Integrin cross-talk modulates stiffness-independent motility of CD4+ T lymphocytes

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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 on surfaces expressing intracellular adhesion molecule-1 (ICAM-1) through interaction with leukocyte function-associated antigen-1 ($\alpha_L\beta_2$) (LFA-1) integrins. LFA-1 likely behaves 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 CD4+ T lymphocytes on polyacrylamide gels with predetermined stiffnesses containing ICAM-1, vascular cell adhesion molecule-1 (VCAM-1), or a 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, but not on VCAM-1 or VCAM-1/ICAM-1 mixed, surfaces as a function of matrix stiffness. The mechanosensitivity of T-cell motility on ICAM-1 is overcome when VLA-4 (very late antigen-4 [$\alpha_4\beta_1$]) is ligated with soluble VCAM-1. Last, 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 cross-talk of VLA-4 and LFA-1 can compensate for deformable substrates. Interestingly, CD4+ T lymphocytes migrated upstream on ICAM-1 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 ranges from pascals to gigapascals (Yang *et al.*, 2017) in locations varying from lymph nodes to inflamed or diseased tissues such as a tumor microenvironment (Paszek *et al.*, 2005; Teijeira *et al.*, 2017). Varying degrees of stiffness can alter the morphology of T cells and their activation or proliferation rates (Judokusumo *et al.*, 2012; Saitakis *et al.*, 2017). T-cell activation involving CD3 and CD28, which are nonintegrin glycoprotein receptors mediating T-cell activation, or the ligation of leukocyte function-associated antigen-1 (LFA-1,$\alpha_L\beta_2$) integrins, are often reported to be mechanosensitive processes, affecting proliferation and chemokine production (O'Connor *et al.*, 2012; Bashoura *et al.*, 2014; Basu *et al.*, 2016; Hickey *et al.*, 2019; Wahl *et al.*, 2019; Blumenthal *et al.*, 2020).

Stiffness also affects integrin-mediated adhesion and migration in circulation (Stroka and Aranda-Espinoza, 2010, 2011). Stroka and Aranda-Espinoza (2009) 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 (Stroka and Aranda-Espinoza, 2009). Stroka and coworkers also observed a stiffness-dependent increase in the fraction of neutrophils undergoing transmigration (Stroka and Aranda-Espinoza, 2011). They showed that the substrate stiffness affects cell–cell adhesion to

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Abbreviations used: APC, antigen presenting cell; BSA, bovine serum albumin; FBS, fetal bovine serum; ICAM-1, intracellular adhesion molecule-1; LFA-1, leukocyte function-associated antigen-1 ($\alpha_L\beta_2$); MI, migration index; PHA, phytohemagglutinin; VCAM-1, vascular cell adhesion molecule-1; sVCAM-1, soluble VCAM-1; TCR, T-cell receptor; VLA-4, very late antigen-4 ($\alpha_4\beta_1$).

the endothelial cell layer which is mediated by myosin light-chain kinase (Stroka and Aranda-Espinoza, 2011). The immunological synapse, a well-studied cell–cell complex seen when a T-cell contacts an antigen presenting cell (APC), requires F-actin network whose assembly is mediated by LFA-1 and is known to generate mechanical forces (Comrie *et al.*, 2015). Rigidity of APCs (Bufi *et al.*, 2015) and endothelial cells (Ley *et al.*, 2007; Stroka and Aranda-Espinoza, 2011)—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 expression levels of intracellular adhesion molecule-1 (ICAM-1) (Yang *et al.*, 2005; Jalali *et al.*, 2015; Xu *et al.*, 2019), which could directly alter T-cell migration on direct contact.

Leukocytes often are exposed to 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 (Ley *et al.*, 2007). Shear flow affects T cells, indirectly by stimulating mechanical or chemical changes in endothelial cell which T cells make contact. Shear flow influences endothelial cell alignment which is critical in blood vessel formation (Wiig and Swartz, 2012; Polacheck *et al.*, 2013; Wang *et al.*, 2013). 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 endothelium 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 (Schaefer and Hordijk, 2015).

Shear flow can also influence T lymphocytes directly. Recently, it has been shown that T lymphocytes migrate in the opposite direction of flow on surfaces presenting ICAM-1 (Valignat *et al.*, 2013; Dominguez *et al.*, 2015; Hornung *et al.*, 2020). The upstream migration of T lymphocytes is driven by biophysical and intracellular biochemical factors (Valignat *et al.*, 2014; Kim and Hammer, 2019; Roy *et al.*, 2020). Valignat and co-workers first reported the upstream migration of T cells in vitro, and proposed a biophysical mechanism of 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 Macrophage-1 antigen (Mac-1, $\alpha_M\beta_2$) integrins (Buffone *et al.*, 2017, 2019; Tedford *et al.*, 2017). Ability of T cells to migrate upstream has also been observed in vivo (Bartholomäus *et al.*, 2009). Our group also observed that T cells migrating upstream on human umbilical vein endothelial cell (HUVEC) monolayers seem better able to transmigrate, suggesting a physiological role of upstream migration (Anderson *et al.*, 2019). *In vivo* observations of T lymphocytes migrating upstream (Bartholomäus *et al.*, 2009) 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 (Hind *et al.*, 2015; Mackay and Hammer, 2016) to examine whether LFA-1 or very late antigen-4 (VLA-4, $\alpha_4\beta_1$) integrin-mediated migration of human primary CD4+ T lymphocytes is mechanosensitive. These studies are done in the absence of T-cell receptor (TCR) signaling in T-cell activation, in which mechanosensitive responses have been clearly identified (Judokusumo *et al.*, 2012; O'Connor *et al.*, 2012; Bashoura *et al.*, 2014; Hong *et al.*, 2015; Hu and Butte, 2016; Jankowska *et al.*, 2018; Blumenthal and Burkhardt, 2020). We also address whether the substrate stiffness affects the upstream migration of CD4+ T lymphocytes under flow.

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 also 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 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 1 presents representative images of individual cells, tracks, and scattergrams of cells migrating on gels at indicated elastic moduli functionalized with ICAM-1. Under static conditions, CD4+ T cells on ICAM–1-functionalized gels all adhered and polarized, but ones on softer hydrogels were less spread than cells on stiffer gels (Figure 1, D and E).

