise process that describes how leukocytes from flowing blood searches an appropriate location to transmigrate to effectively reach its destination, such as inflammation sites or to home to immune organs such as lymph nodes. By expressing selectins and adhesion molecules and releasing chemokines, endothelial cells capture leukocytes and mediate their rolling and subsequently firm adhesion. Each step of the LAC involves different cell membrane receptors and ligand interactions under hydrodynamic flow (26). Figure 2.3 illustrates an overall scheme of LAC.



Figure 2.3. Simplified schematic of the Leukocyte Adhesion Cascade. Leukocytes (purple) are recruited to migrate onto the endothelial layer by selectins, chemokines and adhesion molecules presented by endothelial cells (blue). Adapted from (26) with permissions.

Initial capture and subsequent rolling of leukocytes are mediated by selectins. Endothelial cells express selectins, such as P- or E- selectin, that interact with surface receptors on leukocytes, such as P-selectin glycoprotein ligand-1 (PSGL-1), CD44, or Eselectin ligand-1 (ESL-1) (27). Selectin binding has high on- and off- rates, forming a very short-lived bond at a fast pace for the capture. Selectin binding is a catch-slip bond, meaning the off rate of the complex is force-dependent (28). Catch-slip bonds have intrinsic on and off rates under no stress. However, the off rate decreases and bond time increases as force is applied, until at some critical force, the off rate will increase. Shortlived selectin interactions allow endothelial cells to catch and slow down leukocytes (29– 31). Selectin bonds also increase integrin affinity to promote slow rolling and adhesion (27,32,33).

During slow rolling, leukocytes become further activated by chemoattractants, such as CXCL12 (also known as stromal cell-derived factor 1 α , SDF-1 α), with high affinity to G-protein coupled receptors (GPCRs), such as CXCR4. GPCR signals then induce intracellular signaling network remodeling, such as polarization of PI3K and PIP2 on cell membranes, to define the front of the cell (34). Activation of GPCRs also triggers inside-out signaling of integrins by recruiting actin-nucleating proteins to mediate actin polymerization. Inside-out signaling and adaptor proteins recruited near the cell membrane converts integrins from low to high affinity states (35). More details of integrin signaling are discussed in the 'Downstream of outside-in signaling of integrins: Role of lamellipodial signaling in upstream migration' sub-section of the Background.

When integrins on leukocytes attain a high-affinity conformation, they strongly bind to cell adhesion molecules (CAMs), which further slows down rolling leukocytes (36). Specifically, T cells employ lymphocyte function-associated antigen-1 (LFA-1, $\alpha_L\beta_2$) and very late antigen-4 (VLA-4, $\alpha_4\beta_1$) integrins to bind to intracellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), respectively. The β_2 subunit of LFA-1, similar to selectins, exhibits a catch-slip bond with a much lower off rate and a longer bond life time (37–40), supporting firm adhesion and migration. In inflammation, endothelial cells upregulate the expression of ICAM-1 to support adhesion and transmigration (41,42) of innate immune cells, such as neutrophils. ICAM-1 dimerization and clustering are often results of endothelial cell activation, which directly correlates with enhanced binding to LFA-1 and leukocyte recruitment (43,44). Finally, ICAM-1 clustering and additional CAMs, such as platelet endothelial cell adhesion molecule-1 (PECAM-1) or junctional adhesion molecule (JAM), guide leukocyte transmigration (45,46).

Conformation states and activation mechanism of integrins

Integrins are heterodimeric receptors that bind to specific immunoglobulin (Ig) proteins with their extracellular domains and recruit intracellular adaptor proteins and actin polymers with their cytoplasmic tails, the cellular membrane to foster cellular movement. Immune cells express 10 members of the integrin family, including β_2 , β_7 and β_1 . The β_2 and β_7 subunits are exclusive to leukocytes, while β_1 integrins are expressed in other cell types (47). Mutations in β_2 integrins cause Leukocyte Adhesion Deficiency (LAD) -I or -III, which are respectively characterized by complete deletion of β_2 integrins (48,49) or a mutation in the binding site of kindlin-3, an adaptor protein linking integrins

to the actin cytoskeleton (50). Both forms of LAD inhibit proper cell migration and leukocyte recruitment to inflammatory sites or immune organs (51,52).



Figure 2.4. Structure of LFA-1 integrins. LFA-1 has 3 conformations: closed (A), extended, closed (B), and extended, open (C). The closed, bent conformation represents an inactive integrin with low ligand affinity. Both forms of extended conformations have higher affinities. The open, fully extended conformation (C), the highest affinity form of LFA-1, is characterized by an extended extracellular domain and cytoplasmic tails separated by interacting cytosolic adaptor proteins. Adapted from (53) with permissions (This article was published in PNAS, Vol 107, X Chen, C Xie, N Nishida *et al*, Requirement of the openpiece conformation for activation of leukocyte integrin $\alpha x\beta 2$, pages 14727-14732, Copyright Elsevier (2010)).

The α and β integrin subunits each consist of several extracellular subdomains, a transmembrane domain and a short cytoplasmic tail containing binding sites for adaptor proteins (Fig. 2.4) (54). Multiple interfaces between secondary structures from the subdomains give rise to their flexible nature. The α I domain of the α subunit recognizes specific peptide sequence of cognate ligands (55). The key ligand binding head, which consists of α I domain and β -propeller domain from the α subunit and β I domain from the β subunit, determines the specificity of the integrin. The headpiece contains adhesion sites, such as metal ion-dependent adhesion sites (MIDAS), ligand-induced metal ionbinding site (LIMBS), and adjacent to metal ion-dependent adhesion site (ADMIDAS), which are stabilized by divalent cations and contribute to integrin affinity and conformation (47). Addition of Mn^{2+} , Ca^{2+} or Mg^{2+} alters the affinity of integrins and induces conformational changes (56). It has been reported that LIMBS mediates a synergistic effect of low concentrations of Ca²⁺ with suboptimal Mg²⁺ concentrations to support adhesion (57). ADMIDAS serves as a negative regulatory site at high concentrations of Ca^{2+} , competed by Mn^{2+} (58,59). Metal ions binding to integrin subunits induce conformational changes by moving subdomains and remodeling the interfaces between subdomains, ultimately shifting the equilibrium of conformational states of integrin (47).

Integrins have multiple conformations in equilibrium; closed, intermediate and open conformation states (53,60,61). Each state is associated with morphologically different structure and ligand affinity. In a bent conformation, MIDAS and the headpiece are stabilized by interfaces formed by the headpiece and the lower legs, and by α and β transmembrane and cytoplasmic domains. Structurally tucking away the ligand-binding

headpiece, the closed, bent conformation represents physiological low-affinity state (60,62). Integrins extend by remodeling the interfaces and destabilizing the bent conformation. The intermediate and fully-extended conformations correlate to intermediate and high-affinity states accompanied by a large-scale global conformational rearrangement in which the integrin extends with a switchblade-like motion (63,64). Integrin with intermediate affinity extends its headpiece for ligand binding but the α and β subunits remain in close proximity. In the high affinity state, integrins further extend and there is separation of the α and β subunits. Activation-dependent antibodies have demonstrated that extension is sufficient to induce integrin adhesiveness and to enable a substantial proportion of integrin molecules to equilibrate to the high-affinity, open head piece conformation (65,66). The set point for the equilibria between each conformation is integrin-dependent (67–70).

Small perturbations, such as inside-out or outside-in signaling, can readily shift the equilibrium between integrin conformational states towards one conformation or another (71,72). Integrin affinity can be increased extracellularly via divalent cations or ligands (47). Integrin affinity can also be modulated from inside of a cell as a downstream result of PKC signaling or by chemoattractant-triggered GPCR. Outside-in or inside-out signaling signaling result in a separation of α and β subunits and the conformational change. When an integrin extends, intracellular adaptor proteins, such as talin and kindlin-3, are recruited to the cytoplasmic domain of integrins, stabilizing the separation of α and β subunits in the activated state (73,74).

Downstream of outside-in signaling of integrins: Role of lamellipodial signaling in upstream migration

To trigger cellular movement, integrins recruit adaptor proteins and trigger downstream intracellular signaling pathways to rearrange the cytoskeletal network. Cells reconfigure their actin cytoskeletal structure and by localizing intracellular proteins in a polarized fashion. For circulating leukocytes in the bloodstream to adhere and migrate, leukocytes must be able to undergo several processes: 1. Process extracellular information received from the hydrodynamic flow and cellular adhesion molecules and chemokines on surfaces, 2. reorient and polarize themselves, and 3. recruit proper adaptor proteins needed to assemble actin polymerization. These steps require signaling downstream of integrins, which has been an extensive area of research.

Engagement of LFA-1 integrins on T lymphocytes to ICAM-1 on apical surfaces of endothelium initiates outside-in signaling cascade of LFA-1, which recruits a series of adaptor proteins to the cytoplasmic domain of LFA-1. Talin and kindlin-3 are key adaptor proteins for strengthening integrin-based adhesion. Upon activation of LFA-1, talin induces the extension of LFA-1 to the intermediate conformation, separating the α_L and β_1 subunits of LFA-1 (51,75). Kindlin-3 is subsequently recruited to switch LFA-1 to an open, extended conformation, further strengthening LFA-1-mediated adhesion and integrin clustering (76,77). A mutation in the gene FERMT3, which encodes for kindlin-3, causes a third class of Leukocyte Adhesion Deficiency (LAD III) and has been characterized to impair stable integrin activation and platelet aggregation (50).

Binding of talin and kindlin-3 recruits downstream molecules, such as PIP2, for polarization (78,79). PIP2 is a membrane-bound second messenger, synthesized by

PIP5K and is used as substrates of PI3K to produce PIP3 (80). Most of the proteins that regulate PIP5K activity belong to the Rho family of small GTPases, which are critical regulators of actin remodeling and vesicular (81). In T cells, localization of PIP2 and its associated kinases mediates cell polarization and migration, thereby stabilizing integrinand/or TCR-mediated adhesion and activation (82). Polarized localization of PIP2 controls the selective localization of downstream scaffolding molecules and actinnucleating proteins, such as WASp and Arp2/3, linking the actin network to the plasma membrane (83–85). PIP5K and PIP2 also play a key role in neutrophils by maintaining asymmetry in phosphoinositide localization (86). PI3K & PIP2 are localized to the lamellipod (87,88). Some spliced isoforms of PIP5K are reported to be concentrated at the uropod, mediated by Rho-family of small GTPases (89–91). In T cells, contrasting data has been reported with isoforms of PIP5K. Bolomini-Vittori et al reported in PIP5KC selectively regulates high-affinity state of LFA-1 to favor T cell arrest and adhesion (92), whereas Wernimont et al show that CD4+ T cells from PIP5Kc90deficient mice have increased LFA-1 adhesion to ICAM-1 and enhanced LFA-1 polarization at the immunological synapse (IS) between APC and T cell (93).

In addition to recruiting PIP2 to establish cell polarization, talin also contains a binding site for vinculin to guide actin polymerization near the cytoplasmic tail of integrins. Vinculin is a scaffolding protein that contains binding sites for talin, PIP2 and other adaptor proteins that help to control cell adhesion and polarization (94). Vinculin, in concert with talin, recruits Arp2/3 to the plasma membrane (95). WASp mediates the binding of actin filaments to activate Arp2/3, thereby promoting actin polymerization at
the leading edge (96,97). Arp2/3 then initiates branched actin nucleation on an existing F-actin filament (98).

Another key set of molecules that mediate recruitment of WASp are the CT10 regulator of kinase (Crk) proteins (96). Crk proteins in T cells modulate Ras-related protein 1 (Rap1)-dependent integrin affinity in response to TCR or chemokine stimulation (74,99,100). Defects in Crk proteins lead to impaired integrin-dependent adhesion and chemotaxis (101,102). Activated T cells lacking Crk proteins fail to polymerize actin downstream of LFA-1, and migrate slower and less directionally than the control group (103). Roy *et al.* propose a model in which the engagement of LFA-1 to immobilized ICAM-1 activates Src family kinases, which induces the binding of Crk to further downstream scaffolding proteins, activates PI3K catalytic function, and promotes the production of PIP3 (103).

Role of extracellular matrix in T cells

All tissues are constructed with specific types of cells and the extracellular matrix (ECM) produced by fibrous proteins that cells secrete. Elasticity of the ECM depends on the composition, density, and degrees of crosslinking these fibrous proteins. In the human body, elastic moduli of tissues range from the order of hundred pascals in brain, to the order of gigapascals in bone (Fig. 2.5) (104).



Figure 2.5. Different tissues with various stiffness. Reproduced from (104) with permissions.

Tissue-dependent stiffness of the ECM provides mechanical cues for cellular function and viability and affects integrin-dependent force exertion (105–107). Global substrate rigidity or extracellular tensions can trigger different mechanosensory responses in cells, which could phenotypically translate into different morphologies, expression of receptors, and proliferation (108,109). For example, mechanical properties of ECM affect stem cell differentiation (110–112). Olivares-Navarette *et al* showed that mesenchymal stem cells (MSCs) commit to the osteogenic lineage when cultured on stiff substrates, but undergo chondrocytic differentiation on softer substrates, and they identified β_1 integrin as a critical receptor in stiffness-dependent differentiation (110). Substrate stiffness also has been highly implicated in aging and diseases such as cancer. Breast cancer ECM is associated with higher, more aligned collagen deposition and denser tumor microenvironment, hindering infiltration of leukocytes and often facilitating cancer metastasis (107,113). B16 melanoma lymphatic drainage was associated with alterations in composition, such as lymph node hyaluronic acid and collagen, and an increase in lymph node tissue stiffness and viscoelasticity (114).

Not surprisingly, mechanical properties of substrates are reported to also affect T cell function, such as cellular morphology, spreading, activation and proliferation. One could imagine that, unlike most tissue-specific cell types, leukocytes are subjected to interactions with various tissues, cells and ECM. Upon hematopoiesis, T cells leave the bone marrow, enter the circulation and arrive to the lymph nodes for further maturation and activation. They then leave lymphoid tissues to activate other leukocytes and govern the immune response. Naturally, as T cells interact with various types of cells and locations, T cells constantly come in direct contact with a wide ranges of stiffnesses. T

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cells respond to stiffness morphologically; they are typically less spread on softer substrates, and more widely spread on stiffer substrates (115).

T cell function can also be affected by ECM stiffness. T cells on ICAM-1 coated polyacrylamide (PA) gels of increasing stiffness display increased production levels of key cytokines, such as IFN δ and TNF β (115). Numerous reports show that TCRmediated signaling is a mechanosensitive process (116). Wahl et al reported a biphasic mechanosenstivity of CD3-mediated cell spreading of lymphocytes (117). Bashour et al measured that T cells generate much greater traction force (pN) per pillar when stimulated through both CD3 and CD28 rather than CD3 alone (118). The study also reported that wortmannin, a PI3K inhibitor, reduced traction force of T cells upon costimulation of CD3 and CD28 to levels comparable to CD3 stimulation alone. The PDK inhibitor, GSK2334470, completely abrogated the force generation. Their results indicate that TCR signaling is stiffness-dependent which also affects the downstream signaling intracellularly. Though the literature is divided about specifics, it is clear that substrate stiffness affects IL-2 secretion and the proliferation rate of T cells. Judokusomo et al and Blumental *et al* reported stiffness-dependent IL-2 secretion and proliferation, respectively (119,120). O'Connor et al also reported the opposite with CD4+ T lymphocytes on varying ratio of the base and the crosslinker of PDMS, but in inverse correlation to stiffness (121). Softer PDMS substrate coated with OKT3, a monoclonal antibody activating CD3, led to higher production of IL-2, and higher cell proliferation on softer PDMS substrate with OKT3 and anti-CD28 (121).

In the circulation, the endothelium acts as a physical platform to modulate contact-guided migration of leukocytes. For efficient transmigration, endothelial cells

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express CAMs and modify local stiffness (122). A higher local stiffness is associated with the initial capture and the rolling of leukocytes. A gradient in stiffness was suspected to mediate leukocyte crawling and migration, eventually leading to regions with low local stiffness for transmigration (122,123). Stiffer matrix results in increased ICAM-1 expression (124), and a different elasticity and morphology of HUVECs (125). Applying mechanical forces on ICAM-1 clusters has been shown to enhance Rho activation and MLC phosphorylation, suggesting that contractile forces exerted by endothelium and leukocytes in concert facilitates diapedesis and uses ICAM-1 as a force sensor. Clustering of ICAM-1 naturally leads to clustering of integrins, which strengthens integrin outside-in signaling for adhesion (105). Some diseases are associated with stiffening of the blood vessel or remodeling endothelial ECM. For example, Hutchinson Gilford Progeria Syndrome (HGPS), a chronic premature aging disease, is accompanied by stiffening of the artery and less strictive physical diameter of artery (126).

Current studies of T cell migration under shear flow

When leukocytes circulate and migrate on the endothelium, some immune cell types can detect and reorient themselves in response to the direction of flow (127–129). Valignat *et al* reported that T lymphocytes can migrate in the direction opposite of flow, that is, upstream, when are migrating on ICAM-1 coated surfaces (130). Valignat *et al* followed up with a proposed biophysical mechanism to explain this behavior. According to this model, the uropod acting as a "wind-vane", passively steering the cell in response to shear flow (131). The upstream migration of T cells under flow is mediated by LFA-1

integrins binding to ICAM-1. T cells on ICAM-1 surfaces migrate upstream, whereas T cells on VCAM-1 migrate downstream (132).

The Hammer lab has reported that the upstream migration is not limited to T cells, but also seen in hematopoietic stem and progenitor cells (HSPCs) (132,133). Neutrophils migrate upstream on ICAM-1 surface when Mac-1 integrin is blocked (134). Leukocytes *in vivo* were also shown to migrate in response to shear flow with T cells and *ex vivo* with marginal zone B cells (127,135,136). However, our fundamental understanding of upstream migration is still incomplete. While cell migration and signaling studies are very active under static conditions, it has been challenging to decouple the hydrodynamic stimulus and receptor-ligand interaction during migration.

