MCP-1 Induces Rapid Formation of Tethered VLA-4 Bonds with Increased Resistance to Applied Force in THP-1 Cells

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MCP-1 INDUCES RAPID FORMATION OF TETHERED VLA-4 BONDS WITH INCREASED RESISTANCE TO APPLIED FORCE IN THP-1 CELLS

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The chemokine, Monocyte Chemoattractant Protein (MCP-1), enhances integrin-mediated monocyte adhesion to the vascular endothelium during inflammation. In this study, we demonstrate that MCP-1 promotes rapid sub-second adhesion of THP-1 cells to Vascular Cell Adhesion Molecule-1 (VCAM-1), but not to Intercellular Cell Adhesion Molecule-1 (ICAM-1). MCP-1 activates membrane tethered Very Late Antigen 4 (VLA-4, \( \alpha_4 \beta_1 \)), but not necessarily cytoskeleton anchored VLA-4. Activated tethered VLA-4 bonds tremendously increased the period of time monocytes remain bound from hundreds of milliseconds to several seconds and also increased the distance over which immunologic surveillance occurs from several microns up to 20 microns along the endothelium. Lastly at the single molecule level, MCP-1 stimulated tethered VLA-4 bonds exhibit increased resistance to pulling force. In conclusion MCP-1 increased tethered VLA-4 bond resistance to force providing a mechanism for monocyte recruitment to the endothelium.
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TABLE OF CONTENTS

LIST OF FIGURES ........................................................................................................... viii

LIST OF TABLES ............................................................................................................ xiii

Chapter 1 Introduction ................................................................................................. 1

1.1 Overview of monocyte recruitment to the endothelium ................................. 1

1.1.1 Integrins ............................................................................................................. 4

1.1.1.1 Integrin Conformation States .................................................................... 6

1.1.1.2 Integrin Association with Cytoskeletal Adaptor Proteins..................... 12

1.1.2 Immunoglobulin-related Cell Adhesion Molecules (IgCAMs).............. 14

1.1.3 Chemokines .................................................................................................... 15

1.1.3.1 Overview of chemokines ........................................................................ 15

1.1.3.2 Monocyte Chemoattractant Protein-1 .................................................... 16

1.1.4 VLA-4/VCAM-1 and LFA-1/ICAM-1 Roles in Monocyte Adhesion ...... 17

1.1.4.1 Very Late Antigen-4 (VLA-4)/VCAM-1 adhesion complex ................. 17

1.1.4.2 Leukocyte Function associated-Antigen-1 (LFA-1)/ICAM-1 ............... 21

1.2 Atomic force microscope ................................................................................... 21

1.2.1 AFM components ......................................................................................... 22

1.3 Measurement of forces .................................................................................... 25

Chapter 2 Specific Aims ........................................................................................... 28
Chapter 5 MCP-1 increases the occurrence of tethered VLA-4/VCAM-1 bonds................................................................. 51

5.1 Description .................................................................................................................. 51

5.2 Single molecule force spectroscopy background ............................................. 53

5.3 Data Acquisition and Processing ................................................................. 56

5.3.1 Acquiring single molecule measurements .................................................. 56

5.3.2 Data Analysis ......................................................................................................... 57

5.3.2.1 Cytoskeleton anchored bonds ................................................................. 57

5.3.2.2 Tethered bonds ......................................................................................... 59

5.4 Results ......................................................................................................................... 60

5.4.1 Single tethered VLA-4/VCAM-1 ruptures are the most predominant adhesive bond ................................................................. 60

5.4.2 Investigating single tethered VLA-4/VCAM-1 ruptures ...................... 62

5.4.2.1 MCP-1 does not affect effective tether viscosity ......................... 62

5.4.2.2 MCP-1 increases binding strength of individual tethered VLA-
4/VCAM-1 ruptures ......................................................................................... 65

5.4.3 MCP-1 does not affect cytoskeleton anchored VLA-4/VCAM-1 ruptures ........................................................................ 68

5.5 Statistical validation of curve fits to the force spectra............................... 70

5.5.1 Description of the Bootstrap method ......................................................... 70

5.5.2 Bootstrap Data Analysis ..................................................................................... 71
5.5.3 Bootstrap Results ........................................................................................................72

5.5.3.1 Confidence Limits of Parameters from Single Tethered VLA-4/VCAM-1 ruptures .................................................................72

5.6 Discussion ..................................................................................................................75

Chapter 6 Conclusions .................................................................................................81

Bibliography .....................................................................................................................84

Appendix: Calibration of AFM cantilevers for force measurements ..................105

A.1 Calibrating photodiode position sensitivity ......................................................105

A.2 Thermal Fluctuation Method ..............................................................................106
LIST OF FIGURES

Figure 1-1: Different steps of the monocyte adhesion cascade are illustrated. The first stage of monocyte-endothelium adhesion cascade is monocyte rolling which is mainly facilitated by selectin bonds. Biochemical signals trigger the adhesion of monocyte expressed integrins to IgCAMs expressed on the endothelium................................................................................................................. 2

Figure 1-2: Diagram showing all the discovered heterodimeric integrin subunit combinations. Asterisks indicate alpha subunits containing the A-type binding domain. (Figure adapted Figure 1 from Shimaoka et al 200253). ............................................................................................................................... 5

Figure 1-3: Integrin architecture of the different domains. The β-propeller and I domains are the two major domains coordinating ligand binding in integrins32,44,57,61. The interaction between EGF domains 2 and 3 are responsible for the bending of the integrin tails62. Only the domains relevant to integrin activation and binding will be discussed in greater detail in this work. Half of the integrin subunits contain an I-domain (a) and the other half do not (b). Structural conformational changes of the extracellular integrin domains are shown in for an integrin with an alpha subunit containing an I-domain (a) and (b) an integrin without an I-domain in the alpha subunit. Integrins exist in three different conformational states: 1) bent low affinity, 2) extended low affinity, and 3) extended high affinity. The low and high affinity states are equivalent to the closed and open headpiece states, respectively. (Figure is adapted from Luo et al 200732). .............................................. 7

Figure 1-4: The crystallized beta-propeller structure of the αV integrin subunit. Disulfides (sticks) and glycans (spheres) are shown in red. Ca²⁺ ions are green. (Taken from Figure 2 from Xiong et al 200157). ......................................................................................................................................................... 9

Figure 1-5: Illustration of an IgCAM. This IgCAM contains 5 Ig-like domains/folds formed due to a disulfide bond between cysteine residues in each domain. Two fibronectin like domains are also pictured as rectangles proximal to the cellular membrane. (Adapted from Sandi et al 2004 Figure 1120) ............................................................................................................................................................. 15
Figure 1-6: The immunoglobulin domains of the two expressed forms of VCAM-1. The binding domains to VLA-4 are shown in dark gray. VCAM-7D contains two VLA-4 binding sites in Ig-like domains 1 and 4. VCAM-6D lacks the Ig-like domain 4 from VCAM-7D. (Adapted from Figure 1 from Osborn et al 1994).

Figure 1-7: Illustration showing the layout of the essential AFM components. A ~635 nm laser (red line) is reflected off of a cantilever into a photodiode. The amount of cantilever deflection is measured as the voltage difference between the photodiode segments A and B. The piezoelectric element controls the distance between the tip and the sample.

Figure 1-8: Side and bottom view of an MLCT-AUHW (C) cantilever. A V-shaped or triangular cantilever is shown with a pyramidal at the end. (Thickness: ~ 0.55 µm, Width: 20 µm, Length: 310 µm).

Figure 1-9: Detection of motion with a two segmented PSPD. The gray circle represents the laser spot reflected from the cantilever. The voltage difference between segments indicates the direction in which the cantilever is deflected.

Figure 1-10: Cantilever deflection during a force curve. Approach curve (dashed blue) and the retraction curve (solid red). Solid triangles indicate the direction of displacement of the piezo electric element. The y-axis represents either the negative voltage difference from a two segmented photodiode measuring cantilever deflection or the amount of force applied to the cantilever, and the x-axis represents the displacement of the piezoelectric element.

Figure 3-1: AFM components and the system layout. The laser and the photodiode combination are used to measure the cantilever deflection. The piezoelectric module maintains the distance between the cantilever and the sample. The thermoelectric module regulates the sample temperature.

Figure 3-2: Adhesion system measured with the AFM. The adhesion of unstimulated or MCP-1 stimulated THP-1 cells to VCAM-1 or ICAM-1 functionalized substrates was quantified using the AFM. THP-1 cells were attached to cantilevers via poly-L-lysine.

Figure 4-1: Whole cell adhesion parameters extract from a force curve. AFM force curve measuring adhesion between a THP-1 cell and substrate functionalized with VCAM-1 and MCP-1 retracted at
Figure 4-2: VCAM-1 supports larger adhesion than ICAM-1 in unstimulated cells. Top trace: AFM force curves of THP-1 cells adhering to either ICAM-1 or VCAM-1. Bottom Traces: Adhesion parameters quantified from whole cell adhesion curves a) Detachment force b) Detachment energy c) Detachment time/distance. Two-tailed nonparametric Mann-Whitney tests were performed (*p<0.1, ***p<0.001).

Figure 4-3: Whole cell adhesion force curves measured from THP-1 adhesion. a) VCAM-1 b) ICAM-1 c) control surfaces.

Figure 4-4: MCP-1 enhances THP-1 adhesion to VCAM-1 not ICAM-1. Quantification of adhesion parameters measured from AFM whole cell adhesion force curves a) detachment force b) detachment energy c) detachment time/distance. Two-tailed Mann-Whitney tests were performed between ICAM-1 and VCAM-1 measurements (***p<0.001). MCP-1 was exposed to cells via two different methods (left) immobilized on the dish (right) applied to the bath.

Figure 4-5: The response of different mechanical materials being pulled at a constant velocity. When an elastic element is pulled at a constant velocity, the amount force exerted to the probe increases with pulling distance. On the other hand, pulling a viscous element at a constant velocity results in a constant force applied to the probe.

Figure 4-6: MCP-1 increases VCAM-1 bond formation. The number of cytoskeleton anchored and tethered VLA-4/VCAM-1 ruptures was determined for unstimulated and MCP-1 stimulated cells for each curve. Measurements were obtained from at least 10 force curves from 4 different cells for each condition. Nonparametric Mann Whitney tests were performed comparing unstimulated to MCP-1 stimulated measurements (***p<0.001).

Figure 4-7: VLA-4 facilitates THP-1 binding to VCAM-1. Detachment forces of THP-1 cells adhering to VCAM-1 were quantified from untreated cells or after blocking with β1 or β7 subunits. Forces for all the conditions were normalized by non-treated cell detachment forces (No antibody).
Nonparametric Mann-Whitney tests were performed comparing the blocking antibody measurements to cells which were not treated.

Figure 4-8: The β7 subunit is not expressed on THP-1 cells and MCP-1 does not affect the expression integrin levels. Flow cytometry experiments examining THP-1 cell surface expression of the α4 (A), β1 (B), and β7 (C) subunits for unstimulated (green) and MCP-1 stimulated (green) THP-1 cells. Histograms plotting counts vs mean channel fluorescence are displayed.

Figure 5-1: Linear energy barrier of the dissociation of a molecular bond. The changes in the energy barrier height of the system in the absence of force (f=0) and an applied force (f>0). \( \gamma \) is the energy barrier width and \( E_b \) is the energy barrier height, and \( \tau_0 \) is the intrinsic lifetime of the bond.

Figure 5-2: Single cytoskeleton anchored bonds. Determining the loading rate \( (\dot{r}) \) and rupture force \( (F) \) from the force curve of a cytoskeleton anchored VLA-4/VCAM-1 bond.

Figure 5-3: Single tethered bonds. Determining bond lifetime \( (\tau) \) and rupture force \( (F) \) from force curves exhibiting tethered VLA-4/VCAM-1 bonds.

Figure 5-4: Force spectrum of single tethered VLA-4/VCAM-1 bond ruptures. (Inset) Mean tether lifetimes for each velocity group were obtained by fitting lifetimes (dashed line) to the cumulative probability distribution function (Eqn. 5-3). Force spectrum was fit to the Bell model Eqn. 5-1.

Figure 5-5: Frequency of adhesion events in single molecule measurements a) Force curves exhibiting different types of VLA-4/VCAM-1 bonds measured at 30% adhesion frequency. Top trace: tethered VLA-4/VCAM-1 bond. Middle trace: Cytoskeleton anchored VLA-4/VCAM-1 bond. Bottom trace: multiple VLA-4/VCAM-1 bonds. b) Frequency of each type of bond under different stimulation conditions. The actual normalized frequency is listed in each bar.

Figure 5-6: MCP-1 and TS2/16 do not effect tether viscosity. Median tether extraction forces for each velocity group were fit to Eqn. 5-4.

Figure 5-7: Cumulative lifetime probability of measured tethered VLA-4/VCAM-1 rupture lifetimes. Solid lines indicate the 95% upper and lower confidence limits of the cumulative lifetime.
probability distribution function (Eqn. 5-3) to the data. Three different conditions are shown above (left) unstimulated (middle) MCP-1 (right) TS2/16..................................................................................66

Figure 5-8: Force spectra of single tethered VLA-4/VCAM-1 bonds. Force spectra of mean tether lifetimes vs median tether extraction force. Spectra were fit to the Bell Model Eqn. 5-1.............67

Figure 5-9: Dynamic force spectra of cytoskeleton anchored VLA-4/VCAM-1 bonds. Force spectra of median rupture force versus median loading rate were fit to the Bell-Evans model (eqn 5.2)......69

Figure 5-10: Bell-Model fits to the unprocessed tether lifetimes and extraction forces. Symbols represent the unprocessed data and solid black lines represent the Bell-Model fits to the data...73

Figure 5-11: MCP-1 does not affect the energetics or kinetics of single tethered VLA-4/VCAM-1 ruptures. A) Bootstrap estimation of confidence limits of the intrinsic rupture lifetime and width of the energy potential from Bell-Model fits to single tethered VLA-4/VCAM-1 rupture data. B) Intrinsic lifetime and width of the energy potential of single tethered VLA-4/VCAM-1 ruptures determined by fitting to the raw untransformed data in Figure 5-10..................................................74

Figure 5-12: Comparison of conventional and unconventional data sets fit to the Bell-Model fits. The Bell-Model was fit to the total data set without grouping by pulling velocity (unfilled symbols) to data grouped by pulling velocity (solid symbols). Unfilled/open symbols denote intrinsic lifetimes and energy barrier widths were determined from Bell-Model fits to the total set without grouping by velocity (Section 5.5) and filled symbol parameters were obtained from fits to most probable bond lifetimes and median tether extraction forces (Section 5.3.2)...........................79
LIST OF TABLES

Table 5-1: Intrinsic off-rate ($k_{off}$) of the VLA-4/VCAM-1 system measured in response to an applied force. Flow chambers and the AFM were two techniques used to measure the off-rate. .......................... 52

Table 5-2: Experimental parameters for determining the effective viscosity. Fitting Eqn. 5-4 to the data plotted in Figure 5-6. ($\pm$ s.e.m. is shown)............................................................................................................. 64

Table 5-3: Energy barrier widths and intrinsic bond lifetimes of single tethered bonds. Force spectra in Figure 5-8 were fit to the Bell-Model (eqn. 5-1). ............................................................................................................. 68

Table 5-4: Energy barrier widths and intrinsic bond lifetimes of single cytoskeleton anchored bonds. Coefficients from the Bell-Evans model fit to cytoskeleton anchored VLA-4/VCAM-1 bond dynamic force spectra................................................................................................................................................ 70

Table 5-5: Energy barrier width ($\gamma$) and intrinsic bond lifetime ($\tau_0$). Obtained from fitting the Bell-Model to the unprocessed raw data in Figure 5-10............................................................................................................. 73
Chapter 1 Introduction

Monocytes in the immune system protect the human body from harmful foreign substances and aide in the repair of damaged tissue. They circulate in the bloodstream to look for signs of tissue damage. Circulating monocytes are recruited to the vascular endothelium through a series of distinct adhesion events. Biochemical signals released and/or produced from damaged tissue activate monocytes and also activate the endothelium to facilitate monocyte recruitment. One of these biochemical signals is a group of molecules known as chemokines, named for their ability to induce chemotaxis or cellular migration\(^1\). Chemokine induced monocyte activation increases the binding of adhesion molecules expressed on the monocyte cell surface known as integrins to Immunoglobulin-like Cellular Adhesion Molecules (IgCAMs) expressed on the vascular endothelium\(^1-12\). Lastly, these biochemical signals also up-regulate expression of adhesion molecules on the endothelial cell surface\(^13\). The main objective of this paper is to determine how the chemokine, Monocyte Chemoattractant Protein-1 (MCP-1) affects the adhesion of the integrins VLA-4 and LFA-1.

