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The Biophysics Of Leukocyte Adhesion Deficiency

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The Biophysics Of Leukocyte Adhesion Deficiency

Abstract
In order to perform an effective immune response, leukocytes must be able to exit the vasculature and enter the interstitial space. The leukocyte adhesion cascade has evolved to slow and stop cells to allow this access. Despite extensive molecular characterization, there are still significant questions regarding the biophysical constraints of the cascade. In this thesis, we explore the requirements for cells to physically complete the adhesion cascade. In the first aim, we confirm previously published predictions regarding the synergy between E-selectin and ICAM-1, showing that a consistent level of leukocyte adhesion can be maintained using varying ratios of the two molecules. We also show that T cells require $O(10^0)$ sites/µm$^2$ to support tethering, $O(10^1)$ sites/µm$^2$ to support rolling, and $O(10^2)$ sites/µm$^2$ to cause arrest. In addition, we characterized the migration of T cells against the direction of flow. We discovered that cells determine their direction of migration within 30 seconds of arrest. We also found that cells migrating upstream transmigrate across a HUVEC monolayer faster than cells crawling downstream. In the second aim, we determined that cells attached to a surface through a series of linkages show a non-linear decrease in the critical detachment force as the number of linkages increases. We also showed that the intrinsic off rate of the linkages can control the critical force, while the spring constant of the linkages causes less of a change. Finally, in the third aim we used simulations to quantitatively predict the effect of depletion of kindlin-3 on cellular adhesion. We predicted that adhesion would be hypersensitive to kindlin-3 expression, requiring a reduction to below 20% of normal expression levels to see an effect. We also predicted that rolling velocity would be independent of kindlin-3 expression, while both time and distance to stop would increase with decreasing kindlin-3 expression. Experiments using the Jurkat T cell line supported these predictions, with a significant decrease in cell adhesion, no change in rolling velocity, and an increase in the time to stop. Together, these aims suggest that we now have the knowledge to improve leukocyte targeting through engineering the leukocyte adhesion cascade.

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THE BIOPHYSICS OF LEUKOCYTE ADHESION DEFICIENCY

Nicholas Roy Anderson

A DISSERTATION

in

Chemical and Biomolecular Engineering

Presented to the Faculties of the University of Pennsylvania

in

Partial Fulfillment of the Requirements for the

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THE BIOPHYSICS OF LEUKOCYTE ADHESION DEFICIENCY

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To my parents, Michael and Cynthia Anderson
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Although there is a single name on the title page, this thesis is actually the work of many people and without them I would not have been able to finish.

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ABSTRACT

THE BIOPHYSICS OF LEUKOCYTE ADHESION DEFICIENCY

Nicholas R. Anderson

Daniel A. Hammer

In order to perform an effective immune response, leukocytes must be able to exit the vasculature and enter the interstitial space. The leukocyte adhesion cascade has evolved to slow and stop cells to allow this access. Despite extensive molecular characterization, there are still significant questions regarding the biophysical constraints of the cascade. In this thesis, we explore the requirements for cells to physically complete the adhesion cascade. In the first aim, we confirm previously published predictions regarding the synergy between E-selectin and ICAM-1, showing that a consistent level of leukocyte adhesion can be maintained using varying ratios of the two molecules. We also show that T cells require $O(10^0)$ sites/µm$^2$ to support tethering, $O(10^1)$ sites/µm$^2$ to support rolling, and $O(10^2)$ sites/µm$^2$ to cause arrest. In addition, we characterized the migration of T cells against the direction of flow. We discovered that cells determine their direction of migration within 30 seconds of arrest. We also found that cells migrating upstream transmigrate across a HUVEC monolayer faster than cells crawling downstream. In the second aim, we determined that cells attached to a surface through a series of linkages show a non-linear decrease in the critical detachment force as the number of linkages increases. We also showed that the intrinsic off rate of the linkages can control the critical force, while the spring constant of the linkages causes less of a change. Finally, in the third aim we used simulations to quantitatively predict the effect
of depletion of kindlin-3 on cellular adhesion. We predicted that adhesion would be hypersensitive to kindlin-3 expression, requiring a reduction to below 20% of normal expression levels to see an effect. We also predicted that rolling velocity would be independent of kindlin-3 expression, while both time and distance to stop would increase with decreasing kindlin-3 expression. Experiments using the Jurkat T cell line supported these predictions, with a significant decrease in cell adhesion, no change in rolling velocity, and an increase in the time to stop. Together, these aims suggest that we now have the knowledge to improve leukocyte targeting through engineering the leukocyte adhesion cascade.
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CHAPTER 1: Motivation

The immune system is responsible for responding to insults to tissue homeostasis, whether they be from infection or tissue trauma (1). In order to perform these functions, cells of the immune system, known as leukocytes, must be able to leave circulation in the vasculature and enter the interstitial space. In addition, the body must prevent non-specific adhesion to reduce the risk of auto-immune disorders (2). Thus, cells utilize the leukocyte adhesion cascade as a method to perform these contradictory needs. Initially, the cell is traveling through the blood vessel at a speed whereby it is impossible for the cell to immediately stop. Instead, the cell weakly binds to the endothelium, which allows the cell to slow down compared to its initial velocity. As the cell rolls across the surface, it will determine whether to stop at that location. If the cell receives the appropriate signal, it can firmly arrest on the surface. The leukocyte can now transmigrate and access the interstitial space (3–5).

One of the important cells of the immune system is the T cell, which is part of the adaptive immune system. T cells play a role in many immune responses, including stimulating the production of antibodies and killing infected cells (6). Recently, T cells have been engineered as an anticancer therapeutic against several kinds of blood cancers (7, 8). However, there is a great desire to expand the ability of these cells to fight solid tumors as well. One way to improve the efficacy of these engineered anti-cancer cells would be to enhance the ability of these cells to home to the tumors, which would allow fewer cells to have the same level of response (9). We propose that understanding the biophysics of how these cells target specific areas could allow for improved targeting of
these engineered cells, resulting in enhanced efficacy. We can explore this topic using both experiments on engineered recombinant protein and physiologically relevant endothelial cell surfaces, along with computer simulations.

**Specific Aim 1: Determine the state diagram of CD4+ T cells interacting with various densities of ligand using an engineered surface with recombinant proteins.**

Previous theoretical work from our lab has suggested the presence of synergy between selectins and integrin ligands on the recruitment of leukocytes from the blood stream. This synergy implies that a reduction in the level of one of these ligands can be compensated for by an increase in the expression level of the other ligand (10). However, this has never been shown experimentally with primary cells. We hypothesized that this synergy would be visible in “state diagrams,” showing the type of adhesion being used by cells on a surface of defined composition and density. Thus, we determined the site density of E-selectin and ICAM-1 on polystyrene surfaces and then used video microscopy of interactions of primary CD4+ T cells to generate these state diagrams, which confirmed our theoretical predictions.

**Specific Aim 2: Use Adhesive Dynamics to understand the impact of multiple bonds in series on cell detachment.**

Bonds in series are important components of human immune biology as well as experimental techniques used to probe cell-surface interactions. Previous theoretical work has suggested that bonds in series have a linear reduction in apparent bond strength. However, this work was limited by the assumption of large numbers of bonds between the cell and the surface (11), when many attachment processes can be mediated by as few
as $O(10^0)$ bonds (12). Using Adhesive Dynamics allowed us to explore the low bond number regime, where stochastic effects dominate. We hypothesized that multiple bonds in series would show a linear reduction in apparent bond strength for both normal and shear forces. Thus, we simulated cells attached to a surface with varying numbers of linkages to determine the effect of the number of linkages in series. Our simulations also allowed us to alter the biophysical characteristics of the individual linkages to determine their effect on the system as a whole.

Specific Aim 3: Use Adhesive Dynamics to predict the effect of quantitative reductions of kindlin-3 on leukocyte adhesion and confirm those predictions experimentally.

The hematopoietic cell-specific integrin activator kindlin-3 is a key protein in the activation of leukocyte-expressed LFA-1 in response to chemokine stimulation (13). When kindlin-3 is absent or non-functional, a serious medical condition called Leukocyte Adhesion Deficiency Type III occurs where leukocytes are not able to adhere to the vascular wall (14). Our lab previously developed Integrated Signaling Adhesive Dynamics (ISAD), which allows for the simulation of intracellular signaling cascades during the leukocyte adhesion cascade (15). We hypothesized that ISAD simulations would be able to accurately and quantitatively predict the response of cells to reductions in the level of kindlin-3 in the cells. To test the predictions, we used an engineered cell line with reduced kindlin-3 levels and compared their response to a recombinant protein surface with wild type cells.
References


CHAPTER 2: Background

The immune system

The immune system is a complex network of many cell types and specialized structures dedicated to maintaining tissue homeostasis by responding to infections and tissue injury (1). Thus, the cells of the immune system maintain a constant surveillance of tissues throughout the body to search for antigens (2). If an antigen is found, the immune system must be able to respond rapidly, in order to prevent an infection from spreading significantly, and in a manner which does not further harm the surrounding healthy tissue. However, nonspecific responses to infection, such as the release of inflammatory cytokines, can result in further tissue damage, while more specific responses can take days to weeks to develop. In order to meet these competing demands between speed and specificity, the immune system has developed two distinct systems that act in concert to resolve the insult. The innate and adaptive immune systems each prioritize one of these conflicting needs regarding speed and specificity (1). The cells of both the innate and adaptive immune system descend from the same stem cells, termed “hematopoietic stem cells,” as shown in Figure 2.1.

The initial response to infection or tissue trauma is handled by the innate immune system. Many of the cells in this section of the immune system, such as neutrophils and macrophages, are from the granulocyte lineage. These cells rush to the site of infection and begin producing pro-inflammatory cytokines, which aim to minimize the spread of the infection by changing the surrounding environment to be less hospitable to bacteria and viruses. In addition, these immune cells can directly endocytose foreign particles and
Figure 2.1: Blood cell development from a hematopoietic stem cell (HSC). Important cytokines and growth factors that support development and survival of each kind of cell are shown in red. CMP: common myeloid progenitor, CLP: common lymphoid progenitor, MEP: megakaryocytes and erythroid cells, GM: granulocytes and macrophages, TNK: T and natural killer cells, BCP: B cell committed progenitor, MkP: megakaryocyte progenitor, EP: erythrocyte progenitor, MP: monocyte progenitor, GP: granulocyte progenitor, TCP: T cell progenitor, NKP: natural killer progenitor, NK:
natural killer: Reproduced with permission from (5), copyright Massachusetts Medical Society.
destroy them. However, if unchecked for long periods of time, the inflammation triggered by these granulocytic cells can lead to tissue damage and impaired healing, which is detrimental to the completion of the immune response (3, 4).

In order to minimize off-target effects, the adaptive immune system is used if the innate immune system is unable to fully clear the insult. This side of the immune system uses lymphoid cells, such as B and T cells, which have functionalities specific to a particular antigen. However, these cells are initially naïve and require time to be “activated” and start performing their effector functions. The functions can include causing the apoptosis of infected cells, marking pathogens for destruction, or immunomodulation after clearance of the antigen. This specificity allows for resolution of the infection so that the tissue can move on to healing. In addition, the adaptive immune system allows for the creation of “immunologic memory,” which allows for a faster and more forceful response to repeated insults (6).

T cell maturation

One of the important cell types in the adaptive immune system is the T cell. These cells start in the bone marrow as hematopoietic stem cells, which are the progenitors of all blood cells. Some of these stem cells are chosen to become thymic seeding progenitors (TSPs). The selected TSPs enter the thymus in order to begin the T cell maturation process. At this point, the TSPs are uncommitted and retain some ability to become most of the cells in the immune system (7–9). During the journey to become mature naïve T lymphocytes, they will migrate to different areas of the thymus which support specific maturation steps (10). The exact stage of maturation for these cells can
be determined by using surface expression of various molecules (11). Figure 2.2 highlights the important stages of T cell maturation.

The immature T cells at this point lack surface expression of CD4 and CD8, which will later become markers for the two major subsets of T cells. Thus, the cells are referred to as double negative (DN) cells. Typically, there are thought to be four major steps in DN maturation, labeled DN1-DN4, which also contain smaller subfractions (11). Starting as DN1 cells, the cells undergo significant proliferation as well as restriction of their lineage commitment (12). Next, the DN1 cells migrate to a different location in the thymus, where they receive signals to differentiate to DN2 thymocytes (10, 13). The cells begin the genetic rearrangement of T cell receptor (TCR) genes, thus beginning the DN3 stage (14, 15). A pro-survival signal via the new TCR will allow the cells to become DN4 cells (16).

The DN4 cells will upregulate the TCR co-stimulatory molecules CD4 and CD8, which causes the cell to become double positive (DP) (12). During the DP stage, there are additional genetic rearrangements to the TCR. The binding strength and specificity of the complete TCR is then tested against major histocompatibility complex (MHC) ligands. Cells that interact with the MHC at an intermediate affinity survive, while those which bind too strongly or too weakly are apoptosed. As the final step of maturation, the T cell then down regulates the expression of either CD4 or CD8, resulting in single positive naïve CD8+ or CD4+ T cells, respectively (17). These two cell types each have a different function in the immune response. CD4+ cells are generally responsible for modulating the
Figure 2.2: Overview of T cell maturation in the thymus. Important steps included (1) restriction of progenitor cells to the T cell lineage, (2) rearrangement of the TCR genetic loci, and (3) the single positive selection checkpoint. HSC: hematopoietic stem cell, TSP: thymic seeding progenitors, DN: double negative, DP: double positive, SP: single positive. Republished with permission of Annual Reviews, Inc. from (11); permission conveyed through Copyright Clearance Center, Inc.
activity of other immune cells, while CD8+ cells directly engage and kill infected or transformed cells (18).

**T cell activation**

Naïve T cells are not able to respond to an infection immediately. These cells must first be activated by an antigen presenting cell (APC) before the T cells can perform their effector functions. APCs take up peptide chains and proteins from the extracellular space via endocytosis and then process them into polypeptides around a dozen amino acids in length. The polypeptides are “loaded” onto the MHC molecules of the APC, thus becoming peptide-MHCs (pMHCs), and then presented on the surface of the APC in order to react with TCRs. In addition, the APC may change the expression of surface receptors and its migratory capacity as a result of finding antigenic peptide. This process is termed “activation,” although it should not be confused with T cell activation (19).

Activated APCs and naïve T cells are concentrated into secondary lymphoid organs, such as lymph nodes or Peyer’s patches, which allow for the naïve T cells to efficiently explore the space of pMHC complexes by migrating across many APCs. Once the T cell finds a pMHC that carries a peptide which strongly interacts with the TCR, the T cell stops and begins the process of T cell activation (20, 21).

T cell activation is a complex process which results in the clonal expansion of a single T cell and will only be briefly covered here. The TCR of the appropriate T cell binds to the pMHC and undergoes a conformational change to allow the recruitment of numerous intracellular signaling molecules. In parallel, a secondary stimulatory signal is passed to the naïve T cell through CD28 (20). Without both components of signaling, the
T cell will become anergic, which hinders activation in the future (22). If both signals are provided, a complex cascade of intracellular signaling occurs, which results in changes in the surface expression of receptors and the induction of proliferative capacity. The ability to proliferate allows the T cell to create many clones which can respond to the infection and increase the rate of the response. In addition, the newly activated T cell will also change its expression of certain genes in order to complete the activated phenotype (23). These activated cells can then exit the secondary lymphoid organ and home in on the site of infection. There, the T cell will perform its effector functions as needed. Once an immune response is no longer required, a majority of the T cell clones will undergo apoptosis. However, a small subset will remain in the body as long-lived “memory” cells. These memory cells will allow for a faster and more forceful response to infection if the same antigen is encountered again (24).

An important aspect of T cell activation is the partial activation of integrins on the cell surface. Integrins are key transmembrane proteins that connect the cytoskeleton to the extracellular matrix and are vital for T cell homing and migration. These integrins have different affinity states, which will be discussed in more detail later. Their activation to an increased affinity state allows the cell to spontaneously polarize in response to ligand binding, which facilitates the exit of the activated T cell from the secondary lymphoid organ (21, 25).

**The leukocyte adhesion cascade**

In order to perform effector functions or immune surveillance, cells must be able to exit the vasculature and enter the lymphatic or interstitial space. This is mechanically
difficult, as the cell must initially slow itself from the free stream velocity of the blood to a complete stop on the endothelium. Once this is completed, the cell must then resist the shear forces applied to it by the still flowing blood as the cell searches for an appropriate location to transmigrate. In addition, immune cells must be restricted from entering locations where there is no infection or immune surveillance functions to be performed, as this could result in autoimmune diseases. Thus, there are contradictory needs for a system to be adhesive enough to allow for extravasation but slippery enough to prevent non-specific adhesion (26, 27). In order to meet this challenge, nature has evolved the leukocyte adhesion cascade, a series of interdependent steps which allows for precise control over the location of leukocyte trafficking. Traditionally, this cascade is subdivided into 4 steps: initial capture, rolling, firm arrest, and transmigration. Each of the steps is dependent on the steps before it. In addition, this cascade depends on the proper expression of different adhesive molecules on both the endothelium and the leukocyte to reduce non-specific binding (2). The leukocyte adhesion cascade is outlined in Figure 2.3.