On ICAM-1, CD4+ T lymphocytes exhibited increased motility as the stiffness increased (Figure 2A). CD4+ T cells on ICAM-1 became more motile (had a higher random motility coefficient, μ) as the stiffness increased. Speed (Figure 2B), but not persistence time (Supplemental Figure S1), 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 (Figure 2, C–F), suggesting that when VCAM-1 is present, cells exhibited stiffness-independent random motility. Thus, we have concluded 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.

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

After observing that CD4+ T lymphocytes on VCAM-1/ICAM-1 hydrogels migrate independent of matrix stiffness, we next hypothesized that the cross-talk 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 (Porter and Hogg, 1997; Chan *et al.*, 2000; Uotila *et al.*, 2014; Grönholm *et al.*, 2016), possibly suggesting the role of cross-talk affecting the affinity or downstream signaling. A previous study from our laboratory also reports that the cross-talk of LFA-1 and VLA-4 results in the persistent upstream migration post flow (Kim and Hammer, 2019). To test whether or not the cross-talk of the two integrins are involved in substrate-dependent motility, we modulated the integrin activities with soluble factors.

First, we activated VLA-4 integrins on CD4+ T lymphocytes with soluble VCAM-1 (sVCAM-1) to recreate the cross-talk 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 displayed a similar random motility coefficient at all stiffnesses. This result indicates cells ligated by sVCAM-1 are no longer sensitive to stiffness on ICAM-1 surfaces (Figure 3, A–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 sVCAM-1 to activate VLA-4 and generate the cross-talk.

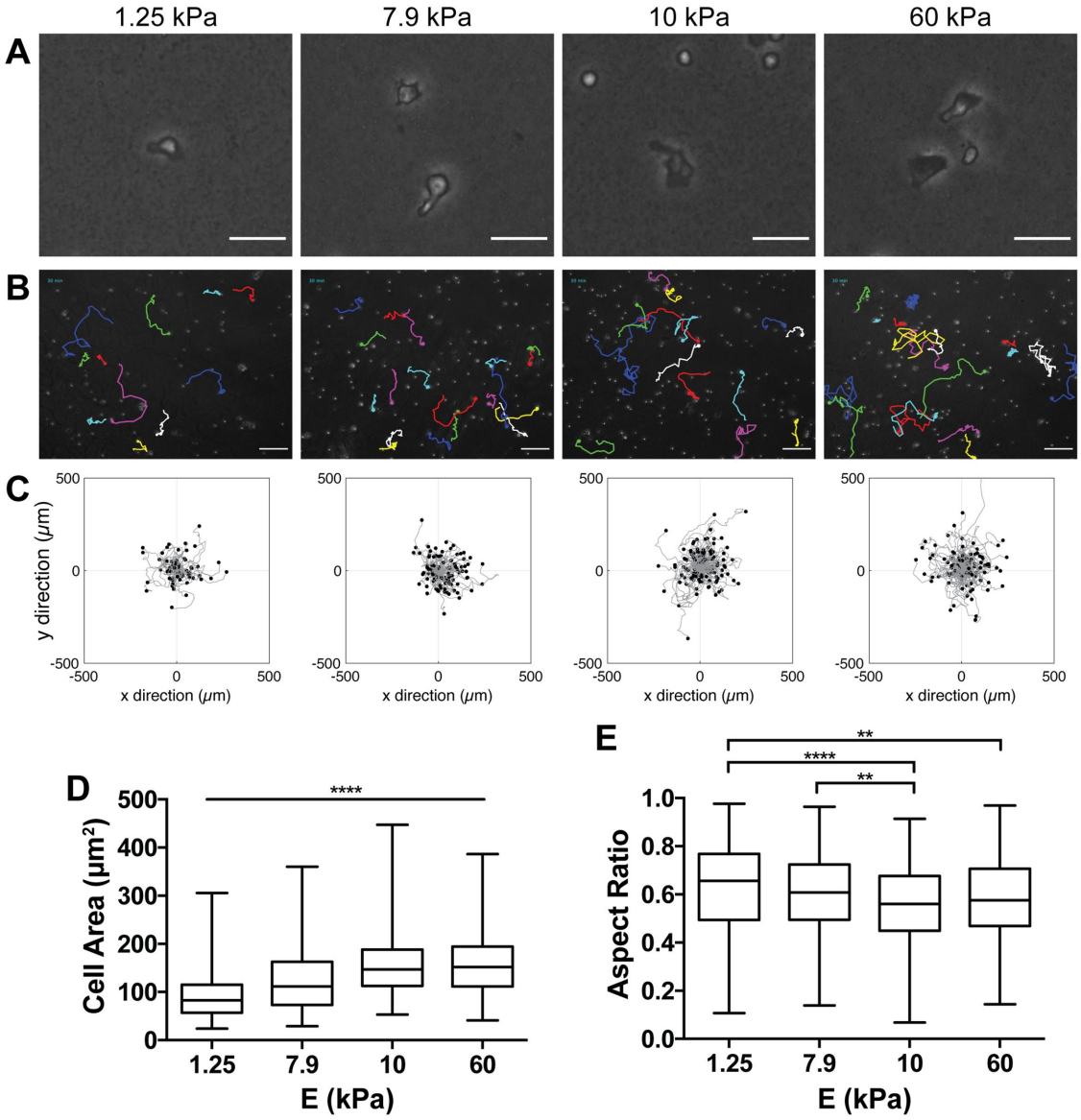

FIGURE 1: CD4+ T lymphocytes on ICAM–1-functionalized gels. Images of cells (A), overlayed cell trajectories (B), and scattergrams (C) of analyzed cell trajectories from a representative experiment of CD4+ T cells on ICAM–1-functionalized gels at indicated substrate stiffness. (D) Cell area and (E) aspect ratio of cells on ICAM-1 gels of indicated moduli. Scale bar = 25 μm (A) and 100 μm (B). $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

Next, we inhibited integrins binding to immobilized cognate ligands to further confirm that the cross-talk 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 mediated by a single integrin–ligand pair. When subunits of VLA-4 integrins are blocked, the motility coefficient and speed of cells on softer gels dropped significantly (Figure 3, D–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 (Figure 3, D–F). Blocking LFA-1 forces CD4+ T lymphocytes to rely on VLA-4, returning the motility to that seen on VCAM-1 surfaces. Cells treated with LFA–1-blocking antibodies showed no significant changes in motility, indicating VLA-4 alone does not distinguish different stiffnesses. Together, these results confirm that the cross-talk 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 hydrodynamic flow and migrate in the opposite direction of flow (Valignat *et al.*, 2013, 2014; Dominguez *et al.*, 2015; Kim and Hammer, 2019; Hornung *et al.*, 2020; Roy *et al.*, 2020). 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 800 s^{-1}. Migration indices (MI) were calculated from cell trajectories to quantify the upstream migration; negative values of the MI indicate upstream migration.