1.1 Overview of monocyte recruitment to the endothelium

Monocytes in circulation are first captured to the vascular endothelium by selectin molecules expressed on the monocyte cell surface\(^14\). Selectins are adhesion molecules which have very high on-rates (association rate) and off-
rates (dissociation rate). The fast on and off-rates allow rapid and reversible binding to the endothelium\textsuperscript{15,16}. In fact, selectins dissociate 100-fold faster than antibodies\textsuperscript{16}. The process of rapid binding and unbinding is known as rolling. During rolling bonds between the leukocyte and the endothelium are constantly being formed and broken due the shear stress exerted at the endothelial from circulating blood flow. The rolling speed is dependent on the dissociation rate of selectin bonds\textsuperscript{16,17}. Rolling allows the monocyte to survey the local endothelial microenvironment for adhesive signals\textsuperscript{15}. Damage to vascularized tissue can release biochemical signals to trigger monocyte adhesion\textsuperscript{8,18}. If adhesive signals are not present, the monocyte can quickly disengage from the endothelium due to the rapid selectin off-rate\textsuperscript{15,16}.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Fig1-1.png}
\caption{Different steps of the monocyte adhesion cascade are illustrated. The first stage of monocyte-endothelium adhesion cascade is monocyte rolling which is mainly facilitated by selectin bonds. Biochemical signals trigger the adhesion of monocyte expressed integrins to IgCAMs expressed on the endothelium.}
\end{figure}
One of the biochemical signals released upon tissue damage is a group of proteins called chemokines. Chemokines activate integrins\textsuperscript{6,12,19,20} and may also increase the expression of IgCAMs on the endothelium\textsuperscript{21-23}. Integrin activation may increase affinity\textsuperscript{6,12,19,20}, expression\textsuperscript{24,25}, and clustering\textsuperscript{26}. Integrins in general exhibit slower intrinsic dissociation rate and are thus capable of resisting larger forces compared to selectin bonds\textsuperscript{15,16}. Since integrins can resist higher forces than selectin bonds, monocytes stop rolling and become arrested on the endothelium\textsuperscript{16}. The major integrin mediating monocyte arrest is Very Late Antigen-4 (VLA-4, $\alpha_4\beta_1$, cd49d/cd29)\textsuperscript{27,28}.

Following arrest, the monocyte firmly adheres to the endothelium. Firm adhesion supports contractile forces necessary for cellular migration. The monocyte will begin to migrate or extravasate across the endothelium and infiltrate the vascularized tissue to inspect for possible tissue damage. Firm adhesion and monocyte extravasation are mainly supported by the integrin, Leukocyte Function-associated Antigen-1 (LFA-1, $\alpha_L\beta_2$, cd11a/cd18)\textsuperscript{29}.

The overall goal of this work is to examine how and whether the chemokine, Monocyte Chemoattractant Protein-1 (MCP-1) enhances the adhesion of the integrins VLA-4 and LFA-1 during monocyte recruitment. The majority of the introduction will focus on the structural and functional properties of integrins, chemokines, and IgCAMs. The last section will describe the Atomic Force Microscope (AFM) and how it is used to quantify monocyte adhesion.
1.1.1 Integrins

The name integrin was first derived in 1986 by Tamkun and co-workers for an integral membrane protein which maintained the “integrity” of the linkage between the actin cytoskeleton and ECM\(^{30-32}\). All cells express at least one type of integrin\(^{31,33}\). The main function of integrins is to facilitate cell-cell and cell-ECM adhesion. Integrins were first identified with monoclonal antibodies that blocked cellular adhesion to extracellular matrix proteins (ECM)\(^{34-36}\) and also affinity chromatography of ECM proteins bound to integrins\(^{37}\). Integrins are unique in that they can transduce both inside-out and outside-in signaling\(^{31,33}\). Inside-out signals result from chemokine and cytokine stimulation of cell surface receptors which trigger intracellular regulation of integrin adhesiveness\(^{1,3-7,20,26,38-41}\). Outside-in signals are triggered by extracellular ligand binding to Integrins\(^{42-47}\).

Integrins are hetero-dimers composed from one single alpha and beta subunit with a 1:1 stoichiometry\(^{31,32,48}\), except for a integrin receptor for fibronectin expressed in chicken fibroblasts which is a trimer containing an integrin complex with 1 beta subunit and 2 alpha subunits\(^{34,49}\). The alpha and beta subunits contain around 1000 and 750 amino acids, respectively\(^{50-52}\). There are 18 \(\alpha\) and 8 \(\beta\) subunits which combine together to form 24 known combinations of heterodimers\(^{31-33,50}\). The known integrin heterodimeric subunit combinations are shown in Figure 1-2. Each heterodimer binds to different ligands\(^{31,32,48}\). The numerous combinations of integrin subunits allow integrins to not only bind to a diverse number of ligands, but also to signal through a plethora of cytoskeletal elements\(^{31-33,48}\).
Early electron microscope images of purified integrins revealed that integrins contain an extracellular globular ligand binding domain (8x12 nm) connected to two tails (14-20 nm long). The ligand binding domain is formed from the non-covalent interface between the N-termini of the alpha and beta subunits in the extracellular space. Each tail consists of a C-terminus from a single subunit. The two tails extend from the extracellular ligand binding domain across the cellular membrane into the cellular cytoplasm. The extracellular portion of the two tails is roughly 12-15 nm long. Both the alpha and beta subunits are predicted to span the membrane with a single alpha helix.

The subsequent sub-sections will give general overviews of the conformational states of integrin extracellular domain (Section 1.1.1.1), the ways in which integrins become activated (Section 1.1.1.2), and lastly a brief
description of the two integrins of principle importance in this work: VLA-4 (Section 1.1.4.1) and LFA-1 (Section 1.1.4.2) will be described.

1.1.1.1 Integrin Conformation States

Integrins exist in three distinct conformational states (Figure 1-3): 1) a closed low affinity state 2) an extended low affinity state and 3) an extended high affinity state. Much of what is known today regarding the conformational changes in the extracellular integrin domain is mostly based on the first crystallization of the complete extracellular domain of the integrin αVβ3 in 200157. These distinct conformational states have also been visualized in electron microscope images of purified integrins44,59,60.
The β-propeller and I domains are the two major domains coordinating ligand binding in integrins. The interaction between EGF domains 2 and 3 are responsible for the bending of the integrin tails. Only the domains relevant to integrin activation and binding will be discussed in greater detail in this work. Half of the integrin subunits contain an I-domain (a) and the other half do not (b). Structural conformational changes of the extracellular integrin domains are shown in for an integrin with an alpha subunit containing an I-domain (a) and (b) an integrin without an I-domain in the alpha subunit. Integrins exist in three different conformational states: 1) bent low affinity, 2) extended low affinity, and 3) extended high affinity. The low and high affinity states are equivalent to the closed and open headpiece states, respectively. (Figure is adapted from Luo et al 2007).
The extracellular ligand binding domain for all integrins is homologous to the A domain of the von Willebrand Factor\textsuperscript{63}, a soluble protein that mediates the thrombus formation by adhering to cells and other ECM proteins\textsuperscript{64}. The A domain of the von Willebrand Factor is also referred to as an A-type domain or the I-domain in integrins. For the rest of this paper the A-type binding domain will be referred to as the I-domain. All beta integrin subunits are predicted to contain an I-domain\textsuperscript{65,66}. On the other hand, only half of the integrin alpha subunits contain an I-domain, which are denoted by asterisks in Figure 1-2\textsuperscript{32,48,67}, hence some integrins may contain more than one I-domain.

The I-domain was first identified in the integrin Mac-1 by sequence alignment of the Mac-1 primary amino acid sequence in 1988\textsuperscript{63}. In 1995, the I-domain from the αL integrin subunit was crystallized confirming the predicted structure\textsuperscript{68}. A beta-propeller domain from the integrin alpha subunit also participates in ligand binding for certain integrins\textsuperscript{69}. The beta-propeller domain is composed of seven beta-sheets oriented like a circle to resemble the blades of a propeller.
The majority of integrins expressed exists in the bent low affinity conformation and are not constitutively active\textsuperscript{16,57,70}. In the context of leukocytes, if integrins were constitutively active, large amounts of leukocytes would adhere to the endothelium causing the formation of adhesion plaques which would deter blood flow. Integrins in immune cells by design are only activated in response to tissue damage and/or harmful foreign particles\textsuperscript{16,33,48,71}. Early Adhesion experiments between integrins and their ligands exhibited a two-state kinetic process, providing the first experimental evidence that ligand binding to integrins induces a conformational change from the low affinity to high affinity state in integrins\textsuperscript{46,47}. Crystal structures of the complete extracellular integrin domains demonstrated that the inactive integrin existed in a conformational state where the integrin ligand binding domain was bent towards the cellular membrane at angle of 60 degrees\textsuperscript{57,72}. Bending of the two integrin tails conceals the ligand binding from cognate ligands, hence integrins expressed on inactivated cells

\begin{figure}[h]
\centering
\includegraphics[width=0.8\textwidth]{image1.png}
\caption{The crystallized beta-propeller structure of the $\alpha_v$ integrin subunit. Disulfides (sticks) and glycans (spheres) are shown in red. Ca$^{2+}$ ions are green. (Taken from Figure 2 from Xiong et al 2001\textsuperscript{57}).}
\end{figure}
confer very little adhesion\textsuperscript{19,26,41,73,74}. The interactions in the beta subunit between the Epidermal Growth Factor (EGF)-2 and 3 domains with the hybrid domain are responsible for facilitating the bent conformation (Figure 1-3). The hybrid domain interaction with the EGF domains was discovered using NMR\textsuperscript{62} and integrin domain specific monoclonal antibodies\textsuperscript{75,76}.

The separation of the ligand binding domain from the cellular membrane upon first stage of integrin activation was also confirmed by Förster Resonance Energy Transfer (FRET) measurements between a fluorophore donor on the cell membrane and an acceptor fluorophore on the integrin ligand binding site\textsuperscript{77}. In the inactivated state, the integrin ligand binding domain (acceptor fluorophore) was in close proximity to the cell membrane (donor fluorophore). Upon chemokine induced activation of integrin VLA-4, the integrin ligand binding site separates from the cellular membrane as there was no detected energy transfer\textsuperscript{77}. The separation of the integrin ligand binding site from the cellular membrane is thought to undergo a switchblade like motion\textsuperscript{62}.

Upon extending away from the cellular membrane, the integrin ligand binding domain becomes accessible for binding\textsuperscript{26,77}. However, one final stage of activation is required to induce a high affinity state. Electron microscopy\textsuperscript{59} and the crystal structures\textsuperscript{58} of the inactive and activated states revealed that both ligand binding and outward swinging of the hybrid domain induces the opening of the I-domain into a high affinity state (Figure 1-3 from a-2 to a-3 and b-2 to b-3). Immediately following ligand binding, swinging of the hybrid domain is thought to pull apart the two integrin tails as well (Figure 1-3 from a-2 to a-3 and b-2 to b-3).
Furthermore leukocyte adhesion measurements also demonstrated that prior cellular pre-treatment/exposure to integrin ligands increased leukocyte adhesion to ligands immobilized on tissue culture plastic compared to untreated cells.\textsuperscript{78}

In addition to ligand binding; divalent cations\textsuperscript{79}, cytokine and chemokine induced activation\textsuperscript{6,19}, the application of a physical force\textsuperscript{80,81}, and physical separation of the integrin tails via association with the cytoskeleton\textsuperscript{76,82-84} can also induce the opening of the I-domain into a high affinity state. Chemokine-induced integrin activation is the most physiological of the three types of activation. All Integrin I-domains contain a Metal Ion Dependent Adhesion Site (MIDAS)\textsuperscript{32}. X-ray crystal structure of $\alpha_v\beta_3$ integrin in complex with an R-G-D ligand revealed that three amino acid residues on the MIDAS in the integrin I-domain coordinate the binding to divalent cations\textsuperscript{85}. A glutamic\textsuperscript{86} or aspartic acid\textsuperscript{85} residue on the integrin ligand binds to the MIDAS-divalent cation complex. Mg\textsuperscript{2+} and Mn\textsuperscript{2+} are two divalent cations which aide in the coordinate of ligand binding to the integrin MIDAS domain. Ca\textsuperscript{2+} on the other hand sequesters integrins in an inactive state\textsuperscript{87}. Chemokine induced inside-out signaling is induces both the extended low\textsuperscript{11} and high affinity\textsuperscript{3,4,6,19} state through inside-out signaling via G-protein coupled receptor. However, it is not known whether a single chemokine can trigger both extended low and high affinity states. Cytoplasmic regulation of integrins was recognized when deletion of the cytoplasmic integrin domains induced a constitutively active high affinity integrin\textsuperscript{83,84}. 
1.1.1.2 Integrin Association with Cytoskeletal Adaptor Proteins

Cytoplasmic domains of integrins do not possess any enzymatic activity\textsuperscript{50,88}. Since integrins have no intrinsic enzymatic activity, the intracellular domains serve as platforms to recruit and coordinate the assembly of cytoskeletal molecules and signaling complexes\textsuperscript{50,89-91}. The cytoplasmic domains of the alpha and beta subunits are ~8 nm which is relatively short\textsuperscript{33,50,57}. As the heterodimeric subunit combination determines extracellular ligand specificity, the subunit combination also determines the distinct cytoplasmic proteins associated with each individual integrin\textsuperscript{32,50,67}. More importantly, the diversity and immensity of integrin ligands is also conferred in the cytoplasmic protein interactions.