The first step in the cascade is initial capture, where the free-flowing leukocyte first interacts with the endothelial layer. Selectins, such as P- or E-selectin (28), can interact with various surface receptors on the leukocyte, including PSGL-1 (29), CD44, or ESL-1 (30). In addition, the leukocyte itself is coated with L-selectin, which can interact with proteins on the endothelial cell (28, 31). In all cases, the association of selectin and receptor is mediated by sialyl-Lewis X (sLeX), which is a carbohydrate-based motif found on these cells. Without this structure, the selectins are unable to associate
Figure 2.3: Diagram of the leukocyte adhesion cascade. Leukocyte-borne receptors are in red and endothelial cell ligands are in blue. PSGL-1: P-selectin glycoprotein ligand-1, LFA-1: leukocyte function-associated antigen-1, ICAM-1: intercellular adhesion molecule-1. Adapted and reprinted from (2) with permission from Springer-Nature.
with their receptors and the adhesion cascade cannot continue. Thus, the body can control the location of leukocyte extravasation by controlling the location of selectin expression on the endothelium (32). Selectin binding is controlled by fast intrinsic on and off rates, as required for efficient interaction between the ligand and receptor due to the minimal interaction time caused by the convective motion of the cell (33, 34). Selectin binding is also a “catch-slip” bond, meaning the off rate of the complex is dependent on force. Under zero stress, the off rate is some intrinsic value with some bond lifetime. Increasing the force of the bond by stretching it counterintuitively decreases the off rate, resulting in increased bond lifetimes. However, at some critical force, the increase in bond stress starts to cause the off rate to increase, leading to bonds which break more quickly under larger forces (35, 36). These interactions help to bring the cell closer to the surface, where additional selectin molecules can exert additional force on the cell (29). As the cell approaches the endothelium, additional selectin interactions help to continually slow the cell and ensure that the cell maintains continuous contact with the blood vessel wall (37). These rolling interactions can trigger intracellular signaling cascades which can impact the later steps of the adhesion cascade (38–40).

Activated endothelial cells will also express integrin-activating chemokines, such as CXCL12 (also known as SDF-1α), and cell adhesion molecules, such as intercellular cell adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) (41, 42). The presence of chemokines on the surface of endothelial cells begins an intracellular signaling cascade in the leukocyte, which results in the activation of integrins on the leukocyte surface (43, 44). The integrins on the surface move from a
bent, inactive conformation to an open, extended, highly active conformation that allows for binding to appropriate ligands (45). The pathway of this activation will be discussed in additional detail later. On T cells, the two main integrin receptors on the cell surface are leukocyte function associated antigen-1 (LFA-1, $\alpha_L\beta_2$) and very late antigen-4 (VLA-4, $\alpha_4\beta_1$). LFA-1 binds to ICAM-1, while VLA-4 binds to VCAM-1 (46). Integrin bonds, similar to selectins, exhibit a catch-slip bond behavior, although the base off rate is lower and the critical force is higher (47). Once the leukocyte has formed enough integrin-ligand bonds to resist the applied shear force, it stops rolling and arrests on the endothelium (48).

After the cells stop, they spread and migrate in order to find a suitable location for transmigration (2). Cellular migration is a complex process, requiring coordinated adhesion and deadhesion in order to remain attached to the surface at all times. In a typical T cell migrating on ICAM-1, the front of the cell forms a broad, thin, actin rich sheet termed the lamellipodium. This structure includes actively polymerizing actin, which allows for the extension to new locations. LFA-1 in the lamellipodium binds to the surface and is gradually moved to the back of the cell by the motion of the cell body. Most of the surface bound LFA-1 on T cells is concentrated towards the rear of the cell, providing a firm base for the lamellipodium to extend and move the cell forward. The rear of the cell, called the uropod, causes the detachment of the bound LFA-1 and also acts as a storehouse for LFA-1 which has been internalized after its deadhesion. The internalized LFA-1 is then recycled to the front of the T cell to allow migration to continue through the formation of new adhesive contacts (49–52). Since the LFA-1
stored in the uropod is not attached to the surface, the uropod typically comes off of the surface and “floats” above the rest of the cell body (53). In addition, the cell can decide to migrate in a preferred direction in response to cues from its environment, resulting in directional migration. One type of directional cue which is undoubtably present in the vasculature is shear forces caused by the flowing blood, which can direct the migration of T cells. Previous work has shown that T cells migrate against the direction of flow, resulting in the net migration upstream (54, 55). It has been suggested that the uropod is the controlling structure in determining the direction of migration for these cells (53). Although this behavior has been shown in vivo (56), the exact biological reason for this response remains unknown.

Once the T cell finds a suitable location on the endothelium, it begins the process of transmigration. This is a highly complex and regulated process whereby the leukocyte disrupts the endothelial layer in order to access the interstitial space. This process requires a large amount of coordination between the transmigrating leukocyte and the endothelial cell (57, 58). A full explanation of the directional communication between cells is beyond the scope of this introduction. A leukocyte can transmigrate in two ways: transcellularly or paracellularly. Most transmigration is through the paracellular route, which occurs when a cell transmigrates through the junction of two or more endothelial cells (58). Transcellular migration happens when an endothelial cell, in concert with the leukocyte, constructs a “tunnel” through the individual endothelial cell (59). Although most transmigrating cells prefer the paracellular route, the molecular interactions controlling the choice between the two modes is still the subject of much research (60).
Integrin activation

Control of the activation state of integrins is critical to both an effective immune response as well as prevention of unnecessary immune reactions. Integrin activation can be controlled through two pathways, which differ in the initial location of the activating signal. “Inside-out” signaling requires the use of intracellular signaling cascades, while “outside-in” signaling causes integrin activation based on external ligand binding and application of force. The “inside-out” pathway is the focus of this section, as it is the more important pathway in the leukocyte adhesion cascade (61). This pathway is shown in Figure 2.4.

Integrin activation during the leukocyte adhesion cascade begins with the binding of chemokines on the endothelial cell surface to chemokine receptors on the leukocyte. Chemokines are a family of small signaling proteins secreted by multiple types of cells. These chemokines can be pro- or anti-inflammatory, as well as homeostatic. The wide variety of chemokines found in humans have been grouped into four main families, CXC, CC, CX3C, and XC, which are organized based on the amino acid sequence of the chemokine. Despite the large numbers, all chemokines signal through G-protein-coupled receptors (GPCR) on the immune cell surface. Each receptor has a repertoire of chemokines that it will bind to, with some being highly specific and others more promiscuous (62). However, upon binding to an appropriate chemokine, all chemokine receptors transmit the binding signal in a remarkably similar way (63). GPCRs are transmembrane receptors with a structure that crosses the plasma membrane seven times. The receptor protein, upon ligand binding, activates a trimeric G protein consisting of an
α, β, and γ subunit. The activation of the G protein causes the α subunit to release the currently bound GDP and exchange it for GTP. The GDP-GTP exchange causes a significant conformational change in the α subunit. This change can cause the α and the βγ subunits to dissociate, or in certain cases, allow the subunits to remain bound but reveal cryptic binding sites to allow for binding to their targets (64). After a short time, the GTPase activity of the α subunit hydrolyzes the bound GTP to GDP, greatly decreasing the activity of the subunit. The three subunits can then rebind and are ready for the next signal from the receptor (65).

Activated G proteins can then activate a number of proteins inside the cell. For the purposes of integrin activation, the two most important are phospholipase C (PLC) and a family of proteins called regulator of G protein signaling (RGSs). PLC is a constitutively membrane-bound protein which becomes activated by GTP-bound G proteins (66, 67). Once activated, PLC cleaves the membrane phospholipid phosphatidylinositol 4,5-bisphosphate (PIP2) to inositol trisphosphate (IP3) and diacylglycerol (DAG). IP3 is a freely diffusing small molecule which binds to receptors on the endoplasmic reticulum, leading to Ca2+ release from intracellular stores. IP3 is rapidly degraded to IP2 or IP4 to prevent further calcium release (68, 69). DAG remains in the plasma membrane, where it can activate molecules such as protein kinase C (PKC) by serving as a localization signal. The activity of PKC is increased when both Ca2+ and DAG are present, causing the activation of additional downstream molecules (70). Over time, DAG is degraded by the diacylglycerol kinase (DGK) family of proteins, reducing pro-adhesion signaling in the cell (71). To further reduce the amount of signaling
activating the cell, the GRSs in the cell phosphorylate the GPCRs in order to prevent additional G protein activation events (72).

One of the targets of PKC activation is the GTPase Rap1. GTPases are a family of small proteins found in the cytosol of all eukaryotic cells. These proteins can bind GTP or GDP and are active when bound to GTP. They possess an intrinsic GTPase activity, which causes bound GTP to slowly be converted to GDP. This process can be accelerated by interactions with GTPase-activating proteins. However, GTPases cannot convert GDP to GTP. In order to exchange the nucleotide, GTP exchange factors (GEF), which are also proteins, are required. Thus, the activated PKC in turn activates a GEF of Rap1, which exchanges the bound GDP for GTP (70, 73, 74). Once Rap1 is activated, it can be recruited to the plasma membrane. Once there, Rap1 interacts with Rap1-GTP-interacting adapter molecule (RIAM), which will lead to integrin activation (75).

In the inactive, resting form, integrins such as LFA-1 are bent over so the ligand binding pocket is close to the plasma membrane (76). In addition, the cytoplasmic tails of the α and β subunits are in close proximity, creating a salt bridge between the two (77). Talin, as an integrin activator, initially disrupts this close association of the cytoplasmic tails, which moves the complex into an intermediate affinity form of the integrin. However, talin requires activation of Rap1 and its association with RIAM before it is able to perform this function (75, 78, 79). When Rap1 is inactive, the rod-shaped talin is maintained as an anti-parallel, autoinhibitory dimer in the cytosol (80, 81). The intermediate affinity integrin has a ligand binding site that is further from the cell surface compared to the inactive form. However, the ligand binding site is still prevented from
being fully open, preventing ligand from binding easily. In order to move the integrin to the final highly active form, the integrin-talin complex requires the addition of an additional integrin activator of the kindlin family of proteins. In T cells, this protein is the hematopoietic cell-specific protein kindlin-3, also known as FERMT3. Kindlin-3 binds only after talin is already bound to the integrin (82). Upon its binding, the cytoplasmic tails of the integrin move even further apart. This lifts the heads of the integrin even further from the cell surface and fully unmarks the ligand binding site (83). In addition, kindlin-3 also plays a role in the post-binding clustering of integrins, which increases the apparent affinity of the cell for ligand and thus increases binding strength (84). This transition between intermediate and high affinity states is also enhanced by the application of force to the integrin (50). All of these events, in addition to changing the affinity of the integrin for ligand, also change the response of the integrin-ligand bond to force. Each of the steps increases the catch-slip nature of the bond, helping cells to resist shear forces applied by the blood (47).

**Leukocyte Adhesion Deficiencies**

A family of immune disorders termed Leukocyte Adhesion Deficiencies (LAD) is caused by mutations which prevent the function of the leukocyte adhesion cascade. Since each step of the cascade is dependent on the steps preceding it, the failure of one step can doom the ability of the cell to arrest on endothelium. There are three established LADs, each of which impacts a single protein in the cascade (85). There is also growing acceptance of a fourth LAD in the literature (86). Below is a brief summary of each LAD.

LAD I was the first LAD to be described in the literature. It is caused by the
mutation or deletion of the ITGB2 gene, which encodes the β2 subunit (also known as CD18) of LFA-1. Initially, the disorder was characterized in patient samples completely lacking the β2 subunit. However, other studies later showed a similar phenotype with in patient samples with quantitatively normal levels of CD18 which were functionally defective (87, 88). Without this integrin, leukocytes are unable to arrest on the endothelium, as they cannot create adhesions strong enough to resist the applied shear forces by creating integrin-ligand bonds (89). Clinical symptoms of LAD I include leukocytosis (increase in the number of circulating leukocytes), recurrent infections in the skin and mucosal surfaces, absent pus formation in response to infection, impaired wound healing via chronic inflammation (90, 91), periodontitis (92), and even necrosis (93). Clinically, the severity of the disorder appears to be correlated with the expression levels of the β2 subunit, assuming it is able to function normally. Patients with less than 2% of normal expression levels of CD18 are categorized has having “severe deficiency” and typically die in infancy if not treated with a bone marrow transplant. Patients with higher levels of expression (2-30% of normal) have fewer and less severe infections and can survive into adulthood without treatment, suggesting an improvement in their infection-fighting ability (94).

LAD II is caused by the mutation of the SLC53C1 gene, which encodes a fucose transporter in the Golgi membrane. Without this transporter, cells are unable to synthesize the sLe^x structures required for the initial selectin binding (95, 96). Since the reaction rate of integrins with their ligands is significantly slower than that of selectins, the rapid convective motion in the blood vessel prevents the formation of integrin-ligand
bonds. Thus, the cell again cannot stop and extravasate in order to reach sites of infection or trauma, even though these cells are able to migrate normally. This restricts leukocyte extravasation to sites with low shear stresses, which allows for some innate immune surveillance. Patients with LAD II are reported to have less severe and fewer infections than patients suffering from severe LAD I, although they have many of the same clinical manifestations (97). Interestingly, many patients with LAD II show a rapid decrease in the frequency of infections as the adaptive immune system matures. LAD II also has an associated metabolic dysregulation component, which results in developmental delays and musculo-skeletal abnormalities (96).

LAD III, like the previous examples, involves the mutation of a gene, in this case *FERMT3*, which encodes the kindlin-3 protein (98). As discussed previously, kindlin-3 is imperative for the strengthening of integrin-ligand adhesive bonds. Since LFA-1 is no longer able to transition to the high affinity form or to cluster after binding, the intermediate affinity form of the integrin is the strongest bond that can form. This is unable to allow the cells to extravasate, as the shear forces blow cells off of the endothelium (99). In addition, kindlin-3 has been shown to be required for the activation of integrins on platelets (100, 101), with the result that LAD III has a life-threatening bleeding disorder along with the immune deficiencies of the LAD family (98). LAD III has also been shown to lead to an osteoporosis-like condition due to defects in osteoclast adhesion (102).

The most commonly cited LAD IV occurs in patients with cystic fibrosis. Interestingly, the mutation in the cystic fibrosis transmembrane conductance regulator
which causes the disease can cause adhesion deficiencies in monocytes, but not neutrophils or lymphocytes. The defect results in an inability for the integrins to activate, although the exact molecular mechanisms are not understood (86, 103).

Each of the established LADs affects only a single protein, yet the failure of this individual protein results in the failure of the entire cascade. This allows us the opportunity to gain insights into leukocyte adhesion biology by studying the similarities and differences between them. In addition, a quantitative understanding of how these LADs impact the leukocyte adhesion cascade will only increase the impact of this area of research.

**Adhesive Dynamics**

Adhesive Dynamics is a modeling framework first published by Hammer and Apte in 1992, who used it to study the influence of bond properties on cellular motion. The general form of the Adhesive Dynamics algorithm is as follows. First, bonds are created or destroyed as needed. This could occur from a combination of the translational and rotational motion of the cell changing whether bonding points are close enough to the surface to react, or also from the stretching of a bond increasing its likelihood of failure. Once the bonds have been updated appropriately, the forces and torques from the bonds, along with nonspecific forces like shear and van der Waals forces, are summed to determine the net force acting on the cell. Using the net force, together with the equations of motion governing the motion of a sphere near a wall, the velocity matrix can be constructed. Finally, using the velocity matrix, it is trivial to update the location and orientation of the cell according to established mathematical principles. A schematic of

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an adhesive dynamics simulation is shown in Figure 2.5. The adhesive dynamics framework allowed for the accurate simulation of cells stably rolling on a surface of ligand, which in turn suggested that the main factor controlling the motion of cells attached to a surface was the biophysical properties of the bonds themselves. Thus, by accurately simulating the way the bonds form, stretch, and break, Hammer and Apte could accurately simulate the motion of a cell (104).

This algorithm has proven to be quite powerful, as it has been used to study a wide variety of phenomena. For example, Adhesive Dynamics has been used to predict the behavior of functionalized beads rolling and stopping on surfaces (105), recapitulate the shear threshold effect of leukocytes (106), predict the interactions of multiple rolling cells (107), as well as the binding of an HIV particle to a CD4+ T cell (108). The algorithm has been improved from the initial formulation to account for the effect of convection on the rates of reaction of the receptors on the cell surface (109). Additional work has included the addition of deformable microvilli on the surface of the cell (110), along with the inclusion of deterministic (111) and stochastic signaling cascades (71, 112, 113). Possibly the most important update to the base Adhesive Dynamics model has been the integration of signaling into adhesive dynamics, as first done by Beste and coworkers (113). This technique, termed Integrated Signaling Adhesive Dynamics (ISAD), allows for the cell to change its receptor expression profile in response to chemical stimulation. ISAD combines the exact physical model of cell rolling of adhesive dynamics with the next subvolume method, which allows for stochastic and spatial simulation of the intracellular signaling cascade. In the simulation, the cytosol of the cell
Figure 2.5: Diagram of Adhesive Dynamics simulation. The cell is modeled as a hard sphere studded with receptors. Receptors bind and unbind stochastically to a ligand-coated surface. Bound receptors exert translational forces and torques on the cell body. These forces are summed and used to update the position and orientation of the cell, restarting the simulation loop. Reprinted from (104) with permission from Elsevier.
is divided into a large number of smaller subvolumes, which are then populated with proteins in an intracellular signaling cascade. Each subvolume is assumed to be a well-mixed reactor so that there is no spatial inhomogeneity of concentration within a single compartment. However, protein species are allowed to diffuse between adjacent subvolumes, creating concentration gradients over larger distances within the cell. Finally, protein species can be restricted to certain compartments, such as binding to the plasma membrane, in a biologically relevant way (113).