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CHAPTER 3: Aim 1: Migrational memory of CD4+ T lymphocytes

Adapted from: Sarah Hyun Ji Kim and Daniel A Hammer, "Integrin crosstalk allows CD4+ T lymphocytes to continue migrating in the upstream direction after flow", *Integrative Biology*, Volume 11, Issue 10, October 2019, Pages 384– 393, doi.org/10.1093/intbio/zyz034

Abstract

In order to perform critical immune functions at sites of inflammation, circulatory T lymphocytes must be able to arrest, adhere, migrate and transmigrate on the endothelial surface. This progression of steps is coordinated by cellular adhesion molecules (CAMs), chemokines, and selectins presented on the endothelium. Two important interactions are between Lymphocyte Function-associated Antigen-1 (LFA-1, $\alpha_L\beta_2$) and Intracellular Adhesion Molecule-1 (ICAM-1) and also between Very Late Antigen-4 (VLA-4, $\alpha_4\beta_1$) and Vascular Cell Adhesion Molecule-1 (VCAM-1). Recent studies have shown that T lymphocytes and other cell types can migrate upstream (against the direction) of flow through the binding of LFA-1 to ICAM-1. Since upstream migration of T cells depends on a specific adhesive pathway, we hypothesized that mechanotransduction is critical to migration, and that signals might allow T-cells to remember their direction of migration after the flow is terminated. Cells on ICAM-1 surfaces migrate against the shear flow, but the upstream migration reverts to random migration after the flow is stopped. Cells on VCAM-1 migrate with the direction of flow. However, on surfaces that combine ICAM-1 and VCAM-1, cells crawl upstream at a shear rate of 800 s^{-1} and continue migrating in the upstream direction for at least 30 minutes after the flow is terminated—we call this

'migrational memory'. Post-flow upstream migration on VCAM-1/ICAM-1 surfaces is reversed upon the inhibition of PI3K, but conserved with cdc42 and Arp2/3 inhibitors. Using an antibody against VLA-4, we can block migrational memory on VCAM-1/ICAM-1 surfaces. Using a soluble ligand for VLA-4 (sVCAM-1), we can promote migrational memory on ICAM-1 surfaces. These results indicate that, while upstream migration under flow requires LFA-1 binding to immobilized ICAM-1, signaling from VLA-4 and PI3K activity is required for the migrational memory of CD4+ T cells. These results indicate that crosstalk between integrins potentiates the signal of upstream migration.

Introduction

Cell migration is a crucial for T lymphocytes to home and reach sites of inflammation (1). T lymphocytes need to stop, roll and adhere on the endothelial layer to reach the sites of inflammation (2,3). This process, known as the leukocyte adhesion cascade, is regulated by multiple adhesive interactions and intracellular signaling cascades. In response to inflammatory stimuli, vascular endothelial cells express chemokines and cellular adhesion molecules (CAMs) on their surface to recruit T lymphocytes to the sites of inflammation (4,5). To facilitate firm adhesion and migration along the endothelium, T lymphocytes express the integrins Lymphocyte Functionassociated Antigen-1 (LFA-1, $\alpha_L\beta_2$) and Very Late Antigen-4 (VLA-4, $\alpha_4\beta_1$) to bind to Intracellular Adhesion Molecule-1 (ICAM-1) or Vascular Cell Adhesion Molecule-1 (VCAM-1), respectively, on the endothelial surface. After firm adhesion, T lymphocytes become migratory, first migrating on the apical surface of endothelial cells, and then transmigrating. Despite the obvious importance of hydrodynamic interactions in the vasculature, T lymphocyte migration is often studied in the absence of external shear flow. Not only do leukocytes have to withstand shear forces within the blood vessel while rolling and adhering, but migration involves spreading and cell reorganization under hydrodynamic forces to generate directional motion.

The complex signaling cascade activated downstream of integrin binding has been well studied in T lymphocytes in the absence of shear flow. Upon ligand binding, integrins recruit adaptor proteins to the cytoplasmic tail of integrins, connecting the integrins to cytoskeleton for force transmission and movement (6–13). Scaffolding molecules propagate the extracellular signal to Phosphoinositide 3-kinase (PI3K) and

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Rho-family small GTPases for migration by subsequent recruitment and activation. PI3K near the plasma membrane promotes further recruitment and activation of GTPases to the plasma membrane (14,15). Rho-family small GTPases are often involved in establishing cell polarity and fostering actin polymerization (16). Cdc42 and Rac localize at the leading edge of cells promoting Arp2/3-dependent activation of ther respective effector molecules, WASp and WAVE2, and WAVE in the case of Rac (15,17–19). Rho, on the other hand, regulates myosin-dependent contractility at the rear end of cells (20,21). However, it is unknown how these signaling molecules affect the directionality of migration in response to shear flow.

Interestingly, it has been reported that T cells can crawl upstream against the direction of flow, depending on the chemistry of the surface. Valignat and coworkers reported that primary T lymphocytes orient themselves against the direction of flow and crawl upstream on surfaces coated with ICAM- 1 and the chemokine SDF-1 α (22). Upstream migration has been observed in vivo; effector T cells exhibited intravascular upstream migration in a rat model of neurological autoimmune lesions (23). A study in our own group showed that only a small amount of LFA-1-ICAM-1 interactions were necessary for the upstream migration of T lymphocytes; on surfaces coated with both VCAM-1 and ICAM-1, T lymphocytes migrated upstream even when ICAM-1 represented less than 10 % of the substrate ligand, reinforcing the idea that the upstream migration on ICAM-1 is not limited to T lymphocytes but has also been demonstrated for hematopoietic stem and progenitor cells (HSPCs), marginal zone B-cells, and neutrophils when Mac-1 was blocked to isolate LFA-1/ICAM-1 interactions (25–28).

We have started to search for clues regarding the mechanisms behind upstream migration. Previously, we showed that after subjecting cells to repeated cycles in which the flow was maintained for 10 minutes and then stopped for 10 minutes, cells on ICAM-1 surfaces moved randomly when the flow was stopped, while cells on surfaces presenting a mixture of VCAM- 1/ICAM-1 persisted in crawling against the direction in which the flow had been, even after the flow was stopped (24). In other words, the cells displayed directional persistence, which we call 'migrational memory', after the flow was terminated on surfaces in which both VCAM-1 and ICAM-1 are mixed.

Here, we aim to expand this investigation and address how CD4+ T lymphocytes maintain their directionality. Previously, we found that migrational memory was maintained for 10 minutes after the flow was terminated on surfaces that were functionalized with both VCAM-1 and ICAM-1 (24). We ask now, how much longer do cells migrate upstream after flow, and what role does intracellular signaling and integrin crosstalk play in persistence? We find that although the engagement of LFA-1 with ICAM-1 is absolutely required for upstream migration, the independent activation of VLA-4 can stimulate a persistent memory of the direction of upstream migration on ICAM-1 surfaces. Furthermore, we show that the activity of P13K is needed for persistent memory; inhibitors of P13K induce CD4+ T cells to revert to random migration when the flow is terminated. Surprisingly, based on studies using appropriate inhibitors, GTPases and Arp2/3 have no effect on the ability of T cells to remember the direction of upstream migration. Therefore, we propose that directional memory requires crosstalk between LFA-1 and VLA-4, and signaling through P13K.

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Materials and Methods

Cell culture and reagents

Human Primary CD4+ and CD8+ T lymphocytes were acquired from the Human Immunology Core at the University of Pennsylvania (P30-CA016520). Cells were activated with PHA (MP Biomedicals, Santa Ana, CA) in RPMI-1640 with 10 % FBS for 3 days, followed by subsequent activation with IL-2 (Corning, Corning, NY) in RPMI-1640 with 10 % FBS and penicillin and streptomycin.

For shear flow experiments, cells were kept in RPMI-1640 supplemented with 2 mg/ml D-glucose and 0.1 % BSA. CD4+ T cells were introduced into a flow chamber using a syringe, adhered for 30 minutes to surfaces prior to shear flow, and imaged. CD4+ T lymphocytes were treated with inhibitors 30 minutes prior to seeding, unless noted otherwise. The inhibitors were present in media at all times throughout the experiment. Pharmacological inhibitors used for experiments were the following: Wortmannin (Sigma, St. Louis, MO) LY294002 (CST, Beverly, MA), CT-04 (Cytoskeleton, Denver, CO), NSC23766 (Millipore, Temecula, CA), ML-141 (Millipore), and CK666 (Sigma). For modulating VLA-4 ligand binding, cells were incubated with the following for 15 minutes: soluble VCAM-1 (R&D Systems, Minneapolis, MN) and blocking antibodies, isotype control, anti- α 4 (9F10) or anti- β 1 (P5D2) (Biolegend, San Diego, CA).

Substrate preparation

Substrates were prepared as explained previously (29–31). Briefly, 25 mm \times 75 mm \times 1 mm glass slides (Thermo-Fisher, Hamp- ton, NH) are spin-coated with degassed

Polydimethylsiloxane (PDMS) cross-linked in 10:1 ratio with its curing agent (Dow Corning, Midland, MI). Spin-coated PDMS slides are then allowed to cross-link at 65 °C for 1 hour. PDMS slides were then treated with UV for 8 minutes prior to printing.

Preparation of chimeric areas using microcontact printing

A 10:1 PDMS base:curing agent mix was used to make PDMS stamps for microcontact printing. Degassed PDMS solution was cured over a flat silicon wafer for an hour at 65 °C. Then, cured PDMS was cut into cubes with 1 cm² flat stamping surface area. Trimmed stamps were sonicated in 200 proof ethanol for 10 minutes and rinsed with diH2O. Surfaces were prepared by microcontact printing 2 µg/ml of protein A/G (Biovision, San Francisco, CA) onto UV ozone-treated PDMS spin-coated glass slides at room temperature. Surfaces were then blocked with 0.2% (w/v) Pluronic F-127 (Sigma). Subsequently, 10 µg/ml of ICAM-1 Fc chimera (R&D Systems), 10 µg/ml of VCAM-1 Fc chimera (R&D Systems), or 10 µg/ml consisting 1:1 concentration of VCAM- 1 and ICAM-1 Fc chimera adhesion molecules were adsorbed at 4 °C overnight to create ICAM-1, VCAM-1 or VCAM-1/ICAM-1 mixed surfaces, respectively.

Cell tracking and data acquisition

Images were acquired every minute for an hour using a Nikon TE300 with custom environmental control chamber at 37 °C and 5 % CO2. Cells were exposed to first 30 minutes of shear flow, followed by 30 minutes of no flow (denoted as 'post- flow'). Two shear rates, 100 s^{-1} and 800 s^{-1} , were used. While static conditions last 30 minutes, flow and post-flow groups were taken for an hour. Images were analyzed using Manual

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Tracking (https://imagej.nih.gov/ij/plugins/track/track.html) in ImageJ

(https://imagej.nih.gov/ij/, NIH, Bethesda, MD) and MATLAB (The MathWorks, Natick, MA). Centroids of cells were tracked using Image plug-in Manual Tracking. Cells only stayed in the field-of-view for an entire hour were included in the analysis. With (x, y) coordinates of cells, Migration Index (MI), speed, and persistence time were calculated using a custom MATLAB script. MI describes the directionality of cells by quantifying a ratio of a cell's axial displacement to total distance it has traveled. With flow from left to right of field-of-view, negative MI represents cells traveling upstream, while positive MI denotes cells traveling downstream. Persistence time was obtained by calculating mean squared displacement (MSD) of each cell and fitting to Dunn Equation (32).
Results

CD4+ T lymphocytes retain directional persistence post flow at a high shear rate using both VLA-4 and LFA-1

It has been previously shown that T lymphocytes migrate against the direction of flow on surfaces with immobilized ICAM-1 (22,24,28). Dominguez and coworkers had tested cells on various ratios of ICAM-1 and VCAM-1 at a shear rate of 800 s⁻¹, and discovered that cells migrated against the direction of flow whenever there was any ICAM-1 on the surface. Upstream migration with the combinations of ICAM-1 and VCAM-1 did not depend on the concentration of VCAM-1 present. Furthermore, when the flow at a shear rate of 800 s⁻¹ was stopped, only cells on surfaces in which VCAM-1/ICAM-1 had been mixed maintained upstream direction (24). Here, we expand on this study and further investigate the factors that control T cell memory of directional motion in the absence of flow. Based on our previous results (24), we hypothesized that VLA-4 engagement is important for the persistence of motion when the flow is terminated. On three different surfaces, ICAM-1, a mixture of VCAM-1/ICAM-1, and VCAM-1, CD4+ T lymphocytes were exposed to shear flow for 30 minutes, then tracked for another 30 minutes after the flow was terminated. Figure 3.1 shows cells traces from a representative experiment.



Figure 3.1. CD4+ T lymphocytes exhibit directed migration during and post flow. CD4+ T lymphocytes on ICAM-1, VCAM-1/ICAM-1, or VCAM-1 surfaces migrate according to the direction of flow, but only cells on surfaces with a mixture of VCAM-1/ICAM-1 maintain upstream directionality after the flow is terminated, and only after having been exposed to flow at a shear rate of 800 s⁻¹. (A) CD4+ T lymphocytes on ICAM-1 under flow at a shear rate of 800 s⁻¹ at time points 0, 15 and 30 minutes, followed by 30 minutes of no flow at time points 0, 15 and 30 minutes. (B) CD4+ T lymphocytes on VCAM-1/ICAM-1 surfaces under flow at a shear rate of 800 s⁻¹ at time points 0, 15 and 30 minutes, followed by 30 minutes of no flow at time points 0, 15 and 30 minutes. (C) CD4+ T lymphocytes on VCAM-1 mixed surface under flow at a shear rate of 800 s⁻¹ at time points 0, 15 and 30 minutes of no flow at time points 0, 15 and 30 minutes. (D) CD4+ T lymphocytes on VCAM-1 mixed surface under flow at a shear rate of 800 s⁻¹ at time points 0, 15 and 30 minutes, followed by 30 minutes of no flow at time points 0, 15 and 30 minutes. Points indicate the end of the cell track. Scale bar = 150 μ m.



Figure 3.2. CD4+ T lymphocytes remember shear flow. CD4+ T lymphocytes on ICAM-1 or VCAM-1/ICAM-1 mixed surfaces display upstream migration, and downstream migration on VCAM-1, but only cells on VCAM-1/ICAM-1 mixed surface display migrational memory after having been exposed to flow at a shear rate of 800 s⁻¹. (A) Scattergrams of cells on ICAM-1 at low (100 s⁻¹, a and b) and high (800 s⁻¹, c and d) shear rates. (B) Scattergrams of cells on VCAM-1/ICAM-1 at low (100 s⁻¹, a and b) and high (800 s⁻¹, c and d) shear rates. (C) Scattergrams of cells on VCAM-1 at low (100 s⁻¹, a and b) and high (800 s⁻¹, c and d) shear rates. Migration Index of cells on (D) ICAM-1, (E) VCAM-1/ICAM-1 mixed surface, and (F) VCAM-1. Migration Index of cells on (G) ICAM-1, (H) VCAM-1/ICAM-1 mixed surface, and (I) VCAM-1, plotted over time after

the flow is turned off. Asterisk: P < 0.05 compared to MI = 0. Red tracks indicate cells moving against the direction of flow, and gray tracks are cells moving with the direction of flow. The direction of flow is from left to right.

CD4+ T lymphocytes on ICAM-1 surfaces migrated upstream at shear rates of 100 s⁻¹ and 800 s⁻¹ (Fig. 3.2A-a,c) during flow. Such upstream migration, represented by a negative Migration Index (MI) (Fig. 3.2D and Fig. A1), is more prominent at a shear rate of 800 s⁻¹, which is consistent with previously published studies (24). However, on ICAM-1 surfaces, after the flow is turned off, CD4+ T lymphocytes show no memory of their directional motion and migrate randomly (Fig. 3.2A-b,d, D and G). No significant differences in speed and persistence times were detected (Fig. A1A–C) after the flow was terminated. While LFA-1 engaging ICAM-1 is required for upstream migration, the binding is not sufficient to maintain directional persistence once the flow is stopped.

On surfaces in which VCAM-1/ICAM-1 are mixed, CD4+ T lymphocytes migrated against shear flow at shear rates of 100 and 800 s⁻¹ (Fig. 3.2B-a,c). Similar to CD4+ T lymphocytes on ICAM-1 surfaces, cells on VCAM-1/ICAM-1 mixed surfaces showed a shear rate dependence of upstream migration (Fig. 3.2E), crawling more avidly upstream at higher shear rates. While upstream migration requires from LFA-1-ICAM-1 interactions, VLA-4-VCAM-1 binding does not interfere with upstream migration. After the flow is turned off, CD4+ T lymphocytes on VCAM-1/ICAM-1 mixed surfaces maintained the upstream orientation (Fig. 3.2B-b,d and E). This upstream migration can persist for as long as 30 minutes, indicated by negative MI over this time (Fig. 3.2H). To observe persistent migration in the upstream direction, a shear rate of 800 s⁻¹ was needed. At 100 s⁻¹ shear rate, cells exhibited no collective memory of upstream migration, even on surfaces in which VCAM-1 and ICAM-1 were mixed. Thus, we conclude that while the direction of migration during flow is directed by LFA-1, the persistence of memory of direction post flow is dependent on VLA-4-VCAM-1 interactions and requires a high level of stimulus (higher shear rates).

We additionally confirmed that CD4+ T cells on VCAM-1 surfaces exhibit a strong downstream migration under flow at shear rates of 100 and 800 s⁻¹ (Fig. 3.2C), as demonstrated by a positive MI (Fig. 3.2). This observation was consistent with previous results (24). Cells on VCAM-1 migrated with the direction of flow and continue its direction of motion post flow (Fig. 3.2I). Since cells on VCAM-1 never migrate upstream, they do not have any upstream migration to remember.

CD8+ T cells also migrated upstream under flow on surfaces containing ICAM-1. However, unlike CD4+ T cells, CD8+ T cells exhibited random migration once the flow was turned off on all surfaces (Fig. A2). These results highlight that while upstream migration on ICAM-1 is consistent with CD4+ and CD8+ subsets, only CD4+ T cells remember the upstream migration on VCAM-1/ICAM-1 mixed surfaces. To concentrate on studying how cells remember the upstream migration post flow, we focused solely on CD4+ T lymphocytes on surfaces containing ICAM-1 or a mixture of VCAM-1/ICAM-1 at a shear rate of 800 s⁻¹ for the remainder of the study.



