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Cell Mechanics of Leukostasis in Acute Leukemia

by

Wilbur Aaron Lam

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

BIOENGINEERING

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

AND

UNIVERSITY OF CALIFORNIA, BERKELEY
Cell Mechanics of Leukostasis in Acute Leukemia

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by

Wilbur Aaron Lam
Dedicated to my father, who has been gone for 17 years now.

Even during my teens, he encouraged me to embark on a career combining medicine and technology.

Despite my best intentions, it seems as if I’ve taken his advice.
Acknowledgements

First and foremost I would like to express my gratitude to the leukemia patients – especially to the few who succumbed to leukostasis – and their parents who participated in these studies. During the most trying time of their lives, when they are suddenly faced with the possibility of losing their child, grandchild, sibling, niece or nephew, they granted us permission to take additional blood for our research with the full knowledge that they themselves would not derive any direct benefit. It is in their honor that I spent the last 5 years trying to further our understanding of leukostasis and it is in their honor that I will continue to plug away at this problem. I also owe a debt of gratitude to Patient M.O., the first leukostasis patient I ever encountered as a clinical fellow. He will forever be, in my mind, a 2-year-old with newly diagnosed leukemia, crying and clinging to his mother for comfort, who suddenly developed seizures and died of leukostasis within a day. His case was the inspiration for this project. At times when experiments failed or equipment was breaking down (typical for any graduate student), his memory always reenergized me and allowed me to refocus. I will always be indebted to him and his family.

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always willing to “hold my hand” and patiently teach me whatever scary mathematical or engineering concept eluded me at the time. He has taught me how to think more like an engineer and not to be daunted by the complexity of biological systems. I could not have asked for a better mentor and I can honestly say that when I grow up (even though he’s only a year older than I am), I want to be just like him.

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Use of published material in this dissertation

The experimental research presented in this work comes primarily from two published papers, and combined with the three originally written chapters, forms a document with comparable content to a standard PhD dissertation. Daniel Fletcher supervised all research and co-wrote all published manuscripts used in this work. The text of Chapter 2 is a reprint of the material as it appears in Biophysical Journal. Michael
Rosenbluth and I developed the atomic force microscopy technique to measure non-adherent leukemia cells and co-wrote the manuscript. We both performed the technique characterization, data collection, and data analysis. The text of Chapter 5 is a reprint of the material as it appears in *Blood*. Michael Rosenbluth and I collected and analyzed all experimental data and co-wrote the manuscript.
Abstract

Cell Mechanics of Leukostasis in Acute Leukemia

by

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Doctor of Philosophy in Bioengineering

University of California, San Francisco and University of California, Berkeley

Professor Daniel A. Fletcher, Chair

Leukostasis, a complication of acute leukemia, occurs when leukemia cells aggregate in and obstruct the microvasculature, leading to profound damage of vital organs like the brain and lungs. The underlying mechanisms are poorly understood but are likely related to the abnormal mechanical properties of leukemia cells. Little improvement has been made in our understanding of leukostasis in the last several decades due in part to a lack of tools to sensitively measure leukemia cell biophysical properties, such as cell stiffness and adhesiveness. Accordingly, no reliable methods currently exist to predict, treat or prevent leukostasis.

Using an atomic force microscopy (AFM) technique I developed to measure leukemia cell deformability, I found that leukemia cells taken from patients with leukostasis were significantly stiffer than leukemia cells taken from asymptomatic patients. To bring single-cell deformability measurements towards more clinical use, I developed a high-throughput microfluidic device to quantify cell deformation through
capillary-like microchannels, which is referred to as biophysical flow cytometry. Leukemia cells from leukostasis patients had higher cell transit times and rates of microchannel occlusion than leukemia cells from asymptomatic patients. These results suggest that increased leukemia cell stiffness may be an independent leukostasis risk factor and could be useful for predicting and diagnosing leukostasis.

Using AFM and biophysical flow cytometry, I found that chemotherapy, the standard treatment for leukemia, increases leukemia cell stiffness by two orders of magnitude and therefore, may actually increase leukostasis risk. Toward therapies that reduce the risk associated with altered biophysical properties, I found that the phosphodiesterase inhibitor pentoxifylline substantially decreased leukemia cell stiffness and adhesiveness, and this drug may therefore be a possible therapy or prophylaxis for leukostasis. Overall, these studies show that cell mechanics – and alterations thereof – are relevant in disease pathophysiology. In addition, tools that measure cell mechanics are useful diagnostic or drug discovery platforms for predicting and treating disease states such as leukostasis in acute leukemia.
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1. Introduction
1.1. Overview

Over the last several decades, the underlying genetic and biochemical mechanisms of numerous diseases have been characterized at the cellular and molecular levels. More recently, researchers have begun to investigate how the mechanical and physical properties of cells and subcellular structures influence normal biological processes as well as disease (Discher, et al 2005). With the development of new tools that enable mechanical measurements at the single-cell and nanoscale levels, this nascent field of cell mechanics has already made remarkable strides in increasing our understanding of human biology and disease. In fact, mechanical cues have recently been shown to influence cell signaling pathways and play significant roles in processes as diverse as cancer metastasis, osteoarthritis, and stem cell differentiation (Engler, et al 2006, Ng, et al 2007, Suresh 2007).