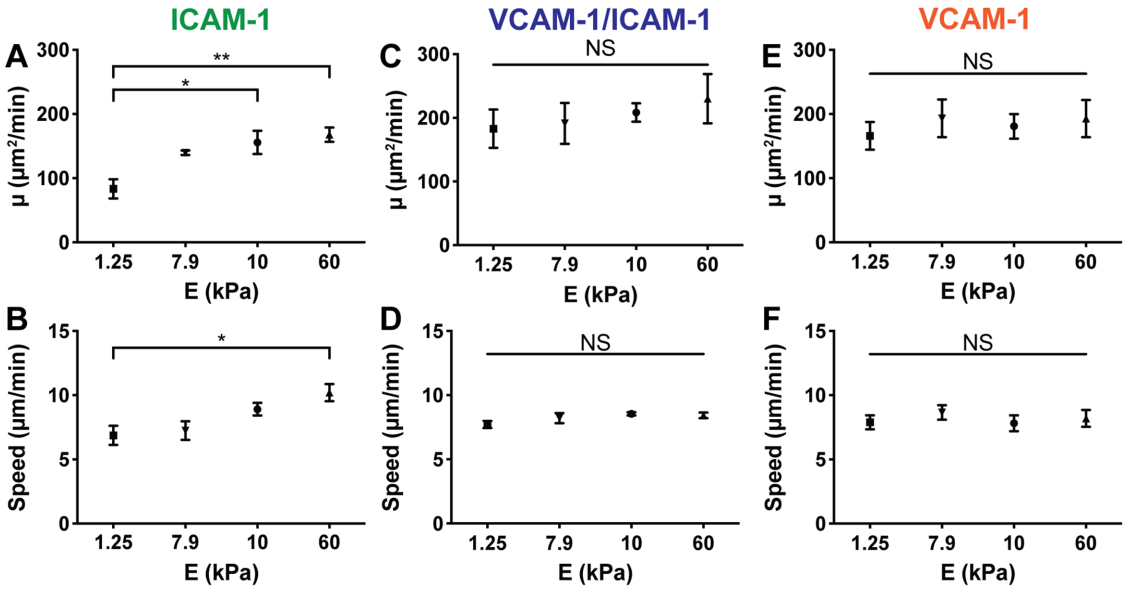

FIGURE 2: CD4+ T lymphocytes show stiffness-dependent motility on ICAM-1, but not on VCAM-1/ICAM-1 mixed or VCAM-1 gels. Random motility coefficient (μ) and speed of cells on ICAM-1 (A, B), VCAM-1/ICAM-1 mixed surfaces (C, D), and VCAM-1 (E, F). NS, not significant; *$p < 0.05$, **$p < 0.01$.

At a shear rate of 800 s^{-1}, the MIs ranged from –0.33 to –0.52 across different substrates (Figure 4, A and B). We further confirmed that more than 75% of CD4+ T cells on all values of matrix stiffness tested migrate upstream (Figure 4C). This result illustrates that, regardless of the stiffness, the direction of shear-dependent migration is unaffected. We note an increase in the magnitude of the upstream migration with cells on stiffer matrices. This observation is mostly from the increase in speed, not the persistence time, as the stiffness increased (Figure 4, D and E). Together, we conclude that CD4+ T lymphocytes exhibit upstream migration on ICAM–1-functionalized hydrogels with different elastic moduli ranging from 1.25 to 60 kPa.

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 previously reported that T cells on ICAM–1-coated PDMS surfaces showed an increasing directional preference in the upstream direction as shear rates increased (Dominguez *et al.*, 2015; Kim and Hammer, 2019). Similar to their findings, CD4+ T cells on ICAM–1-functionalized 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 (Figure 5, A and B). On both soft and stiff gels, MI of cells decreased as the shear rate increased, indicating a stronger upstream migration response at higher shear rates (Figure 5A and Supplemental Figure S2). As shear rates increased from 100 to 800 s^{-1}, MI decreased from –0.11 to –0.35 on soft substrates and from –0.15 to –0.40 on stiff substrates (Figure 5A). 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. The shear rate-dependent increase in persistence time was more apparent in cells on stiff hydrogels (Figure 5D). The slight increase in persistence time with cells on soft gels as a function of shear rate was not statistically significant (Figure 5, C and 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.

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

Under static conditions, we observed that ligating VLA-4 with sVCAM-1 increased the motility of CD4+ T lymphocytes on hydrogels functionalized with ICAM-1 (Figure 3, A–C). Here, we investigated whether ligating VLA-4 and initiating the cross-talk 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 (Figure 6A). The addition of sVCAM-1 to cells migrating on ICAM-1 did not have any significant effect on the upstream migration of T cells. Activating VLA-4 with sVCAM-1 did not affect the direction of migration, indicated by negative MI and the percentage of cells migrating upstream (Figure 6, A–C). On soft substrates, a slight increase from 70 to 82% of cells migrating upstream was detected (Figure 6C). The increase with the addition of sVCAM-1 is comparable to the percentages of cells migrating upstream on stiff substrates with or without sVCAM-1. This suggests that the cross-talk of VLA-4 and LFA-1 contributes to an enhanced upstream migration response of cells. Cells on stiffer gels exhibited higher speed, but the persistence time of cells migrating upstream was not affected (Figure 6, D and E). Consistent with previous studies (Valignat *et al.*, 2013; Dominguez *et al.*, 2015; Kim and Hammer, 2019; Roy *et al.*, 2020), upstream migration of CD4+ T lymphocytes is a direct downstream event of LFA-1 engaging immobilized ICAM-1 on the surface.