In the case of a rolling leukocyte, the simulation can start with low affinity integrins that are shifted to higher affinity levels after the cell is exposed to chemokine using the signaling cascade in Figure 2.6. Using this technique, Beste and coworkers developed an ISAD model which could accurately recapitulate the dynamics of rolling and adhesion of the Jurkat T cell line. They were then able to explore the effect of changes to the system in order to predict the effect on the ability of the cells to adhere. First, they found that increasing concentrations of chemokine on the surface would result in faster stopping of cells due to increasing activation of the signaling cascade. In addition to altering chemokine density, they altered the expression levels of selectin, chemokine, and adhesion receptors on the surface of the cell and studied both the time to arrest and the distance to stop under these different receptor profiles. They found that the distance to stop was highly sensitive to the expression level of the selectin receptor, but this did not change the time to stop the cells in a significant way. In contrast, altering either chemokine or adhesion receptor expression resulted in no significant change in the distance to stop. Decreasing expression of these receptors led to increases in the time to
Figure 2.6: Intracellular signaling cascade used in ISAD simulations. Reprinted with permission from (113). Copyright 2012 American Chemical Society.
stop. Finally, Beste and coworkers found that when multiple chemokine receptors acted through a single G protein, the total receptor occupancy is the controlling factor governing adhesion of cells to surfaces. More selective control of lymphocyte adhesion could then be obtained through the use of multiple cross-reactive low affinity chemokines (113).

Later, Lee and coworkers used ISAD to investigate the role of the intracellular signaling modulator diacylglycerol kinase ζ (DGKζ) on T lymphocyte adhesion. DGKζ converts the important intracellular second messenger diacylglycerol (DAG) to the physiologically inactive phosphatidic acid (PA). This conversion, in turn, reduces the driving force for LFA-1 activation by lowering the level of Rap1 GEF activity. Their simulations showed that removing DGKζ from the cell would result in an increase in intracellular DAG concentration, which would in turn cause lymphocytes to arrest faster on a surface. These simulation results were then shown experimentally, using DGKζ KO murine lymphocytes. The KO cells showed a significant decrease in both the time and distance to stop and no change in the rolling velocity, as was predicted by the ISAD model. Together, these results highlight the predictive potential of ISAD simulations regarding mutations in the integrin activation cascade (71).
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CHAPTER 3: The State Diagram of T Cell Adhesion


Abstract

The leukocyte adhesion cascade is important for the maintenance of homeostasis and the ability of immune cells to access sites of infection and inflammation. Despite much work identifying the molecular components of the cascade, and numerous simulations to predict the relationship between molecule density, identity, and adhesion, these relationships have not been measured experimentally. Using surfaces functionalized with recombinant ICAM-1 and/or E-selectin along with immobilized SDF-1α, we used a flow chamber to measure rates of tethering, rolling and arrest of primary naïve human CD4+ T lymphocytes on different surface densities of ligand. Cells required a minimum level of ligand density to progress beyond tethering. E-selectin and ICAM-1 were found to have a synergistic relationship in promoting cell arrest. Surfaces with both ligands had the highest levels of arrest, while surfaces containing only E-selectin hindered the cell’s ability to progress beyond rolling. In contrast, surfaces of ICAM-1 allowed only tethering or arrest. Cells maintained constant rolling velocity and time to stop over large variations in surface density and composition. In addition, surface densities of only $O(10^1)$ sites/µm² allowed for rolling while surface densities of $O(10^2)$ sites/µm² promoted arrest,
approximately equal to previously determined simulated values. We have systematically and experimentally mapped out the state diagram of T-cell adhesion under flow, directly demonstrating the quantitative requirements for each dynamic state of adhesion, and showing how multiple adhesion molecules can act in synergy to secure arrest.
Introduction

T cells must adhere in the vasculature to traffic into the lymphatics to perform immune surveillance and maintain homeostasis (1). In addition, T cells are often called upon to exit the bloodstream to enter the interstitial space in order to perform effector functions at sites of inflammation and injury (2). T cell adhesion is thought to follow the canonical leukocyte adhesion cascade where cells progress from tethering to rolling to firm adhesion on the endothelium as a prerequisite for transmigration (2, 3). During tethering, cells within reactive distance of the endothelial layer begin to interact with P- and E-selectins, which begin to slow the cell from the free stream velocity and encourage closer contact between the cell and the endothelium (4). Specifically, E-selectins on the endothelial layer can interact with a number of receptors on the leukocyte, including PSGL-1, ESL-1, and CD44 (5). During rolling, the cell forms and breaks multiple bonds with the P- and E-selectins and also partially activated integrins with their respective ligands (6, 7). As the cell translates across the surface using these relatively weak adhesions, chemokine receptors on the cell scan the surface for the presence of activating chemokines (8). Upon chemokine receptor binding, the β2 integrins on the T cell, such as LFA-1, become fully activated and able to rapidly and strongly bind their cognate ligands, such as ICAM-1 (9–12). The integrin-ligand interaction causes the firm arrest and activation of the T cell, which can then crawl (13). Finally, transmigration occurs after the cell has found an appropriate location between endothelial cells to enter the lymphatics or interstitial space (2, 3).
Despite extensive study on the individual molecular components of the adhesion cascade (14–17), there have been few experiments to measure how the combination of selectins, integrins, and chemokine affect the dynamics of tethering, rolling and firm arrest (18). Extensive computer simulations (19–22) using Adhesive Dynamics and other techniques have been performed to predict the interrelationships between molecular type, density and the dynamics of adhesion. These results have been rendered into state diagrams, a consolidated representation in which one can map densities to dynamic states of adhesion (19, 23). An interesting prediction of Adhesive Dynamics is that there would be a synergy between selectins and integrins in securing firm arrest (19). The idea is that by slowing down a cell, selectins enable slow reacting integrins to secure firm binding. This synergy was borne out in experiments using cell-free systems, combining selectin mediated-rolling and antibody-mediated firm arrest (24).

Despite these predictions, there have been no reports an experimentally determined state diagram of adhesion, in which one has measured the effect of changing ligand density type or amount on the type of adhesion observed experimentally. Thus, this paper presents an experimentally determined state diagram of CD4+ T cells interacting with a surface containing recombinant E-selectin and ICAM-1, with the addition of the chemokine SDF-1α, as shown in Figure 3.1.
Figure 3.1: Schematic representation of T cell dynamic adhesion with a surface containing recombinant ICAM-1/Fc and E-selectin/Fc chimeras with SDF-1α.
Materials and methods

Adsorption of Protein A/G and SDF-1α

Non-tissue culture treated polystyrene petri dishes (Corning, Corning, NY) were enclosed using a single well FlexiPerm gasket (Sigma-Aldrich, St. Loius, MO). A solution of 2 µg mL⁻¹ of Protein A/G (Themo-Fisher Scientific, Waltham, MA) and 1 µg mL⁻¹ of SDF-1α (R&D Systems, Minneapolis, MN) was applied and incubated overnight at 4°C. The surfaces were then washed three times with PBS before a 30 minute incubation of a 0.2% w/v solution of Pluronic F-127 (Sigma-Aldrich). The surfaces were washed again three times with PBS. Next, a solution of ICAM-1/Fc and/or E-selectin/Fc chimera (R&D Systems) was applied to the surface and incubated for three hours at room temperature. The completed surfaces were then washed again three times with PBS before use. For experimentally tested points, molar ratios of 1:0, 10:1, 1:1, 1:10, and 0:1 E-selectin/Fc:ICAM-1/Fc were chosen to highlight differences between similar surfaces. In addition, total protein concentrations were chosen to yield total site densities of 4-5, 35-45, 130-160, 310-360, 550-610, and 1200-1300 sites/µm².

CD4⁺ T Cells

Purified primary human CD4⁺ T cells from anonymized donors were obtained from the University of Pennsylvania Human Immunology Core. Cells were resuspended in RPMI-1640 media supplemented with 0.1% BSA and 2 mg mL⁻¹ of glucose and used immediately.

Flow Chamber Assays

Flow chamber experiments were performed in a circular parallel plate flow
chamber (GlycoTech, Gaithersburg, MD) with a gasket width of 0.25 cm, thickness of 127 µm, and length of 2 cm. Before flow chamber experiments, a functionalized dish was washed with prewarmed PBS and media to remove air bubbles in the flow path. The assembled flow chamber was mounted on an inverted Axiovert 200 (Carl Zeiss, Gottigen, Germany) enclosed by a XL-3 microscope incubator (PeCon, Ulm, Germany). All experiments were performed at 37°C. T cells were suspended at a concentration of 5 x 10^5 mL^-1 and were perfused into the flow chamber via syringe pump (Harvard Apparatus, Holliston, MA) at a flow rate corresponding to a calculated wall shear rate of 100 s^-1. Rolling and adhesion of T cells on the immobilized ICAM-1 and E-selectin was observed via phase contrast microscopy under a 10X objective (NA = 0.2, Type A-Plan, Carl Zeiss). All points were tested twice on three different days with different anonymized donors and the results averaged.

Data Acquisition and Cell Tracking

A CCD camera (QImaging, Surrey, BC, Canada) was used to monitor T cell adhesion events with adhesive E-selectin/ICAM-1/SDF-1α substrates. Adhesion of T cells was recorded on DVD+RW discs for cell tracking analyses. Cell adhesion videos were redigitized to 640×480 pixels at 29.97 frames s^-1 and deinterlaced with HandBrake software (http://handbrake.fr/) then converted to image stacks with MATLAB (MathWorks, Natick, MA). The stacked images were thresholded and converted to binary images. The coordinates of the centroid of interacting T lymphocytes with a surface every video frame were then acquired using the MTrack2 plugin.

**Analysis of Cellular Adhesion**

MATLAB was used to analyze the tracked centroids obtained from the MTrack2 plugin. State diagrams were created in MATLAB using the Curve Fitting Toolbox. Surfaces were fit to experimental points with linear interpolation between points. Rolling was defined as the movement of an interacting cell over the substrate at an instantaneous velocity in any frame less than 20% of the calculated free stream velocity of a noninteracting cell\(^{(25)}\) while remaining in the field of view. Cell stopping was defined as reduction of its instantaneous velocity below 5% of the free stream velocity. Adherent T lymphocytes were further classified as tethering, rolling or firmly arrested cells based on their instantaneous velocities and the duration of their stopping. A tethering cell is defined as a cell that “rolls” less than 30 video frames (1 s) in a rolling period within the field of view. A firmly arrested cell is defined as a cell that “stops” stably more than 90 frames (3 s). Time and distance to stop were both calculated based on the initial time and location of the cell rolling. Values for figures were calculated based on cells that interacted with the surface. Cells which were tracked but did not interact with the surface were excluded from the analysis. A single flow chamber experiment resulted in 200-250 cells interacting with the surface on surfaces containing \(O(10^2)\) or more sites/\(\mu m^2\) to around 10 cells on surfaces containing \(O(10^0)\) sites/\(\mu m^2\). The number of cells interacting with the surface was also somewhat dependent on surface composition. To highlight
differences in relative rates of adhesion, data are presented as fraction of cells undergoing a specific kind of motion.

**Determination of Site Densities**

A solution of 2 µg mL⁻¹ of Protein A/G (Thermo-Fisher Scientific) and 1 µg mL⁻¹ of SDF-1α (R&D Systems) was added to the wells of a non-tissue culture treated 96 well polystyrene plate (Corning). This solution was allowed to coat the wells overnight at 4°C. The wells were then washed three times with PBS before blocking with 0.2% Pluronic F127 (Sigma-Aldrich) for 30 minutes. The wells were again washed three times with PBS and then serial dilutions of human IgG1 (Invitrogen, Carlsbad, CA) were added and allowed to incubate for 3 hours. Wells were washed three times with PBS and remaining binding sites on Protein A/G were blocked using 50 µg mL⁻¹ murine IgG1 (Invitrogen) for two hours at 37°C. Next, an AlexaFluor 488-tagged anti-human IgG hinge antibody (Southern Biotech, Birmingham, AL) at 2.5 µg mL⁻¹ was added to the wells and allowed to bind for one hour at RT in the dark. After the incubation, wells were washed three times with PBS. Wells were then filled with PBS and fluorescence compared to dilutions of fresh AlexFluor-tagged antibody using a Tecan M200 Infinite plate reader. Fluorescence readings from the IgG1 treated wells were converted to an equivalent concentration and then into number of molecules per well, assuming two AlexaFluor 488 antibodies per IgG1 molecule, due to the two hinge regions available for binding and molar excess of antibody. The resulting number concentration was converted to a surface density by dividing by the wetted area of the well. This was performed on three different days and the results averaged. To determine site densities of the experimental conditions,
the experimentally determined site densities were fitted to a four parameter logistic regression using R (R Core Team) and the drc package. Site densities are reported as monomer molecules per $\mu$m$^2$ in order to facilitate comparison to previously reported studies.
Results

Determination of Site Densities

As described in the Materials and Methods section, we determined the site densities of surfaces using an isotype-matched IgG1 as a probe. The results of these experiments are shown in the Appendix as Figure A.1. When using chimeric molecules, it was assumed that the chimeras had the same binding affinity with Protein A/G as the IgG1. In the case of mixtures of two different chimeras, it was assumed that they partitioned onto the surface at the same ratio as their molar ratio in the bulk. To confirm this assumption, diffusivities for each of the chimeras were calculated according to the procedure in Young, et al (26), which showed a difference in diffusivities of only 5%. In addition, since both chimeras utilize the same IgG base, we assumed that the resulting KDs were the same. When plotting the state diagrams, the reported densities are derived from these calibration curves. This system allows us to study E-selectin concentrations above and below levels provided by stimulated HUVECs (100-150 sites/µm²) (27). In addition, the system can reach levels roughly equivalent to activated HUVEC expression of ICAM-1 (1000-2000 sites/µm²) (28–30).

Flow chamber adhesion experiments

We conducted a number of flow chamber experiments using surfaces functionalized with ICAM-1, E-selectin, and SDF-1α. These surfaces allowed for visualization of three states of T cell adhesion - tethering, rolling, and firm arrest. Because we could specifically vary the density and composition of the surfaces, we could probe the effects of different surface densities on the types of adhesion observed. For
each combination of densities of ICAM-1 and E-selectin, along with a fixed concentration of SDF-1α, we mapped the fraction of cells displaying each kind of motion, as shown in Figure 3.2. Surfaces containing ICAM-1 but lacking SDF-1α did not show significant levels of arrest, with fewer than 7% of cells arresting on these surfaces (data not shown). The presence or absence of SDF-1α did not change the cellular response to surfaces containing only E-selectin (data not shown). An additional set of experiments were carried out at a much higher ligand density, but these did not show any significant change from the points presented here (Figure A.2 in the Appendix).

*Tethering*

Surfaces presenting low densities of molecules (O(10^0) sites/µm²) allowed only tethering. Overall, the rate of tethering decreased as surfaces contained increasing surface densities of ligand. In addition, surfaces containing only ICAM-1 showed higher rates of tethering at all densities compared to surfaces with similar levels of ICAM-1 paired with E-selectin.

*Rolling*

Rolling required a surface density of O(10^1) sites/µm². Surfaces displaying only E-selectin exhibited the highest levels of rolling at all surface densities, due to a decrease in the level of firm arrest. T cells proved to be largely unable to roll on surfaces presenting only ICAM-1, with less than 5% of cells rolling during their interaction with the surface, compared to 40-50% of cells on surfaces with E-selectin.

* Arrest*

Surfaces containing both ICAM-1 and E-selectin at sufficient densities (O(10^2))
Figure 3.2: State diagrams of tethering, rolling, and firm arrest. Fraction of CD4+ T cells undergoing (a) tethering, (b) rolling, or (c) firm arrest. Red dots indicate experimentally tested points. All experiments were performed at a calculated wall shear rate of 100 s⁻¹.
sites/µm²) were found to be the most efficient at allowing cells to firmly adhere. Surfaces containing an equal density of ICAM-1 and E-selectin as well as surfaces with a 10-fold higher density of ICAM-1 compared to E-selectin were the most efficient at causing the arrest of cells. Surfaces containing a 10-fold higher density of E-selectin compared to ICAM-1 had a modestly lower level of arrest, with a concomitant increase in the level of rolling on these surfaces. Surfaces containing only E-selectin did not support robust levels of arrest. In addition, when cells did arrest, surfaces containing only E-selectin did not support significant levels of cell spreading in comparison to surfaces that also contained ICAM-1, as shown in Figure A.3 in the Appendix.

**Synergy of arrest**

As seen in the state diagrams, reductions in the level of one of the ligands could be rescued by an increase in the level of the other ligand. This indicates the presence of synergy between the molecules. Surfaces with more E-selectin than ICAM-1 showed slight reductions in the level of firm arrest, but increases in ICAM-1 density fully rescued reductions in the level of E-selectin. However, fully removing one of the ligands reduced the level of firm adhesion on the surface, regardless of the remaining ligand density.