Cell mechanics is particularly important in the study of hematologic disorders, as blood is a suspension of trillions of cells dynamically interacting with each other and the blood vessel wall while constantly subjected to varying degrees of shear stress due to blood flow (Fung 1997a). Leukocyte rolling and transmigration, platelet activation, clot formation, and atherosclerosis are all examples of biological processes that are dependent upon biofluidic dynamics and blood cell mechanical properties, such as cell deformability and adhesion. In addition, in hematologic diseases, alterations in blood cell mechanical properties can profoundly affect the cells’ ability to normally flow in the microvasculature (Lipowsky 2005). Therefore, a comprehensive understanding of blood
pathology requires the ability to analyze biophysical properties of blood and its component elements: blood cells and plasma with its constituent proteins.

Although bulk measurements of blood rheology have been in use for the last half-century (Baskurt and Meiselman 2007), the sensitivity of single-cell measurements have proven to be more clinically useful as pathological processes originate at the single-cell level (Davey and Kell 1996, Nolan 2006). For hematologic diseases, flow cytometry has been an effective tool for single-cell analysis and has been widely used to quantify cell surface protein expression and signaling activity, among others (Irish, et al 2006). Flow cytometry, however, cannot measure cellular mechanical properties. As single mechanically altered blood cells are theoretically sufficient to induce microvascular pathology (Aprelev, et al 2007, Bagge, et al 1980), a clinical need exists in hematology and hematopathology for improved single blood cell mechanical analysis. The archetypal bulk methods as well as more recently developed single-cell techniques for measuring blood cell mechanics are reviewed in this chapter.

This dissertation focuses on the development and application of biophysical tools for single-cell mechanical measurements to further our understanding of leukostasis, a poorly understood complication of acute leukemia in which altered cell mechanical properties are thought to lead to microvascular occlusion. More specifically, the goal of this work has been to explore whether new technologies can improve our methods of predicting, diagnosing, and determining the most effective therapy for this dangerous complication.
1.2. **Techniques for measuring blood cell mechanics**

The biophysical properties of blood have been assessed with bulk methods since the 1960’s (Meiselman and Baskurt 2006) while single-cell mechanical measurements have only been in use the last few years. Both types of techniques have their advantages and disadvantages. In general, single-cells techniques are much more sensitive and obviously have higher resolution, but are low-throughput processes and may not have the statistically power to assess the overall biophysical behavior of the entire cell population. In contrast, bulk measurements by definition assess cellular characteristics at the population level and are thus able to determine average biophysical properties of cells. However, they are not capable of single-cell measurements, and therefore, unable to identify any differentiating characteristics of cellular subpopulations.

1.2.1. **Bulk techniques**

**Microfiltration**

Microfiltration techniques to measure blood cell deformability through capillary-sized tubes has been in use for decades and has been most widely used for biophysical properties of leukocytes (Chien, *et al* 1983, Downey and Worthen 1988, Lowe 1987). Blood cell suspensions are flowed into micropore filters under either constant pressure while the flow rate is measured over time. Alternatively, the opposite experimental setup can be employed as well, in which the flow rate is kept constant while the pressure differential across the filter is measured. Flow rate (or alternatively, pressure increase if the constant flow rate is used) is directly related to cell deformability, cell size, and cell adhesiveness. However, these three variables are convolved, and the inability to independently assess cell deformability, size, and adhesion is a major limitation to this
In addition, microfiltration cannot directly identify biophysically distinct cellular subpopulations within the entire suspension population. More recent advances include the use of optical microscopy to directly visualize cells (Nishino, et al 2005), improved geometric modeling of in vivo microvascular networks, and measurement of cell impedance, which has the sensitivity to account for fast flowing, but not slow flowing, cells (Nash, et al 1988). However, these improvements are not sufficient enough to yield subpopulation measurements.

**Ektacytometry**

Ektacytometry uses a Couette viscometer to expose red blood cells to increasing shear stress as a laser is directed onto the cell suspension. A diffraction pattern through the cell suspension is generated and recorded. As cells stretch due to increasing shear, the diffraction pattern changes from circular to elliptical. From these measurements a deformability index for the cells can be derived (Bessis and Mohandas 1974, Bessis, et al 1980). Disorders of hemoglobin structure and synthesis, such as sickle cell disease and thalassemia, respectively, lead to reduced red cell deformability and can be easily distinguished from normal red cells. In addition, an osmotic gradient within the cell suspension can also be established within the device to measure the cells’ ability to deform under varying osmotic conditions (Clark, et al 1983). This modified technique has been especially useful in diagnosing disorders of the red cell membrane in which the normal red cell surface area to volume ratio is altered. This leads to more “osmotically fragile” cells that lyse under osmotic stress, causing a laser diffraction pattern that is distinct from that of normal, more deformable cells (Johnson 1994). Osmotic gradient
Ektacytometry has been used as a clinical assay for diagnosing diseases such as hereditary spherocytosis, hereditary elliptocytosis, hereditary pyropoikilocytosis, and autoimmune hemolytic anemia (Johnson and Ravindranath 1996).

**Platelet Aggregometry/Platelet Function Analyzer**

Platelet aggregometry uses spectrophotometry to measure platelet aggregation with agonists common in the hemostatic process, including ADP, epinephrine, collagen and ristocetin. Some platelet aggregation tests use platelet-rich plasma, while others use whole blood with sodium citrate as the anticoagulant (Shah and Ma 2007). The basis of measurement is the decrease in optical density that occurs in solution as platelets aggregate. This change is represented graphically as a platelet aggregation curve. Though it has become the mainstay of the clinical coagulation laboratory, platelet aggregometry is expensive, labor intensive, does not use shear to activate platelets and requires highly-specialized technicians to perform and interpret test results (Michelson, et al 2006).