While the cross-talk of VLA-4 and LFA-1 integrins contributes to more negative MI and stronger upstream motility than activation of LFA-1 alone, establishing the upstream direction of motion in response to shear flow is dictated by cells engaging immobilized ICAM-1 on the surface. CD4+ T cells on VCAM-1 surfaces do not migrate upstream, indicated by MI of +0.73 (Supplemental Figure S3). Triggering the cross-talk of VLA-4 and LFA-1 on cells migrating on VCAM-1 also did not trigger the upstream migration

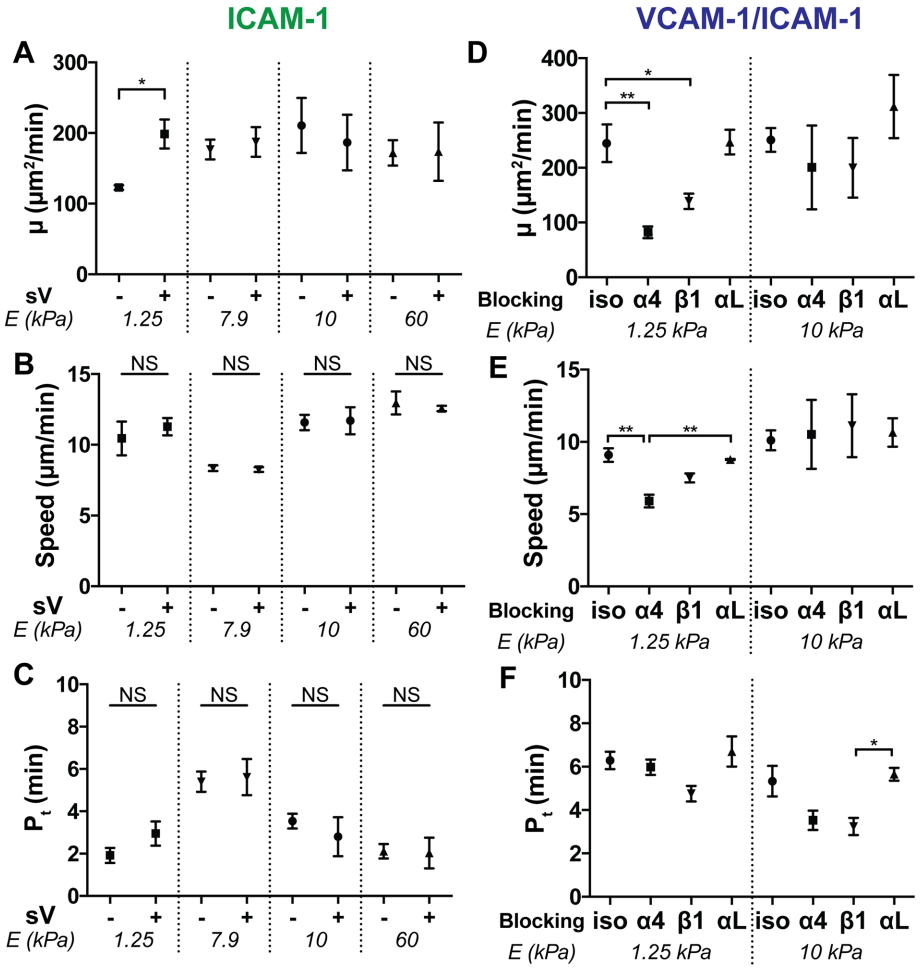

FIGURE 3: Cross-talk of VLA-4 and LFA-1 make CD4+ T lymphocytes more motile, but reduces sensitivity to stiffness. (A–C) Random motility coefficient (μ), speed, and persistence time (P_t), respectively, of CD4+ T lymphocytes on ICAM-1 with or without sVCAM-1 at indicated matrix stiffness. (D–F) Random 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. NS, not significant; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

(Supplemental Figure S3). In conclusion, we show that the cross-talk of LFA-1 and VLA-4 may enhance the upstream migration under shear flow and reinforce that the upstream direction of motion is dependent on LFA-1-ICAM-1 interaction.

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 (Saitakis *et al.*, 2017; Blumenthal and Burkhardt, 2020; Blumenthal *et al.*, 2020). 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 (Valignat *et al.*, 2013; Dominguez *et al.*, 2015; Kim and Hammer, 2019; Roy *et al.*, 2020). Studies from the Theodoly group suggest that the detection of the direction of flow and the direction of migration is mediated by the uropod structure of the cell, acting like a wind vane (Valignat *et al.*, 2014). While past studies elucidate fundamental biophysical and biochemical mechanisms of the upstream migration (Valignat *et al.*, 2013; Kim and Hammer, 2019; Roy *et al.*, 2020), in vitro platforms do not accurately recreate the in vivo microenvironment cell experience partially due to the lack of physiological mechanistic properties in the in vitro set up. In this study, we used polyacrylamide gels (Hind *et al.*, 2015; Mackay and Hammer, 2016) to create substrates of different compliances and to measure integrin-dependent motility of CD4+ T lymphocytes in response to shear flow.

Previous work has reported that matrix stiffness significantly affects integrin-mediated cell spreading, migration and force generation (Pelham and Wang, 1997; Dembo and Wang, 1999; Lo *et al.*, 2000; Reinhart-King *et al.*, 2005). Similar to previously published reports, stiffness of the hydrogels affected T-cell spreading and motility (Figure 1). Furthermore, CD4+ T lymphocytes exhibited mechanosensitive migration on hydrogels functionalized with ICAM-1 (Figure 2). However, cells became more motile, but stiffness independent when gels contained VCAM-1 (Figure 2). We then showed that the simultaneous engagement of LFA-1 and VLA-4 made cells more motile, but insensitive to matrix stiffness. We ligated VLA-4 using sVCAM-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 (Figure 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 (Figures 4–6). A slight increase in speed as a function of matrix stiffness (Figure 4) was observed. However, the persistence of the upstream migration of CD4+ T cells is dependent on the shear rate, not the matrix stiffness (Figure 5) nor soluble ligation of VLA-4 (Figure 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 (Bartholomäus *et al.*, 2009; Valignat *et al.*, 2013; Dominguez *et al.*, 2015; Anderson *et al.*, 2019; Kim and Hammer, 2019; Roy *et al.*, 2020), we show here that CD4+ T lymphocytes migrate consistently upstream on ICAM-1 substrates. Unlike other immune cell types (Smith *et al.*, 2007; Jannat *et al.*, 2011; Henry *et al.*, 2015; Hind *et al.*, 2015), T cells do not exert as much traction force (Zhang *et al.*, 2002; Nordenfelt *et al.*, 2016; Li *et al.*, 2017). Using an intracellular tension sensor, Nordenfelt *et al.* (2016) have reported an average force per LFA-1 on Jurkat T cells on ICAM-1 to