The first evidence for integrin interaction with the cytoskeleton through talin was reported in 1986\textsuperscript{92}. Since then at least 90 so-called adaptor/scaffolding proteins have been implicated to interact with integrin cytoplasmic tails\textsuperscript{50}. A review of all cytoplasmic proteins and their roles would be extremely exhaustive; hence a brief overview of only the most important concepts and proteins prevalent to the MCP-1 signaling pathway will be discussed.

Paxillin is one of the cytoskeletal adaptor proteins that interacts with integrins and is phosphorylated by Py2k via MCP-1 signaling to coordinate the binding of α4 subunit containing integrins. In this section, the major properties of Py2k, paxillin and their roles in integrin regulation. The exact steps and other signaling molecules involved in paxillin activation by MCP-1 will be discussed later in Section 1.1.3.2.
Paxillin is a 68kDa protein that becomes phosphorylated from tyrosine kinase in chicken fibroblasts\textsuperscript{93}. The name paxillin is derived from the Latin word “paxillus” meaning a small stake or peg\textsuperscript{94}. Paxillin is indeed a small stake that plays a role in linking together the cytoskeleton and adaptor proteins to integrins\textsuperscript{88,91,94-103}. A zinc finger domain in the C-terminus of paxillin contains the tyrosine kinase phosphorylation which regulates paxillin activity\textsuperscript{102}. This phosphorylation site regulates the ability of paxillin to recruit other adaptor and/or structural proteins like vinculin\textsuperscript{102}, tyrosine phosphotases\textsuperscript{104}, and tubulin\textsuperscript{105}. The N-terminus contains the domains which mediate signaling functions of paxillin\textsuperscript{102,106}. Paxillin contains many different tyrosine, serine, and threonine phosphorylation sites making it an exceptional signaling molecule capable of integrating and disseminating multiple signaling pathways that govern cellular adhesion and/or cell viability\textsuperscript{88,101}. Paxillin binds to a phosphorylated serine-988 in the cytoplasmic tail of the α4 integrin subunit\textsuperscript{98,107} and participates in leukocyte recruitment to the endothelium\textsuperscript{98,100,107,108}.

When an adherent cell line is plated on a tissue culture dish it first resembles a sphere. As the spherical cell comes into contact with the dish, it begins to flatten out or spread on the dish due to the formation of adhesive contacts. The process of cell spreading is the initial stage of cellular migration, where nascent adhesive contacts are formed. Spreading must occur before migration can happen\textsuperscript{109}. Time-lapse fluorescent imaging of a GFP tagged paxillin revealed that it is one of the first proteins found in early cell adhesion.
contact sites\textsuperscript{110}, suggesting paxillin coordinates the cell spreading by regulating the molecular composition of the adhesion complex.

The paxillin-\(\alpha_4\) subunit association was blocked by mutating the serine-988 to a non-phosphorylatable alanine. Blocking paxillin-\(\alpha_4\) association reduced both leukocyte cell spreading and cell migration compared to the wildtype. On the other hand, mutation of serine-988 to a phosphorylatable aspartic acid resulted in an increase in cellular spreading, but surprisingly suppressed cellular migration, showing that paxillin mediates the initial events of cellular adhesion\textsuperscript{100}. Lastly, paxillin mediates cytoskeleton association of integrins containing \(\alpha_4\) subunit to increase integrin adhesion without affecting integrin affinity\textsuperscript{97,108}, suggesting that paxillin plays a role in inducing the extended low affinity state in integrins.

### 1.1.2 Immunoglobulin-related Cell Adhesion Molecules (IgCAMs)

Immunoglobulin-related Cell Adhesion Molecules (IgCAMs) are set of proteins which are expressed on cellular membranes that mediate cell-cell adhesion. IgCAMs can mediate either homotypic (IgCAM to IgCAM) or heterotypic adhesion to integrin receptors on an opposing cell. They are named Ig-like because they contain 1-6 repeated immunoglobulin domains each 100 amino acids in length\textsuperscript{31}. Each domain contains a pair of cysteine residues roughly 55-75 residues apart\textsuperscript{31,111-114}. The cysteine residues of each domain form a disulfide bridge to create an Ig-fold which is often depicted as a globular
structure (Figure 1-5)$^{31,115}$. There is no particular naming scheme for IgCAMs. They were initially named after the cell type from which they were isolated from, for instance V-CAM (vascular) and N-CAM (neuron). The I-CAM family on the other hand binds to integrins$^{31}$. The Ig-like folds mediate adhesion$^{116-119}$, which is calcium dependent$^{31}$.

![Figure 1-5: Illustration of an IgCAM. This IgCAM contains 5 Ig-like domains/folds formed due to a disulfide bond between cysteine residues in each domain. Two fibronectin like domains are also pictured as rectangles proximal to the cellular membrane. (Adapted from Sandi et al 2004 Figure 1$^{120}$)](image)

### 1.1.3 Chemokines

#### 1.1.3.1 Overview of chemokines

Chemokines are named as such due to the chemotactic response that they illicit upon stimulating cells$^{1,9,10,18}$. They vary in size from $\sim$ 8kDa to 15kDa. There are four families of chemokines: CC, CXC, C, and CX3C$^{8,13,121}$. They are
grouped according to the number and spacing of cysteine residues in the amino acid sequence, where the C denotes cysteine residues, and the X any amino acid other than a cysteine residue separating the two cysteine residues. Chemokines stimulate cells by binding to G-protein coupled receptors\textsuperscript{122}.

As briefly mentioned in the previous sections, chemokines can enhance adhesion of the monocyte cell surface expressed integrins to IgCAMs expressed on the endothelium\textsuperscript{20,123}. There are several proposed mechanisms through which chemokines can enhance integrin mediated adhesion. Increase in integrin affinity is one of the major mechanisms by which chemokines enhance integrin mediated adhesion\textsuperscript{3,6,39,124-126}. In addition to affinity regulation, chemokines also enhance adhesion by increasing integrin clustering or cell surface integrin expression\textsuperscript{7,26}. Chemokine stimulated adhesion has also been implicated to induce integrin extension, which is a conformational change in the $\alpha$ and $\beta$ chains, the non-binding region of the integrin which properly orients the integrin binding head towards the integrin ligand\textsuperscript{11}. Lastly, chemokines may also decrease cellular stiffness allowing the cell to form larger contact areas with the substrate.

\textit{1.1.3.2 Monocyte Chemoattractant Protein-1}

Monocyte chemoattractant protein-1 (MCP-1, CCL2) is an 8 kDa protein that triggers the recruitment of monocytes to the endothelium. MCP-1 was first
cloned in 1988\textsuperscript{127} and its receptor was cloned in 1994\textsuperscript{128}. MCP-1 binds to the CCR2, a G-protein coupled receptor to enhance integrin mediated adhesion.

MCP-1 is involved in the progression of monocyte related inflammatory diseases which include atherosclerosis\textsuperscript{129}, allergic reactions\textsuperscript{130}, diabetic nephropathy\textsuperscript{131}, and neuronal inflammation in both peripheral\textsuperscript{132} and central nervous systems\textsuperscript{18}. It also promotes neuronal regeneration in the peripheral nervous system\textsuperscript{133}. Over-expression of MCP-1 in normal mice tissue also induces monocyte extravasation into the host tissue\textsuperscript{129}, causing the accumulation of fatty atherosclerotic plaques in the blood vessel walls narrowing the arteries and also reducing arterial wall compliance to constrict blood flow. The role of MCP-1 activation of integrins in the monocyte adhesion cascade will be discussed in the next section.

1.1.4 VLA-4/VCAM-1 and LFA-1/ICAM-1 Roles in Monocyte Adhesion

1.1.4.1 Very Late Antigen-4 (VLA-4)/VCAM-1 adhesion complex

The name “Very Late Antigen” is derived from the fact that VLA integrins are not expressed on the lymphocyte cell surface several days after lymphocyte activation\textsuperscript{134}. All of the VLA integrins contain the $\beta_1$ subunit\textsuperscript{51,135,136}. VLA-4 facilitates leukocyte recruitment to the endothelium by binding to VCAM-1 expressed on the endothelium and also fibronectin\textsuperscript{3,6,7,19,27,74,125,137-139}. The VCAM-1 and fibronectin binding sites on VLA-4 are on distinct epitopes\textsuperscript{140}. Since the VLA-4 adhesion to VCAM-1 is the major interaction mediating monocyte
adhesion to the endothelium, this work will not focus on the VLA-4/fibronectin interaction.

VLA-4 consists of an α4 and β1 subunit. VLA-4 only contains one I-domain the β1 subunit (Figure 1-3a)\textsuperscript{32,33,48,70}. Neither the complete extracellular VLA-4 domain nor the individual subunits have been crystallized yet. However, the α4 subunit has been predicted to fold into a beta propeller domain based on the secondary structural similarities with other integrin alpha subunits\textsuperscript{69}. As mentioned previously, the beta propeller domain of the α4 subunit helps coordinate the binding integrin ligands along with the I-domain on the β1 subunit\textsuperscript{32,33,48,70}.

VCAM-1 is expressed on endothelial cells and is the major ligand facilitating VLA-4 mediated leukocyte adhesion during the immune response\textsuperscript{27,28,140-144}. VCAM-1 binds to both VLA-4 and α\textsubscript{4}β\textsubscript{7} integrins, where VLA-4 is the primary ligand\textsuperscript{145}. Monocytes however, only express β7 subunit after transmigration from the bloodstream into the vascularized tissue and differentiate into macrophages\textsuperscript{146}. Hence VLA-4 is the primary integrin that binds to VCAM-1 during the first stages of the leukocyte adhesion cascade. Alternative splicing leads to the expression of VCAM-1 (VCAM-7D) with 7 Ig-like domains and another with 6 Ig-like domains (VCAM-6D)\textsuperscript{147-149}. Monoclonal antibodies specific to VCAM-1 domains 1 and 4 blocked VLA-4 mediated binding to VCAM-1 substrates (pictured in dark gray Figure 1-6), demonstrating that VCAM-1 contains two independent binding sites in domains 1 and 4\textsuperscript{150}. Site-directed mutagenesis of VCAM-1 revealed that an identical Q-I-D-S-P-L amino acid
sequence in both domains 1 and 4 coordinates VLA-4 binding to VCAM-1. More importantly the Q-I-D-S-P-L sequence is homologous to the CS-1 binding site of fibronectin.\textsuperscript{150}

Figure 1-6: The immunoglobulin domains of the two expressed forms of VCAM-1. The binding domains to VLA-4 are shown in dark gray. VCAM-7D contains two VLA-4 binding sites in Ig-like domains 1 and 4. VCAM-6D lacks the Ig-like domain 4 from VCAM-7D. (Adapted from Figure 1 from Osborn et al 1994\textsuperscript{143})

Flow chambers were one of the devices used to investigate leukocyte adhesion systems. Essentially, a suspension of cells is perfused over a functionalized substrate to mimic the function of blood flow in leukocyte recruitment to the endothelium. The observation of leukocyte rolling on VCAM-1 substrates in parallel plate flow chambers\textsuperscript{27,28}, was the first evidence that the VLA-4/VCAM-1 facilitates the early adhesion events in the leukocyte adhesion cascade (Figure 1-1). These early flow chamber observations of leukocyte
rolling on VCAM-1 substrates suggested that VLA-4/VCAM-1 adhesion complex has fast on and off-rates to facilitate the constant breakage and formation of bonds that mediates the rolling behavior\textsuperscript{27}. Flow chamber assays of leukocytes expressing VLA-4 have demonstrated that chemokines are capable of inducing both the extended low\textsuperscript{7} and high affinity states\textsuperscript{3,4,6,39,124}.

MCP-1 induces and inside-out activation of VLA-4 cells in monocytic cell lines\textsuperscript{38,41,123,129,151-154}. MCP-1 binds to a heterotrimeric G-protein called CC chemokine Receptor 2 (CCR2), which contains a Gi subunit\textsuperscript{155}. CCR2 can mediate signaling through the protein tyrosine kinase, Proline-rich tyrosine kinase-2 (pyk2) in monocytic cell lines\textsuperscript{156}. Pyk2 mediates the early events of cellular adhesion and leukocyte activation like cell spreading by amplifying biochemical signals to strengthen integrin adhesion\textsuperscript{157}. MCP-1 stimulation of VLA-4 increases paxillin association with the α4 subunit of VLA-4\textsuperscript{151,156}. As mentioned before, paxillin is only found in nascent adhesion complexes suggesting a role for paxillin in the events of cellular spreading and not migration\textsuperscript{110}. In addition, MCP-1 induced inside out signaling has also been shown to induce an extended high affinity state in VLA-4\textsuperscript{3,4}. Taken together, most of the studies on MCP-1 implicate that it is mainly involved in inducing rolling and arrest of monocytes on the endothelium by inducing an extended low affinity VLA-4 state.
1.1.4.2 Leukocyte Function associated-Antigen-1 (LFA-1)/ICAM-1

The integrin Leukocyte Function-associated Antigen-1 facilitates the firm adhesion and transmigration of leukocytes from the endothelium into the surrounding vascularized tissue by binding to InterCellular Adhesion Moelcule-1 (ICAM-1). LFA-1 contains an I-domain in both the alpha and beta subunits (Figure 1-3b). The I-domain on the α-subunit coordinates ligand binding. Divalent cation binding to the MIDAS domain is believed to stabilize the binding of LFA-1 to ICAM-1. Crystal structure of the I-domain of the LFA-1 alpha subunit bound with Mg$^{2+}$ showed that five oxygenated amino residues (Asp137, Ser139, Ser141, Thr206, and Asp239) constitute the MIDAS motif on the I-domain to coordinate divalent cation binding. A sixth residue is believed to be contributed by from ICAM-1. In addition to the MIDAS domain, point mutations of amino acid residues on top of the I-domain (Leu-205, Glu-241, Met-140, Glu-146, Thr-243, and Ser-245) also significantly reduced LFA-1 binding to ICAM-1. ICAM-2 and ICAM-3 also bind to LFA-1, but to other sites on the alpha subunit distinct from ICAM-1.

1.2 Atomic force microscope

The interest in cellular adhesion has led to the fabrication of numerous devices capable of resolving and quantifying adhesion between one adhesion receptor and its ligand. Some of these devices include the optical tweezers, the biomembrane force probe, and the atomic force microscope (AFM). One of the major advantages of the AFM over other adhesion measurement
techniques is the capability to apply and resolve forces over a range from ~5 pico-Newton to several nano-Newton. This allows the resolution of the breakage of not only one single bond (~20pN)\textsuperscript{165,167,168}, but also a few bonds at a time\textsuperscript{169,170}. In addition, sub-second (100 ms) temporal control of cell-substrate interactions and also the resolution of sub-second adhesion events can also be achieved with the AFM, making it a highly sensitive machine. This section gives a basic overview of the principles of AFM operation.