**1.2.2. Single-cell techniques**

**Atomic force microscopy (AFM)**

Invented in 1986, the atomic force microscope was originally designed as an extremely sensitive tool to image and characterize the mechanical properties of materials at the nanometer scale. In the last decade, this technique has been used extensively in cell biology to measure the mechanical properties of live single cells and protein-ligand interactions (Costa 2003, Radmacher 2002, Radmacher 2007). An AFM consists of a cantilever, a sample stage and an optical beam deflection system which consists of a laser diode and a position sensitive photodiode (Figure 1-1). Mechanical measurements
acquired using AFM rely on measuring the cantilever deflection as the cantilever tip is pushed toward, indented into, and retracted from the sample or cell surface in this case. A piezoelectric driver is used to bring the cantilever tip into contact with the sample surface. The force is measured by recording the deflection (vertical bending) of the cantilever after calibration to determine cantilever stiffness. The cantilever deflection is detected by a laser beam focused on the free end of the cantilever and reflected into a photodiode; this deflection is directly proportional to the force.

![Atomic force microscopy of living blood cells.](image)

**Figure 1-1.** Atomic force microscopy of living blood cells. AFM uses a flexible cantilever to both measure and apply forces. As the cantilever interacts with a surface, like a cell in culture medium, its deflection can be measured to sub-nanometer precision by reflecting a laser onto the end of the cantilever and into a quadrant photodetector. The sample’s position relative to the cantilever can be controlled to nanometer precision via a piezoelectric positioning platform. Cell stiffness can be measured by indenting a cell with a cantilever at a constant speed with respect to the sample.

Force-displacement curves are obtained by monitoring the deflection of the cantilever (Figure 1-2). From there, an elastic model can then be fit to the force versus indentation data to determine cell stiffness, or Young’s modulus. The Hertzian mechanics model is often selected because of its extensive use with AFM and it accurately models the deformation of a semi-infinite elastic medium by a spherical indentor (Radmacher 2002, Radmacher, *et al* 1996, Wojcikiewicz, *et al* 2004).
Figure 1-2. Typical data curves obtained during AFM measurements. The stiffness, or elastic modulus, $E$, can then be extracted using a Hertzian mechanics model. The slope of the deflection-distance curve is steeper with stiffer cells. Here, the stiffness of a HL60 promyelocytic leukemia cell is 100 times higher than that of a Jurkat T-cell leukemia cell. The open circle denotes the contact point between the cantilever and cell.

A particular advantage of AFM in cell biology is the capability of studying live cells in an aqueous environment, which allows for the acquisition of real-time biophysical properties of living cells. Serial measurements conducted over time will yield kinetic information as well. In Chapter 5, the biophysical alterations of leukemia cells via pharmacological agents are tracked with AFM.

The majority of AFM research in cell biology involves adherent cells, such as fibroblasts, bone cells, or endothelial cells (Costa 2003). Blood cells, which are non-adherent when inactivated, tend to slip from under the AFM cantilever and must be immobilized for AFM measurements. This can be accomplished with biochemical or mechanical means. In Chapter 2, a new technique using microfabricated wells that laterally immobilize blood cells for AFM stiffness measurements is described. Although
AFM is an extremely sensitive technique for measuring cell mechanics, a major disadvantage is low throughput. In our laboratory and with our current AFM setup, our maximum rate of data acquisition is approximately 20 cells per hour. A solution to this throughput problem is addressed in Chapter 3.

**Micropipette Aspiration**

Micropipette aspiration has extensively been used for measuring cell mechanical properties at the single-cell level. This technique has been used to study adherent cells such as endothelial cells, chondrocytes, and fibroblasts as well as blood cells including neutrophils, red blood cells, and even leukemia cells (Chien, *et al* 1987, Discher, *et al* 1994, Hochmuth 2000, Lichtman 1970, Riveline, *et al* 2001, Rowat, *et al* 2006, Sato, *et al* 1987).


Similar to AFM, micropipette aspiration is extremely sensitive technique, but also suffers from being low-throughput. In addition, successful measurements depend on
matching the micropipette size with the size of the cell of interest and as cell size may not be constant throughout an experiment, especially in the case of blood cells, this poses as a technical challenge for the researcher.

**Microfluidic Systems**

Microfluidic devices – so-called “lab-on-chip” technologies – have had a profound impact on biomedical research in the last 10 years. By applying techniques that the computer and semiconductor industries use to design and produce micro- and nanoscale computer chips, bioengineers have developed microfluidic devices that integrate multiple biochemical assays on a single chip millimeters in size and that are capable of handling extremely small fluid volumes in fluidic channels as small as 1 micrometer in size. As the geometry and layout of the microfluidic channels in these devices are fairly straightforward to design and manipulate and the pressure and fluid flow rate can be easily controlled, microfluidic systems are particularly useful for studying blood cell mechanics in a hydrodynamic environment similar to that of the physiologic microvasculature (Shevkoplyas, *et al* 2006).