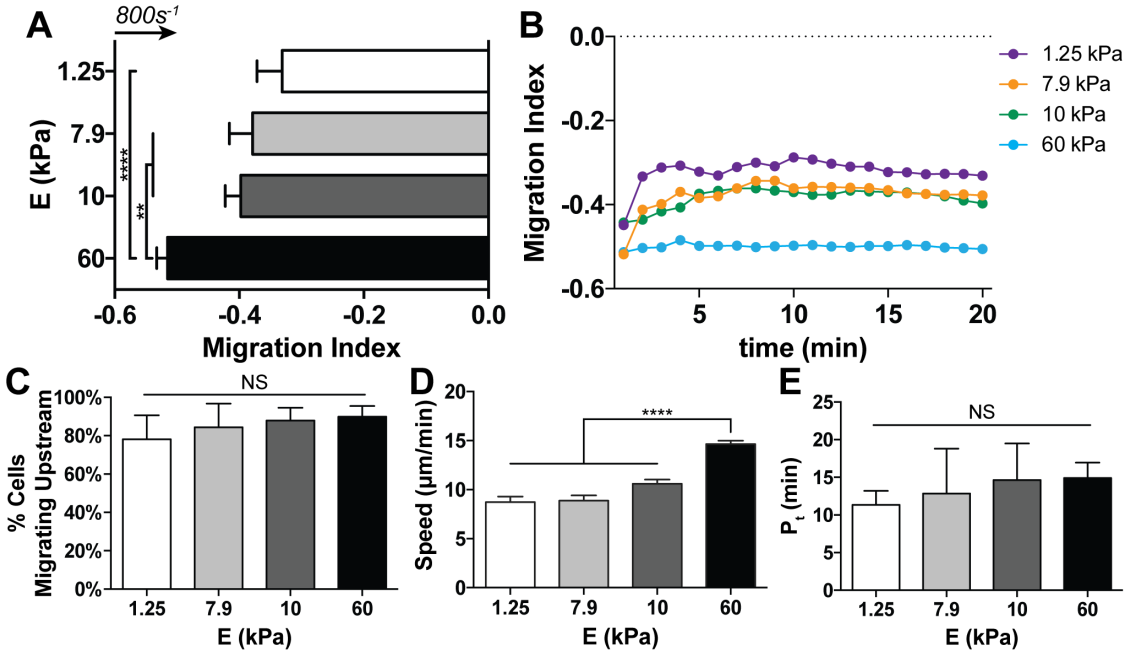

FIGURE 4: CD4+ T lymphocytes robustly migrate upstream on polyacrylamide gels of different stiffness. Migration index at 20 min (A) and over time (B), percentage of cells migrating upstream (C), speed (D), and persistence time (E) are reported to show that CD4+ T cells migrate upstream under flow (shear rate = 800 s^{-1}) on substrates of varying elastic moduli. NS, not significant; $^{**}p < 0.01$, $^{****}p < 0.0001$.

be ~1 pN (Nordenfelt *et al.*, 2016). 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 (Hickey *et al.*, 2019; Blumenthal *et al.*, 2020), 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 on 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.

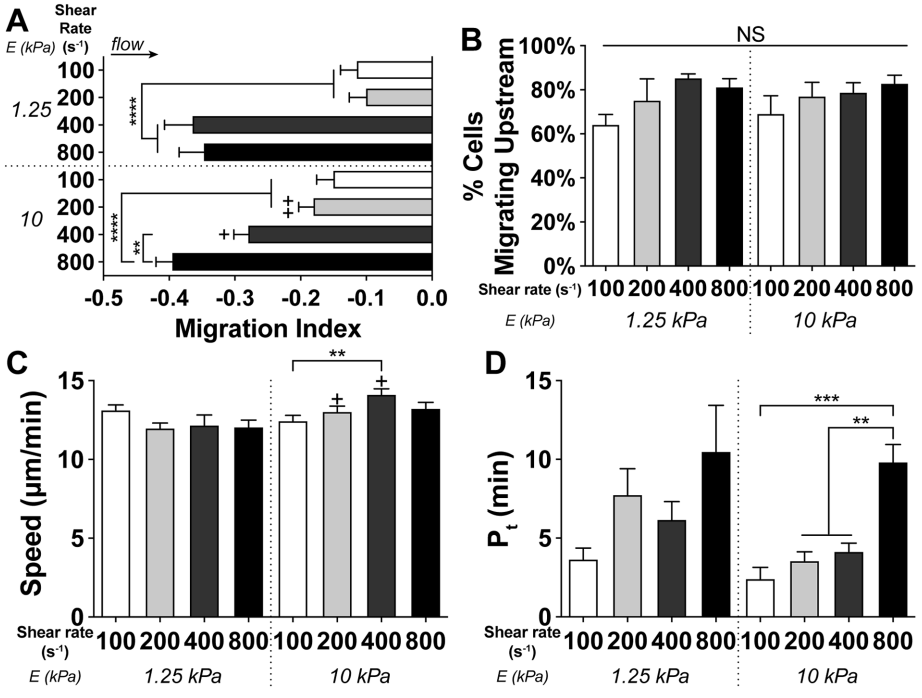

FIGURE 5: CD4+ T lymphocytes exhibit shear rate-dependent upstream migration on both soft (1.25 kPa) and stiff (10 kPa) ICAM-1 gels. Migration index at 20 min (A), percentage of cells migrating upstream (B), speed (C), and persistence time (D) of cells migrating under flow with varying shear rates on soft (1.25 kPa) and stiff (10 kPa) substrates. NS, not significant; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. $^{+}p < 0.05$, $^{++}p < 0.01$ compared with corresponding shear rates on 1.25 kPa.