### 1.2.1 AFM components

The AFM consists of several components: cantilever, laser, position sensitive photodiode (PSPD), and a piezoelectric element shown in Figure 1-7.

![Figure 1-7: Illustration showing the layout of the essential AFM components. A ~635 nm laser (red line) is reflected off of a cantilever into a photodiode. The amount of cantilever deflection is measured as the voltage difference between the photodiode segments A and B. The piezoelectric element controls the distance between the tip and the sample.](image)

Arguably, the most important component of the AFM is the cantilever as it is the transducer through which forces to the sample are applied and measured.
Cantilevers are flexible and behave like springs. All springs tend to oscillate at a natural frequency when there are no external perturbations exerted on the system. This frequency is commonly referred to as the resonant frequency. The cantilever used in this study is a triangular or V-shaped Veeco MLCT-AUHW (C) with a nominal spring constant of 0.01 N/m and a resonant frequency of ~1 kHz (Figure 1-8). The MLCT-AUHW (C) cantilever is composed of a thin layer silicon nitride (Si₃N₄) ~0.55 µm thick. At the apex of a triangular cantilever lies a small pyramidal tip. The tip or bottom side of the cantilever comes into contact with the substrate. The apex of the tip is between 2.5-8.5 µm in height away from the cantilever. A 635 nm laser is emitted onto the top side of the cantilever and reflected onto a photodiode (UDT sensors 0526-1), as pictured in Figure 1-7. To increase the measured reflected laser signal, the top side of the cantilever is coated with a thin layer of gold; hence the top side is also commonly referred to as the reflective side. The cantilever deflection changes as a force is applied to the cantilever, resulting in a displacement of the reflected laser signal.

Figure 1-8: Side and bottom view of an MLCT-AUHW (C) cantilever. A V-shaped or triangular cantilever is shown with a pyramidal at the end. (Thickness: ~ 0.55 µm, Width: 20 µm, Length: 310 µm).
One of the simplest and most common methods to detect displacement or deflection in one dimension is through a two-segmented photodiode. Photons from incident light on the photodiode impart energy onto a silicon semiconductor material. This causes the movement of electrons resulting in a measured current proportional to the incident photon power. The two segments of the photodiode are commonly referred to as A and B. One dimensional motion can be detected by determining the difference in measured light intensity measured as a voltage difference between segments A and B (A-B signal) (Figure 1-9). The sign of the voltage difference indicates the direction of motion, and the magnitude of the difference indicates the degree of displacement relative to the center of the photodiode. As the light is centered on the photodiode the voltage difference between segments A and B is zero.

![Figure 1-9: Detection of motion with a two segmented PSPD.](image)

Lastly, the piezoelectric element controls the separation distance between the cantilever and the functionalized substrate. An electric field applied to a piezoelectric material causes either the expansion or compression of the material depending on the polarity of the electric field. The amount of expansion or
compression is monitored with a position sensor, in this case a strain gauge. The resistance across the strain gauge changes as it is deformed or displaced from the expansion/compression of the piezoelectric element. This measured resistance is proportional to the amount of displacement in the strain gauge\textsuperscript{171}.

1.3 Measurement of forces

The previous section gave a brief overview of the basic components of an AFM. This section will describe how molecular forces are measured with an AFM. A force applied to the cantilever causes the displacement or deflection of the cantilever. The amount of cantilever deflection is measured using a two segmented photodiode.

Since the cantilever behaves like a spring and the deflection is measured, the force applied to the adhesion system can be determined from Hooke’s Law, $F = kx$, where $F$ is the force, $k$ is the spring constant, and $x$ is the amount of deflection. The spring constant can be determined from the thermal fluctuation method (Appendix A.2).
Figure 1-10: Cantilever deflection during a force curve. Approach curve (dashed blue) and the retraction curve (solid red). Solid triangles indicate the direction of displacement of the piezoelectric element. The y-axis represents either the negative voltage difference from a two segmented photodiode measuring cantilever deflection or the amount of force applied to the cantilever, and the x-axis represents the displacement of the piezoelectric element.

Figure 1-10 depicts deflection of a cantilever during an AFM force curve exhibiting an adhesion event in the retraction curve. Once the spring constant is known the amount of force applied to the cantilever can be determined from the measured deflection. Initially the cantilever is far away from the substrate, and there is virtually no force applied to the cantilever, hence the measured cantilever deflection and force applied to the cantilever is zero (dashed trace Figure 1-10 (i)). As the piezoelectric element expands, the cantilever is lowered and the cantilever tip eventually comes into contact with the surface resulting in a negative deflection (dashed trace Figure 1-10 (iii)). The cantilever is then retracted from the substrate. If there is no adhesion between the cantilever tip
and the substrate the retraction trace will look exactly like the dashed blue trace. If there is adhesion between the cantilever tip and the substrate however, the adhesion complex applies a positive force to the cantilever (Figure 1-10 (iv)). Eventually as the bond breaks, the force applied to the cantilever almost instantaneously returns to zero force, hence the sharp vertical jump.
Chapter 2 Specific Aims

Chemokines play an important role in facilitating leukocyte adhesion to the vascular endothelium by enhancing integrin adhesion during inflammation via G-protein coupled receptor signaling. MCP-1 has been proposed to enhance the adhesion of the integrins LFA-1\(^\text{10}\) or VLA-4\(^ {\text{41,123,153,172}}\). These integrins play distinct roles during monocyte recruitment. VLA-4 mediates the initial leukocyte contact or recruitment to the endothelium\(^ {\text{27}}\) whereas LFA-1 mediates the firm adhesion of leukocytes\(^ {\text{29}}\). The abundance of chemokines during inflammation may implicate either distinct or overlapping roles. Monocyte Chemoattractant Protein-1 (MCP-1) is an abundant and highly potent chemokine present during monocyte mediated inflammation. The majority of the studies investigating MCP-1 implicate that MCP-1 enhances VLA-4 adhesion to VCAM-1, and not LFA-1 to ICAM-1\(^ {\text{41,123,151,154-156}}\). There has only been one study to date that suggests MCP-1 enhances LFA-1 adhesion to ICAM-1\(^ {\text{20}}\). It can definitely be concluded from these studies that MCP-1 does not enhance both VLA-4 and LFA-1 mediated adhesion simultaneously. Furthermore, the mechanism(s) by which MCP-1 enhances integrin mediated adhesion is still unknown.

The overall objective of this thesis is to investigate the distinct role of MCP-1 during monocyte recruitment to the vascular endothelium, and also to elucidate the mechanism(s) by which MCP-1 enhances integrin mediated monocyte adhesion. We hypothesize that MCP-1, more importantly MCP-1
facilitates early monocyte recruitment by enhancing VLA-4 adhesion to VCAM-1 mediated adhesion.

**General aim:**

To uncover the mechanism by which MCP-1 mediates the early recruitment of monocytes to the endothelium.

**Specific aims:**

1. To examine whether MCP-1 enhances VLA-4, LFA-1 or both integrins.
2. To investigate the mechanism(s) by which MCP-1 increases integrin mediated adhesion.
Chapter 3 Methods

3.1 AFM setup

Whole cell adhesion and single molecule rupture force measurements were both conducted using a custom built AFM depicted in Figure 3-1. The major components in the system include a 90 µm piezoactuator (Physik Instrument P-841.10.SYS), a pigtail Laser (Oz optics), and a two-segment photodiode (UDT sensors 0526-1); refer to Chapter 1 for a more detailed functional description of each component. All measurements were conducted at 37°C by modulating the sample temperature with a thermoelectric module (LairdTech 66156-501). AFM cantilevers (Veeco MLCT-AUHW (C)) with a nominal spring constant of ~ 0.01 N/m were used in all experiments and were calibrated at 37°C using the thermal fluctuation method to determine spring constant and optical lever sensitivity of the cantilever (Appendix A.1).

Figure 3-1: AFM components and the system layout. The laser and the photodiode combination are used to measure the cantilever deflection. The piezoelectric module maintains the distance between the cantilever and the sample. The thermoelectric module regulates the sample temperature.
3.2 Tissue culture of THP-1 cells

THP-1 cells (ATCC #TIB-202), a human monocytic cell line are an ideal cell line to study the effects of MCP-1 on monocyte adhesion because they have been shown to express a similar amount of the MCP-1 receptor, CCR2, as primary human monocytes\textsuperscript{174}. Furthermore, THP-1 cells have also been used to examine the effect of MCP-1 on integrin mediated adhesion\textsuperscript{20,123}.

THP-1 cells were split every 48 hours in RPMI-1640 medium supplemented with 10\% fetal bovine serum and penicillin/streptomycin (50\(\mu\)g/mL CellGro) at 5\% CO\(_2\) at 37\(^\circ\)C. Cells used in experiments were split 24 hours prior to experimentation.

3.3 Attaching THP-1 cells to AFM cantilevers

Cantilevers were cleaned with acetone for 5 minutes and then irradiated with UV light for 10 minutes. Following UV irradiation cantilevers were then soaked in NHCO\(_3\) (pH 9.0) then air dried. After air-drying, cantilevers were coated with 0.1 mg/mL of Poly-L-lysine (PLL) overnight (Sigma P4832) at 4\(^\circ\)C in a humidified chamber. PLL is extensively used to attach cells to substrates\textsuperscript{175}. Cantilevers were then rinsed three times in PBS and stored in PBS at 4\(^\circ\)C for up to 5 days.
Cantilevers were mounted on a custom made AFM cantilever holder and the cantilever spring constant was determined (Appendix A.1), the functionalized cantilever was pressed against the cell to immobilize it to the cantilever.\textsuperscript{169}

### 3.4 Functionalizing substrates with adhesion molecules

The basic principle behind functionalizing substrates with adhesion molecules is to increase the substrate surface pH to at least 9.0 imparting a partial negative charge to the substrate. At a pH $\geq 9.0$, the primary amine groups in the adhesion molecule bind to the negatively charged dish.

Hydrophobic Petri dishes (Falcon #35-1008) were briefly coated with a 20 µL drop of NaHCO$_3$ (pH 9.0). The NaHCO$_3$ drop was then removed leaving thin film fluid. A 20 µL drop of protein solution was plated directly on top of the NaHCO$_3$ film, and allowed to incubate in a humidified chamber at 4°C at least overnight. Prior to experimentation, functionalized dishes were rinsed three times with PBS. Dishes were then blocked with 1% w/v Pluronic® F108NF Prill Proloxamer 338 (BASF) in PBS for 30 minutes. Pluronic® is a block copolymer which blocks hydrophobic surfaces; it has been used in other work to block adhesion of adherent cell lines.\textsuperscript{176} The blocked dishes were rinsed three times with PBS.
3.5 Acquiring cell adhesion data with the AFM

THP-1 cells were attached to AFM cantilevers (Section 3.3) and dishes were functionalized with desired molecules (Section 3.4). Adhesion between THP-1 cells and functionalized dishes were carried out by pressing the cell against the substrate at a desired indentation force and contact time (Figure 3-2). After the desired contact time, the cell is retracted away from the substrate and the adhesion is measured from the retraction force curve.

The indentation force and contact time influence the number of bonds formed between the cell and the substrate. As the indentation force increases, the cell can form a larger contact area with the substrate. Similarly, increasing the contact time, increases the amount of time the cell can interact with the substrate. Both increases in indentation force and contact time increase the probability of bond formation. Lastly, the density of cell adhesion molecules functionalized on the dish also influences the number of bonds formed between the cell and substrate.

Single molecule and whole cell adhesion data are two main types of cell adhesion data acquired with the AFM in this study. Whole cell adhesion is the measurement of multiple bond ruptures between the cell and the substrate, and single molecule experiments measure only one visible rupture in the AFM force curve. By reducing the indentation force, contact time, and more importantly adhesion molecule density on the
functionalized substrate whole cell adhesion measurements can be titrated to a point where only one single rupture is observed. These measurements are commonly referred to as single molecule rupture force measurements.

Figure 3-2: Adhesion system measured with the AFM. The adhesion of unstimulated or MCP-1 stimulated THP-1 cells to VCAM-1 or ICAM-1 functionalized substrates was quantified using the AFM. THP-1 cells were attached to cantilevers via poly-L-lysine.

3.6 Cell surface expression of integrins

Flow cytometry was used to compare the relative THP-1 cell surface expression of the α4 and β1 subunits of VLA-4. The β7 subunit expression was also investigated. Cells were either unstimulated or treated with MCP-1 for 10 minutes at 37°C. Fc receptors were blocked with Fcγ receptor binding inhibitor (eBioscience 14-9161-71). Cells were stained in flow cytometry staining buffer (eBioscience 00-4222-57) with the following integrin subunit antibodies: PE-α4 (eBioscience 12-0499-71), PerCP-β1 (eBioscience 46-0299-41), FITC-β7 (BioLegend 321213) and isotype controls PE-mouse IgG1,κ isotype control (eBioscience 12-4714-71), and FITC-Rat IgG2a, κ (eBioscience 11-4321-71). Cells were fixed with 2% paraformaldehyde. Flow cytometry was performed on a FACS Calibur flow cytometer (BD Biosciences), and histograms of plotting
counts vs mean channel fluorescence were constructed using CellQuest software (BD Biosciences).

3.7 Adhesion blocking experiments

Adhesion blocking experiments were conducted to examine the specificity of VLA-4 binding to VCAM-1. THP-1 whole cell adhesion to VCAM-1 (0.5 µg/mL) dishes co-immobilized with MCP-1 (5 µg/mL) was quantified in response to adhesion blocking antibodies. Adhesion curves of unblocked THP-1 cells adhering to VCAM-1/iMCP-1 dishes were acquired. Cells were then blocked with anti-α₄ antibody (R&D BBA37) 10µg/mL for 10 minutes, and adhesion curves were acquired. Cells were blocked a final time with either anti-β₁ 10µg/mL (R&D MAB17781) or 25µg/mL anti-β₇ (Biolegend 321218) antibodies for 10 minutes and a final set of adhesion curves were measured.
Chapter 4  MCP-1 increases VLA-4 adhesion, but not LFA-1

4.1 Description

MCP-1 enhances integrin mediated monocyte adhesion\textsuperscript{20,123}. VLA-4 and LFA-1 are the two major integrins facilitating monocyte adhesion. It is unclear whether MCP-1 enhances the adhesion of VLA-4, LFA-1, or both integrins. MCP-1 has been shown to enhance VLA-4 adhesion without enhancing LFA-1\textsuperscript{123}. On the other hand, MCP-1 has also been shown to increase LFA-1 adhesion via a PI3K pathway without affecting VLA-4 adhesion\textsuperscript{20}. Furthermore, the effect of MCP-1 on integrin mediated adhesion is unknown. To identify the integrins affected and to examine the effect of MCP-1 on monocyte adhesion, whole cell adhesion measurements between MCP-1 stimulated THP-1 cells and substrates functionalized with either ICAM-1 or VCAM-1 were obtained. The detachment distance, detachment time, maximum detachment force, number of ruptures, and detachment energy can be quantified from whole cell adhesion curves. In this study, we find that MCP-1 increases the formation of tethered VLA-4/VCAM-1 bonds tremendously enhancing THP-1 adhesion.