Most microfluidic devices used for biomedical applications are comprised of the biologically inert silicon-based polymer polydimethylsiloxane (PDMS). The PDMS structure is cast from a mold with the desired geometry, which in turn is microfabricated onto a silicon wafer using standard fabrication and lithography techniques (Kartalov, *et al* 2006, McDonald, *et al* 2000, McDonald and Whitesides 2002). Cell suspensions can then be flowed into the system, with flow rate or pressure as key control parameters, and
because PDMS is transparent, individual cells traversing the microfluidic network can be detected with optical microscopy (Sia and Whitesides 2003).

With microfluidic devices, blood cell mechanical properties can be evaluated in physiologically-relevant flow conditions similar to that of the in vivo microcirculation (Lam, et al 2007, Shevkoplyas, et al 2003, Shevkoplyas, et al 2006). Similar to what occurs in the microcirculation in vivo, as blood cells compress into the smallest microfluidic channels of the device, the deformation of single cells can be recorded with video microscopy. As the cell suspension can flow through multiple parallel channels, all of which can be tracked and recorded simultaneously, microfluidic devices can serve as high-throughput, devices for measuring blood cell mechanical properties at the single-cell level. Cellular transit time, cell stretching, cell velocity, microchannel clogging and pressure have all been experimental endpoints (Abkarian, et al 2006, Guck, et al 2005, Lam, et al 2007, Nishino, et al 2005, Shevkoplyas, et al 2003, Shevkoplyas, et al 2006). We demonstrate the use of metrics in microfluidic systems in Chapters 3 and 5. A disadvantage of these techniques, however, is the lack of a standardized physical measurement that can be compared between different systems.

Adhesion Assays (Static and Shear Flow)

In addition to cell deformability, adhesion is another cellular mechanical property that is vital for the biological processes of blood cells. The adhesive interactions between circulating blood cells and vascular endothelial cell lining the blood vessel wall are necessary for normal processes such as inflammation (leukocyte-endothelial cell adhesion) and hemostasis (platelet-endothelial cell or platelet-extra-cellular matrix
adhesion). However, inappropriate blood cell adhesion to endothelium can be detrimental as it may impair blood flow, reduce oxygen delivery or lead to pathologic thromboses (Hebbel and Vercellotti 1997). To investigate adhesive interactions between blood and endothelial cells, several in vitro experimental models have been used over the years.

In static adhesion assays (or gravity sedimentation method), washed blood cells are allowed to settle on and adhere to endothelial monolayers in culture medium. Then, the two populations are co-incubated for minutes to hours. After the co-incubation, unattached blood cells are removed with repeated washes and the remaining blood cells are counted (usually with optical or fluorescence microscopy if the blood cells are fluorescently tagged) for measuring cellular adherence to endothelial cells (Shiu and McIntire 2003). Although this assay is simple and high-throughput, as hundreds of cells can be counted simultaneously, it does not take into account the dynamic variables present in the vascular flow environment. Therefore, this method may not accurately represent the microcirculatory flow conditions in vivo.

The use of parallel-plate flow chambers allows for investigation of blood cell-endothelial interaction in the presence of flow and shear stress. In general, the chamber consists of a polycarbonate base, a gasket, and a glass slide or cover slip where endothelial cells are cultured. The cover slip forms the floor of the chamber and the polycarbonate base forms the top and has entrance and exit for perfusion medium; the geometry is designed to ensure that laminar flow is present throughout the entire chamber, thus mimicking the flow environment of the microvasculature (Montes, et al 2002). Cell suspensions are perfused through the chamber over endothelial cells and by increasing flow rate, shear stress is increased as well (Barabino, et al 1987a). In general,
a flow experiment with this type of device consists of a short perfusion period to preshear the endothelial cells with cell-free medium. Then a cell suspension is perfused through as blood cells are allowed to interact with the endothelial cells at a controlled level of shear stress and adherent cells are visualized and quantified. Throughout the entire flow study, the endothelial cell is exposed to well-defined flow conditions (Shiu and McIntire 2003).

Variations of the aforementioned AFM and micropipette techniques have also been used to measure cell-cell adhesion. With AFM, using biochemical methods such as concanavalin A-mediated linkages, the cantilever tip/cell complex is lowered onto an endothelial cell monolayer. Once contact is established and confirmed by visualization, the cantilever/cell complex is steadily retracted with continuous measurement of the adhesive force until the bond is broken and the cells separate (Zhang, et al 2002). Using micropipettes, blood cells can be partially aspirated and can be brought into controlled contact with an endothelial monolayer. Then the cell is released and adheres to the endothelial cell. The adhesive strength of this interaction is then measured by re-aspiring the cell into the micropipette at increasing pressures until it is pulled away from the endothelial cell (Mohandas and Evans 1985). In addition to measuring single-cell adhesive interactions, the ability to control cell-cell contact time and contact force as experimental variables is another powerful capability of AFM and micropipette aspiration as tools to measure cell adhesion. However, as mentioned above for measurement of cell deformability, both are low throughput techniques compared to other assays of cell adhesion.
1.3. **Cell mechanics in hematologic diseases**

Cell mechanics are extremely important in diseases of the blood, as flowing blood cells are constantly interacting with each other and the endothelium of the vascular wall. Blood cell mechanical properties are especially relevant in hematologic diseases affecting the microcirculation. Here, blood cells are in intimate contact with the vessel wall of the microvasculature and must deform to pass through the vascular network (Lipowsky 2005, Somer and Meiselman 1993). Any alterations in cell deformability, adhesiveness, or transmigration may lead to microvascular occlusion, tissue ischemia, hemorrhage, and ultimately failure of vital organs like the brain and lung. Although the scope of this dissertation is to investigate the mechanisms underlying microvascular occlusion in leukostasis in acute leukemia, altered blood cell mechanical properties contribute to the pathophysiology of several other important hematologic diseases.