MATERIALS AND METHODS

Cell culture and reagents

Human primary CD4+ T lymphocytes were acquired from Human Immunology Core at the University of Pennsylvania (P30-CA016520). Cells were activated with phytohemagglutinin (PHA, MP Biomedicals, Santa Ana, CA) in RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS). Cells were further cultured in RPMI–1640-supplemented 10% FBS, IL-2 (Cat. #354043 Corning, Corning, NY) and penicillin and streptomycin (Life Technologies, Gaithersburg, MD). During experiments, cells were kept in RPMI-1640 with D-glucose

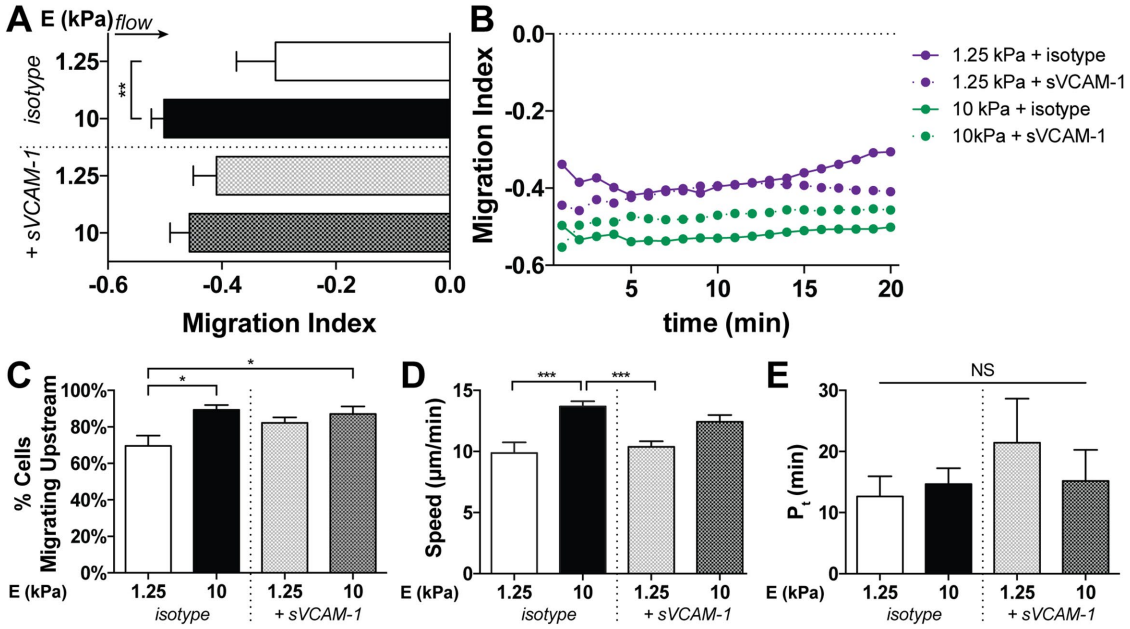

FIGURE 6: Cross-talk of VLA-4 and LFA-1 has no effect in the upstream migration on substrates with varying stiffness. Migration index at 20 min (A) and over time (B), percentage of cells migrating upstream (C), speed (D), and persistence time (E) of cells migrating under flow (shear rate = 800 s^{-1}) on ICAM-1 gels at indicated stiffness. NS, not significant, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

and bovine serum albumin (BSA). CD4+ T cells were given 30 min to adhere to surfaces prior to imaging (Dominguez *et al.*, 2015; Kim and Hammer, 2019). To activate or block integrins, cells were incubated with the following for 15 min prior to adhesion: sVCAM-1 (Cat. #809-VR, R&D Systems, Minneapolis, MN), isotype control (Cat. #400166, MOPC-21), anti-α_L (Cat. #301214, clone: HI111), anti-α_4 (Cat. #304310, clone: 9F10), or anti-β_1 (Cat. #921304, clone: P5D2) (BioLegend, San Diego, CA). For shear flow experiments, RPMI-1640 with D-glucose and BSA was introduced to the parallel flow chamber with a pump to establish indicated shear rates (Dominguez *et al.*, 2015).

Substrate preparation

Polyacrylamide gels with acrylamide and bis-acrylamide (Bio-Rad, Hercules, CA) were prepared as explained previously (Yeung *et al.*, 2005; Henry *et al.*, 2015; Hind *et al.*, 2015; Mackay and Hammer, 2016). Briefly, gels with elastic moduli of 1.2, 7.9, 10, and 60 kPa, all containing N-6-((acryloyl)amino)hexanoic acid cross-linker (N6) and 0.1% wt/vol TEMED (Bio-Rad), were polymerized at a final concentration of 0.1% wt/vol ammonium persulfate (Bio-Rad). On initiating polymerization, gel solutions were carefully pipetted onto silanized glass slides. Rain-X-coated cover glasses were placed on top to create flat surfaces. Flattened gels were allowed to polymerize for 30–45 min under nitrogen. After polymerization, gels were detached from the top coverslips under water, followed by rinsing and functionalization.

Gel functionalization

To functionalize gels, N6 cross-linker was added into all gel solutions prior to polymerization. After washing gels with water, gels were conjugated with 2 μg/ml protein A/G (Cat. #6502-1, BioVision, Milpitas, CA) for 2 h at room temperature. Then, gels were rinsed with 1/100 parts ethanolamine in 50 mM HEPES to block unreacted N6, followed by the addition of 10 μg/ml ICAM-1 and/or VCAM-1 Fc Chimeras (Cat. #720-IC and 862-VC, respectively, 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 20× 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 (https://imagej.nih.gov/ij/, 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 min under static and 20 min under flow using a Nikon TE300 with a Nikon 10×, numerical aperture 0.25, objective. During the course of experiments, cells were kept in 37°C and 5% CO_2. 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 (P_t) by calculating mean squared displacement of each cell fitted to the Dunn equation (Dunn, 1983). MI is defined as the ratio of the cell's axial displacement over the total length of the cell trajectory. Negative MI represents upstream migration, and positive MI represents downstream migration. MI of –1 indicates the upstream migration at a perfectly straight line. Data are presented as mean ± SEM, with at least three biological repeats (i.e., independent donors). 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.