4.2 Whole Cell Adhesion Data Acquisition and Analysis

4.2.1 Acquiring whole cell adhesion force curves

Dishes were functionalized with 0.5 µg/mL of either human IgCAM-1 (R&D systems 720-IC) or VCAM-1 (R&D systems 862-VC) and/or 5 µg/mL of human
MCP-1 (Peprotech 300-04). Additional adhesion experiments of cells stimulated with 25 ng/mL of soluble MCP-1 (applied to the bath) were also conducted. Adhesion was measured between the cell and the functionalized substrate, as pictured in Figure 3-2. After the cell was immobilized to the cantilever, it was pressed against the functionalized petri dish for ~0.1s with a force of 500-800 pN and then retracted from the substrate. The approach and retract velocity were held constant at around ~3 µm/s.

### 4.2.2 Whole cell adhesion data analysis

The number of ruptures, detachment distance, detachment time, maximum detachment force, and detachment energy can all be quantified from whole cell adhesion curves (Figure 4-1). Sharp vertical jumps in the force curve represent the breakage of at least one molecular bond. The detachment distance signifies the total retraction distance required to completely detach the adhered cell from the substrate. Since the cell is retracted at a constant velocity of ~3µm/s, the detachment distance is proportional to the detachment time. The detachment distance/time may indicate the association between the adhesion molecule and the underlying cytoskeleton or a bond exhibiting longer lifetimes. In addition, the detachment force is the maximum amount of force required to completely detach the cell from the substrate. The detachment force reflects the number of ruptures formed between the cell and substrate. The more ruptures formed between the cell and the substrate the larger the detachment force. Lastly, the detachment energy indicates the amount of energy required to
completely detach the cell and is calculated as the integral of the approach trace over the detachment distance. The detachment energy is dependent on both the detachment force and also the detachment distance/time.

![AFM force curve](image)

**Figure 4-1: Whole cell adhesion parameters extract from a force curve.** AFM force curve measuring adhesion between a THP-1 cell and substrate functionalized with VCAM-1 and MCP-1 retracted at a constant velocity of ~3µm/s. Both retract (solid black trace) and approach (solid gray line) traces are shown. Shaded area represents the detachment energy. Vertical arrows show the molecular ruptures in the force curve.

### 4.3 Results

#### 4.3.1 VCAM-1 supports larger adhesion than ICAM-1 in unstimulated THP-1 cells

To investigate integrin mediated adhesion, AFM force curves of MCP-1 stimulated THP-1 cells adhering to VCAM-1 or ICAM-1 functionalized substrates were obtained (Figure 4-2). Physiologically, Monocytes only have 1s or less to sense the local endothelial microenvironment before the blood flow dislodges
them from the endothelium. To mimic this short physiological exposure time, THP-1 cells attached to AFM cantilevers were exposed to functionalized substrates for 100ms of contact time with an indentation force of ~600 pN.

To quantify the adhesion between THP-1 cells and ICAM-1 or VCAM-1 functionalized substrates, the detachment force, detachment energy, and detachment time/distance were determined from whole cell adhesion curves. VCAM-1 supports much larger adhesion at short contact times (~100 ms) for unstimulated THP-1 cells as there was a 5 fold increase in detachment energy, and time/distance to completely detach THP-1 cells from VCAM-1 substrates compared to ICAM-1 (Figure 4-2 b-c). Interestingly, VCAM-1 only supported a slight increase in the detachment force compared to ICAM-1.
40

Figure 4-2: VCAM-1 supports larger adhesion than ICAM-1 in unstimulated cells. Top trace: AFM force curves of THP-1 cells adhering to either ICAM-1 or VCAM-1. Bottom Traces: Adhesion parameters quantified from whole cell adhesion curves a) Detachment force b) Detachment energy c) Detachment time/distance. Two tailed nonparametric Mann-Whitney tests were performed (* p<0.1, ***p<0.001).

4.3.2 MCP-1 increases THP-1 binding to VCAM-1, but not to ICAM-1

To examine the effect of MCP-1 on integrin mediated THP-1 adhesion, AFM whole cell adhesion measurements of MCP-1 stimulated cells adhering to VCAM-1 or ICAM-1 were obtained. This was a highly specific adhesion system, as THP-1 cells did not bind to MCP-1 immobilized on the dish or to the pluronic blocked dish itself (Figure 4-3c). More importantly, MCP-1 tremendously enhanced THP-1 adhesion to VCAM-1 substrates (Figure 4-3a; Figure 4-4), but did not have a noticeable effect on THP-1 adhesion to ICAM-1 (Figure 4-3b;
The same trend is apparent for THP-1 cells stimulated with soluble MCP-1 (Figure 4-4 right panels).

**Figure 4-3: Whole cell adhesion force curves measured from THP-1 adhesion.** a) VCAM-1 b) ICAM-1 c) control surfaces.

MCP-1 increased the detachment force, detachment energy, and detachment distance/time of THP-1 adhesion to VCAM-1 (Figure 4-4). MCP-1 increases the number VCAM-1 bonds formed as reflected by a ~3.5 fold increase in detachment force compared to unstimulated VCAM-1 (Figure 4-4a). MCP-1 stimulated VCAM-1 bonds required a ~6-fold increase in pulling distance in order to completely detach the cell from the substrate (Figure 4-4c). The increase in detachment force and distance led to a ~10 fold increase in the detachment energy required to completely detach MCP-1 stimulated THP-1 cells from VCAM-1 substrates (Figure 4-4b).
Figure 4-4: MCP-1 enhances THP-1 adhesion to VCAM-1 not ICAM-1. Quantification of adhesion parameters measured from AFM whole cell adhesion force curves a) detachment force b) detachment energy c) detachment time/distance. Two-tailed Mann-Whitney tests were performed between ICAM-1 and VCAM-1 measurements (***, p<0.001). MCP-1 was exposed to cells via two different methods (left) immobilized on the dish (right) applied to the bath.

Two different types of bonds are also visible in the whole cell adhesion measurements. These different bonds are most visible in MCP-1 stimulated THP-1 adhesion to VCAM-1 (Figure 4-3a). The most obvious bonds are the ones which rupture following a constant force plateau or commonly referred to as
tethered bonds. Tethered bonds are not strongly associated with the cytoskeleton. More importantly, treatment of THP-1 cells with cytochalasin D (a molecule that disrupts the actin cytoskeleton) drastically increased the formation and detachment distance of membrane tethers. Membranes tethered to adhesive bonds were visualized by labeling the cell membrane with a fluorescent dye. When pulling a tethered bond, the force is applied to stretching the cell membrane which is highly viscous. A force applied to a viscous element results in a constant applied force over time giving rise to a force plateau (Figure 4-5 left trace). The second type of bond ruptures almost instantaneously following a ramp or linear increase in force. These bonds are anchored to cytoskeleton, and will be referred to as cytoskeleton anchored bonds. The cytoskeleton is extremely elastic. A force applied to an elastic element increases over time (Figure 4-5 right trace). Hence, bonds anchored to the cytoskeleton break almost instantaneously following a ramp in force. Cytoskeleton anchored bonds are generally the first bonds to break in the whole cell adhesion force curves. On the other hand, tethered bonds need to be pulled over large distances in order to break and generally occur towards the end of the force curve.
Figure 4-5: The response of different mechanical materials being pulled at a constant velocity. When an elastic element is pulled at a constant velocity, the amount force exerted to the probe increases with pulling distance. On the other hand, pulling a viscous element at a constant velocity results in a constant force applied to the probe.

MCP-1 increases the number of VLA-4/VCAM-1 tethered ruptures by almost 4 fold, and the number of cytoskeleton anchored VLA-4/VCAM-1 ruptures by ~2 fold (Figure 4-6). It is important to note that each rupture maybe supported by more than one bond, so the number of bonds may be larger than the quantified number of ruptures. An increase in bond formation increases the amount of force required to completely detach the MCP-1 stimulated cell, in this case both the detachment force (Figure 4-4a) and number of tethered VLA-4/VCAM-1 bonds (Figure 4-6) increase by the same proportion, roughly ~4 fold. The increase in detachment force can be mainly attributed to the increase in tethered VLA-4/VCAM-1 bond formation. Furthermore, the increase in tethered VLA-4/VCAM-1 bond formation also most likely accounts for the 6-fold increase in detachment distance (Figure 4-4c), as tethered bonds require a much larger pulling distance to rupture compared to cytoskeleton anchored bonds.
4.3.3 THP-1 binding to VCAM-1 is facilitated by VLA-4 and not α₄β₇

VLA-4 (α₄β₁) and α₄β₇ are two integrins known to bind to VCAM-1. Both VLA-4 and α₄β₇ share a common α₄ subunit. To examine whether THP-1 binding to VCAM-1 measured in the previous sections is mediated by VLA-4 and/or α₄β₇ adhesion blocking controls were performed. Adhesion blocking antibodies specific to the β₁ and β₇ subunits were applied to block adhesion (see Section 3.7). Detachment forces were quantified from AFM force curves after application of the adhesion blocking antibodies. Anti-β₁ antibodies blocked adhesion by greater than 50%, whereas anti-β₇ did not have a significantly reduce THP-1 adhesion to VCAM-1 (Figure 4-7).
Figure 4-7: VLA-4 facilitates THP-1 binding to VCAM-1. Detachment forces of THP-1 cells adhering to VCAM-1 were quantified from untreated cells or after blocking with β₁ or β₇ subunits. Forces for all the conditions were normalized by non-treated cell detachment forces (No antibody). Nonparametric Mann-Whitney tests were performed comparing the blocking antibody measurements to cells which were not treated.

Only monocytes activated by cytokines express mRNA encoding the β₇ subunit. Just to confirm that the β₇ subunit is not expressed in the THP-1 cells used in this study, flow cytometry experiments were also conducted to examine the expression levels of both VLA-4 and α₄β₇. THP-1 cells were incubated with fluorophore conjugated antibodies specific to the α₄, β₁, and β₇ subunits and the expression levels were quantified by flow cytometry. β₇ subunit expression was minimal as there was not a noticeable increase compared to the isotype control expression levels (Figure 4-8c). On the other hand, there was significant β₁ subunit expression (Figure 4-8b). The major integrin facilitating THP-1 adhesion to VCAM-1 substrates is VLA-4 confirmed by adhesion blocking antibodies (Figure 4-7) and low levels of β₇ subunit expression (Figure 4-8).
Figure 4-8: The $\beta_7$ subunit is not expressed on THP-1 cells and MCP-1 does not affect the expression integrin levels. Flow cytometry experiments examining THP-1 cell surface expression of the $\alpha_4$ (A), $\beta_1$ (B), and $\beta_7$ (C) subunits for unstimulated (green) and MCP-1 stimulated (green) THP-1 cells. Histograms plotting counts vs mean channel fluorescence are displayed.

4.3.4 MCP-1 does not affect VLA-4 cell surface expression

One of the possible mechanisms facilitating the large increase observed in cell adhesion in MCP-1 stimulated cells (Figure 4-3 and Figure 4-4) is a possible increase VLA-4 expression. To examine this possibility flow cytometry experiments were conducted to examine whether MCP-1 increases VLA-4 cell surface expression. MCP-1 did not noticeably affect expression levels of either the $\alpha_4$ or $\beta_1$ subunits (Figure 4-8 a-b).
4.4 Discussion

The two aims of this chapter were 1) to identify the integrins enhanced by MCP-1 and 2) to examine the effect of MCP-1 on integrin mediated enhancement. MCP-1 has previously been shown to increase the adhesion of VLA-4 to VCAM-1 in monocytes without affecting LFA-1 adhesion to ICAM-1\(^\text{123}\). MCP-1 has also been reported to increase LFA-1 adhesion to ICAM-1 without affecting VLA-4 adhesion\(^\text{20}\). AFM whole cell adhesion measurements were conducted to identify whether MCP-1 enhances the adhesion of VLA-4, LFA-1 or both integrins. Whole cell adhesion measurements provide a tremendous amount of information regarding the adhesion system. The relative number of bonds supporting cellular adhesion, and the association strength between the cytoskeleton and the bond can be obtained from whole cell adhesion measurements.

The VLA-4/VCAM-1 interaction is the predominant adhesion complex mediating monocyte adhesion at subsecond contact times (~100ms) in unstimulated cells, whereas LFA-1/ICAM-1 adhesion complex exhibited minimal adhesion (Figure 4-2). VLA-4 is the major integrin facilitating monocyte adhesion during the processes of rolling and arrest, the initial stages of monocyte recruitment\(^\text{27}\). LFA-1 on the other hand is involved further downstream in the monocyte adhesion cascade by supporting firm monocyte adhesion and migration/infiltration into damaged host tissue\(^\text{29}\). Several possibilities could account for the contrasting roles between VLA-4 and LFA-1. The expression levels of VLA-4 may be tremendously greater than LFA-1 during the early
adhesion events, leading to increase in the probability of VLA-4 binding. On the other hand, VLA-4 and LFA-1 expression levels maybe comparable, but VLA-4 may be more densely clustered proximal to adhesive contact sites, making VLA-4 more accessible for binding. Lastly, VLA-4 may have a faster on-rate compared to LFA-1. In any case, if MCP-1 affected LFA-1/ICAM-1, an increase in the adhesion parameters should have been observed. Hence it is safe to conclude that sub-second exposure of THP-1 cells to MCP-1 does not affect the LFA-1/ICAM-1 adhesion complex.

Sub-second exposure (~100ms) of THP-1 cells to MCP-1 significantly enhanced VLA-4 adhesion, but not LFA-1 (Figure 4-3 and Figure 4-4). Increased detachment force, energy, and pulling distance were required to completely detach THP-1 cells from VCAM-1 substrates (Figure 4-4). The major contribution to the increase in these whole cell adhesion parameters was due to the increase in formation of the number of VLA-4/VCAM-1 bonds, namely tethered VLA-4/VCAM-1 bonds. MCP-1 increased the formation of tethered VLA-4/VCAM-1 ruptures by 4 fold, as opposed to only 2 fold for cytoskeleton anchored VLA-4/VCAM-1 ruptures (Figure 4-6). A subpopulation of cytoskeleton anchored VLA-4/VCAM-1 bonds may be weakly associated with the cytoskeleton and require a minimum extraction force to detach from the cytoskeleton, but still remain bound to VCAM-1. Once dislodged from the cytoskeleton, this subpopulation of bonds form tethered VLA-4/VCAM-1 bonds. This conversion of cytoskeleton anchored to tethered bonds may account for the larger increase in tethered bond formation compared to cytoskeleton anchored bonds. The remainder of the VLA-4/VCAM-
1 bonds more strongly associated with the cytoskeleton ruptures when the lifetime of the VLA-4/VCAM-1 bond is exceeded.