**Sickle cell disease**

As the causative genetic mutation leading to sickle hemoglobin was discovered over 50 years ago, the cell mechanical alterations of sickle cell disease are well characterized, although understanding of its pathophysiology is still far from complete (Serjeant 2001). Recent research revealed that sickle cell microvascular occlusion is a complicated process involving not only the sickle red cells, but also leukocytes, platelets, endothelial cells and coagulation and inflammatory mediators as well (Frenette and Atweh 2007).

The increased rigidity of sickle red blood cells due to polymerized sickle hemoglobin was originally thought to be the sole cause of vaso-occlusion by physical
obstruction of small blood vessels (Frenette 2002). Repeated cycles of cell sickling (due to the deoxygenated venous environment) and unsickling (after re-oxygenization in the arterial environment) of reversibly sickled cells (RSCs) in addition to dehydration via pathologic cation channels ultimately lead to the dense, stiff and permanently deformed irreversibly sickled cells (ISCs), which were thought to be the obvious instigators of vaso-occlusion (Bunn 1997). However, as red cells continuously circulate in the vasculature, this model failed to completely explain the episodic nature of this disease (Frenette 2002).

It is now accepted that adhesion between sickle red cells and the vascular endothelium and extracellular matrix (ECM) also contribute to vaso-occlusion (Bunn 1997, Hebbel, et al 2004, Hebbel, et al 1980). Surface molecules on the red cell including \( \alpha_4\beta_1 \) and \( \alpha_v\beta_3 \) integrins, CD36, CD47, sulfated glycolipids, and basal cell adhesion molecule/Lutheran blood group (BCAM/Lu), vascular cell adhesion molecule-1 (VCAM-1), and intercellular adhesion molecule-4 (ICAM-4) and P-selectin on the endothelial cell, as well as soluble ligands like thrombospondin (TSP) and von Willebrand factor (vWF) have all been implicated in mediating these adhesive interactions (Hebbel, et al 2004). Phosphatidyl serine, abnormally exposed on the outer leaflet of the cell membrane due to repeated sickling/unsickling, likely increases the adhesiveness of the sickle RBCs as they age (Frenette and Atweh 2007). Some studies revealed that immature sickle reticulocytes were more adherent to endothelium than mature sickle cells like ISCs, while other studies showed that ISCs may retain or regain their adhesiveness, (Barabino, et al 1987b, Kaul, et al 1989, Mohandas and Evans 1985, Stone, et al 1996) implying that both reticulocytes and ISCs may initiate vaso-occlusion. Recent attention has been paid to
BCAM/Lu, which increases sickle cell adhesion to the ECM protein laminin as cells become denser even as BCAM/Lu expression decreases on older cells.

Recent studies have also revealed an important role for leukocytes in sickle cell vaso-occlusion (Okpala 2004). As leukocytes are larger, less deformable, and more adherent to the endothelium than red cells, they are more likely to adhere and impair blood flow (Chiang and Frenette 2005). This may promote RBC sickling and vaso-occlusion by increasing sickle RBC transit-time in the deoxygenated post-capillary environment. In addition to adhering to the endothelium, leukocytes may bind to each other, to platelets, and to red cells to form cell aggregates, which could obstruct the lumen of small blood vessels more effectively than single cells (Okpala 2004). Sickle cell adhesion to the endothelium may also activate endothelial cells by upregulating endothelial adhesion molecules, including E-selectin, P-selectin, VCAM-1, and ICAM-1, potentially allowing further interactions with sickle RBCs as well as recruitment of circulating leukocytes (Chiang and Frenette 2005).

In addition, the inflammatory response that results in local tissue injury and secretion of the cytokines tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) may also contribute to vaso-occlusion by activating endothelium leading to further adhesion to sickle red cells and leukocytes (Okpala 2004). TNF-α and IL-1β also activate leukocytes themselves by upregulation of adhesion molecules, increasing the adhesion affinity to both endothelium and sickle red cells.
Malaria

Malaria is caused by protozoan parasites of the genus *Plasmodium*. In humans, malaria is caused by four species: *P. falciparum*, *P. malariae*, *P. ovale*, and *P. vivax*. *P. falciparum* is the most common cause of infection and is responsible for most malaria cases and deaths (Wongsrichanalai, *et al* 2007). Blood cell mechanical alterations occur as the parasite invades the host’s red cells, leading to symptoms of disease (Opoka, *et al* 2008).