**Rolling velocity**

In addition to types of adhesion, we measured the rolling velocity and time to stop, as shown in Figure 3.3. Surprisingly, the rolling velocity of the naïve CD4⁺ T cells was nearly constant across large changes in surface density and surface composition. The two exceptions were on surfaces containing very low surface densities of molecules, where cells rolled much faster, and on surfaces consisting of only ICAM-1, where the
Figure 3.3: Calculated surfaces of rolling and time to stop. Calculated surfaces showing the effect of ICAM-1 and E-selectin densities on (a) rolling velocity and (b) time to stop. Red dots indicate experimentally tested points.
very few cells rolled (typically fewer than five cells per flow chamber). These few cells had a lower rolling velocity compared to other cells, but not enough cells were in this category to make a definitive determination.

*Time to stop*

A metric of the strength of arrest is the time to stop for rolling cells to stop – that is, how long they roll until arrest. Cells which did not stop were excluded from this metric. Cells on surfaces containing only E-selectin rolled a very long time before they stopped, if at all. However, as the amount of ICAM-1 on the surface increased, the time for cells to stop showed a concomitant decrease. The shortest time to stop occurred on surfaces containing only ICAM-1, on which cells stopped extremely rapidly when compared to other surfaces. An additional metric, distance to stop (a convolution of rolling velocity and time to stop), is shown in Figure A.4 in the Appendix.
Discussion and conclusions

In this work, we present the first experimentally determined state diagram for the adhesion of naïve CD4+ T cells on varying densities of ICAM-1 and E-selectin.

Synergy

Our results demonstrate the presence of a synergy between ICAM-1 and E-selectin, as predicted by simulation (19), as both ligands were required for the highest levels of arrest on the surfaces. In addition, reductions in the level of one ligand were compensated by increases in the density of the other, thus maintaining an overall constant density. In contrast, surfaces containing only E-selectin were less efficient at causing cells to arrest, perhaps due to the fast on-off rates of selectins and their ligands (14, 23, 31, 32), coupled with the fact that the bonds are relatively weak (4). Consistent with numerous other reports, LFA-1/ICAM-1 interactions are needed for firm arrest (9, 33). Surfaces displaying only ICAM-1 could support low levels of arrest, but the level of arrest was greatly enhanced by the presence of E-selectin interactions. Because the ICAM-1 only surfaces had a higher rate of tethering and a very low rate of rolling, we suggest that in this system, cells on an ICAM-1 only surface have a binary state space. In the brief time that these cells are initially interacting with the surface, either the requisite number of bonds for firm arrest is reached and the cell stops, or it is not achieved and the bonds that have formed are broken due to the shear force, thus resulting in a tethering event. With the addition of E-selectin, the cells interact with the surface for a longer period of time and are more able to form enough bonds to resist the shear forces applied.

Rolling velocity
We measured the velocity of cells rolling on various surfaces. We found that rolling velocity of these cells was nearly constant across a large variety of surface densities and compositions, provided some amount of E-selectin was present. However, surfaces displaying solely ICAM-1 supported a much lower rolling velocity than surfaces containing ICAM-1 and E-selectin. ICAM-1-only surfaces, as mentioned previously, did not support robust levels of rolling. It has been shown previously that neutrophils, another type of leukocyte, can use LFA-1/ICAM-1 interactions to support rolling on surfaces with P-selectin (15, 34). However, we did not see a significant difference in the rolling velocity between surfaces containing only E-selectin and surfaces with both ligands. We did not see these “slow rolling” interactions in our system, as the rolling velocity of cells on surfaces with ICAM-1 and E-selectin was comparable to surfaces containing E-selectin only. Our experiments do not allow us to determine whether E-selectin does not support the intracellular signaling required for slow rolling or if naïve human T cells do not support this functionality.

**Time to stop**

We also tracked the time to stop of the cells, which was the difference in time between when cells initiated rolling and when they became arrested. Once again, the cells showed a surprising consistency in the time to stop across a wide range of surface densities and compositions. However, surfaces containing only ICAM-1 or E-selectin had divergent responses. For cells on surfaces containing only E-selectin, the time to stop was much higher than similar surfaces to which ICAM-1 was added. This once again highlights the importance of ICAM-1 in mediating the transition from rolling to firm attachment.
arrest. In contrast, cells interacting with surfaces of only ICAM-1 stopped essentially immediately upon contact. This result highlights the binary state space that ICAM-1-only surfaces create.

In summary, we show that surfaces combining ICAM-1 and E-selectin allow for the most efficient arrest. This indicates the presence of synergy between these two molecules for the purposes of completing the leukocyte adhesion cascade. Surfaces containing only one of these molecules were not as efficient, but still managed a basal level of adhesion. These results point out that the bonds between E-selectin and its sialylated and fucosylated T-cell surface ligands and between LFA-1 and ICAM-1 are biomechanically different and tuned to support, by themselves, different dynamics of adhesion. Like many other adhesion systems (6, 15, 24), nature has evolved a system in which multiple molecules act in synergy to support robust and multi-level control of the dynamics of adhesion, and that one cannot compensate for mechanochemical deficiencies by increasing molecular density. In addition, our results can provide important information for the verification of computer models of cell rolling and adhesion, such as Adhesive Dynamics, especially regarding the effect of changing ligand identity and density.
References


374: 539–542.


CHAPTER 4: T Cells Migrate Upstream After Completing the Leukocyte Adhesion Cascade

Abstract
The leukocyte adhesion cascade is of critical importance for both the maintenance of immune homeostasis and the ability of immune cells to perform effector functions. Here, we present data showing CD4+ T cells migrating against the direction of flow after completing the leukocyte adhesion cascade on surfaces containing ICAM-1 or ICAM-1 and VCAM-1 but migrate downstream on surfaces containing only VCAM-1. Cells completing the cascade on HUVECs initially migrate upstream before reverting to more random migration, partly caused by transmigration of cells migrating against the flow. Furthermore, cells migrating upstream transmigrate faster than cells migrating downstream. Blocking LFA-1-ICAM-1 interactions resulted in downstream migration and slower transmigration. These results further suggest a possible physiological role for upstream migration in vivo.
**Introduction**

All immune cells, including T cells, need to be able to access the interstitial space from the vasculature to maintain immune homeostasis or to perform effector functions at the sites of infection and inflammation (1). In order to perform these duties, immune cells need to specifically stop on the endothelium near sites requiring an immune response while avoiding sites where no response is needed. In response to these challenges, nature has evolved the leukocyte adhesion cascade, which is typically separated into four steps, each dependent on the ones before it: tethering, rolling, firm arrest and migration, and transmigration (2). During the initial tethering stage, cells interact with P- or E-selectin molecules expressed on the activated endothelial surface. These initial interactions begin to subtly slow the cell while also allowing more intimate contact between the cell and the endothelial surface (2, 3). As the cell velocity slows, additional selectins and other adhesive molecules are engaged, leading to sustained or slow rolling. During rolling, the T lymphocyte can bind activating chemokines, which initiate a number of intracellular signaling cascades whose end result is the activation of integrins such as LFA-1 on the T cell surface. Once fully activated, these integrin-based adhesions become mechanically stronger, stop the rolling of the cell, and firmly arrest it on the endothelial surface (2, 4, 5). Along these lines, we recently recreated the leukocyte adhesion cascade *in vitro* and identified the requirements for rolling and firm adhesion in the form of a concise state diagram (6). After adhesion, T cells spread and migrate along the apical surface of endothelium to find an appropriate location to transmigrate across the endothelium (2, 7).
Previously, it has been shown that lymphocytes will crawl upstream against the direction of flow on surfaces coated with ICAM-1. On surfaces containing ICAM-1, T and B cells, along with hematopoietic stem and progenitor cells (HSPCs), will migrate against the direction of flow, that is, upstream. On surfaces of VCAM-1, these same cells migrate with the direction of flow (8–11). In the case of T and B cells, small amounts of ICAM-1 on a surface containing mostly VCAM-1 provide enough stimulus for directed migration upstream. However, these migration studies were conducted on surfaces of purified protein where cells were allowed to settle prior to the initiation of shear flow. Thus, it was unknown whether chemokine-mediated arrest or selectin-mediated tethering and rolling might interfere with upstream migration. Previous in vivo studies in rats showed that T-cells indicated a preference for upstream migration in meningeal CNS structures (12), suggesting these molecules might not interfere with upstream migration. In addition, previous reports have shown the importance of LFA-1-ICAM-1 interactions in mediating upstream migration on a model of the murine brain microvasculature, showing that only ICAM-1 can support upstream migration while ICAM-2 only allows cells to migrate perpendicular to the direction of flow (13). Here, we show that primary human CD4+ T cells migrate upstream after completing the leukocyte adhesion cascade on surfaces of purified protein and stimulated endothelium, highlighting the possible biological relevance of this behavior.
Materials and methods

Adsorption of Protein A/G and SDF-1α

Non-tissue culture treated polystyrene petri dishes (Corning, Corning, NY) were enclosed using a single well FlexiPerm gasket (Sarstedt, Nümbrecht, Germany). A solution of 2 µg mL⁻¹ of Protein A/G (Thermo-Fisher Scientific, Waltham, MA) and 1 µg mL⁻¹ of SDF-1α (R&D Systems, Minneapolis, MN) was applied and incubated overnight at 4°C. The surfaces were then washed three times with PBS before a 30 minute incubation of a 0.2% w/v solution of Pluronic F-127 (Sigma-Aldrich, St. Louis, MO). The surfaces were washed again three times with PBS. Next, a solution of ICAM-1/Fc and/or VCAM-1/Fc and P-selectin/Fc chimera (R&D Systems) was applied to the surface and incubated for three hours at room temperature. The completed surfaces were then washed again three times with PBS before use.

CD4⁺ T Cells

Purified primary human CD4⁺ T cells from anonymized donors were obtained from the University of Pennsylvania Human Immunology Core. Cells were resuspended in RPMI-1640 media (Gibco, Gaithersburg, MD) supplemented with 10% FBS (Sigma-Aldrich, Cat. F2442) and used immediately.

HUVECs

Human Umbilical Vein Endothelial Cells (HUVECs) were maintained in EBM-2 growth media with BulletKit supplement (Lonza, Basel, Switzerland). For experimentation, HUVEC were seeded in a 35mm x 10mm TC treated dish (Corning) and
grown to confluence. HUVECs were then stimulated with 200 ng/mL TNFα (BioLegend, San Diego, CA) for 4 hours or 48 hours prior to experimentation.

**LFA-1 Blocking**

Cells were resuspended at a concentration of $1 \times 10^7 \text{mL}^{-1}$. Cells were blocked using a functional blocking anti-CD11a antibody (Clone HI111, BioLegend) at a final concentration of 50 µg mL$^{-1}$ for 30 minutes at 37°C and 5% CO$_2$. Cells were then diluted to $5 \times 10^5 \text{mL}^{-1}$ and used immediately.

**Flow Cytometry**

Immunofluorescence staining and flow cytometric analysis of cells were performed as described previously (10). Cells were washed twice in PBS and resuspended at $1 \times 10^6 \text{mL}^{-1}$. Samples were incubated with combinations of fluorescently labeled antibodies MOPC-21-PE (IgG1κ control), MOPC-21-FITC (IgG1κ control), MOPC-21-APC (IgG1κ control), UCHT1-APC (anti-CD3), RPA-T4-PE (anti-CD4), JS-83-FITC (anti-CD45RA, eBioscience, San Diego, CA), KPL-1-PE (anti-PSGL-1), and V S056-PE (anti-CD62L). All antibodies were from BioLegend unless otherwise noted. Flow cytometric analysis was performed on a Accuri C6 using the Accuri C6 Analysis software (BD Biosciences, San Jose, CA). Histograms were generated using the FlowJo software package (FlowJo, Ashland, OR).

**Flow Chamber Assays**

Flow chamber experiments were performed in a circular parallel plate flow chamber (GlycoTech, Gaithersburg, MD) with a gasket width of 0.25 cm, thickness of 127 µm, and length of 2 cm. Before flow chamber experiments, a functionalized dish was
washed with prewarmed PBS and media to remove air bubbles in the flow path. The assembled flow chamber was mounted on an inverted Axiovert 200 (Carl Zeiss, Gottigen, Germany) enclosed by a XL-3 microscope incubator (PeCon, Ulm, Germany). All experiments were performed at 37°C. T cells were suspended at a concentration of 5 x 10^5 mL^-1 and were perfused into the flow chamber via syringe pump (Harvard Apparatus, Holliston, MA) at a flow rate corresponding to a calculated wall shear rate of 100 s^-1. Rolling and adhesion of T cells on the immobilized adhesive ligands was observed via phase contrast microscopy under a 10X objective (NA = 0.2, Type A-Plan, Carl Zeiss). All recombinant protein surfaces were tested in triplicate on three different days with different anonymized donors and the results averaged. HUVEC surfaces were tested in duplicate on 4 different days with different anonymized donors.

Data Acquisition and Cell Tracking

A CCD camera (QImaging, Surrey, BC, Canada) was used to monitor T cell adhesion events with adhesive substrates. Adhesion of T cells was recorded on DVD+RW discs for cell tracking analyses. Cell adhesion videos were redigitized to 640×480 pixels at 29.97 frames s^-1 and deinterlaced with HandBrake software (http://handbrake.fr/) then converted to image stacks with MATLAB (MathWorks, Natick, MA). Images for every 30 seconds were obtained and migrating cells were manually tracked using the MTrackJ plugin (https://imagescience.org/meijering/software/mtrackj/) in the ImageJ program (http://imagej.nih.gov/ij; National Institutes of Health). Cells on surfaces of recombinant proteins were tracked from the time of their spreading to the end of the experiment or
when the cell stopped migrating, whichever was sooner. Cells on HUVEC monolayers were tracked from initial spreading to transmigration, as indicated by phase brightness change. These points were then analyzed using a custom MATLAB script to determine the migration index of each cell. Statistical tests were performed using a paired Student’s t test.
Results

Figure 4.1 shows scattergrams of paths taken by migrating CD4+ T cells on surfaces containing ICAM-1 only, VCAM-1 only, and ICAM-1 and VCAM-1 (Figure 1A-C). These surfaces also displayed P-selectin to support recruitment from the free stream, along with CXCL12 to cause integrin activation. For simplicity, we will refer to surfaces based solely on the cell adhesion molecule presented. We also characterized the cells using flow cytometry, finding that they were >98% pure CD4+ T cells (Figure A.5 in the Appendix). Most of the cells chose to migrate upstream (against the direction of flow) on surfaces containing ICAM-1, as highlighted by the large fraction of red tracks. In contrast, surfaces containing only recombinant VCAM-1 did not support robust upstream migration, as shown by the large fraction of blue tracks, indicating the cells travel with the direction of flow. Surfaces containing both recombinant ICAM-1 and VCAM-1 show a similar ratio of cells migrating upstream as surfaces containing only ICAM-1. We then quantified the directedness of the migration with or against the direction of flow using the Migration Index (MI) in Figure 4.1D. The MI is the ratio of motion against the direction of flow to the total distance migrated by the cell, with negative values indicating upstream migration. On ICAM-1-only or VCAM-1-only surfaces, the migration index does not change significantly over the time course of the experiment. Although some cells initially migrate downstream on ICAM-1 surfaces, cells normally begin to migrate upstream within the first 30 seconds of their migrating. Cells on surfaces containing only VCAM-1 showed no upstream migration at any time point, as indicated by the positive MI. Surfaces containing both ICAM-1 and VCAM-1
Figure 4.1: T cells migrate upstream after completing the leukocyte adhesion cascade. Scattergrams of cell tracks of cells migrating on surfaces containing (A) ICAM-1, (B) VCAM-1, or (C) ICAM-1 and VCAM-1. Tracks have been adjusted to all start at the same point. Red tracks indicate cells migrating against the direction of flow and blue tracks are cells migrating with the direction of the flow. All images have flow from left to right at 100 s⁻¹. (D) Plot of migration index over time for surfaces of recombinant protein. Upstream migration is indicated by a negative migration index, downstream migration by positive values, and random migration by values near zero. ICAM-1 surfaces support upstream migration while VCAM-1 surfaces do not. Surfaces containing ICAM-1 and VCAM-1 support upstream migration to a similar extent as ICAM-1-only surfaces. Data presented is mean ± SEM, n = 4 independent experiments.
did not show a difference compared to ICAM-1-only surfaces at long time points. However, cells on the surfaces containing both ligands took longer to reach the maximal response. In summary, these results show that the presence of chemokine and selectin do not affect the ability of CD4+ T cells to migrate against the direction of flow while also highlighting the importance of ICAM-1-LFA-1 signaling for this phenotype.