Tethered VLA-4/VCAM-1 ruptures exhibit a much longer bond lifetimes (up to ~3.5s) compared to cytoskeleton anchored bonds (hundreds of ms). This prolonged bond lifetime in tethered bonds tremendously extends the time THP-1 cells remain bound to functionalized substrates in turn requiring a much larger pulling distance to rupture the VLA-4/VCAM-1 adhesion complex. More importantly, the large increase in detachment distance due to tethered VLA-4/VCAM-1 bonds is a major contributing factor to the ~10 fold increase in the detachment energy (Figure 4-4).
Chapter 5  MCP-1 increases the occurrence of tethered VLA-4/VCAM-1 bonds

5.1 Description

In the previous section we identified that only VLA-4/VCAM-1 binding is enhanced by MCP-1, and not LFA-1/ICAM-1. In this section, we will examine whether MCP-1 affects the intrinsic kinetics and/or binding strength of single VLA-4/VCAM-1 bonds. Since LFA-1/ICAM-1 adhesion was not affected by MCP-1, we will only focus on the VLA-4/VCAM-1 system. The dissociation kinetics of the VLA-4/VCAM-1 adhesion complex in response to an applied force have been measured using flow chamber assays\textsuperscript{7} and the Atomic Force Microscope (AFM) measurements\textsuperscript{139}. Furthermore, the study by Zhang et al 2004\textsuperscript{168} is the only study investigating the energetics of the VLA-4/VCAM-1 adhesion system. The flow chamber assay measurements were conducted to examine whether the chemokine Stromal Derived Factor-1 (SDF-1)\textsuperscript{7} enhanced VLA-4 adhesion to VCAM-1. The AFM was used to acquire single molecule rupture force measurements to examine the kinetics and energetics of high affinity VLA-4 adhesion to VCAM-1. The high affinity VLA-4 state was induced by TS2/16 antibody\textsuperscript{139}. The TS2/16 antibody binds to the β1 subunit of VLA-4 on the extracellular domain\textsuperscript{181} and induces a high affinity conformational state in VLA-4\textsuperscript{27,74,168,182,183}. 
Table 5-1: Intrinsic off-rate (k\textsubscript{off}) of the VLA-4/VCAM-1 system measured in response to an applied force. Flow chambers and the AFM were two techniques used to measure the off-rate.

<table>
<thead>
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<th>Technique</th>
<th>Stimulation</th>
<th>k\textsubscript{off} (1/s)</th>
<th>Reference</th>
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<tr>
<td>Flow chamber</td>
<td>none</td>
<td>7.3</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>SDF-1</td>
<td>8.3</td>
<td></td>
</tr>
<tr>
<td>AFM</td>
<td>none</td>
<td>1.1</td>
<td>168</td>
</tr>
<tr>
<td></td>
<td>TS2/16</td>
<td>0.04</td>
<td></td>
</tr>
</tbody>
</table>

Stimulation with the chemokine, SDF-1 did not increase VLA-4 adhesion by inducing a high affinity VLA-4 affinity state as there was no significant increase in the off-rate compared to unstimulated cells (Table 5-1)\textsuperscript{7}. Induction of a high affinity VLA-4 state with TS2/16 antibody increased the off-rate by two orders of magnitude. In comparing the two sets of measurements, the intrinsic off-rates for unstimulated VLA-4/VCAM-1 determined from the flow chamber assay (~7.3-8.3/s) were 8-fold greater than those acquired from the AFM (1.1/s). This difference may be attributed to lower resolution of the flow chamber assay in comparison with the AFM which is much more precise.

In this section, single molecule force spectroscopy (SMFS) of the VLA-4/VCAM-1 bonds was obtained using the AFM at an operational temperature of 37\textdegree C. Force spectra of both cytoskeleton anchored and tethered VLA-4/VCAM-1 bonds were investigated. Single tethered VLA-4/VCAM-1 bonds occurred more frequently than cytoskeleton anchored VLA-4/VCAM-1 bonds and exhibited a higher binding strength and intrinsic bond lifetime compared to single cytoskeleton anchored bonds. Surprisingly, MCP-1 did not have a statistically
significant effect on the binding strength or intrinsic kinetics of single tethered or cytoskeleton anchored VLA-4/VCAM-1 bonds.

5.2 Single molecule force spectroscopy background

SFMS is a technique that interrogates the lifetimes of single molecule bonds in response to a range or spectrum of applied forces. Intrinsic kinetics, energetics, and the binding strength of molecular bonds can be determined from force spectra. All of the theories developed to characterize force spectra are all derived from Kramers’ transition state theory for a chemical reaction escaping over an energy barrier\textsuperscript{184}. The Bell and Bell-Evans models were two theories used in this study to characterize VLA-4/VCAM-1 bond force spectra.

George I. Bell was one of the first to apply kinetic models to investigate biological bonds rupturing under applied forces\textsuperscript{185}. The kinetic model which Bell applied is commonly referred to as the Bell-Model (Eqn. 5.1), where \( \tau \) is the bond lifetime, \( \tau_0 \) is the intrinsic bond lifetime or the bond lifetime at zero force, \( \gamma \) is the width of the energy barrier, \( F \) is the applied force, \( k_B \) is Boltzmann's constant \((1.38*10^{-23}\text{m}^2\text{kg}\cdot\text{s}^{-2}\text{K}^{-1})\), and \( T \) is the temperature in Kelvins.

\[
\tau = \tau_0 \exp \left(-\frac{\gamma F}{k_B T}\right) \quad \text{Eqn. 5-1}
\]

The Bell model uses a single energy barrier to describe the lifetime of a single molecular adhesion complex stressed under a mechanical force (Figure 5-1).
The free energy changes in a single molecular bond in response to an applied force can be characterized from the Bell-Model.

At equilibrium, the two adhesion molecules exist in the bound state because there is a much lower energy configuration than the unbound state. The bond will dissociate once the applied energy exceeds the energy barrier height ($E_b$) (Figure 5-1). The barrier height cannot be determined experimentally, and is commonly lumped into the intrinsic bond lifetime ($\tau_0$) term, hence, the intrinsic bond lifetime calculated from the Bell model factors in the barrier height. The intrinsic bond lifetime ($\tau_0$) gives an indication of how long the complex remains in the bound state in the absence of an applied force. It is important to note that the inverse of the intrinsic bond lifetime is equivalent to the off-rate or dissociation rate of the bound adhesion complex. The energy barrier width ($\gamma$) represents the minimum separation distance required to completely dissociate the bound complex. The energy barrier width scaled by the applied force ($-\gamma*F$) indicates...
the amount of work required to tilt or lower the energy barrier. Therefore mechanical bond strength of the adhesion complex can be determined from the energy barrier width.

Bonds can dissociate in the spontaneously absence of an applied force, mainly due to thermal energy imparted by the surroundings (Figure 5-1). The application of force increases the probability of bond dissociation. A force applied to an adhesion complex tilts the energy barrier width by a factor of the energy barrier width scaled by the applied force ($-\gamma^* F$). This factor is equivalent to the amount of work required to tilt the barrier. This will lower the energy barrier reducing the amount of energy required to completely dissociate the bond (Figure 5-1). The dissociation rate of the bond also increases as the slope becomes shallower.

The Bell model is only applicable to bonds stressed under a constant amount of force over time. Physiologically however, forces are dynamic. The Bell-Evans model was developed to interrogate the energy barrier of bonds breaking under dynamic forces, namely a linearly increase in force over time. The Bell-Evans model (Eqn. 5-2); is an extension of the Bell model proposed by Evans and co-workers, where $F$ is the rupture force of the bond, $r$ is the loading rate, all other terms are the same as in Eqn. 5-1.

**Bell-Evans Model:**

$$F = \frac{K_B T}{\gamma} \ln \frac{\tau_0 yr}{2k_B T}$$

(Eqn. 5-2)

The loading rate refers to the rate of force application and is dependent on the pulling velocity and the mechanical stiffness of the system which includes the
stiffness of the bond itself and the probe/transducer (the cantilever spring constant in this case) which pulls the bond\textsuperscript{189}. The advantage of interrogating the force spectra using the loading rate is that a much larger range of forces can be applied to the molecular bond compared to using a constant force. However, since the bond lifetime is dependent on the force, the disadvantage of using the dynamic force is that the bond lifetime is not constant throughout the application of force. More importantly, the bond lifetime cannot be directly measured from force scans exhibiting bond ruptures in response to dynamic forces. The major disadvantage of both the Bell and Bell-Evans models are that the energy barrier height cannot be directly measured in each case and is simply lumped into the intrinsic bond lifetime term\textsuperscript{187}.

5.3 Data Acquisition and Processing

5.3.1 Acquiring single molecule measurements

Several experimental factors can be controlled to increase the probability of encountering a single molecule adhesion event: 1) protein concentration on the dish 2) indentation force and 3) the interaction/contact time between the cell and the substrate. Changes in the indentation force and contact time, will affect the contact area formed between the cell substrate. Contact time also affects the binding probability, which increases when the association or on-rate exceeds the contact time.
The main difference between single molecule and whole-cell adhesion measurements is that single molecule measurements generally require lower amounts of protein concentration on the dish, smaller indentation forces and contact times. To increase the probability of encountering single molecule adhesion events, the indentation force and contact time were reduced compared to whole cell adhesion. Dishes were functionalized with 0.25-1.0 µg/mL huVCAM-1 and/or 5 µg/mL huMCP-1. Force curves were acquired measuring the interaction between the THP-1 cell attached to the cantilever and VCAM-1 immobilized on the Petri dish. The indentation force varied between 50-200 pN, and the interaction time varied between 0-0.333 seconds to maximize the probability of encountering a single molecule adhesion event. Roughly 30% of the measurements exhibited cellular adhesion. At a 30% adhesion frequency, there is a higher probability of measuring a single bond\textsuperscript{164,187}. Lastly, to interrogate bond lifetimes over a range of forces or loading rates single VLA-4/VCAM-1 bonds were pulled at velocities of 3, 5, 15, and 25 µm/s.

5.3.2 Data Analysis

5.3.2.1 Cytoskeleton anchored bonds

Cytoskeleton anchored bonds exhibited one visible rupture breaking instantaneously following a linearly increasing force. The loading rate ($r$) and bond rupture force ($F$) are two parameters determined from force curves exhibiting single cytoskeleton anchored bond ruptures. The loading rate is
determined as the slope of the force curve just before rupture. The bond rupture force is calculated as the peak force in the force curve.

![Figure 5-2: Single cytoskeleton anchored bonds.](image)

**Figure 5-2: Single cytoskeleton anchored bonds.** Determining the loading rate \( r \) and rupture force \( F \) from the force curve of a cytoskeleton anchored VLA-4/VCAM-1 bond.

VLA-4/VCAM-1 cytoskeleton anchored bonds were pulled over a range of loading rates from 100 pN/s to 10 nN/s to construct dynamic force spectra to interrogate the kinetics of the bond. Loading rates and rupture forces were grouped according to equally spaced logarithmic intervals of the loading rate. Each group contained at least 50 data points. Grouping by logarithmically spaced intervals of the loading rate also ensured that each group contained a similar number of data points. Force spectra were constructed from the median rupture force and loading rate for each group. The intrinsic bond lifetime and energy barrier widths were determined by fitting the Bell-Evans model to the force spectra (Eqn. 5.2).
5.3.2.2 *Tethered bonds*

Single tethered bonds exhibited one single rupture breaking after a constant force plateau persisting longer than 0.25 µm. Tethered bond lifetime and extraction forces were directly determined from force curves exhibiting single tethered VLA-4/VCAM-1 ruptures. The tether extraction force is the amount force required to extract or pull the tethered bond.

![Force vs Time/Distance Graph](image)

**Figure 5-3: Single tethered bonds.** Determining bond lifetime ($\tau$) and rupture force ($F$) from force curves exhibiting tethered VLA-4/VCAM-1 bonds.

Tethered bond lifetimes and rupture forces were grouped according to the velocity at which the bond was pulled. The median tether forces for each velocity group were determined. The mean tether lifetimes for each velocity group were determined from the cumulative lifetime probability distribution ((Eqn. 5-3; Figure 5-4 inset), where $t$ is the time, $P$ is the probability a bond survives longer than time $t$, $\tau$ is the mean bond lifetime, and $A$ is the y-intercept$^{180}$.)
Force spectra of single tethered VLA-4 bonds were constructed from mean tethered bond lifetimes versus median tethered bond rupture forces (Figure 5-4) and fit to the Bell model Eqn. 5-1.

**Figure 5-4:** Force spectrum of single tethered VLA-4/VCAM-1 bond ruptures. (Inset) Mean tether lifetimes for each velocity group were obtained by fitting lifetimes (dashed line) to the cumulative probability distribution function (Eqn. 5-3). Force spectrum was fit to the Bell model Eqn. 5-1.

### 5.4 Results

**5.4.1 Single tethered VLA-4/VCAM-1 ruptures are the most predominant adhesive bond**

To examine whether MCP-1 affects the intrinsic kinetic properties and/or binding strength of individual VLA-4/VCAM-1 bonds, SMFS was conducted on
MCP-1 stimulated THP-1 cells adhering to VCAM-1 functionalized substrates. In order to encounter single VLA-4/VCAM-1 bonds using the AFM, the adhesion frequency was reduced to 30% to increase the probability of encountering single bonds. THP-1 cells were stimulated with TS2/16 antibody. The TS2/16 antibody binds to the extracellular region of the β1 subunit of VLA-4 and induces a high affinity state in VLA-4 (see Section 5.1 for more details). SMFS has shown that TS2/16 stimulation of VLA-4 expressed on U937 cells, a monocytic cell line increases the intrinsic off-rate by two orders of magnitude.