As the parasites divide within the red cells, proteins are exported from the parasite to the host cell membrane, leading to structural changes as they bind to the spectrin cytoskeleton of the red cell. Upon infection, red cells become stiffer, more spherical (compared to the normal biconcave shape), and more adhesive (Suresh, *et al* 2005). Increased adhesion is due to expression of the parasite protein PfEMP1 (*Plasmodium falciparum* erythrocyte membrane protein 1) on the red cell surface. This adhesion protein causes infected red cells to stick to the microvascular wall, which may lead to decreases in microcirculatory flow. In addition, expression of PfEMP1 allows the sequestering of the parasite from passage through the spleen, where infected cells and parasites would ordinarily be destroyed (Opoka, *et al* 2008). These increases in cell stiffness and adhesion ultimately lead to microvascular obstruction and tissue ischemia, ultimately leading to the symptoms of severe malaria, which include coma, renal and respiratory failure.
Diabetes

Diabetes mellitus is due to either insufficient production of the hormone insulin (Type I) or insulin resistance, in which the body fails to properly respond to insulin (Type II). Both types lead to increased blood glucose levels, which in turn causes long term health complications such as cardiovascular disease, chronic renal failure, retinopathy, neuropathy, poor wound healing and microvascular damage (Hatzitolios, et al 2008). For reasons that remain unclear, diabetes also affects blood cell mechanical properties. Red cells and leukocytes are less deformable than those taken from healthy subjects, and for leukocytes, increased f-actin production is thought to be involved in this stiffness increase (Miyamoto, et al 1997, Pecsvarady, et al 1994). In addition, increased red cell and platelet aggregation has been observed as well (Miyamoto, et al 1997). Leukocytes are also more activated and adhesive in diabetics than normal controls (Chibber, et al 2007). Leukostasis, similar to that which occurs in acute leukemia, has been observed as well, most commonly in the retinal microcirculation (Abiko, et al 2003). Although the underlying mechanisms remain unclear, endothelial and leukocyte dysfunction both occur in diabetes and are likely to be involved in diabetic retinopathy and blindness (Chibber, et al 2007).

Platelet-type Von Willebrand Disease

Platelet-type Von Willebrand Disease is caused by gain of function mutations of the alpha chain of glycoprotein Ib, which is the platelet receptor for von Willebrand Factor (vWF) receptor on platelets. Glycoprotein Ib is part of the larger complex (GPIb/V/IX) which forms the full vWF receptor on platelets. These mutations lead to
spontaneous binding to platelets and a characteristic increase in platelet aggregation. This is turn, in leads to increased clearance of platelets as well vWF from the circulation and a subsequent risk for bleeding (Franchini, et al 2008). Platelet aggregometry assays show a consistent increase in aggregation and platelet adhesion to vWf matrices are enhanced in terms of cell tethering, slower rolling velocity, and decreased detachment with increasing shear rate (Tait, et al 2001).

**Acute Inflammation/Sepsis**

An acute inflammatory response is a complex set of biological processes that occur when the immune system reacts to the detection of pathogens, injured cells, or exogenous irritants. During acute inflammation, a cascade of biochemical events propagates, which primarily involves the local microvascular system, leukocytes, and the affected tissue or organ. Inflammatory cytokines such as TNF-α and IL-1β are secreted, activating the vascular endothelium and nearby leukocytes. Leukocytes are then recruited to the area of injury, and transmigration through the endothelium and into the extravascular space occurs (Libby 2007). There, the activated leukocytes sequester and remove pathogens or diseased cells via phagocytosis and release of additional biochemical inflammatory mediators. In addition, as leukocytes are activated, actin polymerization and cell stiffening occur, leading to leukocyte retention and clogging in the microcirculation (Worthen, et al 1989).

In sepsis, the usual inflammatory processes are exaggerated in response to an overwhelming infection (Alves-Filho, et al 2006, Greenhalgh, et al 2007). The immune system becomes diffusely and uncontrollably activated, leading to hemodynamic
consequences and damage to vital organs. Part of this process involves increased leukocyte activation, stiffening, and obstruction of the microcirculation. This specific complication usually occurs in the lungs and can lead to respiratory failure (Downey, *et al* 1993). In addition, leukocyte and endothelial cell adhesion molecule expression is diffusely upregulated in sepsis, which further exacerbates leukocyte retention in the microcirculation and compromises vascular flow (Linderkamp, *et al* 2006). Clinical studies have confirmed the correlation between increased cell stiffness and adhesion with prognosis in patients with sepsis (Drost, *et al* 1999, Skoutelis, *et al* 2000b).

1.4. **Acute leukemia**

The leukemias are a group of disorders characterized by the accumulation of malignant leukocytes in the bone marrow and the blood. These abnormal cells cause symptoms because of bone marrow failure (i.e. anemia, low leukocytes, or low platelets) and infiltration of organs (e.g. liver, spleen, lymph nodes, meninges, brain, skin, or testes).

1.4.1. **Classification of Acute Leukemia**

The main classification of leukemia is into four types – acute and chronic leukemias which are further subdivided into lymphoid and myeloid. “Acute” leukemias are usually aggressive diseases in which the malignant transformation causes accumulation of early bone marrow hematopoietic progenitors, called blast cells, in the bone marrow and subsequently the blood vessels (Figure 1-1). The dominant clinical feature of these diseases is usually bone marrow failure caused by accumulation of blast cells although tissue infiltration also occurs. If untreated these diseases are usually fairly rapidly fatal but, paradoxically, they are also easier to cure than chronic leukemias (Moricke, *et al* 2008). Acute leukemia is defined as the presence of over 30% of blast cells in the bone marrow at clinical presentation. It is further subdivided into acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL) on the basis of whether the blasts are shown to be myeloblasts or lymphoblasts.
Figure 1-1. Hematopoiesis and acute leukemia. (A) Normal hematopoiesis is a complex, well-orchestrated process in which hematopoietic stem cell differentiation is balanced with the proliferative production of mature lymphocytes, red cells, granulocytes (neutrophils, eosinophils, basophils). (B) In acute lymphoblastic leukemia, multiple genetic mutations (symbolized by lightning bolts) beginning at the stage of the hematopoietic stem cell cause an uncoupling of differentiation and proliferation and the subsequent development of an abnormal clonal population. The capacity to terminally differentiate is lost, resulting in the progressive accumulation of immature, non-functional lymphoid precursor cells with a relatively prolonged life span. (C) Similarly, in acute myeloid leukemia, a clonal population of precursor myeloid cells without the capacity to terminally differentiate develops due to multiple genetic mutations and uncontrollably proliferates.