In order to connect the observation of upstream migration to physiology, we measured the migration of cells on stimulated HUVECs after rolling and arrest (Figure 4.2). Cells interacting with a HUVEC monolayer show upstream migration, similar to T cells migrating on a surface consisting of ICAM-1-only or ICAM-1 and VCAM-1 (Figure 4.2A). However, on the endothelium, although cells initially crawl upstream, upstream migration is not maintained for as long, as T cells approach a more random migration after an initial upstream “burst.” This is in comparison to the recombinant protein results, which show a consistent MI for the entire experiment. Also, we found that blocking LFA-1-ICAM-1 interactions through the use of a blocking antibody abolished any migration directed against the fluid flow, once again highlighting the importance of this interaction to the phenotype. We surmised that one possible cause of the reversion to more random or even downstream migration might be caused by the transmigration of cells across the endothelial layer. Since we stopped tracking cells once they had transmigrated, the change in the population of tracked cells might explain the increase in the migration index. Indeed, it is apparent that cells treated with an LFA-1-blocking antibody remain on the apical surface of the HUVEC monolayer than untreated cells (Figure 4.2B). These cells also have a significantly reduced fraction of cells migrating upstream (Figure 4.2C).
Figure 4.2: T cells migrate upstream on HUVEC surfaces. (A) Plot of migration index over time. Upstream migration is indicated by a negative migration index, downstream migration by positive values, and random migration by values near zero. Blockade of LFA-1 prevents upstream migration on TNFα stimulated HUVECS, while cells with unblocked LFA-1 initially migrate upstream before reverting to downstream migration. ICAM-1-only recombinant protein surface data is provided for comparison. Data presented is mean ± SEM, n = 4 independent experiments. (B) Plot showing the...
remaining fraction of tracked cells at each time point. Cells on HUVEC monolayers were tracked from initial migration to transmigration or the end of the experiment, whichever is sooner. (C) Comparison of fraction of cell which migrated upstream on HUVEC monolayers with or without LFA-1 blockade. Data presented is mean ± SEM, n = 4 independent experiments. (D) Comparison of the time from arrest to transmigration on HUVEC monolayers with or without LFA-1 blockade. Data presented is mean ± SEM, n = 4 independent experiments. * p < 0.05, ** p < 0.005.
We then compared the time to transmigrate across the endothelial layer in Figure 4.2D. On HUVECs stimulated for four hours with TNFα, there was a significant difference in the time to transmigrate between cells migrating upstream and those migrating downstream. This difference also exists for cells with blocked LFA-1. In addition, blocking LFA-1-ICAM-1 interactions also significantly increases the time to transmigrate for cells migrating downstream, as well as the overall time to transmigrate for all cells. Interestingly, the period for transmigration almost exactly matches the time delay for the decay of directional migration to random migration. We also tested the cells on HUVECs which had been stimulated with TNFα for 48 hours to see if changing ratios of ICAM-1 and VCAM-1 would influence the migration, but we did not see any such effect (Figure A.6 in the Appendix).
Discussion and conclusions

To our knowledge, this is the first report of primary human T cells showing upstream migration after completing the entire leukocyte adhesion cascade, and the first demonstration that they crawl upstream after the imposition of shear flow on endothelium, at least initially. T cells choose a direction of migration within 30 seconds of arresting on surfaces containing P-selectin and either ICAM-1, VCAM-1, ICAM-1 and VCAM-1, or a monolayer of HUVECs. This is in line with previous results showing the T cells migrate upstream in the presence of even minimal ICAM-1 (9). In addition, a previous study has suggested the importance of the uropod in determining the direction of migration for T cells under flow (14). However, our CD4+ T cells on surfaces containing only ICAM-1 started to migrate upstream before an obvious morphological polarity was established, suggesting that there may be additional regulatory mechanisms controlling this behavior. In contrast, cells on surfaces containing only VCAM-1 began migrating downstream before polarity was established. Surfaces containing both ICAM-1 and VCAM-1 allowed enough time for cells to establish morphological polarity before reaching a maximal response. We also showed that T cells migrating on HUVECs initially prefer to migrate upstream, before reverting to more random migration. This reversion is driven by cells migrating upstream crossing the endothelial layer faster than cells migrating with the direction of flow. Blocking LFA-1 interactions on a stimulated HUVEC surface resulted in consistent downstream migration, as well as significantly longer time to transmigrate, in line with previous reports suggesting a large role for LFA-1 stimulation in transmigration (15). In summary, these results suggest that migration
against the direction of flow in CD4+ T cells is a robust response to their environment and possibly has a physiologically important function \textit{in vivo} by directing T cells to their recruitment sites faster.
References


CHAPTER 5: Adhesive Dynamics simulations reveal the precise mechanodynamics of the failure of adhesive bonds in series

Abstract

Cells often attach to surfaces or to other cells through bonds in series, where there are multiple points of failure between the two surfaces. Previous results have suggested a linear relationship between the number of linkages and force required for detachment. Here, we use Adhesive Dynamics simulations to determine how bonds in series fail in the small bond number regime. In contrast to previous reports, we find a non-linear reduction in the critical force as the number of linkages between surfaces increases. The amount of reduction depends on the direction of force exerted on the cell. The reduction in critical force is driven by a decrease in the spring constant of the overall complex. Altering the intrinsic off rate of the linkages lowers the critical force, in line with previous reports. Changing the spring constant of the linkages had less of an effect. These results highlight the importance of understanding the role of bond in series on cell detachment from surfaces.
Introduction

Adhesion receptors link biological cells to each other and to surfaces. Often, these linkages consist of multiple bonds in series. One biologically relevant example would be the binding of B or T lymphocytes to peptide on the surface of antigen presenting cells (APCs), where a processed peptide from an antigen is attached to and presented by a receptor known as Major Histocompatibility Complex (MHC). On T-cells, the T-cell receptor, and on B-cells, surface antibody, bind to the peptide-MHC complex in order to determine if the receptor “matches” the peptide held by the MHC (1). To do this, B and T cells are known to exert pulling forces in order to determine the strength of affinity between the receptor and peptide (2, 3). These interactions are bond in series, in which there are two possible points of failure for the complex: the bond between the MHC and the peptide or the bond between the peptide and the lymphocyte surface receptor. If either of these bonds fail, the B or T cell may not receive a signal sufficient to be activated and cause an immune response (3). The importance force in controlling the integrity of immune cell complexes have led to a number of laboratories linking lymphocytes to surfaces using multiple molecular assemblies, often including molecular sensors that can report the force of dissociation (4, 5). An additional example of bonds in series is the use of protein A or A/G coated surfaces to make adhesive substrates using Fc chimeras with variable regions of adhesion ligands that replace the variable regions (6–8).

Despite their importance in biology and biotechnology, there is relatively little work to investigate the dynamical failure of bonds in series. A study by Saterbak and Lauffenburger showed that because of multiple detachment points of bonds in series,
there was an increase in the rate of bond failure, such that bonds in series appeared weaker than of the connections themselves. They attributed the decrease in detachment force to the increased number of potential points of failure between bonds. This calculation employed a probabilistic model of cell detachment, but used an approximation where the forces of cell detachment were equally applied to all adhesion molecules at the same time (9), which is an approximation, owing to the curvature of the cell substrate interface which is exacerbated when the number of molecules is small, as it does when it detaches (10–12). As we have shown in simulations of detachment, forces are focused on the adhesion bonds on the edge of the contact zone (uniformly around the periphery when forces are applied normally, and at the back edge of the contact zone when forces are applied under shear) (13). This focusing of forces at the edge of the contact zone causes the cell to peel from the substrate. Other groups have studied adhesion clusters using stochastic simulation of a small numbers of uniformly stressed bonds, but these simulations did not explore the effect of differing numbers of linkages between the cell and the surface (14, 15).

Here, we present the results using the well-known method of Adhesive Dynamics (AD) to simulate the detachment of spheres from surfaces connected by bonds in series (16, 17). In AD, the position of each bond and its strain are tracked exactly, allowing a detailed spatiotemporal map of the distributions of stresses in the adhesive interface. The method is valid for all densities of molecules, and the failure of bonds is sampled using stochastic sampling. We previously used AD to simulate the detachment of particles from surfaces attached by single bond linkages (13). Here, we show that bonds with multiple
points of failure are weaker than single “linkages,” although the reduction in apparent bond strength is non-linear in the number of points of failure. Two bonds in series are not half the strength of one, and the degree of reduction of detachment force depends on how the force is applied. In addition, we also determine the effects of different intrinsic off rates and bond elasticities on detachment. We found that the intrinsic off rate of the individual linkage could control the detachment behavior of the entire bond. In contrast, the elasticity of the linkages did not have a significant effect on the critical force unless all linkages were of the same type.
Materials and methods

Adhesive Dynamics

The method of Adhesive Dynamics has been described at length in several previous publications (12, 13, 17); here we briefly summarize the method. Cells are simulated as hard spheres of radius $R_c$ on an infinite, flat, uniformly adhesive surface. Bonds on the surface of the cell are placed randomly within an adhesive region in which bond formation is plausible, to increase simulation speed. The size of the cap was determined dynamically based on a calculated “maximum reactive distance” based on the chemical properties of the bonds on the cell. This distance was determined at the start of a simulation and was set so that the probability of bond failure in a single time step was $e^{-1}$, in line with previous reports (13). Bonds were modeled as adhesive Hookean springs with on and off rates dependent on distance $x$. We use the model originally proposed by Dembo and coworkers for bond reaction rates, in which the adhesion molecules are treated as springs (18). Individual bonds have an associated resting length $\lambda$, intrinsic on rate $k_{on}^*$, intrinsic off rate $k_{off}^*$, spring constant $\sigma$, and transition state spring constant $\sigma_{ts}$. These values related to the on and off rate of bonds through the following equations:

$$
 k_{on} = k_{on}^* \exp \left( \frac{-\sigma_{ts}(x - \lambda)^2}{2k_B T} \right) \quad (5.1)
$$

$$
 k_{off} = k_{off}^* \exp \left( \frac{(\sigma - \sigma_{ts})(x - \lambda)^2}{2k_B T} \right) \quad (5.2)
$$

In our simulations, we assumed all bonds were slip bonds, i.e. the value of $(\sigma - \sigma_{ts})$ is always positive so the off rate increases with increasing deviation from the ideal length.
The on and off rates were used to calculate the probabilities of failure in each timestep through:

$$P = 1 - e^{(-k \cdot dt)}$$  \hspace{1cm} (5.3)$$

where $dt$ is the time step of the simulation, $k$ is calculated on or off rate, and $P$ is the resulting probability of an association or dissociation reaction. This probability was determined for each potential bond (for formation) and each existing bond (for dissociation) in the adhesive cap. In addition, nonspecific forces were calculated, including electrostatic, steric stabilization, van der Waals, and buoyant forces. Values of all constants can be found in Table 5.1. Finally, to determine the adhesion strength of the cells, a specified body force can be applied normally, tangentially, or shear to the sphere.

A simulation loop consists of the following steps. First, the size of the adhesive cap is determined based on the length of the bonds in the simulation and, using the assigned molecular density, a number of molecules in the contact zone is determined. If additional molecules are needed in the adhesive cap, they are randomly placed within the cap. Next, each molecule is tested individually for binding or unbinding, depending on its current state. The on rates are determined with the outermost receptor binding immediately under its current position, with the assumption that the substrate is uniformly reactive. Next, the sum of forces acting on the sphere is determined, including forces from bound receptors and nonspecific forces. The resulting force vector is used to determine the velocity vector using the Mobility matrix, derived previously for spheres near a smooth wall (19–21). Finally, the position of the cell and all receptor locations are updated based on the linear relationship between position and velocity by a time step $dt$. 

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<table>
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<tr>
<th>Constant</th>
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<tbody>
<tr>
<td>$R_c$</td>
<td>Cell radius</td>
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<td>$A_h$</td>
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<td>$\chi$</td>
<td>van der Waals constant</td>
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<td>$\sigma_{ts}$</td>
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<tr>
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<td>Steric constant</td>
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<tr>
<td>$\Delta \rho$</td>
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Table 5.1: Values for constants used in the series bonds Adhesive Dynamics simulations.
Adhesive Dynamics allows us to change the parameters of each linkage individually, without affecting the other linkages in the simulation. For example, in a two-linkage system, we can alter the elasticity of one of the linkages without altering the other. This allows us to determine the effect of a single linkage on the behavior of the entire bond. The overall bond resting length ($\lambda$) was maintained at a constant value for all simulations to eliminate differences in binding ability between simulation runs. The outermost linkage of the complex was allowed to bind to the surface, while interior linkages were not. Bonds were only allowed to reform using the same linkages, so there was no cross-reaction between broken bonds. Data points, including position of the cell, locations of every bond (both bound and unbound), bond strain, and number of bonds were collected every 20,000 time steps (each time step is $10^{-6}$ s; approximately 0.02 simulated seconds). The resulting data files were then analyzed using custom written MATLAB (Mathworks, Natick, MA) and R (R Core Team) scripts. Critical forces and slopes were determined using the drc R package (22).
Results

A schematic of the simulated system is presented in Figure 5.1. Using Adhesive Dynamics, we were able to simulate the detachment of cells from surfaces using a variety of forces, including normal, tangential, and shear forces. We focused on normal and shear forces, as these are the most widely used methods for detaching particles from surfaces. In addition, we could alter the number of linkages within a bond between the cell and the surface. Each linkage could have its properties changed independently of the other linkages in the bond, allowing us to explore the effects of different component properties on the dynamics of failure.

First, we explored the effect of increasing number of linkages on the apparent strength of the adhesion between the cell and the surface. Figures 5.2A and B show the results of simulations of detachment for both normal and shear forces, in which dots represent the fraction of cells bound to a surface under an applied force after 5 simulation seconds. These points are fitted to a logistic expression, which allow us to estimate a critical force and slope from the collected data. The critical force is the force required to remove 50% of the initially bound cells from the surface. We interpreted the slope as a measure of sensitivity of detachment to force. Figure 5.2C compares the critical force for normal forces of bonds consisting of one, two, or three linkages in series, where each of the connectors have the same reaction rates and each of the components have the same spring constants. As the number of linkages between the cell and the surface increases, the critical normal force decreases, although this decrease is not linear with the number of linkages. Adding a second linkage decreases the detachment force by 23%, adding a third
Figure 5.1: Schematic of adhesive dynamics. (A) Free body diagram of single bond holding a cell onto the surface. The bond location is specified by $(\theta, \phi)$ coordinates, which can easily be converted to $(x,y,z)$. Forces applied by the bond are axial, which are translated vectorially to the body of the cell, and can apply both a body force and torque to the particle. (B) Example of normal (top) and shear (bottom) forces which can be applied to the cell. (C) Schematic showing the labeling schema used in the manuscript as
well as some of the relevant parameters of the simulation. Linkages at the tip of the complex are allowed to bind to the surface, while interior linkages are not.
Figure 5.2: Critical forces for varying number of linkages. (A) Figure showing simulated fraction of cells bound after 5 seconds of applied force for normal and shear (B) forces. Dots represent simulations of 100 cells. Fitted lines are used to estimate the critical force at which half of the cells detach. (C) Critical force (black bar) and 95% CI (shaded area) of cells detaching under a normal (C) or shear (D) force. $\hat{F}$ is dimensionless force, with scale factor $6\pi\mu k_{c} R_{c}^{2}$. $N = 100$ cells.
decreases it by 38%, both compared to the single linkage case. Finally, we also determined the critical shear force, illustrated in Figure 5.2D. In contrast to normal forces, there is no significant difference in the critical force between one or two linkage bonds, while there is a significant decrease for bonds containing three linkages. We found no significant difference between the slopes of the different conditions, suggesting that the sensitivity to force was the same (Figure A.7 in the Appendix).

The observed differences between the bonds with different numbers of linkages were not due to differences in the number of bonds attaching the cell to the surface, as this value was constant across all conditions (Figure 5.3A). However, the separation distance between the cell and the surface was different based on the number of linkages to the surface (Figure 5.3B). As shown in Figure 5.3C, this difference in separation distance then resulted in differences in the time-averaged strain of the bonds. Finally, we could measure the rolling velocity of the cells in shear flow after they had detached. As shown in Figure 5.3D, cells with a larger number of linkages rolled faster, although the difference between one- and two-linkage cells is small, which corresponds to the effect of bonds in series on the shear force. We also examined the location where bonds failed but saw that all linkages in a series were equally likely to fail (Figure A.8 in the Appendix).

Interestingly, we noticed a difference in dynamics of cell detachment based on the number of linkages, as highlighted in Figure 5.4. Cells which were bound to the surface through a single linkage experienced an exponential decrease in the fraction of cells bound as weaker-bound cells were rapidly removed from the surface. The remaining cells then almost always remained attached for the rest of the simulation. However, with
Figure 5.3: Metrics of cell adhesion to surfaces. (A) Equilibrium number of bonds between the cell and surface under no force conditions. (B) Dimensionless separation distance between the cell and the surface in the absence of force. (C) Time averaged strain of all bonds between the cell and the surface in the absence of force. (D) Dimensionless rolling velocity of cells as a function of shear force for single, double, and
triple linkages. All values are mean ± SEM, N = 100 cells. \( \hat{Z} = Z / R_C \), \( \hat{\dot{F}} = F / 6 \pi \mu k^* R_C^2 \), and \( \hat{\dot{U}} = U / R_C \gamma^* \).
Figure 5.4: Detachment profiles of cells over time. Left hand column indicates normal forces; the right hand column, shear forces. The number of linkages is indicated above the two profiles. Forces increase in strength from blue (weakest) to yellow (strongest). \( \tau \) is dimensionless time, with scale factor \( 1/k_{\text{on}} \).
multiple linkages, even the stronger cells detached; the kinetics of detachment were linear with time. There was no qualitative difference between bonds with two or three linkages, suggesting that any number of multiple linkages lead to this response difference. The effect of multiple contacts on detachment were maintained regardless of the type of force (normal versus shear) acting on the cells.