At an adhesion frequency of 30%, three types of VLA-4/VCAM-1 adhesion events were observed: 1) single tethered VLA-4/VCAM-1, 2) single cytoskeleton anchored VLA-4/VCAM-1, and 3) adhesion events exhibiting multiple ruptures (Figure 5-5a). Of the three types of adhesion events, the multiple adhesion events were the most frequently occurring. Single tethered VLA-4 bonds exhibited a similar adhesion frequency as multiple rupture events were at least 5 times more frequent than cytoskeleton anchored VLA-4/VCAM-1 bonds (Figure 5-5b).
5.4.2 Investigating single tethered VLA-4/VCAM-1 ruptures

5.4.2.1 MCP-1 does not affect effective tether viscosity

Biological tethers are long extensions of the cell membrane supported by an adhesive bond at the end of the tether. The tether breaks when the lifetime of the integrin/adhesive bond is exceeded\textsuperscript{191,192}. The tether extraction force is not the amount force required to break the bond, but rather the force required to extract the tether. The tether extraction force is exerted to slide the membrane along the cytoskeleton producing a viscous force\textsuperscript{16,180,192-196}. As previously mentioned in section 4.3.2, the viscous force is a constant amount of force...
applied to the bond over time (Figure 5-5a). The tether viscosity can be
determined from the tether extraction force and pulling velocity from Eqn. 5-4,
where $F$ is the tether extraction force, $F_0$ is the minimum force required to extract
the tether, $\eta_{eff}$ is the effective tether viscosity, and $V$ is the pulling velocity at
which the tether is extracted$^{194}$. The effective viscosity term characterizes the
strength of association between the cell membrane and the underlying
cytoskeleton, the stronger the association between the two, the larger the
effective viscosity. The cytoskeleton and the membrane are linked together
indirectly through integral membrane proteins.

$$F = F_0 + 2\pi \eta_{eff} V$$

Eqn. 5-4

Tethered VLA-4/VCAM-1 bonds were extracted at different velocities.
Unstimulated, MCP-1, and TS2/16 stimulated VLA-4/VCAM-1 tethers exhibited
effective tether viscosities around 0.15 pN*s/µm (Table 5-2, Figure 5-6). Neither
MCP-1 nor TS2/16 affected tether viscosity.
Figure 5-6: MCP-1 and TS2/16 do not effect tether viscosity. Median tether extraction forces for each velocity group were fit to Eqn. 5-4.

$$\eta_{\text{eff}} \, (\text{pN s/\mu m}) \quad F_0 \, (\text{pN})$$

<table>
<thead>
<tr>
<th></th>
<th>$$\eta_{\text{eff}}$$</th>
<th>$$F_0$$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rest</td>
<td>0.15 ± 0.04</td>
<td>23 ± 4</td>
</tr>
<tr>
<td>MCP-1</td>
<td>0.14 ± 0.05</td>
<td>25 ± 4</td>
</tr>
<tr>
<td>TS2/16</td>
<td>0.14 ± 0.02</td>
<td>25 ± 3</td>
</tr>
</tbody>
</table>

Table 5-2: Experimental parameters for determining the effective viscosity. Fitting Eqn. 5-4 to the data plotted in Figure 5-6. (± s.e.m. is shown)

Since tether extraction force increases linearly with pulling velocity, changes in the pulling velocity can be used to interrogate how the VLA-4/VCAM-1 bond lifetime responds over a range pulling forces.
5.4.2.2 MCP-1 increases binding strength of individual tethered VLA-4/VCAM-1 ruptures

The force applied to single tethered bonds is dependent on the pulling velocity. To investigate single tethered VLA-4/VCAM-1 ruptures in response to a range of forces, tethers were extracted at different pulling velocities of 2, 5, 15, 25 µm/s. For each velocity group, the median tether extraction force and mean tether lifetime were determined from cumulative lifetime probability distributions (Eqn. 5-3). The cumulative lifetime probability were calculated for each pulling velocity group of each condition (Figure 5-7) and fit to a simple exponential cumulative probability function (Eqn. 5-3). For TS2/16 stimulation for the 2 µm/s pulling velocity group, there was a small subpopulation of lifetimes where the cumulative lifetime probability function did not fit. This subpopulation exhibited longer tethered rupture lifetimes greater than ~3.5s and was most likely supported by two or more bonds. The existence of double bonds in the TS2/16 tethered ruptures was further confirmed by fitting a double exponential probability function as opposed to a single exponential probability function (Eqn. 5-3). The second exponential term would account for the proposed second bond. The double exponential function fit to the cumulative lifetime probabilities for the TS2/16 stimulated 2 µm/s pulling velocity group described the entire data set. Finally only ~17% (~20 of the tethered ruptures) of the TS2/16 stimulated 2 µm/s pulling velocity group exhibited single ruptures supported by more than one bond. For the most part the single exponential probability function describes 80% of the TS2/216 stimulated tethered lifetimes pulled at a velocity of 2 µm/s.
Figure 5-7: Cumulative lifetime probability of measured tethered VLA-4/VCAM-1 rupture lifetimes. Solid lines indicate the 95% upper and lower confidence limits of the cumulative lifetime probability distribution function (Eqn. 5-3) to the data. Three different conditions are shown above (left) unstimulated (middle) MCP-1 (right) TS2/16.

The mean lifetime of each pulling velocity group was determined from the simple exponential probability fits in Figure 5-7, and plotted against the median tether extraction forces to produce force spectra (Figure 5-8). The force spectra of mean tether lifetimes versus the median tether extraction force of unstimulated, MCP-1 and TS2/16 stimulated tethered VLA-4/VCAM-1 bonds were fit to the Bell model Eqn. 5-1.
Figure 5-8: Force spectra of single tethered VLA-4/VCAM-1 bonds. Force spectra of mean tether lifetimes vs median tether extraction force. Spectra were fit to the Bell Model Eqn. 5-1.

The energy barrier width ($\gamma$) indicates the binding strength of the adhesion complex\textsuperscript{16,187,188,197}. The smaller the energy barrier width, the more resistant the bond is to force. MCP-1 stimulated tethered VLA-4/VCAM-1 bonds exhibited a decreased the energy barrier width (~0.28 nm) compared to unstimulated force spectrum (~0.39 nm) (Table 5-3). MCP-1 increased single tethered VLA-4/VCAM-1 bond resistance to force. TS2/16 stimulation of single tethered VLA-4/VCAM-1 bonds confirmed increased bond resistance seen with MCP-1 stimulation.
Table 5-3: Energy barrier widths and intrinsic bond lifetimes of single tethered bonds.
Force spectra in Figure 5-8 were fit to the Bell-Model (eqn. 5-1).

<table>
<thead>
<tr>
<th></th>
<th>γ (nm)</th>
<th>τ₀ (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>unstimulated</td>
<td>0.39</td>
<td>4.09</td>
</tr>
<tr>
<td>MCP-1</td>
<td>0.28</td>
<td>2.43</td>
</tr>
<tr>
<td>TS2/16</td>
<td>0.27</td>
<td>2.14</td>
</tr>
</tbody>
</table>

Although MCP-1 stimulated an increase in the binding strength of single tethered VLA-4/VCAM-1 bonds, the intrinsic bond lifetime (τ₀) of MCP-1 treated tethered VLA-4/VCAM-1 bonds did not exhibit an increase. In fact, the intrinsic bond lifetime of both TS2/16 (~2.14s) MCP-1 treated tethered bonds (~2.43s) was smaller than unstimulated tethered bonds (~4.09s).

5.4.3 MCP-1 does not affect cytoskeleton anchored VLA-4/VCAM-1 ruptures

Single cytoskeleton anchored VLA-4/VCAM-1 bonds were pulled at the same velocities as tethered bonds (section 5.4.2) from the exact same batch of cells. These pulling velocities resulted in the cytoskeleton anchored bonds being subjected to loading rates ranging from 200 pN/s to 8 nN/s. Rupture forces were grouped according to logarithmically spaced intervals of the loading rate. Median rupture forces and loading rates were determined for each group. Dynamic force spectra of unstimulated, MCP-1, and TS2/16 treated cytoskeleton anchored bonds (Figure 5-9) were fit to the Bell-Evans model (Eqn. 5-2).
Neither MCP-1 nor TS2/16 treatment noticeably affected the dynamic force spectra compared to unstimulated cytoskeleton anchored bonds (Figure 5-9). Upon closer examination, MCP-1 treated cytoskeleton anchored VLA-4/VCAM-1 bonds appear to exhibit a lower intrinsic bond lifetime. However, upper and lower 95% confidence intervals of the fit of MCP-1 data to the Bell-Evans model overlap with both unstimulated and TS2/16 treated cytoskeleton anchored bond spectra (confidence intervals were removed from the graph for clarity). Overlapping confidence intervals of different demonstrate that there is no significant difference between two fits. Furthermore, there is not a noticeable difference between the fit coefficients from Bell-Evans model fits to the dynamic
spectra of the three different conditions (Table 5-2). Hence, MCP-1 does not
seem to noticeably effect the intrinsic kinetics or binding strength of individual
cytoskeleton anchored bond ruptures.

<table>
<thead>
<tr>
<th></th>
<th>$\gamma$ (nm)</th>
<th>$\tau_0$ (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>unstimulated</td>
<td>0.49</td>
<td>0.80</td>
</tr>
<tr>
<td>MCP-1</td>
<td>0.49</td>
<td>0.93</td>
</tr>
<tr>
<td>TS2/16</td>
<td>0.47</td>
<td>0.68</td>
</tr>
</tbody>
</table>

Table 5-4: Energy barrier widths and intrinsic bond lifetimes of single cytoskeleton anchored bonds. Coefficients from the Bell-Evans model fit to cytoskeleton anchored VLA-4/VCAM-1 bond dynamic force spectra.

5.5 Statistical validation of curve fits to the force spectra

5.5.1 Description of the Bootstrap method

Bootstrapping is a method used to estimate the distribution and/or
certainty limits of a population statistic. by resampling with replacement from
the sample population/data set\textsuperscript{198}. Resampling with replacement implies that the
same data point from the original data point can be resampled more than once in
the resampled data set. Multiple resampled data sets from the sample
population are often created, sometimes even several thousand instances to
estimate the distribution of a sample population parameter or statistic\textsuperscript{198-202}.

Goodness of fit of a function to the sample population can also be evaluated
using the bootstrap method by constructing confidence limits of the fit
parameters\textsuperscript{199,201,202}. It is especially effective at estimating confidence limits of
parameters when the parameter distribution is unknown or non-parametric, when
the statistic does not follow a particular distribution. It is also useful for
estimating confidence limits of parameters from small data sets where data is challenging to obtain\textsuperscript{201}. Estimating the confidence limits of a population statistic and determining the goodness of fit between a model and the data, are just a few applications of the bootstrap method\textsuperscript{198-202}. In this work, the bootstrap method was used to estimate the confidence limits of the parameters obtained from fitting the Bell-Model to tethered rupture force data to evaluate the goodness of fit.

5.5.2 Bootstrap Data Analysis

Tether threshold forces and their corresponding lifetimes were randomly resampled with replacement from the original single tether data sets to create a resampled data set with the same number of points as the original data set. For cytoskeleton anchored ruptures, the loading rates and corresponding rupture forces were resampled. The process was repeated to create 60 different resampled data sets. Each of the resampled data sets from tethered rupture data was individually fit to the Bell-model to determine the corresponding intrinsic lifetime and width of the energy potential. Confidence limits of the intrinsic lifetimes and energy barrier widths were constructed from fits to the resampled data sets.
5.5.3 Bootstrap Results

5.5.3.1 Confidence Limits of Parameters from Single Tethered VLA-4/VCAM-1 ruptures

To evaluate the goodness of fit of the Bell-Model to single tethered VLA-4/VCAM-1 rupture lifetimes and extraction forces, the bootstrap method was applied. Bootstrapped data was resampled from unprocessed tether lifetimes and extraction forces from the three different stimulation conditions in Figure 5-10. Unprocessed tether lifetimes and extraction forces refer to the fact that data have not been grouped or sorted in any way according the pulling velocity (Section 5.3.3.2) to determine mean tether lifetimes and extraction forces for each velocity group. In total, 60 resampled data sets were created from the unprocessed tether lifetime and extraction force data in Figure 5-10, and each was fitted to the Bell-Model. The estimated energy barrier width and intrinsic lifetime of VLA-4/VCAM-1 single tethered ruptures from resampling are plotted in Figure 5-11a.
Figure 5-10: Bell-Model fits to the unprocessed tether lifetimes and extraction forces. Symbols represent the unprocessed data and solid black lines represent the Bell-Model fits to the data.

Bell-Model fits to the unprocessed data demonstrate that there is no significant difference between the energy barrier width and intrinsic bond lifetimes of unstimulated single tethered VLA-4/VCAM-1 ruptures and those stimulated with MCP-1 (Table 5-5). More importantly, TS2/16 stimulation increased the energy barrier width by a factor of two compared to MCP-1 treated and unstimulated conditions. On the other hand, the intrinsic bond lifetime of TS2/16 stimulated tethered ruptures was less than both MCP-1 and unstimulated bonds (Table 5-5).

<table>
<thead>
<tr>
<th></th>
<th>$\gamma$ (nm)</th>
<th>$\tau_0$ (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>unstimulated</td>
<td>0.16</td>
<td>0.96</td>
</tr>
<tr>
<td>MCP-1</td>
<td>0.18</td>
<td>1.04</td>
</tr>
<tr>
<td>TS2/16</td>
<td>0.07</td>
<td>0.71</td>
</tr>
</tbody>
</table>

Table 5-5: Energy barrier width ($\gamma$) and intrinsic bond lifetime ($\tau_0$). Obtained from fitting the Bell-Model to the unprocessed raw data in Figure 5-10.
The confidence limits of the intrinsic lifetimes and energy barrier widths of MCP-1 stimulated single VLA-4/VCAM-1 ruptures were not significantly different from unstimulated ruptures. This demonstrates that MCP-1 does not affect the kinetics or energetics of single tethered VLA-4/VCAM-1 bonds. On the other hand, TS2/16 stimulation decreased the energy barrier width of single VLA-4/VCAM-1 ruptures and intrinsic lifetime compared to unstimulated and MCP-1 stimulated single tethered ruptures Figure 5-11a. This was further corroborated by intrinsic lifetimes and energy barrier widths obtained from fitting the Bell-Model to the unprocessed data (Figure 5-10, Figure 5-11b). Moreover, there appears to be a linear relationship between the intrinsic lifetimes and energy barrier widths of the three conditions Figure 5-11b extracted from the unprocessed tether lifetimes and extraction forces Figure 5-10.

Figure 5-11: MCP-1 does not affect the energetics or kinetics of single tethered VLA-4/VCAM-1 ruptures. A) Bootstrap estimation of confidence limits of the intrinsic rupture lifetime and width of the energy potential from Bell-Model fits to single tethered VLA-4/VCAM-1 rupture data. B) Intrinsic lifetime and width of the energy potential of single tethered VLA-4/VCAM-1 ruptures determined by fitting to the raw untransformed data in Figure 5-10.
5.6 Discussion

The aim of this section was to investigate whether MCP-1 affected the intrinsic bond lifetime and binding strength of individual VLA-4/VCAM-1 bonds. To approach this aim, single molecule force rupture measurements were obtained to characterize VLA-4/VCAM-1 dynamic force spectra of unstimulated and MCP-1 treated THP-1 cells. Single tethered VLA-4/VCAM-1 bonds were the most predominant single molecule adhesion event detected while attempting to obtain single molecule rupture force measurements (Figure 5-6b). There were at least more than 5 times as many single tethered VLA-4/VCAM-1 bonds encountered for each of the three conditions compared to cytoskeleton anchored bonds.

Even though the adhesion frequency was reduced to 30% to obtain single molecule rupture force measurements, an extremely high frequency of multiple rupture events were detected (at least 46% adhesion events) (Figure 5-6b). At an adhesion frequency of 30% it is predicted that 83% of the measured adhesion events are single bond ruptures, and the other 15% of the events are double bonds. These values were determined from a probabilistic model assuming that adhesion molecules are uniformly distributed across the cell surface. This high frequency of multiple events may be due to VLA-4 not being uniformly distributed across the THP-1 cell surface. In T-lymphocytes VLA-4 expressed on the cell surface is non-uniformly expressed and mainly clustered to the microvilli tips. The topographical clustering of VLA-4 to the microvilli tips may explain the low frequency of single cytoskeleton anchored VLA-4/VCAM-1 bonds. Adhesion
molecules expressed at microvilli tips may be more accessible for binding and more prone to support membrane tethers, than cytoskeleton anchored bonds expressed distal to the microvilli on the peripheral cell body.