Figure 3.3. Migrational memory of CD4+ T lymphocytes with PI3K inhibitor,

Wortmannin. CD4+ T lymphocytes on VCAM-1/ICAM-1 mixed surfaces lose migrational memory upon inhibition of PI3K with Wortmannin. (A) Scattergrams of cells with a range of dose of Wortmannin during flow with 800 s⁻¹ shear rate (a–e) and postflow (f–j). Red tracks indicate cells moving against the direction of flow, and gray tracks are cells moving with the direction of flow. The direction of flow is from left to right. (B) Migration Index of cells with DMSO or 50 nM of Wortmannin on VCAM-1/ICAM-1 mixed surface after 30 minutes of each flow condition. (C) Speed of cells with DMSO or 50 nM of Wortmannin on the mixed surface after 30 minutes of each flow condition. (D) Migration Index of cells with DMSO or 50 nM of Wortmannin plotted over the course of 30 minutes of post flow. NS: Not significant compared to DMSO control of corresponding flow conditions. *PI3K is required for persistent upstream migration post flow in CD4+ T lymphocytes*

Integrin-ligand binding initiates a wide range of intracellular signaling pathways which determine cell polarity by rearranging actin or microtubules to power movement (33,34). Using our result that CD4+ T cells persist in the upstream direction after a shear rate of 800 s⁻¹ on surfaces in which VCAM-1 and ICAM-1 are mixed, we investigated the role of PI3K in maintaining upstream migration using a PI3K inhibitor, wortmannin. On VCAM-1/ICAM-1 mixed surfaces, inhibiting PI3K did not block upstream migration during flow at a shear rate of 800 s⁻¹ (Fig. 3.3A-a–e and B). After the flow is removed, CD4+ T lymphocytes on VCAM-1/ICAM-1 surfaces with wortmannin lost their memory of upstream migration and showed no preference in direction (Fig. 3.3A-f-j and B). We measured the MIs continuously during the entire period during and after flow. During the 30 minutes of flow, the MI stayed negative, indicating PI3K inhibition did not block upstream migration (Fig. A3D). After the flow is turned off, the MI rapidly approached zero, unlike the MI of the DMSO control group which remained negative, signifying PI3K inhibition led to random migration after the flow was terminated (Fig. 3.3D). At different concentrations of Wortmannin, CD4+ T cells on VCAM-1/ICAM-1 all preserved upstream migration under flow but lost the directionality post flow (Fig. A3). Thus, on VCAM-1/ICAM-1 surfaces, without proper PI3K activity, CD4+ T lymphocytes lose migrational memory.

Treating CD4+ T cells with Wortmannin on ICAM-1 did not interfere with upstream migration under flow (Fig. A4). Wortmannin also did not affect the memory effect of CD4+ T cells on ICAM-1, as cells on ICAM-1 never remembered the upstream migration to begin with. This indicates that reduction in PI3K activity does not affect

upstream migration when flow is present. Inhibiting with another PI3K inhibitor, LY294002, exhibited consistent results on both types of surfaces as the one with wortmannin (Fig. A5). We conclude that PI3K is not imperative for upstream migration under flow, but necessary for migrational memory.

Upstream migration both during and post flow is maintained independent of GTPases

We next hypothesized that cdc42, which is known to establish polarity in cell migration (15,17-19), would be required to preserve the upstream direction after the flow is removed. We inhibited cdc42 with ML-141 to test whether cdc42 affects post-flow polarity and the persistence of directional migration. Inhibiting cdc42 with ML-141 had no significant effect on CD4+ T lymphocytes migrating upstream on either ICAM-1 or VCAM-1/ICAM-1 surfaces (Fig. 3.4). In the presence of ML-141, CD4+ T lymphocytes on ICAM-1 showed upstream migration under flow (Fig. 3.4A and B), but no preferential direction of migration after the flow was removed (Fig. 3.4C). In the presence of ML-141, CD4+ T cells on VCAM-1/ICAM-1 mixed surfaces preserved their upstream orientation during and post flow (Fig. 3.4D–F). This result suggests that CD4+ T cells with reduced cdc42 activity were still able to migrate upstream. Cdc42 inhibition also had no effect in migration after the flow has turned off. Inhibiting Rho or Rac also had no effect on upstream migration under flow or post-flow migratory behavior on both surfaces (Fig. A6). Together, we conclude that inhibiting Rho, Rac, or cdc42 does not affect the ability to migrate upstream or to remember the upstream migration post flow.



Figure 3.4. Migrational memory of CD4+ T lymphocytes with cdc42 inhibitor, ML-141. Inhibiting Cdc42 with 10 μ M of ML-141 had no effect in migration during or after flow of CD4+ T lymphocytes. (A) Migration Index of cells on ICAM-1 after 30 minutes. (B and C) Migration Index plotted over the course of 30 minutes of each flow condition. (D) Migration Index of cells on VCAM-1/ICAM-1 after 30 minutes. (E and F) Migration Index plotted over the course of each flow condition. *: p < 0.05, **: p < 0.01, ***: p < 0.001, ****: p < 0.0001. NS: Not significant compared to DMSO control of corresponding flow condition.

Actin branching at lamellipodia also has no role in upstream migration or directional persistence in CD4+ T lymphocytes

At the leading edge of a moving cell, actin networks are constantly being restructured to foster cellular movement. Actin filaments are branched into mesh-like networks at lamellipodia, guided by Arp2/3 complexes. Since the actin network is the main driving force of lamellipodial extension in a moving cell, we hypothesized that hindering activity of Arp2/3 and reorganization of actin filaments would obliterate upstream migration and post-flow migrational memory. To investigate the effect of actin branching in migration under shear flow, CD4+ T lymphocytes were treated with different concentrations of CK666 on ICAM-1 or ICAM-1/VCAM-1 mixed surfaces.



Figure 3.5. Migrational memory of CD4+ T lymphocytes with Arp2/3 inhibitor, CK666. CD4+ T lymphocytes maintained upstream migration and displayed migrational memory with 25 μ M of Arp2/3 inhibitor, CK666. (A) Migration Index of cells on ICAM-1 after 30 minutes. (B and C) Migration Index of cells on ICAM-1 plotted over the course of 30 minutes of each flow condition. (D) Migration Index of cells on VCAM-1/ICAM-1 after 30 minutes. (E and F) Migration Index of cells on VCAM-1/ICAM-1 after 30 minutes of each flow condition. Asterisk: P < 0.05 compared to MI = 0. NS: Not significant compared to DMSO control of corresponding flow conditions.

CD4+ T lymphocytes treated with CK666 on ICAM-1 surfaces robustly migrated against the direction of flow (Fig. 2.5A and B). Once the flow was removed, cells no longer retained the direction established during shear flow and displayed no significant difference in post-flow migration (Fig. 2.5C). We noticed a slight decrease in speed upon Arp2/3 inhibition, but no significant discrepancies in persistence time (Fig. A7). CD4+ T cells on ICAM-1 with different concentrations of CK666 all preserved upstream migration under flow and no longer remember the upstream direction post flow (Fig. A7A, G and H). CD4+ T lymphocytes treated with CK666 on VCAM-1/ICAM-1 mixed surfaces also showed upstream migration at a shear rate of 800 s⁻¹ (Fig. 2.5D and E). After the flow was turned off, cells still maintained preferential upstream orientation that was established during flow (Fig. 2.5F). Cells treated with CK666 showed decreased speed, and persistent time was observed during and post flow (Fig. A7E and F). CD4+ T cells on VCAM-1/ICAM-1 in other concentrations of CK666 all migrated upstream during and post flow (Fig. A7D, I, and J), indicating that Arp2/3 inhibition of CD4+ T cells on VCAM-1/ICAM-1 surfaces had no effect on post-flow migrational memory. Sequestering actin polymerization with Latrunculin A (LatA) and Cytochalasin D (CytoD) completely abolished migration on both ICAM-1 and VCAM-1/ICAM-1 surfaces during and post flow (Fig. A8). In conclusion, while actin filaments are required for migration, branch formation with Arp2/3 did not affect migratory patterns during or post flow regardless of which type of integrin was activated.

Altering ligand binding of VLA-4 alone can modulate the directional persistence post flow in CD4+ T lymphocytes

While LFA-1 engaging immobilized ICAM-1 drives upstream migration, we performed additional experiments to verify the role VLA-4 plays in fostering migrational memory. We hypothesized that if a signal is propagated through VLA-4, inhibition of VLA-4 with blocking antibodies would eliminate migrational memory in CD4+ T lymphocytes, whereas independent activation using a soluble ligand (sVCAM-1) would preserve migrational memory.

When CD4+ T cells on VCAM-1/ICAM-1 surfaces were treated with VLA-4 blocking antibodies, forcing cells to only engage ICAM-1 with LFA-1, cells migrated upstream under flow, but no longer retained the directional memory after the flow was turned off (Fig. A9D–H). Blocking VLA-4 had no effect on migrational speed (Fig. A9F). This supports our hypothesis in that VLA-4 binding to VCAM-1 is required for CD4+ T cells to preserve their directional migration after flow.



Figure 3.6. Migrational memory of CD4+ T lymphocytes with soluble VCAM-1. CD4+ T lymphocytes on ICAM-1 surfaces migrate upstream during and post-flow in the presence of soluble VCAM-1. (A) Scattergrams of cells on ICAM-1 during (a and b) and post (c and d) flow at a shear rate of 800 s⁻¹. Red tracks indicate cells moving against the direction of flow, and gray tracks are cells moving with the direction of flow. The direction of flow is from left to right. (B) Migration Index of cells on ICAM-1 plotted over the course of 30 minutes of each flow condition. *: p < 0.05. NS: Not significant compared to isotype control of corresponding flow conditions.

CD4+ T lymphocytes on ICAM-1 surfaces treated with soluble VCAM-1 (sVCAM-1) migrated upstream under flow and continued the upstream direction post flow (Fig. 3.6 and Fig. A9A–C). No immobilized VCAM-1 was present in these experiments. Activating VLA-4 with soluble VCAM-1 had no effect on speed (Fig. A9A). However, soluble VCAM-1 stimulated cells on ICAM-1 surfaces to preserve directional memory after the flow is turned off (Fig. 3.6 and Fig. A9C). This suggests that simultaneous signaling from both LFA-1 and VLA-4 is required for the directional memory of upstream migration. However, it is the signal, not the adhesive interaction, of VLA-4/VCAM-1 that is required. This result further highlights that, while upstream migration is driven by LFA-1 and surface-bound ICAM-1 on the surface, post-flow upstream migration can be potentiated by VLA-4 binding to either immobilized or soluble VCAM-1.

Discussion

Intravascular crawling under shear flow is necessary for lymphocytes in order to effectively migrate along and through blood vessel wall to perform immunological functions. The direction of migration varies depending on types and concentrations of adhesion molecules that the cells engage and the shear rates the cells experience. Numerous studies have shown different types of cells orienting against the direction of flow in vitro and in vivo. For example, upstream migration under flow has been observed with marginal zone B cells, HSPCs, effector T cells on ICAM-1 and in a rat model, and neutrophils when Mac-1 is blocked (22–28). However, the intracellular mechanism of upstream migration for leukocytes remains poorly understood. Previously, we investigated how LFA-1 directs upstream migration of T lymphocytes. Cells on combinations of VCAM-1 and ICAM-1 elicited upstream migration under flow. The study further showed that cells on VCAM- 1/ICAM-1 mixed surfaces migrated upstream during flow at a shear rate of 800 s⁻¹, and that cells maintained the direction of motion when flow was stopped for 10 minutes (24). This study expands this work to understand the mechanisms of how cells persist to migrate upstream.

After the flow is removed, CD4+ T cells on ICAM-1 lost their directional signal and migrated at random. Cells on VCAM-1 migrated downstream under flow and continued in this direction when flow was terminated. On the other hand, on surfaces in which VCAM-1 and ICAM-1 are mixed, cells migrate against the direction of flow a shear rate of 800 s⁻¹ maintained their directionality even after the flow had been stopped. Thus, it appears that while LFA-1-ICAM-1 interactions are needed for upstream migration, the engagement of VLA-4 with VCAM-1 is needed for memory. Preservation

of upstream migration post flow was not observed with CD8+ T lymphocytes, regardless of types of adhesion molecules or shear rates.

Clearly, some signaling pathway is at the root of memory persistence. Numerous studies have elucidated intracellular signal transduction pathways connecting lymphocyte adhesion to movement. Specifically, PI3K and Rho family of small GTPases are involved in cellular migration and cytoskeletal reorganization. PI3K phosphorylates PIP2 to PIP3, which is implicated in cell polarity and migration, and is often found in the lamellipodia of migrating cells (15,35,36). Roy *et al* proposed a model in which the engagement of LFA-1 to immobilized ICAM-1 activates Src family kinases, which induces the binding of Crk/CasL to c-Cbl. This complex then activates PI3K catalytic function and promotes the production of PIP3 (35). However, the kinetics and signaling of PI3K of leukocytes under flow are not well known, because most studies on PI3K have been performed in the absence of shear flow. Here, our data show that inhibiting PI3K has no significant effect on upstream migration under shear flow. During flow, CD4+ T lymphocytes with hindered PI3K activity are still able to orient against the direction of flow on both ICAM-1 and VCAM-1/ICAM-1 mixed surfaces. However, without PI3K, CD4+ T lymphocytes no longer exhibited migrational memory on VCAM-1/ICAM-1 surfaces. Our findings suggest that while VLA-4-VCAM-1 interaction promotes post-flow directional persistence, the ability for VLA-4 to maintain the direction of upstream migration in concert with LFA-1 is PI3K dependent.

Downstream of PI3K, Rho-family GTPases play critical roles in cell migration by reorganizing the cytoskeleton and plasma membrane. Cdc42 and Rho activities are essential for chemotaxis (18,38). Rho and Rac inhibit each other by localizing at

polarizing ends to further establish cell polarity and guide amoeboid motion. Here, we next investigated if GTPases affect migration under shear flow and post-flow directionality. However, inhibiting Rho, Rac, and cdc42 all had no significant effects on upstream migration or post-flow migrational memory. This suggests that the basis for polarized cell movement induced by shear flow is distinct from that induced during chemotaxis. One possibility is that the absence of chemokines in our system also may explain the lack of significant effects upon inhibition of GTPases.

At the leading edge of a moving cell, actin filaments are organized in a mesh-like network within the lamellipodium. Arp2/3 initiates actin nucleation at an existing actin filament, in conjunction with actin-polymerizing and depolymerizing factors. While Arp2/3 is essential for T cell activation at the immunological synapse or migration under static conditions, its role in T cell migration in response to shear flow is poorly understood (39–42). At a shear rate of 800 s⁻¹, CK666 reduced speed and persistence times but had no effect on the directionality of CD4+ T lymphocytes during flow. CK666 had no effect in post-flow migrational memory either; cells on VCAM-1/ICAM-1 mixed surfaces with CK666 still maintained persistent directionality post flow. Surprisingly, perturbing Arp2/3 activity with CK666 did not affect upstream migration under flow via LFA-1-ICAM-1 interactions. Upstream migration post flow is regulated independently of Arp2/3. Perturbing actin polymerization with Latrunculin A and Cytochalasin D completely eliminated migration as both inhibitors disturb actin polymerization and eliminate actin filament formation. As actin filaments are the primary component of the lamellipodial cytoskeleton, and they provide force generation for movement, this result was expected. CD4+ T lymphocytes treated with Latrunculin A or Cytochalasin D were

only able to loosely interact with the surface and roll across the surface, rather than actively migrate. On VCAM-1/ICAM-1 mixed surfaces, a subpopulation of cells would roll and form tether-like membrane extensions while rolling, similar to tether structures seen with neutrophils during rolling (43–45). These results suggest that while actin polymerization is required for any migratory phenotype, forming Arp2/3-guided branches may not be necessary for CD4+ T lymphocytes to migrate against the direction of flow and maintain the directionality after the flow is removed.

The mechanism of upstream migration remains rather mysterious. Valignat *et al* have suggested that a uropod provides a passive self-steering structure in T cells during flow, similar to a wind vane (46). While T cells are using their uropods to sense the direction of flow, determining the direction of migration (perhaps the placement of the uropod) ultimately depends which integrins are engaged. This suggests that in order to orient themselves against the direction of flow with the presence of ICAM-1, not only the physical formation of the uropod structure but also the reorientation of cytoskeletal network and cellular polarization, which are often driven by intracellular signaling pathways, are imperative for cells to reorient themselves and persistently migrate in the opposite direction of flow. This study implies that the simultaneous activation of LFA-1 and VLA-4 integrins could also propagate actin reorganization to support the upstream migration of T cells.

While LFA-1 alone is sufficient to promote upstream migration, we have shown that engagement of VLA-4 and LFA-1 together is essential for directed motion of CD4+ T lymphocytes post flow. VLA-4 alone does not support upstream migration. Although there are several reports of β_1 - and β_2 -subunits cross-talking intracellularly, the details of

the crosstalk have not been fully elucidated (34,47–49). Specifically, several groups have noted that the activation of one integrin phosphorylates the other and thus affects the adhesion and migration on surfaces with the affected integrin's cognate ligands (49–52). Grönholm et al and Porter and Hogg have reported LFA-1 activity affecting VLA-4 adhesion and migration on VCAM-1 (49,51). Chan *et al* have investigated how VLA-4 augments LFA-1-mediated adhesion to ICAM-1 (52). Our results also support integrin crosstalk by highlighting the difference in migration post flow between VCAM-1/ICAM-1 and ICAM-1 surfaces. Specifically, we speculate that integrin crosstalk provides some signal that is required for the continued upstream persistence of cell motion. CD4+ T lymphocytes on VCAM-1/ICAM-1 showed no sign of directional memory when blocked VLA-4 was blocked from binding to VCAM-1, which further supports that cells require both LFA-1 and VLA-4 to remember the direction of flow. However, cells on ICAM-1 with soluble VCAM-1 retained migrational memory, suggesting that both soluble or surface-bound form of VCAM-1 can trigger VLA-4 activity, which then initiates the crosstalk of LFA-1 and VLA-4 and promotes the directional memory. Importantly, this result suggests that occupancy of VLA-4, not its adhesive activity, is critical. While uropods provide physical structure to detect the direction of flow and LFA-1 to promote upstream migration, VLA-4-VCAM-1 binding may strengthen LFA-1 adhesion to ICAM-1, which, in turn, will promote robust upstream migration in CD4+ T cells. This somehow promotes the maintenance of polarization or stabilizes the uropods for longer to maintain upstream migration after flow longer than cells would have with LFA-1 alone.