The proceeding results were obtained with fixed values of two features of the bonds – \( k_{\text{off}}^* \) and \( \sigma \). Next, we tested the effect of altering these parameters on the physics of failure. We altered the intrinsic off rate \( (k_{\text{off}}^*) \) of one or both of the linkages in a two-linkage bond to understand the effect of this parameter on detachment. We doubled or halved the base rate constant and termed those bonds DK (doubled \( k_{\text{off}}^* \)) or HK (halved \( k_{\text{off}}^* \)). Figure 5.5A and B shows a comparison of the critical forces for normal and shear forces, respectively. As expected, increasing the off rate of a linkage reduced the critical force, as bonds were more likely to detach at any given force. Also, the position of the change in kinetics did not affect the response of the overall system. Interestingly, a single changed linkage was enough to alter the critical force to the same level as if the kinetics of both linkages had been changed. In contrast, changing the intrinsic off rate did not alter the sensitivity to force, as shown by the change in detachment with force (Figure A.9). The alteration in the critical force was not caused by a change in the number of bonds to the surface. In addition, the average strain of the bonds was not significantly altered by the change in the off rate. In contrast, bonds with lower intrinsic off rates appeared to have less separation distance from the surface, although the difference was not significant. We also saw that bonds with a lower intrinsic off rate were less likely to
Figure 5.5: Effect of intrinsic off rate on cell detachment. (A) Critical force (black bar) and 95% CI (shaded area) of cells detaching under a normal (A) or shear (B) force. (C)
Dimensionless rolling velocity of cells under a shear force of given magnitude. All values are mean ± SEM, N = 100 cells. \( \hat{v} = \frac{F}{6\pi \mu k^*} R^2_c \) and \( \hat{\gamma} = U/R^* \gamma^\dagger \).
break (Figure A.9). Finally, we determined that the rolling velocity of cells (Figure 5.5C), which showed that bonds with lower off rates generally had slower rolling velocities.

Finally, we altered the spring constant ($\sigma$) of one or both of the linkages to determine how bond stiffness might affect the detachment. The spring constant of one or both of the linkages was increased or decreased by 25%, leading to “stiff” or “soft” linkages. In contrast to the alterations in the intrinsic off rate, there was no obvious trend to the results. Bonds which consisted only of stiff or soft linkages had a significant alteration in the critical force for normal forces (Figure 5.6A). However, bonds with only one altered component did not experience a significant change in the critical force. In contrast, there appears to be no general effect of differences in spring constant when measuring detachment by shear forces (Figure 5.6B). Bonds with a higher proportion of “stiff” linkages had a lower overall bond strain (Figure 5.6C), resulting in a reduced separation distance (Figure 5.6D). Conversely, bonds with softer components had higher levels of strain and increased separation distances. These effects seem to counterbalance, yielding a critical force that was statistically similar to the base two-linkage case. Finally, under shear force, we discovered that softer linkages result in faster rolling when compared to stiffer linkages (Figure 5.6E). We found no significant difference between the distribution of removal forces for either normal or shear forces as a function of spring stiffness. The number of bonds between cells and surfaces was constant regardless of the type of bond linkages used. Stiff and soft linkages were equally likely to lead to bond failure (Figure A.10 in the Appendix).
Figure 5.6: Effect of altered spring constant on cell detachment. (A) Critical force (black bar) and 95% CI (shaded area) of cells detaching under a normal (A) or shear (B) force. (C) Time averaged strain of all bonds between the cell and the surface in the absence of force. (D) Dimensionless separation distance between the cell and the surface in the absence of force. (E) Dimensionless rolling velocity of cells under a shear force of given magnitude. All values are mean ± SEM (unless otherwise noted), N = 100 cells. $\hat{Z} = Z/R_C$, $\hat{F} = F/6\pi\mu k^*_R R_C^2$, and $\hat{U} = U/R_C^2 \gamma^{-1}$. 

$$
\hat{Z} = \frac{Z}{R_C}, \quad \hat{F} = \frac{F}{6\pi\mu k^*_R R_C^2}, \quad \text{and} \quad \hat{U} = \frac{U}{R_C^2 \gamma^{-1}}.
$$
Discussion and conclusions

We used Adhesive Dynamics simulations to understand the effect of the number of linkages between a cell and the surface on detachment. In addition, we used the method to understand the effect of changing the intrinsic off rate or spring constant of one or two linkages in two-linkage bonds. Using these physically accurate stochastic simulations, we showed that having two bonds in series reduces the critical normal force by approximately 23% compared to a single bond with the same properties (off rate and spring constant). Adding an additional linkage (for a total of three) further reduces the critical normal force by 20%, for an overall reduction of 38%. This is in contrast to previously reported results by Saterbak and Lauffernberger, whose calculations suggested that two-linkage bonds have a strength half that of a single linkage bond. However, these calculations were made using a much simpler model in which forces were uniformly distributed among adhesion receptors and the detailed mechanodynamics of adhesion molecules was not considered, so the fact that there are differences is not surprising (9). Interestingly, changing number of linkages in series did not change the sensitivity of detachment to force, suggesting that the distribution of forces sufficient to remove cells from the surface had a similar standard deviation regardless of the number of linkages.

Possibly the most surprising result in these simulations regarded the effect of detachment by shear forces for bonds in series. One- and two-linkage bonds had similar critical shear forces, while three-linkage bonds experienced a much lower critical force. Previous results from our lab suggest leveraging effect, similar to that of a “claw-hammer,” when shear forces are applied to a cell. This effect amplifies the forces on the
back edge of the contact zone, which seems to have a significant effect only when there are three linkages between the cell and surface (13, 17). Our results show that bonds with multiple linkages could maintain higher levels of strain than bonds with a single linkage. This is due to the bond model that we chose, which does not account for the strain placed on the molecule, only the absolute deviation from the ideal length.

The effect of the mechanical strength of the linkages itself is largely thought to be included in a composite spring constant, given:

\[
k_{\text{eq}} = \frac{1}{\frac{1}{k_1} + \frac{1}{k_2} + \cdots}
\]  

(5.4)

where \(k_1\) and \(k_2\) are spring constants of individual springs and \(k_{\text{eq}}\) is the spring constant of the entire complex. Thus, adding a second linkage, even with the same spring constant, halves the spring constant of the entire complex. This causes the cell to sit further from the surface as the bonds require more strain to generate the same level of force. However, this appears to only affect the response of the cells to normal forces. When exposed to shear forces, this difference appears to become relatively unimportant as the extension of the bonds due to the “claw hammer” effect at the rear of the cells overcomes any resistance from the bonds. In fact, the difference in rolling velocity between the one- and two-linkage bonds in shear flow is marginal, consistent with the slight difference in the critical force.

Due to the non-linear nature of the off-rate calculation, small changes in receptor length can result in outsize changes in the propensity to bond or unbind. In addition, when bound to the surface, the cell is under the influence of nonspecific forces from the
surface. The increased separation distance cause when binding using bonds of multiple linkages allows for more of the bond force to be resisting the action of the applied body force, not the repulsive forces from the surface. This allows cells with two-linkage bonds to resist shear forces as efficiently as single linkage bonds. However, the increase in the probability of bond failure as the number of linkages increases eventually results in the bonds being weaker, as we see with the triple linkage bonds under shear forces.

Another interesting finding was the difference in the detachment rates when bonds had different numbers of linkages. There was a significant difference in behavior between normal and shear forces, suggesting that there are fundamentally different dynamics occurring in the two modes when the cells detach from the surface. Under normal forces, weakly bound cells with single-linkage bonds are rapidly removed from the surface; strongly adherent bond remain detached with minimal detachment later. When there are bonds with multiple linkages, there is a constant rate of detachment when force is applied. We believe that the lower $k_{eq}$ of the multiple linkage bonds makes them more susceptible to failure compared to when there is a single linkage. With multiple linkages, the failure of a single bond causes the cell to shift position such that the broken bond cannot reform at any significant rate, which puts additional stress on the remaining bonds. With the increased strain, the remaining bonds are thus more likely to also fail, eventually leading to the detachment of the cell from the surface.

We also explored the effects of changing two of the physical properties of the bonds. We found that, as expected, increasing the intrinsic off rate lowers the critical force of bead detachment. We also found that the bond with the higher off rate controls
the overall strength of the bond. This result confirms our intuition that the bond with the higher off rate would be the controlling factor regarding the critical force, which also impacts the rolling velocity of the cell. In line with these results, higher intrinsic off rates lead to higher rolling velocities.

We also changed the spring constants of the individual linkages and found that changing the spring constant of a single linkage did not significantly change the critical force required for detachment but changing the spring constant of both linkages could alter the critical normal force. We determined that the separation distance decreased with increasing spring constant, which lead to lower levels of strain on those bonds. This also impacted the rolling velocity, with higher spring constants leading to lower rolling velocities, as the bonds were more able to maintain the cell in a position where broken bonds had a chance to reform.

Our results highlight the effect that bond in series might have on both experimental and in vivo processes. In order to make sure that experimental results are representative of in vivo processes, it might be important for researchers to determine which bond failure point is the controlling locus in the experiment. It would be intriguing to apply these methods to recent experiments on engineered T-cell dissociation from surfaces using linkages that involve force sensors to interpret the results in terms of the statistics of failure (5). To do so, it would likely be necessary to include deformable interfaces between cells and surfaces. In addition, many bonds in nature appear to be catch bonds, or catch bonds under some regime of force, meaning that an increase in
applied force decreases the off rate (23). We did not use a catch-slip model for any of our bonds, but it would be informative to see if those changes would impact our results.
References


CHAPTER 6: Adhesive Dynamics simulations quantitatively predict effects of kindlin-3 deficiency on T-cell homing

Abstract

Leukocyte adhesion is important for the proper functioning of the immune system. While leukocyte homing is mediated by adhesion receptors, the activation of these receptors is modulated by intracellular signaling proteins. In Leukocyte Adhesion Deficiency Type 3, the loss of the kindlin-3 prevents the activation of Leukocyte Function-associated Antigen-1 (LFA-1), which leads to a defect in adhesion, causing recurrent infections and bleeding disorders. Here, we use Integrated Signaling Adhesive Dynamics, a computer model of leukocyte rolling and adhesion combined with a simulated intracellular signaling cascade, to predict the response of T cells to depletion of kindlin-3. Our model predicts that cell adhesion is hypersensitive to the amount of kindlin-3 in the cell, while the rolling velocity is independent of kindlin-3 concentration. In addition, our simulation predicted that the time to stop, an important metric of adhesion, would also increase with decreasing kindlin-3 expression. These predictions were all confirmed experimentally in experiments using Jurkat cells with reduced expression of kindlin-3. These results suggest that Adhesive Dynamics is a versatile tool for quantifying adhesion in the immune response and predicting the effects of engineering cellular components.
**Introduction**

To perform necessary homeostasis and effector functions, immune and vascular cells must be able to control when and where immune cells adhere to the vasculature. The multi-step process of cell adhesion in the vasculature is normally referred to as the leukocyte adhesion cascade (1), which is modulated by the inside-out activation of adhesion receptors, most notably integrins (2). In the leukocyte adhesion cascade, immune cells progress from tethering, to rolling, to firm adhesion on the endothelium (1). The steps of initial capture and rolling are controlled by binding between selectins and selectin receptors expressed on the surface of activated endothelium and the leukocyte, respectively (3). While the cell is rolling, it is exposed to chemokines which bind to chemokine receptors on the leukocyte and trigger intracellular signaling cascades which activate integrins on the leukocyte surface (4). These activated integrins then bind to adhesion ligands, such as Intercellular Adhesion Molecule-1 and Vascular Cell Adhesion Molecule-1 (ICAM-1 and VCAM-1), which lead to the firm binding of the leukocyte under shear flow (5). The cascade is then completed when the leukocyte transmigrates across the endothelium to perform appropriate immune functions in adjacent tissues (1).

The details of the intracellular signaling cascade that controls integrin activation is being elucidated. Although the specific molecules involved differ, the basic structure of the cascade is shared similar among all leukocytes (4). Initially, chemokines bind to chemokine receptors, which are typically G-protein coupled receptors (GPCRs), causing exchange of the Gα-bound GDP for GTP and leading to the activation of the G proteins. This activation then leads to the creation of second messengers, such as, but not limited
to, IP$_3$ and diacylglycerol (DAG), causing the activation of GTPases, such as Rap, which activate integrins on the cell surface (6, 7).

The hematopoietic cell-specific integrin activator kindlin-3 is critical to the activation of the integrin LFA-1 (α$_L$β$_2$) (8). Kindlin-3 works with talin to convert integrins from a resting, inactive conformation to an open, extended, highly active conformation. Talin binds to LFA-1 first, partially activating LFA-1 so that it binds ICAM-1 with an intermediate affinity. Kindlin-3 then binds to the partially activated integrin, converting it to the high affinity state (9–11). A loss of function mutation of kindlin-3 causes an immune disorder known as Leukocyte Adhesion Deficiency III (LAD III). LAD III is characterized by neutrophilia and recurrent infections, along with an associated bleeding disorder (12). Previous studies have shown that the stabilization of the high affinity form of LFA-1 by kindlin-3 is required for cells to resist shear forces applied to them both in vitro and in vivo (8, 13–15). In addition, studies using mouse models have suggested adhesion is hypersensitive to the amount of kindlin-3 in cells, with as little as 5% of WT levels allowing for normal cell function (16).

Our lab has previously developed Adhesive Dynamics (AD) to study the biophysics of cell rolling and adhesion (17, 18). Previous studies have highlighted its predictive power regarding the adhesion of leukocytes under flow (19–21). In an important modification, we have developed Integrated Signaling Adhesive Dynamics (ISAD), which integrates a simulated intracellular signaling network within a mechanically accurate simulation of adhesion (22). ISAD modeling was able to recapitulate the rolling and stopping of a lymphocyte cell line on surfaces of recombinant
protein (22), as well as predict that the deletion of diacylglycerol kinase would lead to a gain of adhesive function (23).

Here, we extend ISAD to simulate the effect of reductions in the level of kindlin-3 on lymphocyte adhesion under flow. Our model predicts adhesion is hypersensitive to the amount of kindlin-3, which has been reported in the literature (16) and which we confirm experimentally. ISAD predicted decreases in kindlin-3 will increase the time for a lymphocyte to stop, without decreasing the level of firm binding. In addition, we predict kindlin-3 has no effect on the rolling velocity. Both of these predictions are confirmed experimentally. Our results suggest that ISAD is an accurate simulator of lymphocyte homing which could ultimately be used to improve the targeting of leukocytes to sites of infection and inflammation.
Materials and methods

**Jurkat Cells**

Wild type Jurkat E6.1 cells were maintained in RPMI media supplemented with 10% fetal bovine serum and 100 IU/mL of penicillin/streptomycin. Jurkat E6.1 shkindlin-3 cells were graciously donated by the Burkhardt lab (Children’s Hospital of Philadelphia, Philadelphia, PA) and were maintained in the same media. Cells were stimulated for 48 hours with 1 µg/mL phytohemagglutinin (PHA) and 100 nM phorbol 12-myristate 13-acetate (PMA) to stimulate expression of sialyl-Lewis X (sLex) to allow for true rolling interactions.

**Flow cytometry**

Cells were washed three times in PBS before labeling and resuspended at a final concentration of $10^6$ cells mL$^{-1}$. Samples were then incubated individually with each of the following antibodies: IgG1 κ (clone MOPC-21, Biolegend, San Diego, CA), IgG2 κ (clone MOPC-173, Biolegend), anti-CD11a (αL, clone HI111, Biolegend), anti-CD184 (CXCR4, clone 12G5, Biolegend), anti-CD162 (PSGL-1, clone KPL-1, Biolegend), IgM κ (clone G155-228, BD Biosciences, San Jose, CA), and anti-sialyl-Lewis X (clone CSLex1, BD Biosciences). After incubation according to manufacturer’s directions, cells were washed three times in PBS to remove unbound antibodies. Flow cytometric analysis was performed on a BD Accuri C6 flow cytometer (BD Biosciences) and the CFlow software package. Histograms were generated using FlowJo (FlowJo, Ashland, OR).

**Adsorption of Protein A/G and SDF-1α**

Non-tissue culture treated polystyrene petri dishes (Corning, Corning, NY) were
enclosed using a single well FlexiPerm gasket (Sigma-Aldrich, St. Louis, MO). A solution of 2 µg mL\(^{-1}\) of Protein A/G (Themo-Fisher Scientific, Waltham, MA) and 1 µg mL\(^{-1}\) of SDF-1\(\alpha\) (R&D Systems, Minneapolis, MN) was applied and incubated overnight at 4°C. The surfaces were then washed three times with PBS before a 30 minute incubation of a 0.2% w/v solution of Pluronic F-127 (Sigma-Aldrich). The surfaces were washed again three times with PBS. Next, a solution of 8 µg/mL ICAM-1/Fc and 2 µg/mL P-selectin/Fc chimeras (R&D Systems) was applied to the surface and incubated for three hours at room temperature. The completed surfaces were then washed again three times with PBS before use.