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REFERENCES

Anderson NR, Buffone A, Hammer DA (2019). T lymphocytes migrate upstream after completing the leukocyte adhesion cascade. Cell Adh Migr 13, 164–169.

Bartholomäus I, Kawakami N, Odoardi F, Schläger C, Miljkovic D, Ellwart JW, Klinkert WEF, Flügel-Koch C, Issekutz TB, Wekerle H, Flügel A (2009). Effector T cell interactions with meningeal vascular structures in nascent autoimmune CNS lesions. Nature 462, 94–98.

Bashoura KT, Gondarenko A, Chen H, Shen K, Liu X, Huse M, Hone JC, Kam LC (2014). CD28 and CD3 have complementary roles in T-cell traction forces. Proc Natl Acad Sci USA 111, 2241–2246.

Basu R, Whitlock BM, Husson J, Floc'h AL, Jin W, Oyler-Yaniv A, Dotiwala F, Giannone G, Hivroz C, Biais N, *et al.* (2016). Cytotoxic T Cells Use Mechanical Force to Potentiate Target Cell Killing Article Cytotoxic T Cells Use Mechanical Force to Potentiate Target Cell Killing. Cell 165, 1–11.

Blumenthal D, Burkhardt JK (2020). Multiple actin networks coordinate mechanotransduction at the immunological synapse. J Cell Biol 219.

Blumenthal D, Chandra V, Avery L, Burkhardt JK (2020). Mouse t cell priming is enhanced by maturation-dependent stiffening of the dendritic cell cortex. Elife 9, 1–44.

Buffone A, Anderson NR, Hammer DA (2017). Migration against the direction of flow is LFA-1-dependent in human hematopoietic stem and progenitor cells. J Cell Sci 131, jcs205575.

Buffone A, Anderson NR, Hammer DA (2019). Human neutrophils will crawl upstream on ICAM-1 if Mac-1 is blocked. Biophys J 117, 1393–1404.

Bufi N, Saitakis M, Dogniaux S, Buschinger O, Bohineust A, Richert A, Maurin M, Hivroz C, Asnacios A (2015). Human primary immune cells exhibit distinct mechanical properties that are modified by inflammation. Biophys J 108, 2181–2190.

Chan JR, Hyduk SJ, Cybulsky MI (2000). α4β1 Integrin/VCAM-1 interaction activates αLβ2 integrin-mediated adhesion to ICAM-1 in human T cells. J Immunol 164, 746–753.

Comrie WA, Babich A, Burkhardt JK (2015). F-actin flow drives affinity maturation and spatial organization of LFA-1 at the immunological synapse. J Cell Biol 208, 475–491.

Dembo M, Wang YL (1999). Stresses at the cell-to-substrate interface during locomotion of fibroblasts. Biophys J 76, 2307–2316.

Dominguez GA, Anderson NR, Hammer DA (2015). The direction of migration of T-lymphocytes under flow depends on which adhesion receptors are engaged. Integr Biol 7, 345–355.

Dunn GA (1983). Characterising a kinesis response: time averaged measures of cell speed and directional persistence. Agents Actions Suppl 12, 14–33.

Grönholm M, Jahan F, Bryushkova EA, Madhavan S, Aglialoro F, Hinojosa LS, Uotila LM, Gahmberg CG (2016). LFA-1 integrin antibodies inhibit leukocyte α4β1-mediated adhesion by intracellular signaling. Blood 128, 1270–1281.

Henry SJ, Chen CS, Crocker JC, Hammer DA (2015). Protrusive and Contractile Forces of Spreading Human Neutrophils. Biophys J 109, 699–709

Hickey JW, Dong Y, Chung JW, Salathe SF, Pruitt HC, Li X, Chang C, Fraser AK, Bessell CA, Ewald AJ, *et al.* (2019). Engineering an artificial T-cell stimulating matrix for immunotherapy. Adv Mater 31, 1807359.

Hind LE, Dembo M, Hammer DA (2015). Macrophage motility is driven by frontal-towing with a force magnitude dependent on substrate stiffness. Integr Biol (Camb) 7, 447–453.

Hong J, Persaud SP, Horvath S, Allen PM, Evavold BD, Zhu C (2015). Force-regulated in situ TCR–peptide-bound MHC class II kinetics determine functions of CD4 + T cells. J Immunol 195, 3557–3564.

Hornung A, Sbarrato T, Garcia-Seyda N, Aoun L, Luo X, Biarnes-Pelicot M, Theodoly O, Valignat M-P (2020). A bistable mechanism mediated by integrins controls mechanotaxis of leukocytes. Biophys J 118, 1–13.

Hu KH, Butte MJ (2016). T cell activation requires force generation. J Cell Biol 213, 535–542.

Jalali S, Tafazzoli-Shadpour M, Haghighipour N, Omidvar R, Safshekan F (2015). Regulation of endothelial cell adherence and elastic modulus by substrate stiffness. Cell Commun Adhes 22, 79–89.

Jankowska KI, Williamson EK, Roy NH, Blumenthal D, Chandra V, Baumgart T, Burkhardt JK (2018). Integrins modulate T cell receptor signaling by constraining actin flow at the immunological synapse. Front Immunol 9, 00025.

Jannat RA, Dembo M, Hammer DA (2011). Traction forces of neutrophils migrating on compliant substrates. Biophys J 101, 575–584.

Judokusumo E, Tabdanov E, Kumari S, Dustin ML, Kam LC (2012). Mechanosensing in T lymphocyte activation. Biophys J 102, L5–L7.

Kim SHJ, Hammer DA (2019). Integrin crosstalk allows CD4+ T lymphocytes to continue migrating in the upstream direction after flow. Integr Biol 11, 384–393.

Ley K, Laudanna C, Cybulsky MI, Nourshargh S (2007). Getting to the site of inflammation: The leukocyte adhesion cascade updated. Nat Rev Immunol 7, 678–689.

Li N, Yang H, Wang M, Lü S, Zhang Y, Long M (2017). Ligand-specific binding forces of LFA-1 and Mac-1 in neutrophil adhesion and crawling. Mol Biol Cell 29, 408–418.

Lo CM, Wang HB, Dembo M, Wang YL (2000). Cell movement is guided by the rigidity of the substrate. Biophys J 79, 144–152.