We have also shown that migrational memory is dependent upon PI3K. We speculate that PI3K promotes crosstalk between LFA-1 and VLA-4. As phosphorylation

of integrins subunits is required for the recruitment of integrin-binding proteins such as αactinin for cytoskeletal reorganization (48), simultaneous activity from VLA-4 could provide a long-lived support of LFA- 1 activation through PI3K, thus maintaining upstream migration during and after flow. In addition to integrin-associated adaptor proteins, this mechanism could also involve Crk and c-Cbl, which will be investigated in future work (35). However, the intracellular localization and the time scale of signaling proteins to reorganize actin cytoskeleton in response to shear flow still remain unanswered.

Combining these insights, it appears that both the morphological structure of the cell and intracellular signaling cascades that are initiated by integrin binding to adhesion molecules are required for persistent upstream migration. While the upstream direction is determined by LFA-1, the persistence of the upstream direction is maintained by simultaneous VLA-4 activation and consequent signaling generated from the crosstalk of the two integrins. Because upstream migration in response to flow has been implicated to be a relevant physiological process *in vitro* and *in vivo* (23–26,28,46), this study provides a new platform to uncover how integrin crosstalk influences migration during flow and how cells migrate after the absence of shear flow.

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CHAPTER 4: Aim 2: Signaling mechanism of upstream migration with mouse models

Adapted from: Nathan H. Roy, Sarah Hyun Ji Kim, Alexander Buffone Jr, Daniel Blumenthal, Bonnie Huang *et al* "LFA-1 signals to promote actin polymerization and upstream migration in T cells", *Journal of Cell Science*, September 2020. doi.org/10.1242/jcs.248328

Abstract

T cell entry into inflamed tissue requires firm adhesion, cell spreading and migration along and through the endothelial wall. These events require the T cell integrins, LFA-1 and VLA-4, and their endothelial ligands, ICAM-1 and VCAM-1, respectively. T cells have previously been reported to migrate against the direction of shear flow on surfaces containing ICAM-1, and with the direction of flow on VCAM-1 surfaces, suggesting the two ligands trigger different cellular responses. However, the contribution of specific signaling events downstream of LFA-1 and VAL-4 has not been explored. We found that engagement of LFA-1, but not VLA-4, induces cell shape changes associated with rapid 2D migration. Moreover, LFA-1 ligation results in activation of the phosphoinositide-3-kinase (PI3K and ERK pathways, and phosphorylation of multiple kinases and adaptor proteins, whereas VLA-4 ligation triggers only a subset of these signaling events. Importantly, we uncover that T cells lacking Crk adaptor proteins and the ubiquitin ligase c-Cbl (also known as CBL), both which are downstream of LFA-1, fail to migrate upstream under flow. These studies

identify novel signaling differences downstream of LFA-1 and VLA-4 that lead to distinct T cell migratory behavior in response to shear flow.

Introduction

Migration of leukocytes from the vasculature into peripheral tissue is central to their role in fighting pathogens, promoting tissue repair and attacking solid tumors. This process, called transendothelial migration (TEM), is a key control point in the inflammatory response (1). TEM is a multi-step process that begins with selectindependent cell rolling on the vasculature, followed by chemokine-induced cell arrest. At this point, integrins expressed on the leukocyte interact with their endothelial ligands, resulting in shear-resistant adhesion, cell spreading, and migration along the endothelium (1–3). Leukocyte migration along the vascular wall is a prerequisite for transmigration, and is thought to allow cells to search for ideal sites to cross the endothelial layer, such as three-way junctions (4–8). Intravital imaging of immune responses in vivo has revealed that leukocyte migration along the endothelial monolayer is not random and can be directed by shear flow forces (9,10). Interestingly, leukocytes have been observed to preferentially migrate against the direction of shear flow (9), an unexpected result given the extra energy expenditure needed to oppose head-on shear forces. Although it is not clear why leukocytes display this phenotype, in vitro studies have shown that T cells crawling against the direction of shear on inflamed endothelia are more likely to undergo transmigration (11,12), suggesting a link between these two mechanically demanding processes.

T cell adhesion and migration on the vascular wall are dependent on integrin interactions with their endothelial ligands. The two major integrin ligands expressed on inflamed endothelia are ICAM- 1 and VCAM-1, which serve as binding partners for the T cell integrins LFA-1 (α L β 2) and VLA-4 (α 4 β 1), respectively. Although both of these

integrin–ligand pairs can support adhesion, they seem to promote distinct migratory behaviors. The most striking example of this is observed under shear flow, in which T cells migrating on ICAM-1-coated surfaces preferentially migrate against the direction of shear flow (upstream migration), whereas T cells migrating on VCAM-1-coated surfaces migrate with the direction of shear flow (downstream migration) (13–17). This integrindependent phenomenon has also been documented in B cells (18), hematopoietic precursors (19) and neutrophils (under some conditions; (20)). These data point towards fundamentally different roles for ICAM-1 and VCAM-1 in coordinating leukocyte migration.

A biophysical model has been proposed to explain the ability of T cells to migrate upstream on ICAM-1. The model is based on the finding that LFA-1–ICAM-1 interactions occur toward the front of the cell, and induce the formation of a broadly spread leading edge and a raised, trailing uropod, whereas VLA-4–VCAM-1 interactions take place toward the back of the cell and fail to generate these shape changes (17). The shape changes induced by LFA-1 engagement allow the uropod to act as a 'wind vane' that passively steers the T cell upstream (16). Although this model relies on the idea that localized integrin adhesion results in the relevant cell shape changes, T cell spreading and migration have also been shown to involve integrin signaling. This is best documented for LFA-1; T cells that come into contact with surface-presented ICAM-1 immediately polarize and begin to migrate (21–23).

Recently, we characterized a signaling pathway downstream of LFA-1 that leads to cell spreading, actin polymerization, and migration (24). Central players in this pathway are the Crk adaptor proteins, which coordinate Src-dependent phosphorylation

of the scaffolding ubiquitin ligase c-Cbl (also known as CBL), ultimately leading to phosphoinositide 3-kinase (PI3K) activity and actin responses. Importantly, disruption of this pathway by deleting Crk proteins perturbs LFA-1-dependent migration, showing that LFA-1 signaling is an important factor driving T cell migration on ICAM-1. Because the LFA-1–ICAM-1 interaction triggers strong migratory responses in T cells, we hypothesized that differential integrin signaling events account for the differences in behavior of T cells migrating on ICAM-1 versus VCAM-1. Therefore, we analyzed T cell migration and signaling in response to binding ICAM-1 or VCAM-1, with the goal of determining how these different integrin ligands control T cell migration.

Materials and Methods

Antibodies and reagents

Anti-CD3 clone 2C-11 and the anti-CD28 clone PV1 were obtained from BioXCell. Anti-pTyr clone PY-20 was from Upsate (Millipore). Anti-HEF1 (CasL) clone 2G9 and anti-Pyk2 clone YE353 were obtained from Abcam. Anti-pERK (9101), antipAKT (4060), and anti-c-Cbl (2747) were from Cell Signaling Technology. Anti-AKT (559028) was from BD. Secondary antibodies conjugated to appropriate fluorophores and AlexaFluor- conjugated phalloidin were obtained from Thermo Fisher Scientific. Recombinant mouse ICAM-1-Fc and VCAM-1-Fc were purchased from R&D Systems. Antibodies for flow cytometry were from BioLegend. These included rat anti-CD4 APC (clone RM4-5), anti-CD49d FITC (clone R1-2), anti-CD49e PE-Cy7 (clone 5H10-27), anti-CD49f Pac-blue (clone GoH3), anti-CD11a PE (clone M17/4), anti-CD11b APC (clone M1/70), anti-CD29 488 (clone HMβ1-1), anti-CD18 PE (clone M18/2), and anti-ITGB7 PerCP/ Cy5.5 (clone FIB27). All antibodies were used at a 1:100 dilution for flow cytometry. All antibodies were validated by the manufacturer.

Mice and T cell culture

All mice were housed in the Children's Hospital of Philadelphia animal facility, according to guidelines put forth by the Institutional Animal Care and Use Committee. C57BL/6 mice, originally obtained from The Jackson Laboratory, were used as a source of WT T cells. Mice expressing Lifeact– GFP have been described previously (Riedl et al., 2010). Additionally, mice lacking the Crk adaptor proteins in T cells (herein referred to as DKO) were generated by crossing CD4+ Cre mice with mice in which the two Crk
loci have been floxed (Crkfl/fl:CrkLfl/fl mice) (25,26). To generate DKO mice expressing Lifeact–GFP, Crkfl/fl: CrkLfl/fl mice were crossed with Lifeact–GFP mice, and the resulting Lifeact–GFP: Crkfl/fl:CrkLfl/fl mice were crossed with CD4+ Cre mice. Mice expressing Cas9 (C57BL/6J-congenic H11Cas9; Jackson stock no. 028239) were used for CRISPR studies. All mice were 8–14 weeks of age when killed, and both males and females were used as a source of T cells.

Primary mouse CD4+ T cells were purified from lymph nodes and spleens by negative selection. Briefly, after removing red blood cells by incubation for 1 min in ACK lysis buffer (77 mM NH4Cl, 5 mM KHCO3 and 55 µM EDTA), cells were washed and incubated with anti-MHCII and anti-CD8 hybridoma supernatants (M5/114 and 2.43, respectively) for 20 min at 4°C. After washing, cells were mixed with anti-rat Ig magnetic beads (Qiagen BioMag), incubated for 15 min at 4°C, and subjected to three rounds of magnetic separation using a benchtop magnet. The resulting CD4+ T cells were then immediately activated on 24-well plates coated with anti-CD3 and anti-CD28 (2C11 and PV1, 1 μ g/ml each) at 1×106 cells per well. Activation was done in T cell complete medium, composed of DMEM (Gibco 11885-084) supplemented with 5% FBS, penicillin/streptomycin, non-essential amino acids, Glutamax, and 2 µl 2mercaptoethanol. Unless otherwise indicated, all tissue culture reagents were from Gibco. After 48 h, cells were removed from activation and mixed at a 1:1 volume ratio with complete T cell medium containing recombinant IL-2 (obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH from Dr Maurice Gately, Hoffmann -La Roche Inc), to give a final IL-2 concentration of 20 units/ml. T cells were used at days 5–6 after activation.

Flow cytometry

Cells were first stained with Live/Dead Aqua (ThermoFisher) in PBS following the manufacturer's protocol. Staining was quenched using 1% bovine serum albumin (BSA) solution. For staining, antibodies were diluted 1:100 in FACS buffer (PBS, 5% FBS, 0.02% NaN3, and 1 mM EDTA). Flow cytometry was performed using either a Cytoflex LX or CytoFlex S cytometer (Beckman Coulter) and data was analyzed using FlowJo software (FlowJo LLC). T cells were gated based on size, live cells, and expression of CD4+.

Static adhesion assay

96-well plates (MaxiSorp, ThermoFisher) were coated with 2 µg/ml mouse ICAM-1, VCAM-1, or both ligands mixed (1 µg/ml each), in PBS overnight at 4°C. Plates were then washed three times with PBS, blocked with 1% BSA in PBS for 1 h at room temperature, and washed twice with PBS. Activated CD4+ T cells were used on day 5 after initial isolation. To prepare the cells, they were first labeled with Calcein AM (ThermoFisher) at a final concentration of 2.5 µM for 30 min at 37°C in serum-free DMEM. Cells were then washed and resuspended in T cell complete medium, and incubated at 37°C for 30 min. Cells were washed and resuspended in 2.5%

BSA in PBS (with Ca^{2+} and Mg^{2+}) and 1×105 cells were added to each well on ice in triplicate. After a 20 min incubation, the plate was read on a Bio- Tek Synergy HT fluorescence plate reader to obtain the baseline measurements representing 'maximum adhesion' per well. The plate was then incubated at 37°C for 10 min, then washed and read again. The plate was washed and read a total of 2–4 times, until the signal from the

unstimulated control was stable. To calculate the percentage adhesion, the fluorescence per well after washes was divided by the initial 'maximum adhesion' fluorescence reading per well. Background was subtracted using values obtained from empty wells.

F-actin quantification in migrating *T* cells

Lab-Tek 8-chamber slides (ThermoFisher) were coated overnight at 4°C with 2 μ g/ml ICAM-1, VCAM-1, or both ligands mixed (1 μ g/ml each). For supplemental experiments using excess VCAM-1, VCAM-1 was coated at 10 µg/ml. Activated CD4+ T cells were resuspended in Leibovitz's L-15 medium (Gibco) supplemented with 2 mg/ml glucose and incubated at 37°C for 20 min. T cells were then added to the chamber slides for 20 min at 37°C, followed by fixation in 3.7% paraformaldehyde in PBS. Cells were then blocked and permeabilized in PSG (PBS, 0.01% saponin, 0.05% fish skin gelatin) for 20 min, followed by 45 min with fluorescent phalloidin (Molecular Probes) in PSG. Cells were washed in PSG, mounted, and imaged using a 63× PlanApo 1.4 NA objective on an Axiovert 200 M (Zeiss) with a spinning disk confocal system (Ultraview ERS6; PerkinElmer). Four z-planes spanning a total of 0.75 µm were collected at the cell-surface interface using an Orca Flash 4.0 camera (Hamamatsu). Image analysis of the rendered stacks was conducted using Volocity v6.3 software. Cells were identified using the 'Find Objects' command, using a low threshold on the actin channel, and total phalloidin staining was quantified per cell based on integrated pixel intensity.

For the visualization of F-actin in live cells, Lifeact–GFP mice served as the source of CD4+ T cells. Activated CD4+ T cells were added to Lab-Tek 8-chamber slides for 20 min, gently washed in warm L-15 medium, mounted, and imaged at 37°C using

the spinning disk confocal system. For migration movies, 9 z-planes spanning a total of 2 μ m were collected every 2 s. Projections of the full 2 μ m were prepared in Fiji. For whole-cell reconstruction, 49 z-planes spanning a total of 12 μ m were captured, and rendered using the orthogonal viewing tool in Fiji.

Migration under shear flow

Surfaces were prepared by stamping 2 µg/ml of protein A/G (Biovision, San Francisco, CA) using PDMS stamps, onto UV ozone-treated, PDMS spin-coated glass slides. Surfaces were then blocked with 0.2% Pluronic F- 127 (Sigma), washed, and subsequently incubated with 2 μ g/ml of murine ICAM-1 Fc, VCAM-1 Fc, or a 1:1 mixture at 4°C overnight. Activated CD4+ T cells were resuspended in RPMI-1640 supplemented with 2 mg/ml D-glucose and 0.1% BSA and introduced into a flow chamber using a syringe. T cells were allowed to adhere for 30 min prior to the onset of shear (rate: 100 s^{-1} or 800 s^{-1}). Cells were then imaged every minute for 30 min using a Nikon TE300 with a custom built environmental chamber at 37°C and 5% CO2. For drug experiments, either DMSO or LY294002 (50 μ M) were present for 50 min prior to the onset of shear, followed by 30 min of imaging under shear flow conditions. The movies were exported to ImageJ and cells were tracked and analyzed using the manual tracking plugin in ImageJ and a custom MATLAB script. Only cells that stayed in the field-ofview the whole movie were included in the analysis. Total track length, total displacement, and migration index were calculated. Migration Index (MI) describes the directionality of a cell by dividing the displacement in the x-direction (the axis parallel to the shear flow) by the total track length. An MI value of -1 indicates that cells migrate in

a straight trajectory against the direction of flow, and an MI value of +1 indicates migration in a straight trajectory with the direction of flow. Shear flow methodology, experiments, and analysis were carried out by Hyun Ji Kim.

Migration in static conditions

Lab-Tek 8-chamber slides (ThermoFisher) were coated with 2µg/ml ICAM-1, VCAM-1, or both ligands mixed, overnight at 4°C. Activated CD4+ T cells were washed and resuspended in L-15 medium containing 2 mg/ml D-glucose. T cells were then added to the chambers, incubated for 20 min, gently washed to remove all unbound cells, and imaged using a 10× phase contrast objective at 37°C on a Zeiss Axiovert 200M microscope equipped with an automated x–y stage and a Roper EMCCD camera. Timelapse images were collected every 30 s for 10 min using SlideBook 6 software (Intelligent Imaging Innovations). Movies were exported into ImageJ, and cells were tracked using the manual tracking plugin to calculate speed. Directionality was calculated by taking the ratio of displacement and track length.

Retroviral production

The recently described gRNA retroviral transfer vector MRIG (Huang et al., 2019) was modified to express the puromycin resistance gene in place of GFP. Specific gRNAs were cloned into this vector exactly as described by Huang et al. (2019). The gRNA sequences used in this study were as follows: non targeting (NT), 5'-GCGAGGTATTCGGCTCCGCG-3'; cCbl, 5'-TGTCCCTTCTAGCCGCCCAG-3'; and PI3Kδ, 5'-GGAGCGTGGG-CGCATCACGG-3'. 293T cells (originally obtained from

ATCC and screened periodically for mycoplasma) were transfected with MRIG-puro along with the retroviral packaging vector pCL-eco (Addgene 12371) at a 4:3 ratio using the calcium phosphate method (Kingston et al., 1999) and allowed to incubate overnight. The next morning, the medium was gently replaced, and cells were incubated for 24–30 h. Viral-containing super- natants were harvested and centrifuged at 1000 g for 10 min to remove cellular debris. Polybrene was added to clarified viral supernatants to a final concentration of 8 μ g/ml, and supernatants were used immediately for T cell transductions.

T cell transductions

CD4+ T cells harvested from Cas9-expressing mice were activated on 24-well plates coated with anti-CD3 and anti-CD28 (2C11 5 μ g/ml and PV1 2 μ g/ml) at 1×106 cells per well in T cell complete medium. After 24 h, the conditioned medium was removed and reserved for later use, and gently replaced with viral supernatants (with polybrene), and incubated at 37°C for 10 min. The plate was then centrifuged at 1100 g for 2 h at 37°C. Directly following spinoculation, the plate was placed back in the incubator for 10 min. Viral supernatants were then removed and replaced with a mixture of fresh complete T cell medium mixed with conditioned medium at a 3:1 volume ratio, after which cells were cultured for an additional 24 h. T cells were then removed from activation, and mixed at a 1:1 volume ratio with complete T cell medium containing recombinant IL-2 to yield a final IL-2 concentration of 20 units/ml. After 4 h, puromycin was added to a final concentration of 4 μ g/ml. Cells were used after 3 d of selection (day 5 after isolation).