**Flow Chamber Assays**

Flow chamber experiments were performed in a circular parallel plate flow chamber (GlycoTech, Gaithersburg, MD) with a gasket width of 0.25 cm, thickness of 127 µm, and length of 2 cm. Before flow chamber experiments, a functionalized dish was washed with prewarmed PBS and media to remove air bubbles in the flow path. The assembled flow chamber was mounted on an inverted Axiovert 200 (Carl Zeiss, Gottigen, Germany) enclosed by a XL-3 microscope incubator (PeCon, Ulm, Germany). All experiments were performed at 37°C. Cells were suspended at a concentration of 5 x 10\(^5\) mL\(^{-1}\) and were perfused into the flow chamber via syringe pump (Harvard Apparatus, Holliston, MA) at a flow rate corresponding to a calculated wall shear rate of 100 s\(^{-1}\). Rolling and adhesion of T cells on the immobilized ICAM-1 and P-selectin was observed via phase contrast microscopy under a 10X objective (NA = 0.2, Type A-Plan, Carl Zeiss).
Data Acquisition and Cell Tracking

A CCD camera (QImaging, Surrey, BC, Canada) was used to monitor cell adhesion events with adhesive P-selectin/ICAM-1/SDF-1α substrates. Adhesion of Jurkats was recorded on DVD+RW discs for cell tracking analyses. Cell adhesion videos were redigitized to 640×480 pixels at 29.97 frames s⁻¹ and deinterlaced with HandBrake software (http://handbrake.fr/) then converted to image stacks with MATLAB (MathWorks, Natick, MA). The stacked images were thresholded and converted to binary images. The coordinates of the centroid of interacting cells with a surface every video frame were then acquired using the MTrack2 plugin (http://valexlab.ucsf.edu/~nico/IJplugins/MTrack2.html) in the ImageJ program (http://imagej.nih.gov/ij; National Institutes of Health).

Analysis of Cellular Adhesion

MATLAB was used to analyze the tracked centroids obtained from the MTrack2 plugin. Rolling was defined as the movement of an interacting cell over the substrate at an instantaneous velocity in any frame less than 20% of the calculated free stream velocity of a noninteracting cell (24) while remaining in the field of view. Cell stopping was defined as reduction of its instantaneous velocity below 5% of the free stream velocity. Adherent T lymphocytes were further classified as tethering, rolling or firmly arrested cells based on their instantaneous velocities and the duration of their stopping. A tethering cell is defined as a cell that “rolls” less than 30 video frames (1 s) in a rolling period within the field of view. A firmly arrested cell is defined as a cell that “stops” stably more than 90 frames (3 s). Time and distance to stop were both calculated based
on the initial time and location of the cell rolling. Values for figures were calculated based on cells that interacted with the surface. Cells which were tracked but did not interact with the surface were excluded from the analysis.

**Integrated Signaling Adhesive Dynamics**

In this study, we updated a previously developed ISAD model, which was originally designed to simulate the loss of the protein diacylglycerol kinase in T lymphocytes and its effect on T cell homing (22, 23). The major updates are listed below and the parameters used in the model included in Tables 6.1 and 6.2:

i. An intermediate affinity form of LFA-1 was added to the model to allow for determination of the effect of integrin activation on homing. It is assumed in this model that the binding of talin to the inactive form of LFA-1 converts it directly to the intermediate affinity form (2, 9, 10).

ii. Kindlin-3 was added to convert intermediate affinity LFA-1 to high affinity, fully active LFA-1, in line with previous studies (8, 10). Talin and kindlin-3 are present in equi-molar amounts (16). Kindlin-3 is presumed to have no other role in the cell other than serving as an integrin activator and is assumed to be distributed evenly in the cytosol. Kindlin-3 is also assumed to have the same diffusivity as talin.

iii. The reaction rates of talin binding to LFA-1 remained unchanged from previous iterations of the ISAD model (22, 23). Kindlin-3 was assumed to react with intermediate affinity LFA-1 with a forward reaction rate of $10^3 \mu M^{-1} s^{-1}$ and a reverse reaction rate of 0.1 s$^{-1}$. 

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<table>
<thead>
<tr>
<th>Parameter</th>
<th>Definition</th>
<th>Value</th>
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<tbody>
<tr>
<td>R</td>
<td>Cell radius (µm)</td>
<td>5.75</td>
</tr>
<tr>
<td>Rₜ</td>
<td>Nucleus radius (µm)</td>
<td>4.7</td>
</tr>
<tr>
<td>̇γ</td>
<td>Shear rate (s⁻¹)</td>
<td>100</td>
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<tr>
<td>CXCR4</td>
<td>Chemokine receptor at the plasma membrane (per cell)</td>
<td>4.8×10⁴</td>
</tr>
<tr>
<td>Talin</td>
<td>Talin in the cytosol (per cell)</td>
<td>1×10⁴</td>
</tr>
<tr>
<td>Kindlin-3</td>
<td>Kindlin-3 in the cytosol (per cell)</td>
<td>1×10⁴</td>
</tr>
<tr>
<td>LFA-1Low</td>
<td>Low affinity LFA-1 at the cell surface (per cell)</td>
<td>4.8×10⁴</td>
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<tr>
<td>PSGL-1</td>
<td>PSGL-1 at the cell surface (per cell)</td>
<td>7.5×10⁴</td>
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Table 6.1: Parameters used in ISAD simulation.
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<th>Parameter</th>
<th>Definition</th>
<th>PSGL-1 + P-selectin</th>
<th>LFA-1\textsubscript{Low} + ICAM-1</th>
<th>LFA-1\textsubscript{Int} + ICAM-1</th>
<th>LFA-1\textsubscript{High} + ICAM-1</th>
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<tr>
<td>$\lambda$</td>
<td>Equilibrium bond length (nm)</td>
<td>70</td>
<td>24</td>
<td>44</td>
<td>44</td>
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<tr>
<td>$\sigma$</td>
<td>Bond spring constant (pN nm\textsuperscript{-1})</td>
<td>5</td>
<td>50</td>
<td>50</td>
<td>50</td>
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<tr>
<td>$a$</td>
<td>Radius of reactivity ($\mu$m)</td>
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<td>5*10\textsuperscript{-4}</td>
<td>0.0015</td>
<td>0.003</td>
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<td>$k_{on}$</td>
<td>Intrinsic on rate ($\ast10^5$ sec\textsuperscript{-1})</td>
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<td>0.1</td>
<td>0.1</td>
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<td>Ligand density ($\mu$m\textsuperscript{-2})</td>
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<td>50</td>
<td>100</td>
<td>500</td>
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<td>$k_{1rup}$</td>
<td>Fast pathway dissociation rate (s\textsuperscript{-1})</td>
<td>10</td>
<td>19</td>
<td>13</td>
<td>17</td>
</tr>
<tr>
<td>$k_2$</td>
<td>Unstressed slow pathway dissociation rate (s\textsuperscript{-1})</td>
<td>0.37</td>
<td>0.55</td>
<td>0.41</td>
<td>0.02</td>
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<td>$f_{10}$</td>
<td>Thermal energy divided by the position of the first transition state (pN)</td>
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<td>k_B T/0.056</td>
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<td>$f_{12}$</td>
<td>Difference between the two pathways</td>
<td>5</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>$\Phi_0$</td>
<td>Equilibrium constant between the two states at zero force</td>
<td>100</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Table 6.2: Receptor-ligand kinetic constants used in the ISAD simulation.
iv. The outer radius of the cell was increased to 5.75 µm and the radius of the nucleus increased to 4.7 µm to match previously published values for Jurkat cells (25) and our own measurements (data not shown).

The Next Subvolume Method of ISAD stochastically simulates both the reactions and diffusion of the various molecular components of the signaling cascade. In contrast, Adhesive Dynamics is solely concerned with the physical actions occurring at the cell-substrate interface. The simulations begin with the cell close to, but not interacting with, a surface containing simulated chemokine, P-selectin, and ICAM-1 at a shear rate of 100 s\(^{-1}\). The NSM updates the intracellular reactions due to the binding of chemokine to the receptors on the cell surface. As the reaction proceed, the NSM can also change the number of extracellular receptors as integrins change activation states. Ultimately, AD determines the trajectory of the cell over the surface based on the binding and unbinding of the PSGL-1 and low, intermediate, and high affinity LFA-1 on the surface of the cell.

The two systems share information via shared data structures which are accessible at any point during the simulation. Intracellular protein amounts were updated in each subvolume according to the Gillespie algorithm, while the timestep for AD was fixed at 10\(^{-6}\) s. These simulations were programmed in C++ using Xcode 9 (Apple, Cupertino, CA) and run on a Mac Pro with 3.0 GHz 8-core Intel Xeon processor. Results were then summarized using a custom written R script.
Results

Figure 6.1 shows the general structure of the ISAD simulation. The cell is simulated as a rigid sphere coated with receptors on microvilli moving across a flat surface coated with ligand at set densities. PSGL-1 is clustered on the tips of the microvilli, while LFA-1 is distributed equally across the surface of the cell. The LFA-1-ICAM-1 and PSGL-1-P-selectin bonds are assumed to have catch-slip behavior, which is simulated using the Evans two-state model (26). Microvilli are allowed to deform viscoelastically, in line with previous reports (22, 23, 27). Bonds are tested for formation and breakage in a stochastically, with on and off rates dependent on the deviation from the resting length of each receptor. The cytosol of the cell is discretized into subvolumes based on a spherical lattice. Each subvolume, where appropriate, allows for all reactions of the signaling cascade to occur inside. The signaling cascade is a generalized model of the signaling occurring to activate leukocyte integrins. It begins with the binding of chemokine to chemokine receptors on the cell surface, which triggers the release of G proteins. These proteins can interact with other molecular species to catalyze the creation of second messengers like IP$_3$ and DAG. DAG then activates Rap1 GTPase, which triggers the association of Rap1 and talin, leading to the intermediate activation of LFA-1. Finally, kindlin-3 binds to intermediate affinity LFA-1 to convert it to the fully active, high affinity form of LFA-1. We hypothesized that the quantitative depletion of kindlin-3 would reduce the level of adhesion by inhibiting the creation of high affinity LFA-1.

The effects of kindlin-3 depletion on lymphocyte adhesion are shown in Figure 6.2. Our model predicts that the level of cell adhesion is hypersensitive to the amount of
Figure 6.1: Diagram of Integrated Signaling Adhesive Dynamics simulations. (A) Model geometry for simulation of a T lymphocyte interacting with a functionalized surface in shear flow. The contact zone (highlighted by dashed box) allows for the stochastic formation and breakage of bonds between adhesive receptors on the cell and ligands on the flat surface. Adhesive receptors are located on viscoelastic microvilli. Shear flow is in the X direction, causing a translational velocity $U$ and rotational velocity $\Omega$. (B) Discretization of the lymphocyte membrane and cytosol into subvolumes using a spherical lattice. (C) Signaling cascade for integrin activation. Binding of chemokine to chemokine receptors releases G proteins to activate PLC. PLC then converts $\text{PIP}_2$ to $\text{IP}_3$ and DAG, which binds to a GEF. This molecule can then trigger the exchange of GDP for GTP in Rap1, allowing it to recruit talin to the membrane to initially activate LFA-1. Kindlin-3 then binds to convert LFA-1 into its high affinity form. Species in parentheses are not explicitly enumerated in the model.
Figure 6.2: Results of ISAD simulations. (A) Graph showing the effect of kindlin-3 expression (normalized to WT) on the fraction of cells stopping. (B) Plot showing the effect of kindlin-3 expression on the rolling velocity of cells. (C) Graph showing the effect of kindlin-3 expression on the time to stop of simulated cells. (D) Plot showing the effect of kindlin-3 expression on the distance to stop of simulated cells. All plots are the mean ± SEM of 500 independent simulated cells.
kindlin-3 in the cell. There was no significant effect on adhesion when kindlin-3 was depleted to a level of 20% of normal WT expression levels. Below this level, the level of adhesion significantly decreased such that there was no adhesion in the simulated kindlin-3 KO cells, as highlighted in Figure 6.2A. Our model also predicted that kindlin-3 levels would not affect the rolling velocity of cells (Figure 6.2B). Interestingly, the model predicts an increase in the time to stop for the cells as the level of kindlin-3 decreased in the cell, as shown in Figure 6.2C. The time to stop was inversely logarithmic with the level of kindlin-3. Finally, Figure 6.2D shows how the distance to stop is affected by kindlin-3, which combines the effects of the rolling velocity and the time to stop. The distance to stop showed a linear increase with a small slope kindlin-3 expression as long as the level of kindlin-3 was above 20% of the expression of WT cells. However, the rate of change in distance to stop with kindlin-3 levels was greater once the expression of kindlin-3 fell below 20% of the WT expression levels. These results suggest that expression of about 20% is the “tipping point” below which the ability of the cells to adhere to the endothelial surface is significantly degraded.

In order to confirm our simulation results, we compared the adhesion of WT and a shkindlin-3 knock down Jurkat T lymphocytes for their ability to adhere to a surface coated with recombinant adhesion proteins and chemokines. A representative western blot of kindlin-3 levels in Jurkat cells is shown in Figure 6.3A, showing an approximately 84% reduction in kindlin-3 expression in shkindlin-3 knock down Jurkat T lymphocytes. Figure 6.3B shows the quantification of three independent blots. In addition, shkindlin-3 knock down Jurkat cells had similar levels of expression of both LFA-1 and PSGL-1 to
Figure 6.3: Results of kindlin-3 KD in Jurkat cells. (A) Representative western blot showing knockdown of kindlin-3 in Jurkat cells. (B) Quantification of kindlin-3 knockdown. Overall KD was 83.7 ± 1.6% (mean ± SEM, n = 3). (C) Results of flow cytometry staining of shkindlin-3 (grey) and WT (black) cells. Dashed lines are isotype controls. Plots comparing the fraction of cells stopping (D), rolling velocity (E), time to stop (F), and distance to stop (G). Stars indicate p < 0.05 according to a two-tailed Student’s t test. All plots have shkindlin-3 cells in grey and WT cells in black, showing mean ± SEM (n = 4).
the WT Jurkat cells, as shown in Figure 6.3C. Our experiments showed that there was a significant decrease in the level of adhesion of the shkindlin-3 cells compared to the WT control, as shown in Figure 6.3D. There was no significant difference in the rolling velocity between the shkindlin-3 knock down and WT cells, consistent with predictions (Figure 6.3E). Also, as shown in Figure 6.3F, there was a significant difference in the time to stop when comparing the shkindlin-3 Jurkat cells to the WT Jurkat cells. Finally, although there was an increase in the distance to stop, as predicted by the model, this difference was not statistically significant (Figure 6.3G). A direct comparison between the simulation predictions and experimental results can be seen in Table 6.3.
<table>
<thead>
<tr>
<th>Metric</th>
<th>Simulation prediction (n = 500)</th>
<th>Experimental result (n = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fraction stopping</td>
<td>-13.9%</td>
<td>-17.2 ± 6.6%</td>
</tr>
<tr>
<td>Rolling velocity</td>
<td>-1.0 ± 2.0%</td>
<td>+9.6 ± 5.5%*</td>
</tr>
<tr>
<td>Time to stop</td>
<td>+29.7 ± 2.2%</td>
<td>+62.8 ± 16.1%</td>
</tr>
<tr>
<td>Distance to stop</td>
<td>+27.2 ± 4.4%</td>
<td>+45.4 ± 11.4%*</td>
</tr>
</tbody>
</table>

Table 6.3: Comparison of simulated and actual differences between WT and shkindlin-3 cells. All values mean ± SEM. *: Difference was not statistically significant.
Discussion and conclusions

In this chapter, we have presented a simulation using ISAD which predicts the effect of quantitative reductions of expression of the hematopoietic-cell specific integrin activator kindlin-3. We compared the simulations to experiments using WT and shkindlin-3 Jurkat cells to confirm the predictions of the model. Our results suggest that ISAD modeling can accurately predict the effect of a decrease in the expression level of kindlin-3 quantitatively.

Much work has focused on the effect of a loss of kindlin-3 on the function of leukocytes and platelets (28). Kindlin-3 has been implicated in the activation of LFA-1, along with the clustering of integrins, on platelets (8, 14). While knock-out systems provide a snapshot of what happens when a molecule is eliminated, it is often more instructive to understand what might happen with quantitative reductions which can be achieved through siRNA, shRNA, or engineering of mice. In our simulation, the binding of chemokine to the chemokine receptor triggers an intracellular signaling cascade that results in the activation of LFA-1 on the surface of the cell. This model is somewhat simplified from the known biology, as several intermediate steps known to occur in leukocytes (e.g. Rap1-GTP-interacting adaptor molecule linking talin and Rap1GTP to promote talin membrane localization (29–31)) are not explicitly included. In addition, our model does not incorporate the possibility of outside-in signaling through PSGL-1, which has been shown previously to activate integrins that lead to reductions in rolling velocities of leukocytes (32–34). Ca^{2+} signaling might also to play an important role in integrin activation, but was not explicitly included here (35, 36). Despite these
limitations, the model was able to accurately simulate the rolling and adhesion of cells on a flat surface.

Previous experiments have shown that as little as 5% of WT kindlin-3 expression levels can rescue the LAD III phenotype in mice, and that cell adhesion is hypersensitive to the level of kindlin-3 in the cell (16). These findings are consistent with our simulation results showing that at 5% WT kindlin-3 expression, approximately half of the cells will be able to stop, while showing no effect on cell stopping for kindlin-3 levels above 20% of WT expression. These results also are consistent with our experimental findings that kindlin-3 KD of ~84% results in only a 17% reduction in adhesion. In addition, Moser and colleagues found no change in the in vivo rolling velocities of WT and kindlin-3 KO cells in stimulated mouse cremaster muscles (14). This matches our findings from simulations that the selectin-mediated rolling of leukocytes is not perturbed by the absence of kindlin-3.