Mackay JL, Hammer DA (2016). Stiff substrates enhance monocytic cell capture through E-selectin but not P-selectin. Integr Biol (Camb).

Nordenfelt P, Elliott HL, Springer TA (2016). Coordinated integrin activation by actin-dependent force during T-cell migration. Nat Commun 7, 13119.

O'Connor RS, Hao X, Shen K, Bashour K, Akimova T, Hancock WW, Kam LC, Milone MC (2012). Substrate rigidity regulates human T cell activation and proliferation. J Immunol 189, 1330–1339.

Paszek MJ, Zahir N, Johnson KR, Lakins JN, Rozenberg GI, Gefen A, Reinhart-King CA, Margulies SS, Dembo M, Boettiger D, *et al.* (2005). Tensional homeostasis and the malignant phenotype. Cancer Cell 8, 241–254.

Pelham RJ, Wang YL (1997). Cell locomotion and focal adhesions are regulated by substrate flexibility. Proc Natl Acad Sci USA 94, 13661–13665.

Polacheck WJ, Li R, Uzel SGM, Kamm RD (2013). Microfluidic platforms for mechanobiology. Lab Chip 13, 2252–2267.

Porter JC, Hogg N (1997). Integrin cross talk: Activation of lymphocyte function-associated antigen-1 on human T cells alters α4β1- and α5β1-mediated function. J Cell Biol 138, 1437–1447.

Reinhart-King CA, Dembo M, Hammer DA (2005). The dynamics and mechanics of endothelial cell spreading. Biophys J 89, 676–689.

Roy NH, Kim SHJ, Buffone A, Blumenthal D, Huang B, Agarwal S, Schwartzberg PL, Hammer DA, Burkhardt JK (2020). LFA-1 signals to promote actin polymerization and upstream migration in T cells. J Cell Sci 133, jcs248328.

Saitakis M, Dogniaux S, Goudot C, Bufi N, Asnacios S, Maurin M, Randriamampita C, Asnacios A, Hivroz C (2017). Different TCR-induced T lymphocyte responses are potentiated by stiffness with variable sensitivityDifferent TCR-induced T lymphocyte responses are potentiated by stiffness with variable sensitivity. Elife 6.

Schaefer A, Hordijk PL (2015). Cell-stiffness-induced mechanosignaling - a key driver of leukocyte transendothelial migration. J Cell Sci 128, 2221–2230.

Smith LA, Aranda-espinoza H, Haun JB, Dembo M, Hammer DA (2007). Neutrophil traction stresses are concentrated in the uropod during migration. Biophys J 92, L58–L60.

Stroka KM, Aranda-Espinoza H (2009). Neutrophils display biphasic relationship between migration and substrate stiffness. Cell Motil Cytoskeleton 66, 328–341.

Stroka KM, Aranda-Espinoza H (2010). A biophysical view of the interplay between mechanical forces and signaling pathways during transendothelial cell migration. FEBS J 277, 1145–1158.

Stroka KM, Aranda-Espinoza H (2011). Endothelial cell substrate stiffness influences neutrophil transmigration via myosin light chain kinase-dependent cell contraction. Blood 118, 1632–1640.

Tedford K, Steiner M, Koshutin S, Richter K, Tech L, Eggers Y, Jansing I, Schilling K, Hauser AE, Korthals M, Fischer K-D (2017). The opposing forces of shear flow and sphingosine-1-phosphate control marginal zone B cell shuttling. Nat Commun 8, 2261.

Teijeira A, Hunter MC, Russo E, Proulx ST, Frei T, Debes GF, Coles M, Melero I, Detmar M, Rouzaut A, Halin C (2017). T cell migration from inflamed skin to draining lymph nodes requires intralymphatic crawling supported by ICAM-1/LFA-1 interactions. Cell Rep 18, 857–865.

Uotila LM, Jahan F, Soto Hinojosa L, Melandri E, Grönholm M, Gahmberg CG (2014). Specific phosphorylations transmit signals from leukocyte β2 to β1 integrins and regulate adhesion. J Biol Chem 289, 32230–32242.

Valignat MP, Nègre P, Cadra S, Lellouch AC, Gallet F, Hénon S, Theodoly O (2014). Lymphocytes can self-steer passively with wind vane uropods. Nat Commun 5, 5213.

Valignat MP, Theodoly O, Gucciardi A, Hogg N, Lellouch AC (2013). T lymphocytes orient against the direction of fluid flow during LFA-1-mediated migration. Biophys J 104, 322–331.

Wahl A, Dinet C, Dillard P, Nassereddine A, Puech PH, Limozin L, Sengupta K (2019). Biphasic mechanosensitivity of T cell receptor-mediated spreading of lymphocytes. Proc Natl Acad Sci USA 116, 5908–5913.
Wang C, Baker BM, Chen CS, Schwartz MA (2013). Endothelial cell sensing of flow direction. Arterioscler Thromb Vasc Biol 33, 2130–2136.
Wiig H, Swartz MA (2012). Interstitial fluid and lymph formation and transport: Physiological regulation and roles in inflammation and cancer. Physiol Rev 92, 1005–1060.
Xu Y, Huang D, Lü S, Zhang Y, Long M (2019). Mechanical features of endothelium regulate cell adhesive molecule-induced calcium response in neutrophils. APL Bioeng 3, 016104.
Yang L, Froio RM, Sciuto TE, Dvorak AM, Alon R, Luscinskas FW (2005). ICAM-1 regulates neutrophil adhesion and transcellular migration of TNF-alpha-activated vascular endothelium under flow. Blood 106, 584–592.
Yang Y, Wang K, Gu X, Leong KW (2017). Biophysical regulation of cell behavior—cross talk between substrate stiffness and nanotopography. Engineering 3, 36–54.
Yeung T, Georges PC, Flanagan LA, Marg B, Ortiz M, Funaki M, Zahir N, Ming W, Weaver V, Janmey PA (2005). Effects of substrate stiffness on cell morphology, cytoskeletal structure, and adhesion. Cell Motil Cytoskeleton 60, 24–34.
Zhang X, Wojcikiewicz E, Moy VT (2002). Force spectroscopy of the leukocyte function-associated antigen-1/intercellular adhesion molecule-1 interaction. Biophys J 83, 2270–2279.