Biochemical analysis of cell signaling in response to surface-bound ICAM-1 or VCAM-1

60-mm tissue culture dishes (Corning, 430166) were coated with 2 µg/ml ICAM-1 or VCAM-1 overnight at 4°C. Activated CD4+ T cells were serum starved for 3 h in DMEM lacking all supplements, washed and resuspended in L15 medium supplemented with 2 mg/ml D-glucose, and incubated for 10 min at 37°C. 8.5×106 T cells were then allowed to interact with surfaces coated with ICAM-1 or VCAM-1 for 20 min at 37°C. Cells stimulated on surface-bound ICAM-1 or VCAM-1 were lysed by aspirating the medium and adding 500 μ l 1× ice cold lysis buffer (final composition: 1% Triton X-100, 150 mM NaCl, 50 mM Tris-HCl pH 7.5, 10 mM MgCl2, 5 mM NaF, 1 mM sodium orthovanadate and Roche EDTA-free protease inhibitor cocktail). Unstimulated control cells were lysed in solution by adding an equal volume of $2 \times \text{cold}$ lysis buffer for a final volume of 500 μ l. Lysates were incubated on ice with periodic vortexing for 15 min, followed by centrifugation for 10 min at 16,000 g at 4°C. A 50 µl aliquot of each wholecell lysate was retained, mixed with 4× sample buffer containing DTT (50 mM final), and heated to 95°C for 10 min prior to separation by SDS– PAGE. The remainder of each lysate was used for immunoprecipitation.

For immunoprecipitation, 60μ l of Protein A agarose bead slurry (Repligen) were washed and resuspended in lysis buffer. Beads were then pre-bound to antiphosphotyrosine (PY-20, 2.5 µg per condition) overnight at 4°C, with rotation. Antibodycharged beads were then washed three times with lysis buffer, mixed with 450 µl cell lysates, and rotated at 4°C overnight. Beads were then washed three times with lysis buffer, mixed with 30 µl of 2× sample buffer, boiled, and analyzed using SDS–PAGE.

Western blotting

Proteins were separated by SDS–PAGE using the Invitrogen Novex Mini- cell system with NuPAGE 4–12% BisTris gradient gels. Proteins were transferred to nitrocellulose membranes (0.45 μm, BioRad) and blocked using LI-COR blocking buffer mixed 1:1 with PBS for 1 h at room temperature. Primary antibodies were mixed in TBS containing 0.1% tween- 20 (TBST) with 2% BSA and incubated with membranes overnight at 4°C on a shaker. Primary antibody dilutions were as follows: anti-HEF1 (1:350), anti-Pyk2 (1:1000), anti-pERK (1:1500), anti-pAKT (1:1000), anti-c-Cbl (1:1000), and anti-AKT (1:1000). Membranes were then washed three times for 10 min each in TBST, and incubated with fluorophore-conjugated secondary antibodies in TBST with 2% BSA for 1 h at room temperature. Membranes were washed three times for 10 min in TBST, and imaged using a LI-COR Odyssey imaging system. Quantification of bands was performed using ImageStudio software (LI-COR), and all measurements were within the linear range.

Statistical analysis

Statistics were calculated and graphs were prepared using GraphPad Prism 8. When only two groups were being compared, a t-test was used. When more than two groups were compared, a one-way ANOVA was performed using multiple comparisons with a Tukey correction. *P<0.05; **P<0.01; ***P<0.001

Results

We and others have previously shown that T cells preferentially migrate against the direction of shear on ICAM-1 and with the direction of shear on VCAM-1 (11–17). To verify that our primary mouse T cells display this behavior, activated T cells were allowed to adhere to surfaces coated with ICAM-1, VCAM-1, or both ligands mixed at a 1:1 ratio, and then subjected to shear flow of either 100 s⁻¹ or 800 s⁻¹, corresponding to roughly 1 dyn/cm2 (0.1 Pa) and 8 dyn/cm2 (0.8 Pa), respectively.



Figure 4.1. Primary mouse T cells under shear flow on ICAM-1. Activated primary mouse T cells migrate against the direction of shear on ICAM-1. (A) Representative scattergrams of activated primary mouse CD4+ T cells migrating on ICAM-1, VCAM-1, or mixed surfaces under shear flow conditions (100 s^{-1} or 800 s^{-1}). Red lines, cell tracks with a net migration against the direction of shear (upstream). Blue lines, cell tracks with a net migration in the direction of shear (downstream). (B) Average migration index (MI) pooled from three independent experiments. MI for each cell equals the ratio of the displacement left or right by the total distance traveled. Migration with no directional preference results in a value of 0. Negative values indicate net migration upstream, and positive values indicate downstream migration. (C) Surface levels of CD11a and CD18 (integrins α_L and β_2 , respectively, which compose LFA-1), and CD49d and CD29 (integrins α_4 and β_1 , respectively, which compose VLA-4) on CD4+ T cells at day 5 post

activation (the day used for all migration experiments). Gray shaded lines represent isotype controls. (D) CD4+ T cell adhesion to the indicated ligands was measured using a plate-based adhesion assay. B and D show mean \pm s.d. for three independent experiments. Statistics were calculated using a one-way ANOVA with a Tukey multiple comparisons test. **P<0.01; ***P<0.001. As shown in Figure 4.1, we observed a clear tendency of T cells to migrate upstream on ICAM-1 and mixed surfaces, whereas migration on VCAM-1 was almost completely downstream. Quantification of migration index (MI) from at least three independent experiments confirmed that T cells tend to migrate against the direction of shear on ICAM-1, but not on VCAM-1. T cells on mixed surfaces displayed a phenotype similar to those on ICAM-1 alone, showing that upstream migration upon ICAM-1 ligation is the dominant phenotype (Fig. 4.1A,B).

We reasoned that this upstream/downstream phenotype could result from large differences in LFA-1 or VLA-4 expression levels, or from the ability of the different integrins to promote firm adhesion. To test this, we measured that T cells expressed measurable levels of all four integrin subunits by surface labeling for CD11a and CD18 (integrins α_L and β_2 , respectively, which compose LFA-1), and CD49d and CD29 (integrins α_4 and β_1 , respectively, which compose VLA-4) (Fig. 4.1C, Fig. A10). We confirmed that LFA-1 and VLA-4 are likely the primary integrins that bind to ICAM-1 and VCAM-1, respectively (Fig. 4.1C, Fig. A10). VCAM-1 supported more robust adhesion than ICAM-1, and the mixed surfaces promoted adhesion that was slightly higher than VCAM-1 alone (Fig. 4.1D). Thus, we confirmed that while both integrins act as functional adhesion molecules, there is no correlation between the efficiency of adhesion and upstream/downstream migratory behavior. Taken together, these data show that primary mouse T cells migrate upstream on ICAM-1 and downstream on VCAM-1, and that this phenotype cannot be explained simply by differences in integrin expression or adhesive functions.



Figure 4.2. Murine T cells migrate differently on ICAM-1 vs. VCAM-1. Quantitative and qualitative differences in T cell migration on ICAM-1 and VCAM-1. (A) CD4+ T cells were allowed to migrate on the indicated ligands under static conditions, then were fixed and stained with phalloidin to visualize F-actin. Images are a projection of four z-slices totaling 0.75 μ m, displayed as heat maps based on pixel intensity (high intensity, yellow; low intensity, violet). PolyL, poly-L-lysine control. Scale bar: 10 μ m. (B) Quantification of total F-actin intensity per cell (each circle represents one cell). (C,D) T cells were tracked while migrating on the indicated surfaces in the absence of shear, and the (C) average speed and (D) directionality were calculated (each circle represents one cell). (E)

Orthogonal views of T cells expressing Lifeact–GFP, migrating on surfaces coated with the indicated ligands. Scale bar: 10 μ m. (F) Migrating T cells expressing Lifeact–GFP were imaged using time-lapse confocal microscopy. Images are a projection of a 2 μ m total stack starting at the cell–coverslip interface, and are displayed as heat maps. Scale bar: 10 μ m. See Movies 1 and 2. (G) Kymographs of T cells migrating as in F. Data in B– D are pooled from three independent experiments, and red lines indicate the mean. Statistics were calculated using a one-way ANOVA with a Tukey multiple comparisons test. *P<0.05; **P<0.01; ***P<0.001.

To better understand the differential nature of LFA-1 and VLA-4 dependent migration on ICAM-1 and VCAM-1, the shape and behavior of T cells migrating on ICAM-1, VCAM-1 or mixed surfaces were analyzed in the absence of shear flow. T cells on ICAM-1-coated surfaces formed a broad, actin-rich leading edge with an increase in actin polymer compared to cells on poly-L-lysine control surfaces (Fig. 4.2A,B). This is consistent with what Roy et al previously reported (24). In contrast, T cells on VCAM-1coated surfaces displayed a more elongated shape with a narrower leading edge and less F-actin accumulation compared to cells on ICAM-1 (Fig. 4.2A,B). T cells responding to mixed surfaces were indistinguishable from T cells responding to ICAM-1 alone, consistent with the finding that ICAM-1 induces the wide-spreading morphology and more adhesive phenotype (Fig. 4.2A,E). Cells on ICAM-1 and mixed surfaces migrated significantly faster and more directionally with actin-rich leading edges contacting the surface than cells on VCAM-1 which showed less contact with the surface (Fig. 4.2C-G). This morphology is consistent with observations by others (16,17). Taken together, these data show that engagement of ICAM-1, but not VCAM-1, results in T cell spreading, actin polymerization, and fast migration. This behavior was confirmed by analyzing cell morphology, F-actin levels with fixed staining and LifeAct-GFP transfection, and kymographs.

Differences in static migration of T cells suggest that ICAM-1 triggers specific signaling events that lead to enhanced spreading and F-actin levels. Specifically, we decided to focus on the Crk family adaptor proteins as key signaling intermediates that promote actin polymerization and migration in T cells downstream of LFA-1 (24). The

Crk family consists of three proteins, Crk I, Crk II, and CrkL, which are transcribed from two loci (Crk I and Crk II are splice variants encoded by the Crk locus) (27).



Figure 4.3. Crk deficiency in murine T cell upstream migration. Crk-deficient T cells fail to migrate upstream on ICAM-1. (A,B) WT and DKO T cells were imaged while migrating on ICAM-1 in the absence of shear. (A) Average speed pooled from three independent experiments (each circle represents one cell, and red lines indicate the mean). (B) Representative images of migrating T cells expressing Lifeact–GFP, displayed as in Fig. 4.2. Scale bar: 10 μ m. (C) Representative scattergrams of WT and DKO T cells imaged while migrating on ICAM-1 or VCAM-1 under shear flow (shear rate 800 s⁻¹). Red lines, cell tracks with a net migration against the direction of shear (upstream). (D) Average migration index calculated from three independent experiments, displayed as mean \pm s.d. Statistics were calculated using a one-way ANOVA with a Tukey multiple comparisons test. ***P<0.001.

In the absence of shear flow, T cells lacking all three Crk proteins (DKO T cells) migrate significantly slower than wild-type (WT) T cells on ICAM-1, and fail to spread and polymerize actin at the leading edge (24) (Fig. 4.3A,B). This phenotype is strikingly similar to that of WT T cells migrating on VCAM-1, suggesting that Crk protein signaling might be a determining factor in the cellular response to ICAM-1. To ask whether Crk signaling is also important for upstream migration, we imaged WT and DKO T cells migrating in the presence of shear flow (800 s⁻¹). We found that DKO T cells were unable to migrate upstream on ICAM-1, with no apparent defect in downstream migration on VCAM-1 (Fig. 4.3C,D). This phenotype was not due differences in integrin levels on DKO T cells (Fig. A11). These data support a model in which Crk-dependent LFA-1 signaling drives T cell spreading, actin responses, and upstream migration.

In addition to activating canonical signaling pathways, such as the PI3K–AKT and ERK pathways, engagement of LFA-1 and VLA-4 induces the phosphorylation and activation of several signaling and scaffold proteins, including CasL (also known as NEDD9), cCbl (also known as CBL), FAK (PTK2), and Pyk2 (PTK2B) (28–35). Crk proteins are crucial for multiple signaling events downstream of LFA-1, including phosphorylation of CasL and cCbl, as well as activation of the PI3K pathway (24). Our observation that Crk-deficient T cells do not migrate upstream on ICAM-1 suggests that some of these signaling events may be necessary for upstream migration. If so, we reasoned that these events might occur in cells responding to ICAM-1, but not to VCAM-1. To determine whether ICAM-1 and VCAM-1 differentially activate any of these downstream signaling pathways, lysates of T cells on either ICAM-1 or VCAM-1 coated

surfaces were probed for the activation state of the PI3K and ERK pathways using phospho-specific antibodies and immunoprecipitated with anti-phosphotyrosine antibody, and probed with antibodies to CasL, cCbl, and Pyk2.



Figure 4.4. Differential signaling induced by ICAM-1 and VCAM-1. (A) CD4+ T cells were allowed to settle on ICAM-1- or VCAM-1-coated plates for 20 min and lysed. Lysates were immunoblotted with the indicated antibodies (top panel), or immunoprecipitated with anti-pTyr, and immunoblotted with indicated antibodies (bottom panel). –, lysates from unstimulated cells. (B) Quantification of immunoblots performed as in A. Data represent mean ± s.d., normalized to the ICAM-1 condition from three independent experiments.

ICAM-1 stimulated robust phosphorylation of AKT and ERK (ERK1 and ERK2, also known as MAPK3 and MAPK1), while stimulation with VCAM-1 induced only modest activation of these pathways (Fig. 4.4). Similarly, robust phosphorylation of cCbl was only induced by ICAM-1 (Fig. 4.4A,B). Importantly, phosphorylation of CasL and Pyk2 occurred with similar efficiency after engagement of ICAM-1 and VCAM-1. These data demonstrate that both LFA-1 and VLA-4 are capable of signaling, but the repertoire of downstream signaling events is distinct.

Because of the clear differences in LFA-1 and VLA-4 signaling, we next wanted to directly test whether any of the LFA-1-specific signaling events were important for upstream migration. In particular, we focused on cCbl and PI3K, both of which show defective activation in Crk-deficient T cells. To target these proteins, we implemented a CRISPR knockout (KO) system in primary mouse T cells (36). T cell blasts cultured from Cas9-expressing mice were transduced with retroviral vectors containing nontargeting (NT), cCbl, or PI3K δ gRNAs and selected in puromycin. Reductions in protein levels were confirmed by immunoblotting (Fig. 4.5A,B).



Figiure 4.5. Effect of c-Cbl and PI3K deletion in murine T cell upstream migration. c-Cbl knockout reverses upstream migration on ICAM-1. (A) CD4+ T cells from Cas9expressing mice were transduced with the indicated gRNAs, selected for 3 d in puromycin, and then lysed and immunoblotted for the indicated proteins. NT, nontargeting gRNA control. GAPDH was used as a loading control. (B) Quantification of immunoblots performed as in A. Data represent mean \pm s.d., normalized to the NT control, from three independent experiments. (C,D) T cells expressing the indicated gRNAs were allowed to migrate on ICAM-1-coated surfaces, fixed, and stained with

fluorescent phalloidin. (C) Representative images, displayed as in Fig. 4.2. Scale bar: 10 μ m. (D) Quantification of F-actin intensity per cell, pooled from three independent experiments (each circle represents one cell, and red lines indicate the mean). (E) Migration index of T cells expressing the indicated gRNAs migrating on ICAM-1 under shear flow (shear rate 800 s⁻¹). (F) Percentage of cells migrating upstream from experiments shown in panel E. (G) Distribution of cells with a given MI pooled from three independent experiments. E and F show mean ± s.e.m. for three independent experiments. Statistics were calculated using a one-way ANOVA with a Tukey multiple comparisons test. ns, not significant; *P<0.05; **P<0.01; ***P<0.001.

To assess the role of these proteins in LFA-1-mediated responses under static conditions, we allowed T cells to interact with ICAM-1-coated surfaces and stained for Factin. T cells expressing NT gRNA showed the canonical migratory phenotype, with a broad actin-rich leading edge (Fig. 4.5C). Deletion of cCbl had no effect on this phenotype, whereas loss of PI3K δ moderately reduced both cell spreading and actin polymerization (Fig. 4.5C,D). To test responses under shear flow conditions, we tracked cells migrating on ICAM-1 surfaces with a shear rate of 800 s⁻¹ and calculated MI to measure the upstream migration. Note that the procedures used for retroviral transduction and selection diminished the magnitude of upstream migration in all T cell populations, including the NT control. Nonetheless, T cells expressing either NT or PI3K\delta gRNAs remained capable of migrating upstream on ICAM-1, whereas T cells expressing the cCbl gRNA failed to migrate upstream (Fig. 4.5E). This pattern also held true when analyzing the percentage of cells migrating upstream (Fig. 4.5F) and the number of cells as a function of their MI (Fig. 4.5G). When compared to control NT gRNA-treated T cells, a large proportion of T cells lacking cCbl had an MI between 0.1 and 0.4. This was also seen in representative scattergrams (Fig. A12). The finding that T cells expressing the PI3K8 gRNA showed normal upstream motility was rather surprising, especially because these cells did exhibit diminished spreading and polarization under static conditions (Fig. 4.5C,D), and a modest decrease in track length under shear flow conditions (Fig. A12). We reasoned that this might be because deletion of PI3K δ was incomplete. Alternatively, other isoforms of PI3K could play a role. To address these possibilities, we repeated this analysis using T cells treated with the pan-PI3K inhibitor LY294002. LY294002 resulted in overall decrease in directionality but, as with PI3K δ deletion, treatment with

LY294002 had no effect on upstream migration (Fig. A13). This finding with PI3K inhibition is consistent with previous work in human T cells (14,16). Taken together, these studies identify cCbl, but not PI3K, as a necessary component of the LFA-1 signaling pathway that drives upstream migration.

Discussion

We and others have previously shown that T cells migrate upstream on ICAM-1 and downstream on VCAM-1 (11–17). In this study, we investigated the role of integrin signaling events in driving upstream migration. We found that engagement of LFA-1 by surface-bound ICAM-1 triggered robust activation of many signaling events as well as cytoskeletal remodeling and cell shape changes, whereas engagement of VLA-4 by VCAM-1 failed to trigger some, but not all, of these events. Importantly, upstream migration on ICAM-1 could be reversed by deleting key proteins associated with LFA-1 signaling. Taken together, our data uncover fundamental signaling differences between LFA-1 and VLA-4 that directly affect T cell migration under shear flow.