1.4.2. Acute Lymphoblastic Leukemia

ALL is caused by an accumulation of lymphoblasts and is the most common malignancy of childhood (Pui, et al 2008). ALL can be subclassified on the basis of morphology of immunological markers. Immunologically, ALL can be divided into precursor B-cell ALL, T-cell ALL, or mature B-cell ALL.
Incidence

ALL is the most common form of leukemia in children; its incidence is highest at 3-7 years (Downing and Shannon 2002). The common (CD10+) precursor B-cell type which is the most usual in children has an equal sex incidence; there is a male predominance for T-cell ALL. There is a lower frequency of ALL after 10 years of age with a secondary rise after the age of 40.

Therapy

Combinations of at least three chemotherapeutic agents are now usually used to increase the cytotoxic effect, improve remission rates and reduce the frequency of emergence of drug resistance (Pui, et al 2008). These multiple drug combinations have also been found to give longer remissions in acute leukemias than single agents (Pui, et al 2004).

The aim of cytotoxic therapy is first to induce a remission (absence of any clinical or conventional laboratory evidence of the disease) and then to eliminate the hidden leukemia cell population by courses of consolidation therapy (Hutchinson, et al 2003). Cyclical combinations of two, three, or four drugs are given with treatment-free intervals to allow the bone marrow to recover. This recovery depends upon the differential regrowth pattern of normal hematopoietic and leukemic cells. For ALL, long-term (2-3 years) maintenance therapy has been found to reduce the risk of relapse (Li, et al 2006). The specific chemotherapeutic protocols differ in infants, children, and adults, who are thought to have different prognoses.
The aim of remission induction is to kill rapidly most of the tumor cells and get the patient into a state of remission (defined as less than 5% blasts in the bone marrow, normal peripheral blood counts, and no other symptoms or signs of the disease). Dexamethasone, vincristine, and asparaginase are the drugs usually used and effectively achieve remission in over 90% of children and 80-90% of adults (in whom daunorubicin is also usually added) (Hutchinson, et al 2003). However, in remission a patient may still be harboring large numbers of tumor cell and without further chemotherapy virtually all patients will relapse. Nevertheless, achievement of remission is a valuable first step in the treatment course and patients who fail to achieve it have a poor prognosis.

Consolidation and/or intensification courses use high doses of multidrug chemotherapy in order to reduce the tumor burden to very low levels (Estlin, et al 2001). The doses of chemotherapy are near the limit of patient tolerability and during intensification blocks patients may need a great deal of supportive care. Typical protocols involve the use of vincristine, cyclophosphamide, cytosine arabinoside, daunorubicin, etoposide, thioguanine, or mercaptopurine given as blocks in different combinations. The optimal number of intensification blocks is under trial but two or three is typical in children, with more in adults.

**Prognosis**

There is a great variation in the chance of individual patients achieving a long-term cure based on a number of biological variables. Age is important – around 70-90% of children can expect to be cured whereas in adults this drops significantly to less than 5% over the age of 65 years (Downing and Shannon 2002). Cytogenetics are important,
particular the presence of the Philadelphia chromosome, the incidence of which rises with age. Hyperploidy (multiple sets of chromosomes) and rearrangements of the \textit{TEL} gene are associated with good outcome. When treatment fails, death usually occurs because of resistant disease or from infections due to leukemia-induced bone marrow failure.

\textbf{1.4.3. Acute Myeloid Leukemia}

AML is classified according to morphology and divided into eight variants, which indicate the general lineage and degree of differentiation (non-differentiated or red cell, monoblastic, granulocytic, or platelet precursors)(Ventura, \textit{et al} 1988). Although the distinct AML subtypes are in fact different genetic diseases their grouping together is valid as generally their treatment and prognosis is similar. However, differences in treatment according to subtype have been introduced.

\textbf{Incidence}

AML occurs in all age groups and is the most common form of acute leukemia in adults with increasing incidence with age. AML forms only a minor fraction (10-15\%) of leukemias in childhood. An important distinction is between primary AML which appears to arise \textit{de novo} and secondary AML which can develop from myelodysplasia and other hematological diseases or follow previous treatment with chemotherapy. Both types are associated with distinct genetic markers and have different prognoses. Whereas t(15;17), t(8;21), and inv(16) correlate with a favorable prognosis, deletions of chromosome 5 or 7, Flt-3 or 11q23 mutations, and t(6;9) connate a poor prognosis (Porcu, \textit{et al} 1997).
Treatment

Specific therapy for AML primarily involves intensive chemotherapy. This is usually given in four or five blocks each approximately 1 week, and the most commonly used drugs include cytosine arabinoside, daunorubicin, idarubicin, 6-thioguanine, mitoxantrone, or etoposide (Ravindranath, et al 2005). These drugs are myelotoxic with limited selectivity between leukemic and normal marrow cells and therefore, marrow failure is severe, necessitating prolonged and intensive supportive therapy (Kimby, et al 2001). All the AML subtypes are treated similarly except for the promyelocytic variant associated with the t(15;17) translocation in which all-trans retinoic acid and arsenic is added to the initial chemotherapy (Pui, et al 2004).