Our model also predicted an increase in the time to stop when kindlin-3 expression was decreased. This increase is driven by the local depletion of kindlin-3 closest to the plasma membrane, requiring increased diffusion from interior pools of kindlin-3 in the cytosol. The time delay is due to the time for kindlin-3 to diffuse to the plasma membrane. We also confirmed the increase in the time to stop experimentally, although the magnitude of the change was larger than the model predicted. Our model predicted an increase of about 30%, but the experiments showed an increase of more than twice the prediction. The basis of this discrepancy is unclear but could be related to specific localization of kindlin-3 or a lower diffusivity than was assumed.
In this paper, we directly compared the results of ISAD simulations to results from an *in vitro* experimental system. Our experimental system used a flat surface and defined molecular components to more closely match the simulations. It might be more physiologically relevant to include surface roughness from the shape of endothelial cells, which would require solving for the complex motion of a deformable sphere caused by non-laminar flow. In addition, the wider variety of molecular interactions would require understanding all of the possible interactions, as well as the effect of clustering of adhesion molecules on the apical surface. Also, our model did not include the effect that kindlin-3 has on integrin clustering (9, 37), but we suspect this becomes only a major effect after the leukocyte has come to a halt. Finally, our model does not allow for force applied to integrin-ligand bonds to change the affinity state of the integrins, as has been shown previously (38). These complications would require additional effort and resources and could be the basis of future work.

In conclusion, we have used an ISAD simulation to predict *a priori* the effect of a kindlin-3 depletion on the ability of lymphocytes to adhere to a simulated endothelium. Our results suggest that the hypersensitivity of cell adhesion to kindlin-3 results from its ability to stimulate a sufficient number of integrin interactions to secure adhesion. Finally, this manuscript highlights the ability of ISAD to accurately and quantitatively simulation leukocyte adhesion and highlights it ability to predict the effect of intracellular modifications on cell adhesion.
References


PSGL-1 or integrin αLβ2. J. Biol. Chem. 287: 19585–19598.


CHAPTER 7: Conclusions and Future Work

Specific Aims

The research presented in this thesis has improved our understanding of the biophysics involved in leukocyte adhesion and how Leukocyte Adhesion Deficiency Type III can affect this process. The specific aims of this thesis were as follows:

Aim 1: Determine the state diagram of CD4+ T cells interacting with various densities of ligand using an engineered surface with recombinant proteins.

Aim 2: Use adhesive dynamics to understand the impact of multiple bonds in series on cell detachment.

Aim 3: Use adhesive dynamics to predict the effect of quantitative reductions of kindlin-3 on leukocyte adhesion and confirm those predictions experimentally.

Specific Findings

*Site density of ligand impacts the type of adhesion experienced by primary human CD4+ T cells*

We first investigated the effect of different surface densities on the ability of primary human CD4+ T cells to tether, roll, and firmly adhere to surfaces containing different densities of E-selectin and ICAM-1. Previous work from our lab had suggested that there would be a lower limit to the density of these ligands, below which the cell would be unable to adhere. In addition, this work also predicted that a system containing both selectins and integrin ligands would show a level of synergy, where reductions in the level of one ligand could be compensated for by increases in the other (1). We found that there was indeed a “cutoff” to the level of ligand on the surface required for cell
adhesion. We determined that this level was $O(10^9)$ sites/$\mu m^2$ for tethering, while it was $O(10^1)$ sites/$\mu m^2$ for rolling and $O(10^2)$ sites/$\mu m^2$ for firm adhesion. Surfaces containing both ICAM-1 and E-selectin were the most efficient at allowing cells to firmly adhere, while surfaces containing only E-selectin or ICAM-1 were not as effective recruiting cells. In addition, we did find a level of synergy for the system, as reductions in the level of ICAM-1 could be countered with increasing the level of E-selectin without impacting the ability of the cells to adhere to the surface. This study shows that differing levels of selectin and integrin ligands on a surface impacts the ability of cells to adhere, which may have relevance for improving the targeting of engineered immune cells.

*Primary human CD4$^+$ T cells migrate upstream after completing the leukocyte adhesion cascade*

Previously, our lab and others have shown that activated T cells will migrate against the direction of shear flow on surfaces containing ICAM-1 (2, 3). We also noticed this behavior on cells which had not been activated during the ligand density experiments. We wanted to determine the time scale of the decision-making process in these cells regarding the choice of migration direction. Using a surface of P-selectin and ICAM-1 and/or VCAM-1, along with human umbilical vein endothelial cells (HUVECs), we explored the direction of migration in primary human CD4$^+$ T cells. On surfaces containing ICAM-1 (ICAM-1 only, ICAM-1 + VCAM-1, and stimulated HUVECs), we observed migration against the direction of flow, in line with previous results. Surfaces containing solely VCAM-1 only supported downstream migration, with cells moving in the same direction as the shear force. We determined that the choice of direction of
migration is determined within 30 seconds, before morphological polarity is established in the cells. Finally, we showed that cells migrating upstream transmigrate across the endothelial layer faster than cells migrating downstream. These results further highlight the possible physiological relevance of upstream migration and suggest that engineering T cells to perform this activity might increase their ability to transmigrate.

_Bonds with multiple linkages show non-linear changes in apparent bond strength_ 

Bonds in series are important both in biology and in the lab. One example that is biologically relevant is the binding of the T cell receptor (TCR) with a major histocompatibility complex (MHC) bearing an antigenic peptide. If either the bond between the TCR and the peptide or the MHC and the peptide fails, the T cell may not become activated. Alternatively, an example used in this thesis the experimental system used in the previous sections. Previous research has suggested that two linkages in series have a strength half that of a single linkage with the same properties. Unfortunately, this research was performed at the high bond number limit (4), while many cellular processes occur at lower bond numbers (5). We used Adhesive Dynamics, a physically exact simulation technique, to determine how a cell detaches from a surface with varying numbers and types of linkages to the surface. We found that, under normal forces, there is a nonlinear decrease in the adhesive strength as the number of linkages increases, which is different from previously reported results. Most interestingly, we found that cells attached to the surface via one or two linkage bonds had the same critical force and rolling velocity under shear force. However, the dynamics of the detachment were different between the two conditions, as the cells with multiple linkages detached from
the surface in a more linear response curve to the force. Finally, we found that the intrinsic off rate will influence the critical force required to detach cells, while the spring constant of the bond does not affect that metric significantly. These results highlight the need for addition research into how bonds with multiple linkages fail.

*ISAD simulations quantitatively predict response to kindlin-3 depletion*

Kindlin-3 is a protein involved in inside-out integrin activation in leukocytes (6). Missing or nonfunctional kindlin-3 causes the serious immune disorder Leukocyte Adhesion Deficiency Type III due to the inability of the cell to activate LFA-1 to a high affinity form (7). Previously, our lab developed a version of Adhesive Dynamics which incorporates an intracellular signaling cascade (8). We have updated this system to include kindlin-3 activation of LFA-1 on the surface of the cell. We hypothesized that these simulations would be able to predict the result of a decrease in the level of kindlin-3. Our simulations showed that adhesion of the cells was hypersensitive to the level of kindlin-3 in the cell, while the rolling velocity was not affected. In addition, both the time and distance to -stop increased as the level of kindlin-3 decreased. We then sought to confirm our simulation results by using shRNA knockdown of kindlin-3 in the Jurkat T cell line. We found an approximately 84% knockdown in kindlin-3 in these cells. This resulted in a significant, albeit slight, decline in the level of adhesion with no significant change in the rolling velocity. There was also a significant increase in the time to stop and a statistically insignificant increase in the distance to stop. These results are in line with previously reported results (9, 10). This study highlights the predictive potential of modeling and its possible role in screening ways to enhance T cell targeting.
Future Work

*Influence of endothelial layer topology*

This thesis almost exclusively used flat engineered surfaces to understand the biophysics of leukocyte adhesion. However, cells do not interact with flat surfaces *in vivo*. The endothelial layer is composed of cells, which will impart a certain 3D topology to the surface. It would be useful to understand how that 3D shape might affect the recruitment of leukocytes to specific locations. For example, would cells be more likely to arrest on lower ligand densities if there is such a topology on the surface? Or how does the curvature of the blood vessel wall impact recruitment? It is possible to create engineered 3D structures using lithography to then allow us to test these hypotheses. In addition, it could give us additional insight into T cell migration by showing us if cells are more likely to migrate “over the top” of cells or if they prefer to stay in the “valleys” between cells. This technique would allow us to parse the differential impacts of receptor-ligand signaling and local topology as directional cues for migration by allowing us precise control of the molecular components of the surface while maintaining a constant topology of the surface.

*Effect of bond model and distribution on detachment*

The results presented in Aim 2 of this thesis consist of a single bond detachment model. However, there are a wide variety of bond models available (11–13), which respond to force in different ways. Most obviously, the bonds tested in Aim 2 were solely slip bonds, where force always accelerates detachment. In contrast, many bonds of interest are catch-slip bonds, where an increase in force leads to a decrease in the off rate
before reaching a critical value, above which the bonds act like slip bonds. Understanding how the different types of bonds interact might shed light on how bonds with mixed bond models detach from a surface.

In addition, cells rarely interact with a surface or another cell through a single receptor binding to a single ligand outside of highly controlled laboratory conditions. In fact, cells are attached usually to the surface through a variety of bonds working in parallel. This work did not study how different bonds, acting in parallel, would influence the detachment of the entire system. It is not immediately clear how having the various bonds and linkages would interact, as there could be synergistic effects when the different bonds are combined, and whether those effects are dependent on the mechanics of the overall bonds present or the linkages within those bonds.

More concretely, with additional biophysical characterization of bonds of interest, it should be possible to accurately simulate experiments in the literature to determine where a bond is most likely to fail. For example, Hong and coworkers used a peptide-MHC covalently linked to a DNA force sensor, which was then attached to a surface using streptavidin-biotin interactions to study the role of cell-generated forces in positive and negative selection of thymocytes. However, although they could measure overall bond lifetime, they were unable to determine the exact point of failure within this complex (14). Our simulations could determine the relative likelihood of detachment of the various components, which might then allow for the adjustment of the experimental data to show the “true” strength of the linkage most of interest. These results could then be verified experimentally through labeling of the different components and quantitative
microscopy, as was done by Spillane and Tovar in their studies of B cell antigen extraction (15).

Sensitivity to chemokine signaling network perturbations

Although the intracellular signaling cascade in this thesis is presented as complete, there is still some uncertainty regarding the interaction of the different components. For example, the binding of PSGL-1 to P-selectin can trigger intracellular signaling to integrins on the leukocyte (16). However, the exact molecular mechanisms of this interaction, and thus the network topology, have not been completely determined. Furthermore, there is significant cross talk between the signaling cascades following PSGL-1 ligation and chemokine receptor binding, as shown in Figure 7.1 (17). While it is difficult to determine the missing proteins experimentally, it should be possible to use ISAD simulations in order to screen different network topologies and find those networks which most closely match experimental results. This could then help to narrow the possible identities of the unknown proteins, enhancing our ability to search for them. In addition, these same simulations could also be used to study why such an interconnected network has evolved. One hypothesis could be that these networks might promote more or faster leukocyte adhesion than would be found using a single signaling cascade. The simulations could also identify important proteins involved in the crosstalk, thus suggesting experimental targets for genetic manipulation.

ISAD simulations are also uniquely suited to studying the sensitivity of cellular adhesion to perturbations in protein concentrations, protein activity, or network topology. For example, it is often expensive and time consuming to generate an engineered cell line
Figure 7.1: Diagram of signaling cascades following PSGL-1 ligation (green) or chemokine receptor binding (blue). Molecules in both green and blue are involved in both cascades. The controlling factors of kindlin3 are unknown. Republished with permission of IOS Press, from (17); permission conveyed through Copyright Clearance Center, Inc.
with altered levels of a single protein. In contrast, it is a simple matter to alter that protein concentration in our simulations, as it only involves altering a single number in the program. Thus, we can easily scan over concentrations of proteins in the signaling cascade to find those which cause the largest change in the adhesiveness of the cell. To go even further, we can also search for synergistic effects caused by knock down or knock out of multiple proteins at the same time. Beyond protein concentrations, we can also alter the activity of every protein within the signaling cascade. By studying the changes in cellular adhesion, it might be possible to identify druggable targets which could be acted on by small molecules to either enhance or depress protein activity to cause a desired outcome. With the advent of therapeutics based on engineered immune cells, we are not limited to studying an intracellular signaling cascade as it exists in nature. We could use ISAD to study the effects of adding new control loops to the cascade, such as feed forward or feedback loops. These altered network topologies could result in desirable behavior, such as faster stopping on an endothelial layer or reduced chemokine density requirements.

*Effect of LFA-1 activation by force*

In addition to the chemokine-based inside-out activation presented in this thesis, LFA-1 can also become activated through the application of force (18). Obviously, this outside-in activation is not enough on its own to cause leukocyte arrest, as shown by LAD III patients and model systems (7). However, the role of such outside-in integrin activation during the leukocyte adhesion cascade has not been elucidated. ISAD simulations are uniquely suited to probe this question. By adding this force activation
functionality to the model, we may be able to determine whether this plays a large role in the recruitment of leukocytes to areas of inflamed endothelium. It is easy to imagine that kindlin-3-independent activation of integrins might help arrest cells on a surface by allowing for high affinity integrins while maintaining an elevated local concentration of kindlin-3 to allow for additional integrin binding.
References


Figure A.1: Determination of site densities using an IgG1 probe. Protein A/G and SDF-1α were co-immobilized in polystyrene microwells and incubated with IgG1. Site density was determined by comparing to fresh dilutions of an AlexaFluor 488-tagged anti-human IgG1 hinge antibody. Results shown are mean ± SE of three independent experiments.
Figure A.2: Extended state diagrams of the fraction of cells undergoing (A) tethering, (B) rolling, or (C) firm arrest, including surfaces with high site densities. Red dots indicate
experimentally tested points. All experiments were performed at a calculated wall shear rate of 100 s$^{-1}$.
Figure A.3: Comparison between cell spreading after arrest on (A) E-selectin only surfaces and (B) surfaces with E-selectin and ICAM-1. Both images are the end of 10 min flow chamber experiments and are representative of repeated experiments. Orange arrowheads point to arrested cells that are not spread and green arrowheads identify arrested cells that have spread. Surfaces containing only E-selectin show fewer arrested cells than surfaces containing both E-selectin and ICAM-1. Cells arrested on E-selectin surfaces also did not spread efficiently, shown by the fewer cells marked with green. Cells not marked by arrowheads are rolling or tethering during the moment this frame was taken.
Figure A.4: Calculated surface showing the effect of ICAM-1 and E-selectin densities on the distance to stop. Red dots indicate experimentally tested points. All experiments were performed at a calculated wall shear rate of 100 s⁻¹.
Figure A.5: Flow cytometric characterization of primary human T cells. (A) Cells are > 98% CD3+CD4+ T cells by flow cytometry. (B) Roughly 55% of cells are CD45RA+, indicating that slightly over half of cells are naïve T cells. (C) Cells robustly express L-selectin (CD62L) and (D) PSGL-1, indicating their ability to perform the leukocyte adhesion cascade.
Figure A.6: T cells migrate upstream on HUVECs stimulated for 48 hours. (A) Plot of migration index over time for surfaces of HUVECs stimulated for 48 hours with TNFα. Upstream migration is indicated by a negative migration index, downstream migration by positive values, and random migration by values near zero. Data presented is mean ± SEM, n = 4 independent experiments. (B) Plot showing the remaining fraction of tracked cells at each time point. Cells on HUVEC monolayers were tracked from initial migration...
to transmigration or the end of the experiment, whichever is sooner. (C) Comparison of fraction of cell which migrated upstream. Data presented is mean ± SEM, n = 4 independent experiments. (D) Comparison of the time from arrest to transmigration. Data presented is mean ± SEM, n = 4 independent experiments. * p < 0.05.
Figure A.7: Comparison of fitted detachment slopes for varying numbers of linkages. (A)
Fitted slope (black bar) and 95% CI (shaded area) of cells detaching under a normal (A)
or shear (B) force.
Figure A.8: Comparison of bond failure points. The breakage point of every bond in a single (A), double (B), or triple (C) linkage bond was recorded and the probability that a certain linkage failed was calculated. The bond closest to the cell is “Bond 1,” “Bond 2”
is the next furthest out, while “Bond 3” is the furthest away from the cell. Since all bonds has the same characteristics, there was no difference between the breakage rates. $\tau$ is dimensionless time, with scale factor $1/k_{in}$. 
Figure A.9: Comparison of bonds with differing intrinsic off rates. (A) Fitted slope (black bar) and 95% CI (shaded area) of cells detaching under a normal (A) or shear (B) force. (C) Comparison of the time averaged number of bonds between the cell and the surface.
upon system equilibration. (D) Comparison of the bond strain under no force conditions between the cell and the surface. (E) Comparison of the dimensionless separation distance between the cell and the surface under no force conditions. (F) Comparison of failure points in double linkage bonds containing linkages with different intrinsic off rates. Blue lines are DK linkages, red lines are HK linkages, and grey lines are standard C bonds. All values are mean ± SEM, N = 100 cells. $\hat{z}$ has scale factor $R_C$. $\tau$ is dimensionless time, with scale factor $1/k_{on}$. 

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Figure A.10: Comparison of bonds with differing spring constants. (A) Fitted slope (black bar) and 95% CI (shaded area) of cells detaching under a normal (A) or shear (B) force. (C) Comparison of the time averaged number of bonds between the cell and the surface upon system equilibration. (D) Comparison of failure points in double linkage bonds containing linkages with different intrinsic off rates. Green lines are “soft” linkages, orange lines are “stiff” linkages, and grey lines are standard C bonds. All values are mean ± SEM, N = 100 cells. \( \tau \) is dimensionless time, with scale factor \( 1/k^* \).