Our first indication that LFA-1 signaling drives T cell migration came from our previous studies of the Crk adaptor proteins. We found that T cells lacking Crk proteins have defects in LFA-1 signaling cascades leading to activation of the PI3K pathway and phosphorylation of the ubiquitin ligase scaffold protein cCbl. These defects are associated with impaired T cell spreading, actin polymerization and migration in response to surface-bound ICAM-1 under static conditions (24). Here, we show that Crk-deficient T cells fail to migrate against the direction of shear flow on ICAM-1, suggesting a role for signaling in upstream migration. Importantly, although WT T cells responding to ICAM-1 adopt the canonical, flattened morphology with a low-profile leading edge and a raised uropod (21–24,28), DKO T cells fail to spread; they sit high on the coverslip, even at the front. Thus, even when interacting with ICAM-1, DKO T cells look strikingly similar to WT T cells migrating on VCAM-1.

The phenotype of DKO T cells, which fail to flatten and migrate upstream, fits well with the passive steering mechanism proposed by Valignat and co-workers, who argue that the direction of migration under shear is dictated by the shear forces acting on cells of different shape (16,17). Central to this model is the observation that ICAM-1 induces a spread morphology, with the exception of the aforementioned raised uropod. Shear forces over the spread cell make the uropod act as a 'wind vane', aligning the cell to face against the direction of shear. On the other hand, cells migrating on VCAM-1 adhere mostly at the rear, resulting in a raised front. In this instance, shear forces strike the front of the cell, pushing it downstream. In its simplest form, the passive steering mechanism does not explicitly require integrin signaling. Indeed, a signal-independent mechanism was proposed based on the lack of Ca^{2+} influx in cells migrating on either ICAM-1 or VCAM-1 (17). However, although it is clear that Ca^{2+} is important for neutrophil migration (37-39), a requirement for Ca²⁺ signaling in integrin-dependent T cell migration is not well established. Additionally, the lack of Ca²⁺ signaling does not rule out a role for other signaling events. Numerous kinases and adaptor proteins are phosphorylated downstream of integrin engagement in T cells (40), and at least some of these events depend on Crk protein expression. Thus, we conclude that Crk proteins function as signaling intermediates in the pathway linking LFA-1 engagement and upstream motility. Importantly, signaling and passive steering are not mutually exclusive. Indeed, we believe that the two are inextricably linked. We postulate a two-step mechanism in which Crk-dependent signaling downstream of LFA-1 drives cell shape changes, thereby allowing the cell to undergo passive steering.

To further explore the role of signaling in T cell migration under shear flow, we compared signaling events downstream of LFA-1 versus VLA-4 engagement. We found that engagement of LFA-1 by surface-bound ICAM-1 triggers robust signaling, including the activation of the PI3K and ERK pathways, as well as the phosphorylation and activation of scaffold and signaling proteins such as cCbl, CasL, and Pyk2. In contrast, engagement of VLA-4 by VCAM-1 does not trigger substantial activation of PI3K or ERK, nor phosphorylation of cCbl. We were particularly interested in the LFA-1dependent activation of PI3K and cCbl, because these events are also blunted in Crkdeficient T cells (24). While knockout of PI3K8 or pharmacological inhibition of PI3K had modest effects on track length and displacement, neither manipulation affected the ability of T cells to migrate upstream, consistent with earlier studies (14,16). In contrast, knockout of cCbl did perturb upstream migration. Curiously, however, cCbl KO T cells looked morphologically indistinguishable from WT T cells on ICAM-1. This represents an interesting exception to the idea that signaling works by inducing cell shape changes that allow passive steering, and indicates that cCbl promotes upstream migration through a distinct mechanism.

How does cCbl control T cell migration under shear flow? We showed previously that cCbl interacts with the p85 subunit of PI3K after LFA-1 ligation (24), but this does not seem to contribute to upstream migration, because inhibition of PI3K activity had no effect. In addition to p85, cCbl is known to interact with dozens of different proteins in a cell-type-dependent manner (41). Thus, one or more other binding partners could affect T cell migration. In addition to its scaffold function, cCbl functions as a RING finger ubiquitin ligase. Indeed, most of the work studying cCbl in T cells has focused on the

ubiquitin ligase activity of the protein, and it is generally accepted that cCbl acts as a negative regulator of T cell receptor signaling (42–47). The role of cCbl-dependent ubiquitylation in T cell migration is largely unexplored. Since ubiquitylation can control protein degradation, trafficking or functioning, it is attractive to speculate that cCbl promotes upstream migration under shear flow conditions by ubiquitylating proteins needed for T cell migration. Future work aimed at determining the molecular mechanism of cCbl function will provide valuable new insights into T cell migration.

Taken together, our findings demonstrate a clear role for integrin- dependent signaling during T cell migration, and reveal that LFA-1 and VLA-4 deliver distinct signals that direct different migratory behaviors under both static and shear-flow conditions. Since many vessels express multiple integrin ligands, and ligand levels change with tissue type and inflammatory status, it will be important going forward to understand how T cells integrate integrin signaling pathways. Indeed, integrin crosstalk has already been shown to play a role during upstream migration (14,20). It will also be important to test how differential integrin signaling influences immune cell trafficking in vivo, especially during infiltration into sites of inflammation and solid tumors. A greater understanding of these events could guide the rational design of therapeutics to alter immune cell migration.

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CHAPTER 5: Aim 3: Effect of substrate stiffness on the upstream migration of CD4+ T lymphocytes

Abstract

To carry out their physiological responsibilities, CD4+ T lymphocytes interact with various tissues of different mechanical properties. Recent studies suggest that T cells migrate upstream (i.e. in the opposite direction of shear flow) on surfaces expressing ICAM-1 through interaction with LFA-1 integrins. LFA-1 likely interacts as a mechanosensor, and thus we hypothesized that substrate mechanics might affect the ability of LFA-1 to support upstream migration of T cells under flow. Here, we measured motility of activated primary human CD4+ T lymphocytes on polyacrylamide gels with pre-determined stiffnesses containing ICAM-1, VCAM-1, or 1:1 mixture of VCAM-1/ICAM-1. Under static conditions, we found that CD4+ T cells exhibit an increase in motility on ICAM-1 surfaces as a function of matrix stiffness. In contrast, motility on substrates of VCAM-1 or VCAM-1/ICAM-1 mixed surfaces was independent of elasticity. The mechanosensitivity of T cell motility on ICAM-1 surfaces is overcome when VLA-4 is ligated with soluble VCAM-1. Lastly, we observed that CD4+ T cells migrate upstream under flow on ICAM-1-functionalized hydrogels, independent of substrate stiffness. In summary, we show that CD4+ T cells under no flow respond to matrix stiffness through LFA-1, and that the crosstalk of VLA-4 and LFA-1 can compensate for deformable substrates. Interestingly, CD4+ T lymphocytes under flow migrated upstream on ICAM-1 substrates regardless of the substrate stiffness, suggesting that flow can compensate for substrate stiffness.

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Introduction

Leukocytes interact with various different tissues of different mechanical properties while circulating throughout the body and performing immune functions. The mechanical stiffness displayed across the physiological extracellular matrices (ECM) ranges from pascals to giga-pascals (1) in locations varying from lymph nodes to inflamed or diseased tissues such as a tumor microenvironment (2,3). Varying degrees of stiffness can alter the morphology of T cells and their activation or proliferation rates (4,5). T cell activation involving CD3 and CD28, non-integrin receptors mediating T cell activation, or the ligation of leukocyte function-associated antigen-1 (LFA-1) integrins, are often reported to be mechanosensitive processes, affecting proliferation and chemokine production (6–11).

Stiffness also affects integrin-mediated adhesion and migration in circulation (12,13). Stroka *et al* reported that neutrophils were the most motile on substrates with intermediary stiffness (4 kPa) and proposed a biphasic relationship between ligand concentrations, substrate stiffness and cell speed during neutrophil chemokinesis (14). Stroka and coworkers also observed stiffness-dependent increases in the fraction of neutrophils undergoing transmigration (13). They showed that the substrate stiffness affects cell-cell adhesion to the endothelial cell layer which is mediated by myosin light-chain kinase (MLCK) (13). The immunological synapse (IS), a well-studied cell-cell complex seen when a T cell contacts an antigen presenting cell (APC), requires an F-actin network whose assembly is mediated by LFA-1 and is known to generate mechanical forces (15). Rigidity of APCs (16) and endothelial cells (13,17)—cells with which T cells often interact—can also be altered in response to inflammation. Studies

have shown that endothelial cells exhibit stiffness-dependent morphology and ICAM-1 expression (18–20), which could directly alter T cell migration upon direct contact.

Leukocytes often are exposed to is hydrodynamic shear forces in the circulation. During the leukocyte adhesion cascade, rolling, firm adhesion and transendothelial cell migration of leukocytes are mediated by chemokines and cellular adhesion molecules expressed by endothelial cells (17). Shear flow affects T cells indirectly, by stimulating mechanical or chemical changes in endothelial cell, or in the T cells themselves. Shear flow influences endothelial cell alignment which is critical in blood vessel formation (21– 23). Schaefer and Hordijk also reported that T cells find the hotspots in the endothelial layer to transmigrate; these hotspots are suspected to be places where the endothelian exhibits a lower local stiffness between cell junctions to guide transmigration of T cells. In contrast, higher local stiffnesses have been implicated in facilitating leukocyte capture and rolling (24).

T lymphocytes also are affected by shear flow in a variety of ways. Recently, it has been shown that T lymphocytes migrate in the opposite direction of flow on surfaces presenting ICAM-1 (25–27). The upstream migration of T lymphocytes is driven by biophysical and intracellular biochemical factors (28–30). Valignat and co-workers first reported the upstream migration of T cells *in vitro*, and proposed a biophysical mechanism in which how T cells use their uropods to distinguish the direction of flow and determine their migration and polarity. Upstream migration under flow has been reported in not only T lymphocytes, but also in marginal zone B cells, hematopoietic stem cells (HSPCs), and neutrophils with blocked Mac-1 (31–33). The ability of T cells to migrate upstream has also been observed *in vivo* (34). Our group also observed that T

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cells migrating upstream on HUVEC monolayers seem better able to transmigrate, suggesting a physiological role for upstream migration (35). *In vivo* observations of T lymphocytes migrating upstream (34) also suggest that T cells are able to translate physical and biochemical cues into directional migration. However, there has been no systematic study of the role of substrate mechanical properties in the upstream migration of T cells. Here, we use polyacrylamide hydrogels with finely tuned stiffness (36,37) to examine whether LFA-1 or VLA-4 mediated migration of human primary CD4+ T lymphocytes is mechanosensitive. These studies are done in the absence of TCR signaling, in which mechanosensitive responses have been clearly identified (4,6,8,38–41). We also address whether the substrate stiffness affect the upstream migration of CD4+ T lymphocytes under flow.

Materials and Methods

Cell culture and Reagents

Human Primary CD4+ T lymphocytes were acquired from Human Immunology Core at University of Pennsylvania (P30-CA016520). Cells were activated with phytohaemagglutinin (PHA, MP Biomedicals, Santa Ana, CA) in RPMI-1640 supplemented with 10% FBS. Cells were further cultured in RPMI-1640 supplemented 10% FBS, IL-2 (Corning, Corning, NY) and penicillin and streptomycin (Gibco, Gaithersburg, MD). During experiments, cells were kept in RPMI-1640 with D-glucose and BSA. CD4+ T cells were given 30 minutes to adhere to surfaces prior to imaging. To activate or block integrins, cells were incubated with the following for 15 minutes prior to adhesion: soluble VCAM-1 (R&D Systems, Minneapolis, MN), isotype control (MOPC-21), anti- α_L (HI111), anti- α_4 (9F10) or anti- β_1 (P5D2) (Biolegend, San Diego, CA).

Substrate Preparation

Aqueous solutions of acrylamide and bis-acrylamide (Bio-Rad, Hercules, CA) were prepared as explained previously (36,37,42,43). Briefly, gels with elastic moduli of 1.2, 7.9, 10 and 60 kPa, all containing N-6-((acryloyl)amino)hexanoic acid crosslinker (N6) and 0.1 % w/v TEMED (Bio-Rad), were polymerized at a final concentration of 0.1 % w/v ammonium persulfate (Bio-Rad). Upon initiating polymerization, gel solutions were carefully pipetted onto silanized glass slides. Rain-X coated coverglasses were placed on top to create flat surfaces. Flattened gels were allowed to polymerize for 30-45 minutes under nitrogen. After polymerization, gels were detached from the top coverslips under water, followed by rinsing and functionalization.

Gel functionalization

To functionalize gels, N6 crosslinker was added into all gel solutions prior to polymerization. After washing gels with water, gels were conjugated with 2 μ g/ml protein A/G (Biovision, Milpitas, CA) for 2 hours at room temperature. Then, gels were rinsed with 1/100 parts ethanolamine in 50mM HEPES to block unreacted N6, followed by addition of 10 μ g/ml ICAM-1 and/or VCAM-1 Fc Chimeras (R&D Systems).

Cell Tracking and Data Analysis

To measure cell area and aspect ratio, sample images of CD4+ T lymphocytes on ICAM-1 functionalized gels were acquired using a Nikon TE300 microscope equipped with a Nikon 20x LWD, numerical aperture 0.4 objective. Cell area was measured using Fiji, and aspect ratios were calculated with ellipses based on cell periphery from ImageJ (<u>https://imagej.nih.gov/ij/</u>, NIH, Bethesda, MD). Here, aspect ratio is reported as the ratio of the minor axis to the major axis of the cell ellipse.

For static and flow conditions, images were acquired every minute for 30 minutes under static and 20 minutes under flow using a Nikon TE300 with a Nikon 10x, numerical aperture 0.25, objective. During the course of experiments, cells were kept in 37°C and 5% CO₂. Images were analyzed using Manual Tracking (https://imagej.nih.gov/ij/plugins/track/track.html) in ImageJ (NIH) and MATLAB (The MathWorks, Natick, MA). Centroids of cells present throughout the entire duration of an experiment were tracked using ImageJ plug-in Manual Tracking. Tracks were further analyzed with a custom MATLAB script to calculate speed, random motility coefficient (μ), and persistence time (Pt) by calculating Mean Squared Displacement (MSD) of each cell fitted to Dunn Equation (44). Migration Index (MI) is defined as the ratio of the cell's axial displacement over the total length of the cell trajectory, where negative MI represents upstream migration, and positive MI represents downstream migration. Data presented as Mean \pm S.E.M. Statistics were prepared with a t-test or a one-way ANOVA using multiple comparisons with a Tukey correction. *p < 0.05; **p < 0.01; ***p < 0.001, **** p < 0.0001, NS: not significant.

Results

CD4+ T lymphocytes on ICAM-1 surface are sensitive to stiffness under static conditions

In this study, we were particularly interested in whether the matrix stiffness affects the motility of CD4+ T lymphocytes. We hypothesized that since LFA-1 is often coupled with CD3 during stiffness-dependent activation, LFA-1 could be sensitive to stiffness on its own. We plated PHA-activated CD4+ T lymphocytes onto polyacrylamide hydrogels with different elastic moduli (E) ranging from 1.25 kPa to 60 kPa, with three different functionalized surfaces: ICAM-1, VCAM-1 or 1:1 mixture of VCAM-1 to ICAM-1 (VCAM-1/ICAM-1 surfaces). Figure 5.1 presents representative images of individual cells, tracks and scattergrams of cells migrating on gels at the indicated elastic moduli functionalized with ICAM-1. Under static conditions, CD4+ T cells on ICAM-1 functionalized gels all adhered and polarized, but cells on softer hydrogels were less spreaded than cells on stiffer gels (Fig. 5.1D,E).



Figure 5.1. CD4+ T lymphocytes on ICAM-1 functionalized gels. Images of cells (A), overlayed cell trajectories (B), and scattergrams of analyzed cell trajectories from a representative experiment of CD4+ T cells on ICAM-1 functionalized gels at indicated substrate stiffness. Red and blue tracks indicate cells migrating left and right, respectively, to the field of view. (D) Cell area and (E) aspect ratio of cells on ICAM-1 gels of indicated moduli. Scale bar = $25 \mu m$ (A) and $100 \mu m$ (B).**: p < 0.01, ***: p < 0.001, ****: p < 0.0001.



Figure 5.2. Stiffness-dependent motility of CD4+ T lymphocytes on ICAM-1. CD4+ T lymphocytes show stiffness-dependent motility on ICAM-1, but not on VCAM-1/ICAM-1 mixed or VCAM-1 gels. Random motility coefficient (μ) and speed of cells on ICAM-1 (A,B), VCAM-1/ICAM-1 mixed surfaces (C,D), and VCAM-1 (E,F). NS: Not significant, *: p < 0.05, **: p < 0.01.

On ICAM-1, CD4+ T lymphocytes exhibited increased motility as the stiffness increased (Fig. 5.2A). CD4+ T cells on ICAM-1 became more motile (had a higher random motility coefficient, μ) as the stiffness increased. Speed (Fig. 5.2B), but not persistence time (Fig. A14), increased with increasing stiffness. On VCAM-1 or VCAM-1/ICAM-1 mixed surfaces, cells at all ranges of stiffness exhibited relatively similar motility coefficients and speeds (Fig 5.2C-F), suggesting that when VCAM-1 is present, cells exhibited stiffness-independent random motility. Thus, we conclude that, under static conditions, CD4+ T lymphocytes show stiffness-dependent motility on ICAM-1 and become more motile on stiffer matrices. However, CD4+ T cells no longer display mechanosensitive motility when substrates contain VCAM-1. The results suggest that while LFA-1 is mechanosensitive, VLA-4 is not.

Crosstalk of VLA-4 and LFA-1 makes CD4+ T lymphocytes motile, but independent of stiffness

After observing that migration of CD4+ T lymphocytes on VCAM-1/ICAM-1 hydrogels independent of matrix stiffness, we next hypothesized that the crosstalk of VLA-4 and LFA-1 increases the motility on softer substrates, but negates the stiffness sensitivity of CD4+ T lymphocytes. Published studies have noted that an activation state of one integrin can affect the adhesion of another (45–48), possibly suggesting the role of crosstalk affecting the affinity or downstream signaling. My previous aim also reports that the crosstalk of LFA-1 and VLA-4 results in the persistent upstream migration post flow (28). To test whether or not crosstalk between the two integrins influences substratedependent motility, we modulated the integrin activities with soluble factors.