Not only were single tethered VLA-4/VCAM-1 bonds more frequent, but also exhibited at least a 3 fold increase in the intrinsic bond lifetime (Table 5-1) compared to cytoskeleton anchored bonds (Table 5-2). The energy barrier widths of single tethered VLA-4/VCAM-1 bonds were also smaller than those of single cytoskeleton anchored bonds by at least a factor of 3. The decreased energy barrier width of single tethered VLA-4/VCAM-1 bonds compared to cytoskeleton anchored bonds indicating that tethered bonds have a higher binding strength facilitating much longer bond lifetimes than tethered bonds compared to cytoskeleton anchored bonds.

Conventionally, single VLA-4/VCAM-1 rupture data is fit to the Bell-Model grouping by the pulling velocity/loading rate to determine the intrinsic bond lifetime and energy barrier width for each respective group (Section 5.4)\textsuperscript{180,187,204}. Using the conventional method of grouping by the pulling speed, MCP-1 treatment of THP-1 cells resulted in an apparent increased binding strength of single tethered VLA-4/VCAM-1 bonds. The observed increased binding strength was reflected by a slight decrease in the energy barrier width (~0.28 nm) compared to unstimulated VLA-4/VCAM-1 bonds (~0.39 nm). The slight increase in VLA-4/VCAM-1 binding strength was also confirmed by treatment of THP-1 cells with TS2/16 antibody (Table 5-1) which locks VLA-4 into a high affinity state\textsuperscript{74,168}. 
Although both MCP-1 and TS2/16 treatment increased single tethered VLA-4/VCAM-1 binding strength, the intrinsic bond lifetimes for both treatments (~2.43 s) was less than the unstimulated single VLA-4/VCAM-1 tethered bonds (~4.09 s). The first point in the unstimulated tethered VLA-4/VCAM-1 force spectrum (Figure 5-8) could potentially be an outlier biasing the Bell model fit to the force spectra thus skewing the intrinsic bond lifetime for unstimulated VLA-4/VCAM-1 bonds. This first point also corresponds to the slowest pulling velocities. At slower pulling velocities the probability of encountering a double bond increases. Additionally, the instantaneous rebinding of a dissociated adhesion complex can occur at slower rates of separating the probe from the functionalized substrate, especially if there is sufficient time for the broken bond to rebind. Both the phenomena of double bound formation and instantaneous rebinding may increase the measured tether lifetime, thus skewing the Bell model fit to the dynamic force spectra at lowest tether extraction force.

In addition to determining the intrinsic bond lifetime and energy barrier width from the Bell-Model using the conventional method (previous paragraph and Section 5.3.2), the Bell-Model was also fit to the total ungrouped data (Section 5.5). There was no significant difference between the energy barrier widths and intrinsic bond lifetimes of unstimulated and MCP-1 treated tethered bonds (Table 5-5; Figure 5-10). On the other hand however, TS2/16 treated tethered bonds exhibited a significant decrease in the energy barrier widths compared to MCP-1 treated and unstimulated ruptures, but not the intrinsic bond lifetime.
To evaluate the goodness of fit of the Bell-Model to single tethered VLA-4/VCAM-1 rupture data not grouped by pulling velocity, tethered rupture lifetimes and forces were resampled from the original total data set using the bootstrap method (Section 5.5). Confidence limits of the resampled data (Figure 5-11) confirm that energy barrier widths and intrinsic bond lifetimes of MCP-1 treated tethered ruptures are not significantly different from unstimulated cells. On the other hand, TS2/16 stimulation decreases the energy barrier width compared to MCP-1 treated and unstimulated cells, but does not significantly affect intrinsic bond lifetime. The main conclusion of the bootstrap data is that MCP-1 does not affect intrinsic bond lifetime or the energy barrier width of single tethered VLA-4/VCAM-1 ruptures.

In comparing the Bell-Model parameters extracted from the conventional method (Figure 5-8; Table 5-3) to those from the data not grouped according to convention (Figure 5-10; Table 5-5), the conventional method overestimates the intrinsic bond lifetimes (Figure 5-12) compared to those extracted from fitting to the entire data set. This overestimation may be due a small sub-population of single tethered ruptures supported by more than one bond in each visible rupture. The possibility of multiple bonds supporting a single rupture would effectively increase the observed bond lifetimes, thus biasing the estimated mean tether lifetimes for each pulling velocity (Figure 5-4). This bias would have a pronounced effect on the conventional method for determining Bell-Model fit parameters because the determination of the mean tether lifetimes determined for each pulling velocity are quite sensitive to lifetimes measured from tethered
ruptures supported by multiple bonds. On the other hand, this bias will be less pronounced for the Bell-model parameters from the unconventional method.

Figure 5-12: Comparison of conventional and unconventional data sets fit to the Bell-Model fits. The Bell-Model was fit to the total data set without grouping by pulling velocity (unfilled symbols) to data grouped by pulling velocity (solid symbols). Unfilled/open symbols denote intrinsic lifetimes and energy barrier widths were determined from Bell-Model fits to the total set without grouping by velocity (Section 5.5) and filled symbol parameters were obtained from fits to most probable bond lifetimes and median tether extraction forces (Section 5.3.2).

Neither MCP-1 nor TS2/16 affected bond strength and intrinsic bond lifetime of cytoskeleton anchored VLA-4/VCAM-1, compared to unstimulated THP-1 cells (Figure 5-9; Table 5-4). In contrast, TS2/16 was found to increase the intrinsic bond lifetime of single cytoskeleton anchored VLA-4/VCAM-1 bonds in the slow loading regime in monocytic U937 cells at room temperature\(^{168}\). The TS2/16 binding site on cytoskeleton anchored VLA-4 expressed on THP-1 cells may not be exposed for TS2/16 binding. Alternatively, cytoskeletal regulation of
VLA-4 expressed in THP-1 and U937 monocytic cell lines may be different. Integrin affinity can be regulated by adaptor and scaffolding proteins associated with both the cytoskeleton and the integrin itself. There are cytoskeleton associated proteins that can either induce high affinity states in integrins\textsuperscript{103} or lock the integrin in a closed low affinity state\textsuperscript{205}. The composition or activity of the regulatory adaptor/scaffolding proteins may differ between THP-1 and U937 cells leading to the inability of both MCP-1 and TS2/16 to activate cytoskeleton anchored VLA-4 in THP-1 cells. MCP-1 also increases the association of the α4 subunit of VLA-4 with paxillin\textsuperscript{98-100,103}, which has been found to lock integrins in a low affinity state\textsuperscript{97,103}.

Lastly, MCP-1 did not affect the viscosity of single VLA-4/VCAM-1 tethers. The viscosity of membrane tethers is dependent on the strength of association between the cytoskeleton and the membrane, and also the fluidity of the membrane itself. Stronger association of the cytoskeleton with the membrane and decreased membrane fluidity will both increase the effective viscosity. Both of these properties can affect the expression levels and also the activity of integral membrane proteins, and the lipid composition of the cell membrane\textsuperscript{206,207}. By not affecting the viscosity of single VLA-4/VCAM-1 tethers, MCP-1, most likely does not affect the lipid composition, activity or expression of levels integral membrane proteins regulating the cytoskeleton-membrane interaction.
Chapter 6 Conclusions

The main goal of this study was to investigate the mechanism by which MCP-1 enhances integrin mediated adhesion. To investigate this, single molecule force spectroscopy on adhesive bonds was performed to identify whether MCP-1 affected the intrinsic kinetics and/or binding strength of individual receptor-ligand bonds. Whole cell adhesion measurements were conducted with the AFM to examine the effect of MCP-1 stimulation and to identify the integrins affected by MCP-1. Furthermore, cell surface expression of the integrins expressed on the monocyte cell surface was also performed to investigate whether MCP-1 enhances the cell surface expression of VLA-4.

The major conclusion drawn from this work is that MCP-1 enhances VLA-4 binding to VCAM-1 by increasing the binding strength (Section 5.4.2.2) and formation of tethered VLA-4/VCAM-1 bonds (Figure 4-3; Figure 4-6). The increased binding strength in individual tethered VLA-4/VCAM-1 bonds facilitates extremely long tether extensions seen in whole cell adhesion curves. The MCP-1 induced longer tether extensions require much larger amount of detachment energy to completely rupture the complex (Figure 4-4). The increased binding strength and formation of tethered VLA-4/VCAM-1 complex allows the monocyte to remain bound for longer periods of time so that the adhered monocyte can survey the local endothelial microenvironment.

In addition, to increasing VLA-4/VCAM-1 bond formation in whole cell adhesion measurements, compared to unstimulated cells MCP-1 also increased
the overall frequency of adhesion events when trying to acquire single molecule rupture forces. Increases in bond formation can either be due to an increase in the association rate of the VLA-4/VCAM-1 complex and/or the clustering of VLA-4 to local adhesive contact sites. An increase in the association rate would lead to more rapid binding between VLA-4 and VCAM-1. The clustering of VLA-4 to local adhesive contact sites would increase the amount of VLA-4 available for binding.

The increase in adhesion of MCP-1 stimulated cells in whole cell measurements may also be due to MCP-1 induced activation of VLA-4 from a bent low affinity state to an extended low affinity state (Figure 1-1). This would account for the fact that MCP-1 did not affect intrinsic bond lifetimes single VLA-4/VCAM-1 (Figure 5-8; Figure 5-9; Table 5-5). The VLA-4/VCAM-1 system has long been implicated to simply as a breaking mechanism to facilitate leukocyte arrest on the endothelium. In a biological context, the breaking mechanism would be equivalent to the induction cell spreading on the endothelium. Paxillin is one of the cytoskeletal adaptor proteins that plays a role in cell spreading by acting as a platform to recruit other signaling and structural molecules. It has been implicated that paxillin mainly plays role to increase adhesion by strengthening the association of the adhesion complex with the cytoskeleton in leukocytes. More importantly, paxillin strengthening of VLA-4 adhesion complexes does not alter integrin affinity. MCP-1 stimulation increased paxillin association with the α4 subunit of VLA-4. These studies coupled with the findings in this work offer strong support that MCP-1 induces the
arrest of monocytes on the endothelium by inducing an extended low affinity state in VLA-4.

VLA-4/VCAM-1 binding may also prime LFA-1/ICAM-1 adhesion for cellular migration. It is possible that a prolonged exposure of monocytes to MCP-1 for several minutes or more may induce monocyte migration by activating LFA-1. However, the exposure of monocytes to soluble MCP-1 for 15 minutes only increased VLA-4 binding to VCAM-1 and not LFA-1 to ICAM-1 (Figure 4-4 right panels), similar to MCP-1 immobilized on the dish (Figure 4-2 top traces; and Figure 4-4). This data suggests that another stimulus is required to activate LFA-1/ICAM-1 or that LFA-1 is not as highly expressed on the THP-1 cell surface as VLA-4. Also VLA-4/VCAM-1 is the predominant adhesion complex supporting the monocyte rolling and arrest on the endothelium.

Other mechanisms of MCP-1 enhancement include an increase in cell surface expression of VLA-4. In this study, THP-1 cells were only exposed to MCP-1 for ~100ms, so it is highly unlikely that MCP-1 would affect protein expression at the transcription or translational level. It is possible that MCP-1 can enhance the cell surface expression of VLA-4 either by increasing the clustering of VLA-4 to adhesive contact sites and/or targeting the insertion of VLA-4 into the cell membrane from transport vesicles. MCP-1, however, does not affect VLA-4 cell surface expression on THP-1 cells confirmed by flow cytometry measurements (Figure 4-8). VLA-4 clustering cannot be ruled out from this study however.
Bibliography


103. Alon R, Feigelson SW, Manevich E, et al. alpha(4)beta(1)-dependent adhesion strengthening under mechanical strain is regulated by paxillin


Appendix: Calibration of AFM cantilevers for force measurements

The calibration of AFM cantilevers for force measurements is a two step process that involves the calibration of the position sensitivity of the PSPD, and determination of the cantilever spring constant. Without the spring constant \( k \), the measured cantilever deflection cannot be converted to a force. The spring constant is dependent on the size and shape of the cantilever. Cantilever spring constants reported by manufacturers are determined theoretically from a parallel beam approximation\(^{210}\). Although the cantilevers are manufactured to a certain precision at the microscopic level, small microscopic variations in cantilever thickness will affect the cantilever spring constant. There is an even larger variability between different cantilevers from different batches. Hence, it is necessary to determine the spring constant experimentally for each cantilever. The thermal fluctuation method is the most convenient and non-destructive method to determine the cantilever spring constant experimentally\(^{173}\).

A.1 Calibrating photodiode position sensitivity

As described in more detail in Section 1.2, the raw signal of cantilever deflection measured from a photodiode is the voltage difference between the two segments of the photodiode. It is assumed that as the cantilever comes into contact with a hard/non-compliant surface the deflection of the cantilever is equivalent to the displacement of the piezoelectric element. The calibration
factor converting position sensitivity measured in volts from the photodiode to actual distance is the inverse of the slope of the contact region of an AFM scan (region bound by the open squares in Figure A-1). This calibration factor is commonly referred to as the inverse optical lever sensitivity (InvOLS). Multiplying raw deflection signals in volts by the InvOLS converts measured deflection into actual distance.

![Figure A-1: Determining optical lever sensitivity.](image)

The vertical arrow represents the initial contact point and the open squares represent the contact region over which the InvOLS is determined.

### A.2 Thermal Fluctuation Method

The basic principle behind the thermal fluctuation method is to determine the spring constant from a freely oscillating cantilever which is at equilibrium with its surroundings. The oscillations or fluctuations of the cantilever described above are driven mainly by thermal noise (Figure A-2a). The cantilever spring constant \((k)\) can be determined by measuring the rms displacement of the freely
oscillating cantilever ($q^2$), where $K_B$ is Boltzmann's constant, and $T$ is the temperature in Kelvins:

$$k = \frac{K_B T}{\langle q^2 \rangle} \quad (A.1)$$

Equation A.1 was derived by applying the equipartition theorem to a Hamiltonian approximating the total energy of the oscillating cantilever. From Parseval's Theorem, the r.m.s. displacement of the freely oscillating cantilever can be obtained by integrating the area under the power spectral density of the cantilever oscillations. In practice, the power spectral density of the first resonant peak of the cantilever is fit to a Lorentzian function (Figure A-2b) and the integral of the Lorentzian gives the r.m.s displacement of the freely oscillating cantilever.
Figure A-2: Determination of spring constants thermally induced cantilever fluctuations.

a) Deflection of a freely oscillating cantilever approximately 30 µm away from the surface.  
b) Power spectral density of the cantilever oscillations measured in part a.