Figure 5.3. Crosstalk of VLA-4 and LFA-1 in stiffness-dependent migration. Crosstalk of VLA-4 and LFA-1 make CD4+ T lymphocytes more motile, but reduces sensitivity to stiffness. (A-C) Random motility coefficient (μ), Speed, and Persistence time (P_t), respectively, of CD4+ T lymphocytes on ICAM-1 with or without soluble VCAM-1 at indicated matrix stiffness. (D-F) Random motility coefficient (μ), Speed, and Persistence time (P_t), respectively, of CD4+ T lymphocytes on VCAM-1/ICAM-1 with or without

antibodies blocking indicated subunits on soft (1.25 kPa) and stiff (10 kPa) hydrogels.). , *: p < 0.05, **: p < 0.01, ***: p < 0.001. First, we activated VLA-4 integrins on CD4+ T lymphocytes with soluble VCAM-1 (sVCAM-1), to recreate the crosstalk between LFA-1 and VLA-4 on cells migrating on hydrogels functionalized with ICAM-1. On ICAM-1, cells stimulated by sVCAM-1 showed an increase in motility on softer gels and thus a similar random motility coefficient on all ranges of stiffness. This result indicates cells ligated by sVCAM-1 are no longer sensitive to stiffness on ICAM-1 surfaces (Fig 5.3A-C). Activating VLA-4 with sVCAM-1 similarly resembles our previous result, where motility of cells on VCAM-1/ICAM-1 mixed surface did not depend on stiffness. This confirms that when VLA-4 and LFA-1 are simultaneously ligated, CD4+ T lymphocytes are no longer sensitive to stiffness. While LFA-1 requires an immobilized ICAM-1, VLA-4 can be ligated with substrate-bound or soluble VCAM-1 to activate VLA-4 and generate the crosstalk.

Next, we inhibited integrins binding to immobilized cognate ligands to further confirm that the crosstalk of VLA-4 and LFA-1 affects the mechanosensitivity of CD4+ T lymphocytes. We blocked subunits of VLA-4 or LFA-1 of cells on VCAM-1/ICAM-1 hydrogels to observe motility by a single integrin-ligand pair. When subunits of VLA-4 integrins were blocked, the motility coefficient and speed of cells on softer gels dropped significantly (Fig. 5.3D-F), returning the motility of CD4+ T lymphocytes to their observed motility on soft substrates coated with ICAM-1. The random motility coefficient and speed were not affected by LFA-1 blocking antibodies (Fig 5.3D-F). Blocking LFA-1 forces CD4+ T lymphocytes to rely on VLA-4, returning the motility to that seen on VCAM-1 surfaces, indicating VLA-4 alone do not distinguish different stiffness. Together, these results confirm that the crosstalk between LFA-1 and VLA-4 renders CD4+ T lymphocytes independent of stiffness. This further emphasizes that LFA-1, but not VLA-4, is mechanosensitive.

CD4+ T lymphocytes migrate upstream under flow on hydrogels

Emerging studies indicate that T lymphocytes are able to sense the hydrodynamic flow and migrate in the opposite direction of flow (25–30). This led us to investigate whether or not the mechanical properties of the substrate would also affect the direction of migration under flow. CD4+ T cells were allowed to adhere on ICAM-1 functionalized hydrogels with different ranges of matrix stiffness and exposed to flow at a shear rate of 800s⁻¹. Migration indices (MI) were calculated from cell trajectories to quantify the upstream migration; negative values of the migration index indicate upstream migration.



Figure 5.4. Upstream migration of CD4+ T cells on substrates with different stiffness. CD4+ T lymphocytes robustly migrate upstream on polyacrylamide gels of different stiffness. Migration Index at 30 minutes (A) and over time (B), percent of cells migrating upstream (C), speed (D), and persistence time (E) are reported to show that CD4+ T cells migrate upstream under flow (shear rate = $800s^{-1}$) on substrates of varying elastic moduli. NS: Not significant, **: p < 0.01, ****: p < 0.0001.

At a shear rate of 800s⁻¹, the MIs ranged from -0.33 to -0.52 across different substrates (Fig. 5.4A,B). We further confirmed that more than 75% of CD4+ T cells on all values of matrix stiffness tested migrate upstream (Fig. 5.4C). This result illustrates that, regardless of the stiffness, the direction of shear-dependent migration is unaffected. We note an increase the magnitude of MIs with cells on stiffer matrices. This observation is mostly from the increase in speed, not the persistence time, as the stiffness increased (Fig. 5.4E,F). Together, we conclude that the matrix stiffness does not affect the direction and the persistence of the upstream migration of CD4+ T cells under flow. On substrates with higher elastic modulus, CD4+ T lymphocytes migrate upstream more robustly and faster than cells on lower elastic modulus.

CD4+ T lymphocytes show shear-dependent upstream migration on hydrogels

 β_2 integrin mediated binding to ICAM-1 is a catch-slip bond; increased applied force to the bond will counterintuitively decrease the off rate, resulting in a longer bond lifetime. Our group and my previous aim reported that T cells on ICAM-1-coated PDMS surfaces showed an increasing directional preference in the upstream direction as shear rates increased (25,28).



Figure 5.5. Shear-dependent migration of CD4+ T lymphocytes on compliant substrates. CD4+ T lymphocytes exhibit shear rate-dependent upstream migration on both soft (1.25 kPa) and stiff (10 kPa) ICAM-1 gels. Migration Index at 30 minutes (A), percent of cells migrating upstream (B), speed (C) and persistence time (D) of cells migrating under flow with varying shear rates on soft (1.25 kPa) and stiff (10 kPa) substrates. NS: Not significant, *: p < 0.05, **: p < 0.01, ***: p < 0.001, ***: p < 0.0001.

Similar to their findings, CD4+ T cells on ICAM-1 coated hydrogels at different elastic moduli also exhibited shear rate-dependent upstream migration on both soft (E = 1.25 kPa) and stiff (E = 10 kPa) hydrogels (Fig. 5.5A,B). On both soft and stiff gels, cells exhibited more robust upstream migration as shear rate increased. While the stiffness did not affect the direction of migration in response to shear flow, cells showed an increase in persistence time as a function of shear rate, which contributed to a strong upstream migration behavior. Shear rate-dependent increase in persistence time was more apparent in cells on stiff hydrogels (Fig. 5.5D). The slight increase in persistence time with cells on soft gels as a function of shear rate was not statistically significant (Fig. 5.5C,D). This illustrates that, as shear rate increased, cells migrate more persistently as a function of shear rate on stiff materials, resulting in more persistent and robust upstream migration under flow.

Crosstalk of VLA-4 and LFA-1 do not affect the upstream migration of CD4+ T lymphocytes on hydrogels with different stiffness

Under static conditions, we observed that ligating VLA-4 with soluble VCAM-1 increased the motility of CD4+ T lymphocytes on hydrogels functionalized with ICAM-1 (Fig. 5.2A-C). Here, we investigated whether ligating VLA-4 and initiating the crosstalk of LFA-1 and VLA-4 also affect the motility and the upstream migration of CD4+ T cells under flow. CD4+ T cells on hydrogels with varying elasticity migrated in the opposite direction of flow on both soft (1.25 kPa) and stiff (10 kPa) hydrogels consistent with previous findings (Fig. 5.6A).



Figure 5.6. Crosstalk of VLA-4 and LFA-1 in cells migrating under shear flow on hydrogels. Crosstalk of VLA-4 and LFA-1 has no effect in the upstream migration on substrates with varying stiffness. Migration Index at 30 minutes (A) and over time (B), percent of cells migrating upstream (C), speed (D), and persistence time (E) of cells migrating under flow (shear rate = 800s-1) on ICAM-1 gels at indicated stiffness. NS: Not significant, *: p < 0.05, **: p < 0.01, ***: p < 0.001, ****: p < 0.0001.

Activating VLA-4 with soluble VCAM-1 did not affect the direction of migration (Fig. 5.6A-C). Cells on stiffer gels exhibited higher speed and had a greater number of cells migrating upstream, but the persistence time of cells migrating upstream was not affected (Fig. 5.6D,E). Together, we show that the crosstalk of LFA-1 and VLA-4 does not affect the upstream migration under shear flow.

Discussion

Mechanical cues from the microenvironment impact various cellular processes. Mechanotransduction and downstream signaling events involving actin rearrangement in T lymphocytes are critical and highly implicated in T cell development, such as activation and proliferation (5,10,39). Perceiving and responding to mechanical properties of the extracellular matrix is guided by integrins, specifically LFA-1. However, the role of mechanical cues on migration under shear stress in the blood stream has not been fully explored. In particular, shear-dependent upstream migration of T cells is a direct result of LFA-1 activation, followed by downstream signals such as Crk and c-Cbl (25,26,28,30). Studies from Theodoly group suggest that the detection of direction of flow and direction of migration is mediated by the uropod structure of the cell, acting as a wind vane (29). While more studies have uncovered possible mechanisms mediating the upstream migration (28,30), in vitro platforms do not accurately recreate the in vivo microenvironment cells experience. In this study, we used polyacrylamide gels (36,37) to create substrates of different compliances and measure integrin-dependent motility of CD4+ T lymphocytes in response to shear flow.

Previous work has reported that matrix stiffness significantly affects integrinmediated cell spreading, migration and force generation (49–52). Similar to previously published reports, stiffness of the hydrogels affected T cell spreading and motility (Fig. 5.1). Furthermore, CD4+ T lymphocytes exhibited mechanosensitive migration on hydrogels functionalized with ICAM-1 (Fig. 5.2). However, cells became more motile, but stiffness-independent when gels contained VCAM-1 (Fig. 5.2). We then showed that the simultaneous engagement of LFA-1 and VLA-4 made cells more motile, but

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insensitive to matrix stiffness. We ligated VLA-4 using soluble VCAM-1 to cells migrating on gels containing ICAM-1, and blocked VLA-4—VCAM-1 binding using blocking antibodies to cells on gels with both VCAM-1 and ICAM-1 (Fig. 5.3). While results under static conditions suggest LFA-1 is mechanosensitive, LFA-1 dependent upstream migration under flow was unaffected by the elastic moduli of substrates. CD4+ T cells consistently migrated in the opposite direction of flow on hydrogels of all ranges of stiffness as long as gels were functionalized with ICAM-1 (Fig. 5.4-6). A slight increase in speed as a function of matrix stiffness (Fig. 5.4) was observed. However, the persistence of the upstream migration of CD4+ T cells is dependent on the shear rate, not the matrix stiffness (Fig. 5.5) nor soluble ligation of VLA-4 (Fig. 5.6).

In summary, we have demonstrated that, while LFA-1-dependent motility is mechanosensitive under static conditions, LFA-1-mediated upstream migration of CD4+ T cells is dependent on shear rate, not the elastic moduli of substrates. Parallel to previous studies (25,26,28,30,34,35), we show CD4+ T lymphocyte upstream migration is robust. Unlike other immune cell types (36,42,53,54), T cells do not exert as much traction force (55–57). Using an intracellular tension sensor, Nordenfelt *et al* have reported an average force per LFA-1 on Jurkat T cells on ICAM-1 to be ~1 pN (57). Despite such small reported traction forces, CD4+ T lymphocytes migrate in the opposite direction of flow even on compliant substrates.

How LFA-1 supports the upstream migration and the physiological importance of the upstream migration is still being investigated. Studies investigating T cell function and activation in response to matrix stiffness for immunotherapy applications has been emerging (7,10), suggesting an exciting area of development for improvement in immunotherapy or more optimized production of engineered immune cells for better infiltration. Interesting future work can include establishing a more stable system to study the upstream migration in various substrates with different geometry, depicting different microenvironments lymphocytes contact, such as tumor microenvironment or other cellular matrices in response to different inflammatory response or autoimmune diseases.

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CHAPTER 6: Conclusions and future work

Specific aims

The research presented in the thesis provides more insight in intracellular mechanism to the upstream migration of CD4+ T lymphocytes under shear flow. Three specific aims discussed are below:

Aim 1: Show that CD4+ T lymphocytes remember shear flow under co-stimulation of VLA-4 and LFA-1 and contribute to persistent upstream migration during and/or post flow.

Aim 2: Determine the mechanism of the upstream migration of CD4+ T lymphocytes and downstream signaling of LFA-1.

Aim 3: Determine whether substrates with varying degrees of stiffness affect migratory behavior of CD4+ T lymphocytes.

Specific conclusions

Aim 1: Migrational memory of CD4+ T lymphocytes

In this aim, I investigated how CD4+ T lymphocytes are able to continue migrate in the same upstream direction after the flow is turned off (referred to as 'migrational memory'). While the upstream migration under flow is directed by LFA-1-ICAM-1 interaction, I reported that in order for CD4+ T lymphocytes required specific conditions in order to show persistent upstream migration post flow. I first observed that CD4+ T cells remember shear flow and maintain its upstream direction of motion when migrating on 1:1 mixture of VCAM-1/ICAM-1 surfaces under flow at 800s⁻¹ shear rate. I further confirmed that having both VLA-4 and LFA-1 ligated simultaneously was the key to the migrational memory of CD4+ T cells. The crosstalk was mimicked with soluble VCAM-1, ligating VLA-4, on cells migrating on ICAM-1 surfaces and blocked with VLA-4 blocking antibodies on cells migrating on VCAM-1/ICAM-1 mixed surfaces. Using small molecule inhibitors, I showed that while the upstream migration under flow is robust, the migrational memory of CD4+ T lymphocytes require flow at a high shear rate (800s⁻¹) and the crosstalk of LFA-1 and VLA-4 that is mediated by PI3K.

While LFA-1 alone is sufficient to mediate upstream migration, VLA-4 and LFA-1 together is required for CD4+ T cells to remember and maintain the same direction of motion cells established under flow. Migrational memory may elucidate how CD4+ T lymphocytes migrate at situations where cells need to migrate through environments where flow profile changes abruptly after migrating in blood vessel. For instance, CD4+ T cells transmigrate through the endothelium to arrive to sites of inflammation. Migrational memory, or persistent directional migration post flow, may be essential for cells to reach to the inflammatory site after passing the endothelium and no longer experience blood flow. While PI3K is not involved in the upstream migration per se, I show in this aim that cells with PI3K inhibitors no longer remember shear flow and migrational memory is lost. More future investigation is needed to completely elucidate the application of migrational memory. Specifically, it is important to ask how T cells that were migrating upstream under flow migrate after passing through endothelium and into the extracellular matrix.

Aim 2: Signaling mechanism of the upstream migration with mouse models

I collaborated with Dr. Roy from the Burkhardt lab to answer the intracellular mechanism of the upstream migration. Knocking out the genes encoding all isoforms of CrkI/II and CrkL proteins caused defects in upstream migration; DKO murine T cells were unable to migrate upstream on ICAM-1 coated surfaces. Downstream of LFA-1 and Crk, we focused on PI3K and c-Cbl which were specifically activated downstream of LFA-1, not VLA-4. Under static conditions, levels of cell spreading and F-actin assembly were significantly decreased with CRISPR KO PI3K T cells, while CRISPR KO c-Cbl T cells were comparable to the control group. Interestingly, under flow conditions, CRISPR KO PI3K T cells were able to migrate upstream, but CRISPR KO c-Cbl were unable to migrate upstream on ICAM-1 surfaces. Here I find that, downstream of LFA-1, Crk and c-Cbl play critical roles in determining the directionality of CD4+ T cells under flow.

Consistent with the previous aim, perturbing PI3K activity via CRISPR KO and PI3K inhibitor, LY294002, had no effect in upstream migration murine T cells, whereas knocking out Crk and c-Cbl reversed the direction of migration under flow. This is the first report that shows Crk and c-Cbl, directly downstream of LFA-1, mediates sheardependent migration response. While both c-Cbl and PI3K both become active downstream of Crk, In addition, c-Cbl and PI3K may not share the same signaling pathway. Only c-Cbl is essential in driving upstream migration in response to shear. PI3K inhibition did not affect the upstream migration, consistent with the first aim. This aim suggests that c-Cbl responds to shear and biochemically trigger cells to migrate in the opposite direction of flow. Further experiments investigating the distinctive pathway of c-Cbl and PI3K in shear flow-dependent migration would help elucidate the intracellular

pathway of the upstream migration. Incorporating Valignat and co-workers' proposed model of the upstream migration with the uropod as the wind vane of the cell, Crk and c-Cbl may further establish the polarity and reorganize cytoskeleton to propel cells in the opposite direction, whereas PI3K may be involved in persistent migration in response to shear flow.

Aim 3: Effect of substrate stiffness on the upstream migration of CD4+ T lymphocytes

Aim 3 investigates whether CD4+ T lymphocytes can distinguish different compliant substrates and whether the upstream migration can be manipulated with substrate mechanics. Under static conditions, I confirmed that cells are smaller and less motile on softer hydrogels functionalized with ICAM-1. However, the stiffnessdependent motility disappeared when VCAM-1 was present. While LFA-1 showed mechanosensitive results under static conditions, the upstream migration under flow, which is also LFA-1-mediated, is unaffected by the stiffness. CD4+ T cells showed an increase in directionality (i.e. more robust upstream migration) as shear rates increased. Cells also showed more robust upstream migration on 60 kPa than 1.25 kPa hydrogels, but the directionality was unaffected, confirmed by both Migration Indices and percentage of cells migrating upstream above 75%. This observation captures upstream migration of T cells at a microenvironment which resembles *in vivo* mechanical properties more reliably than 2D parallel flow chamber.

In this aim, I report that cells can sense mechanosensitivity from the matrix and shear. Mechanosensitive motility response under static and shear-dependent upstream migration are both LFA-1 driven. However, when both physical stimuli are presented to

cells, shear-dependent upstream direction of migration does not seem to be affected by matrix stiffness. This suggests that the type of stimuli can trigger different signaling pathway that will lead to directional migration. Further studying how cells decide to commit to c-Cbl or PI3K dependent migratory response and how cells prioritize different stimuli, not limited to high shear and/or soft matrix, could highlight the physiological application of upstream migration *in vivo*.

Future work

Traction force measurement during the upstream migration

Aim 3 uncovered that CD4+ T lymphocytes migrated in the opposite direction of flow on ICAM-1-functionalized polyacrylamide hydrogels at different elastic moduli. In order for a cell to migrate upstream, the cell has to withstand the shear force in order to stay adherent to the surface and generate enough force to propel itself forward in the opposite direction of shear. I hypothesize that this requires a great amount of force that has not been yet reported.

The Hammer lab has reported traction forces of various immune cells, such as neutrophils and macrophages, by calculating the displacement of beads or posts embedded in compliant substrates. Henry *et al* used microfabricated post-array detectors (mPADs) and reported that neutrophils generate 75 pN of protrusive force during spreading (1). Smith *et al* showed that neutrophils undergoing chemokinesis concentrate most of their traction forces at the uropod, ranging about 25 nN (2). Hind *et al* showed that macrophages show stiffness-dependent traction force, ranging from 1000-3000 nN. They found that macrophages show a frontal towing mechanism of motility in which cells extend a pseudopod and attach to the substrate, generating cellular contraction through their actomyosin cytoskeletons, which exerts tension on the substrate, and then release the uropod (3).

Unlike previously studied myeloid cells, T lymphocytes do not exert as much force as previously mentioned cell types. T cells that have been co-activated with anti-CD3 and anti-CD28 generate about 70pN on mPADs (4). However, Li and co-workers' AFM result of LFA-1-ICAM-1 binding and Nordenfelt and co-workers' force

measurement using β_2 -integrin tension sensors report much lower force that is undetectable by conventional traction force measurements with beads (5,6). To measure the traction force of T lymphocytes migrating upstream, one can utilize other measurement methods, such as DNA- or protein- based tension sensors, that can detect lower orders of magnitude of traction forces.

Effect of T cell activation in migration

Often in the literature, T cell activation and migration are studied separately. T cells are often already activated to become more migratory in culture prior to experiments. When studying activation, studies often report genetic and/or epigenetic changes, or cytokine release as a response of activation and proliferation. When activating naïve T cells cause genetic manipulations and lead to further differentiation of T cells, the activation process affect receptors for motility and chemoattractants as well.

While optimizing experimental settings for Aim 2, we noticed that the culture conditions under which CRISPR KO cells are activated and maintained influences the direction of migration in response to shear flow. Specifically, the addition of IL-2 affected the directional migration response (Table 6.1). For the purpose of the project, we had to optimize culturing conditions for CRISPR KO T cells after harvesting from mice. For shear flow experiments with CRISPR KO T cells described in Aim 2, cells were activated with anti-CD3 and anti-CD28, each at 1 μ g/mL, for 48 hours, followed by viral transduction and subsequent activation with 20 units/mL of IL-2 (7).

	Culture conditions				
Trials	Ligands	Activation	Transduction	Selection & Proliferation	Direction of Migration
Roy <i>et al</i> 2018 (7)	Anti-CD3 (µg/mL)	1	_*	-	<i>→</i>
	Anti-CD28 (µg/mL)	1	_*	-	
	IL-2 (IU/mL)	-	_*	20	
Huang <i>et al</i> 2018 (8)	Anti-CD3 (µg/mL)	8	-	-	+
	Anti-CD28 (µg/mL)	8	-	-	
	IL-2 (IU/mL)	-	10	10	
9-24-29	Anti-CD3 (µg/mL)	5	-	-	÷
	Anti-CD28 (µg/mL)	2	-	-	
	IL-2 (IU/mL)	-	10	10	
10-8-19	Anti-CD3 (µg/mL)	1	-	-	→
	Anti-CD28 (µg/mL)	1	-	-	
	IL-2 (IU/mL)	-	10	10	
10-8-19 -NT	Anti-CD3 (µg/mL)	8	-	-	÷
	Anti-CD28 (µg/mL)	8	-	-	
	IL-2 (IU/mL)	-	10	10	
11-7-19 Normal	Anti-CD3 (µg/mL)	1	-	-	<i>→</i>
	Anti-CD28 (µg/mL)	1	-	-	
	IL-2 (IU/mL)	-	-	10	
11-7-19 A	Anti-CD3 (µg/mL)	1	-	-	÷
	Anti-CD28 (ug/mL)	1	-	-	
	IL-2 (IU/mL)	10	10	10	
11-7-19 B	Anti-CD3 (µg/mL)	5	-	-	<i>→</i>
	Anti-CD28 (µg/mL)	2	-	-	
	IL-2 $(\overline{IU/mL})$	-	-	10	

Table 6. 1. Culture conditions and the direction of migration of c-Cbl CRISPR KO murine T cells.

*: Transduction step in Roy *et al* 2018 was not present, since DKO cells are harvested from mice that have been generated by crossing CD4+ Cre mice mice in which the two Crk loci have been floxed (Crk^{fl/fl}:CrkL^{fl/fl}). After activation, primary murine T cells were activated with IL-2 as indicated for 5-6 days prior to shear flow experiments. +: This condition is same as 10-8-19 NT. Table 6.1 summarizes various combinations of culture conditions for CRISPR KO murine CD4+ T lymphocytes upon harvest and reports the direction of migration under flow of DKO Crk or c-Cbl CRISPR KO on ICAM-1 surfaces. Depending on the addition of IL-2 during transduction, spinoculation and proliferation steps of culture, the migration result varied. While WT groups all migrated upstream on ICAM-1 consistently regardless of the culture conditions, Dr. Roy and I noticed a diverging response with DKO Crk and c-Cbl CRISPR KO T cells. Dr. Roy confirmed with immunoblots that in all conditions shRNA knockdown and CRISPR/cas9 successfully knocked down or knocked out, respectively, the protein of interest (data not shown).

The protocol Huang and co-workers developed for CRISPR/cas9-mediated mutagenesis includes activation of T cells at 8 µg/mL of anti-CD3 and anti-CD28 each, followed by spinoculation with IL-2 during viral transduction. In contrast, NHR's method of culture published in 2018, which was used with cells without CRISPR/cas9 system, requires much lower concentration of anti-CD3 and anti-CD28, each at 1 µg/mL. Differences between NHR's and Huang *et al*'s culture conditions for CRISPR/cas9 mutagenesis were the amount of activation signals with anti-CD3 and anti-CD28 and the introduction of IL-2. Through the last iteration, Dr. Roy and I observed that when IL-2 was introduced during the viral transduction, T cells without c-Cbl could still migrate upstream on ICAM-1 surfaces. While we report that Crk and c-Cbl downstream of LFA-1 are critical in the upstream migration of T cells under flow, the effect of c-Cbl deletion can be rescued by manipulating the amount and/or the exposure of IL-2 during activation. This implies that the activation of T cells can also affect the motility of T cells under flow. This process implies that how T Cells are activated in early stage development could also affect the upstream migration. Since activation conditions are also key for T cell development and immune function, understanding the relationship between activation and migration can provide better understanding of the upstream migration of T cells.

Consequence of the upstream migration

The work in this dissertation along with several other studies from the Hammer lab, upstream migration is an LFA-1 dependent migratory response to shear flow. However, as the phenomenon is a fairly newly-discovered with still little understanding, uncovering the physiological importance of this phenomenon is essential. Results from Anderson *et al* may provide an interesting insight to the purpose of the upstream migration. Anderson *et al* observed that T cells that were migrating upstream on a layer of activated HUVECs also tend to transmigrate underneath the endothelial layer sooner than cells that do not migrate upstream (9). The authors were also able to show that by blocking LFA-1, they reduced the number of cells migrating upstream and increased the time cells took to burrow underneath the endothelial layer. This not only provides the possible application of the upstream migration, but also alludes to a possibility a selective, functional advantage. Unlike *in vitro* surfaces in shear flow experiments, inflamed endothelium most likely will have uneven concentrations and geometrical presentation of ligands. In that case, T cells may exhibit heterogeneous responses in response to shear flow. Hammer and Burkhardt labs already observe differences in morphology and migration under static and flow conditions on surfaces containing ICAM-1 vs. VCAM-1 (10–12). T cells on VCAM-1 showed enhanced attachment by the

rear, whereas cells on ICAM-1 adhered strongly by the front (11). Hornung *et al* noticed such bimodal responses even on ICAM-1/VCAM-1 mixed surface at different ratios, suggesting that the local pattern of ligands will affect the mode and the direction of migration in response to flow. Together, literatures suggest a heterogeneous division of mode of migration which will affect T cell transmigration. To answer why cells migrate upstream, investigating what happens to cells that have migrated upstream behave after transmigration would be important. For instance, I suspect that the upstream migration is advantageous for cells that have arrested downstream of ideal target site of transmigration. If so, what would happen to those cells that have migrated upstream and successfully transmigrated? Do they retain the persistence and remember the upstream migration, similar to what I had observed in Aim 1? Could the upstream migration lead to more persistent and faster 3D-migration for cells to reach the site of inflammation? What about in the lymph nodes, or early stages of development? Once subsequent events of upstream migration are discovered, then one could answer the physiological importance of the upstream migration and engineer cells to migrate upstream more robustly for enhanced immune performance.

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APPENDIX



Figure A1. CD4+ T lymphocytes migrate upstream under flow. CD4+ T lymphocytes on ICAM-1 or VCAM-1/ICAM-1 mixed surfaces display upstream migration, and downstream on VCAM-1, but only cells on VCAM-1/ICAM-1 mixed surface preserve post-flow directionality only after having been exposed to flow at a shear rate of 800 s⁻¹. (A-C) Speed, persistence time, and Migration Index plotted over time during the course of flow of cells on ICAM-1. (D-F) Speed, persistence time, and Migration Index plotted over time during the course of flow of cells on VCAM-1. (CAM-1) mixed surface. (G-I) Speed, persistence time, and Migration Index plotted over time during the course of flow of cells on VCAM-1. (CAM-1) mixed surface. (G-I) Speed, persistence time, and Migration Index plotted over time during the course of flow of cells on VCAM-1. (CAM-1) mixed surface. (G-I) Speed, persistence time, and Migration Index plotted over time during the course of flow of cells on VCAM-1. (CAM-1) mixed surface. (G-I) Speed, persistence time, and Migration Index plotted over time during the course of flow of cells on VCAM-1. (CAM-1) mixed surface. (G-I) Speed, persistence time, and Migration Index plotted over time during the course of flow of cells on VCAM-1. (CAM-1) mixed surface. (G-I) Speed, persistence time, and Migration Index plotted over time during the course of flow of cells on VCAM-1.



Figure A2. CD8+ T lymphocytes do not migrate upstream under flow. CD8+ T lymphocytes do not retain the upstream direction after the flow has stopped. (A-C) Migration Index, speed, and persistence times of cells on ICAM-1 after 30 minutes. (D-F) Migration Index, speed, and persistence time of cells on VCAM-1/ICAM-1 mixed surface after 30 minutes. (G-I) Migration Index, speed, and persistence time of cells on VCAM-1 after 30 minutes. *: p < 0.05 compared to MI = 0 or to static conditions for speed and persistence times.



Figure A3. Upstream migration of CD4+ T lymphocytes with Wortamnnin on VCAM-1/ICAM-1 surfaces. CD4+ T lymphocytes on VCAM-1/ICAM-1 with increasing concentrations of Wortmannin migrate upstream, but loses directionality after the flow is turned off. (A-C) Migration Index, speed, and persistence time of cells on VCAM-1/ICAM-1 after 30 minutes of each flow condition. (D-E) Migration index of cells with Wortmannin over time during flow and post flow on VCAM-1/ICAM-1. *: p < 0.05compared to MI = 0 or to static conditions for speed and persistence times. NS: Not significant compared to DMSO control of corresponding flow condition.



Figure A4. Upstream migration of CD4+ T lymphocytes with Wortmannin on ICAM-1. Inhibiting PI3K with increasing concentrations of Wortmannin in CD4+ T lymphocytes on ICAM-1 do not affect the upstream migration. (A) Scattergrams of CD4+ T lymphocytes under flow (a-e) and post flow (f-j). (B-D) Migration Index, speed, and persistence time of cells on ICAM-1 after 30 minutes of each flow condition. (E-F) Migration Index of cells with Wortmannin over time during flow and post flow on ICAM-1. *: p < 0.05 compared to MI = 0 or to static conditions for speed and persistence times. NS: Not significant compared to DMSO control of corresponding flow condition.



Figure A5. Upstream migration of CD4+ T lymphocytes with LY294002. Inhibiting PI3K with increasing concentrations of LY294002 in CD4+ T lymphocytes also do not affect the upstream migration, but eliminate the upstream migration memory. (A-C) Migration Index, speed, and persistence time of cells on ICAM-1 after 30 minutes of each flow condition. (D-F) Migration Index, speed, and persistence time of cells on VCAM-1/ICAM-1 after 30 minutes of each flow condition. (G-H) Migration Index of cells with 10 μ M LY294002 plotted over time during and post flow on ICAM-1. (I-J) Migration Index of cells with 10 μ M LY294002 plotted over time during and post flow on VCAM-1/ICAM-1 mixed surface. *: p < 0.05 compared to MI = 0. #: p < 0.05 compared to DMSO control of corresponding flow condition.



Figure A6. Effect of Rho famliy GTPases inhibition in the upstream migration. Inhibiting Rho, Rac, and Cdc42 with inhibitors CT-04, NSC23766, and ML-141, respectively, do not affect migration of CD4+ T lymphocytes on either surfaces or flow conditions. (A-C) Migration Index, speed, and persistence time of cells on ICAM-1 after 30 minutes of each flow condition. (D-F) Migration Index, speed, and persistence time of cells on VCAM-1/ICAM-1 after 30 minutes of each flow condition. *: p < 0.05. NS: Not significant compared to DMSO control of corresponding flow condition.



Figure A7. Upstream migration of CD4+ T lymphocytes with CK666. CD4+ T lymphocytes maintained upstream migration and post flow migratory phenotype with increasing concentrations of Arp2/3 inhibitor, CK666. (A-C) Migration Index, speed and persistence time of cells on ICAM-1 with CK666. (D-F) Migration Index, speed and persistence time of cells on VCAM-1/ICAM-1 with CK666. (G-H) Migration Index of cells with increasing CK666 plotted over time during and post flow on ICAM-1. (I-J) Migration Index of cells with increasing CK666 plotted over time during and post flow on ICAM-1. (I-J) Migration Index of cells with increasing CK666 plotted over time during and post flow on ICAM-1. (I-J) Migration Index of cells with increasing CK666 plotted over time during and post flow on VCAM-1/ICAM-1. #: p < 0.05 compared to MI = 0. NS: Not significant compared to DMSO control of corresponding flow condition.



Figure A8. Actin polymerization in the upstream migration of CD4+ T cells. CD4+ T lymphocytes no longer migrate properly with low (150 nM) or 1 μ M Latrunculin A (LatA) or 10 μ M Cytochalasin D (CytoD). Scattergrams of cells on VCAM-1/ICAM-1 mixed surfaces during flow with 800s⁻¹ shear rate (A-D) and post flow (E-H). Red tracks indicate cells moving against the direction of flow, and gray tracks are cells moving with the direction of flow. The direction of flow is from left to right.



Figure A9. Actin polymerization in the upstream migration of CD4+ T cells. Altering VLA-4 activity with soluble VCAM-1 or VLA-4 blocking antibodies can affect the directional memory of CD4+ T lymphocytes. (A) Speed of cells with soluble VCAM-1 on ICAM-1 surfaces. (B-C) Migration index of cells with soluble VCAM-1 on ICAM-1 surfaces during and post flow plotted over time. (D) Scattergrams of cells with VLA-4 blocking antibodies on VCAM-1/ICAM-1 surfaces during (a-c) and post (d-f) flow with 800 s⁻¹ shear rate. Red tracks indicate cells moving against the direction of flow, and gray tracks are cells moving with the direction of flow. The flow is from left to right. (E-F) Migration index and Speed of cells with VLA-4 blocking antibodies on VCAM-1/ICAM-

1 surfaces at each flow condition. (G-H) Migration index of cells with VLA-4 blocking antibodies on VCAM-1/ICAM-1 surfaces during and post flow plotted over time.



Figure A10. Additional integrin levels on activated mouse CD4+ T cells. CD4+ T cells were activated as described in the materials and methods, and surface stained for flow cytometry. A) Gating strategy and B) T cells stained for indicated integrin chains. Note that surface levels of CD11b, which when paired with CD18 can bind ICAM-1, are low. The same is true for levels of ITGB7, which when paired with CD49d can bind VCAM-1. Higher expression of CD49e and CD49f is observed, but when paired with CD29 these integrins do not bind VCAM-1.



Figure A11. Integrin levels in murine T cells. Integrin levels on WT and DKO T cells are similar. WT and DKO CD4+ T cells were surface stained for the indicated integrin chains and analyzed by flow cytometry.



Figure A12. c-Cbl knockout in the upstream migration of murine T cells. C-Cbl KO effects upstream migration. A) CD4+ T cells from Cas9 expressing mice were transduced with the indicated gRNAs, selected for protein deletion, and tracked while migrating on ICAM-1 coated surfaces under shear flow conditions (shear rate 800s-1). Scattergrams are shown (representative of three independent experiments). Red lines, cell tracks with a net migration against the direction of shear (upstream). Blue lines, cell tracks with a net

migration in the direction of shear (downstream). Insets are enlarged to better show the behavior of cells with small displacements. B) Track length, C) displacement, and D) directionality were calculated from three independent experiments. Statistics were calculated using a one-way ANOVA, p<0.05; ***p<0.001.



Figure A13. PI3K inhibition in the upstream migration of murine T cells. PI3K inhibition does not affect upstream migration. A) CD4+ T cells were left untreated, treated with DMSO, or with the pan PI3K inhibitor LY294002 for 30mins, and then tracked while migrating on ICAM-1 coated surfaces under shear flow conditions (shear rate 800s-1) in the continued presence of inhibitor. Scattergrams are shown (representative of three independent experiments). Red lines, cell tracks with a net migration against the direction of shear (upstream). Blue lines, cell tracks with a net migration in the direction of shear (downstream). B) Track length, C) displacement, and D) directionality were calculated from three independent experiments. Statistics were calculated using a one-way ANOVA, ***p<0.001.



Figure A14. Mechanosensitivity of CD4+ T lymphocytes. CD4+ T lymphocytes show stiffness-dependent motility on ICAM-1, but not on VCAM-1/ICAM-1 mixed or VCAM-1 gels. Persistence time of cells on ICAM-1 (A), VCAM-1/ICAM-1 (B), and VCAM-1 (C). NS: Not significant.