A recent concept in AML consists of basing the treatment schedule of individual patients on their risk group. Remission after one course of chemotherapy is also favorable. In contrast, monosomy 5 or 7 abnormalities, blast cells with Flt-3 mutations or poorly responsive disease places patients into poor risk groups which may need more intensive treatments (Downing and Shannon 2002). Monoclonal antibodies targeted against CD33 or CD45 are being developed as a possible addition to AML therapy.

Prognosis

AML prognosis has improved steadily over the years, particularly for younger patients (Downing and Shannon 2002). Perhaps 50% of children and young adults may expect a long-term ‘cure’. Cytogenetic abnormalities and initial response to treatment are
major predictors of prognosis. For the elderly the situation is poor and only 5% of those over 65 years of age can expect long-term remission.

### 1.5. Leukostasis

#### 1.5.1. Pathophysiology

Approximately 10% to 13% of ALL cases and 15% to 20% of AML cases present with hyperleukocytosis, which is defined as a peripheral leukocyte concentration exceeding 100,000 cells per microliter (Rheinhold and Lange 2002). Hyperleukocytosis may in turn lead to leukostasis, in which leukemic cells accumulate and aggregate in the vasculature (Figure 1-2). Although this often fatal phenomenon can occur in almost any organ, it most commonly occurs in the brain and lungs, leading to intracranial hemorrhage and respiratory failure, and has an estimated mortality rate of approximately 20-40% (Porcu, et al 2000). Autopsy results in patients who died from leukostasis reveal infiltrates of leukemia cells and hemorrhage in the vasculature, interstitium, and parenchyma of the lungs and brain.

![Figure 1-2. Hyperleukocytosis in acute leukemia.](image) Left panel: a blood smear from a healthy adult with a leukocyte count of 7000 cells/µL. Right panel: a blood smear from a 2 year-old child with newly diagnosed ALL and hyperleukocytosis with a leukocyte count of 1,132,000 cells/µL (>90% of leukocytes were leukemic blast cells). This patient subsequently died of leukostasis after initiation of chemotherapy.
Several potential mechanisms leading to leukostasis have been proposed over the years. Originally, symptoms of leukostasis were thought to be simply due to the high number of leukemic blast cells in the circulation leading to increased blood viscosity. However, this does not explain the entire pathophysiological process, as myeloid leukemias like AML were noted to cause symptoms of leukostasis at lower white blood cell, or leukocyte, counts than lymphoid leukemias (ALL) (Porcu, et al 2002).

Lichtman developed an alternate model of leukostasis in the 1970s contending that the fractional volume of white blood cells, called leukocrit, needed to increase the blood viscosity to the point of leukostasis was approximately (12% to 15%) and this volume is a function of the mean cell volume (MCV) (Lichtman 1973a). Therefore, because the leukemic cells of AML, or myeloblasts, have larger MCVs than the leukemic cells of ALL, or lymphoblasts, fewer myeloblasts than lymphoblasts are necessary to cause symptoms of leukostasis. However, leukemic blast counts are rarely sufficiently high to cause leukocrits of 12% to 15% and in addition, as the leukocrit rises, the number of red blood cells usually decreases, yielding a protective effect against hyperviscosity (Steinberg and Charm 1971). These observations suggested that leukostasis is not simply due to the size, concentration and mechanical properties of the blast cell. Indeed, rare cases of leukostasis have been reported in leukemic patients without markedly elevated white blood cell counts (Lichtman, et al 1987, Soares, et al 1992). In addition, the fact that most cases of leukostasis occur in the brain and lungs suggests that there is some organ specificity and that local factors may play a role as well.

Recent research has show that leukostasis may result from the adhesive interactions between leukemic cells and the endothelium of the vasculature. Myeloblasts,
which are thought to be “stickier” in nature than lymphoblasts, may have a higher propensity to aggregate, possibly accounting for some of the disparities seen between AML and ALL leukostasis cases (Bunin and Pui 1985). Interactions between normal leukocytes and endothelial cells via expression of adhesion molecules have been well characterized and expression of adhesion molecules of leukemic cells has also been investigated (Mielcarek, et al 1997). Stucki, et al, have shown that via secretion of certain cytokines by myeloblasts, expression of several adhesion molecules on the surface of endothelial cells is upregulated resulting in an increased number of myeloblasts attached to the endothelium (Stucki, et al 2001). Once adhered, leukemic cells may then cause endothelial damage by migrating into perivascular space (Mustjoki, et al 2001) (Figure 1-3). Recent studies have shown that at least for AML, upregulation of the adhesion molecule NCAM on the surface of leukemia cells correlated with leukostasis and therefore may have a role in pathophysiology (Novotny, et al 2005, Novotny, et al 2006).

Figure 1-3, Possible mechanisms of leukostasis in acute leukemia. Leukostasis is mostly likely caused by a combination of cell mechanical alterations including decreased cell deformability of leukemia cells compared to their more mature counterparts, aberrant and dysregulated leukemia cell-leukemia cell and leukemia-endothelial cell adhesion, and leukemia cell extravasation into leading to vascular damage and hemorrhage.
1.5.2. Open questions

Numerous questions remain regarding the pathophysiology of leukostasis. Although several studies have investigated whether the stiffness of leukemic cells plays a role in leukostasis, these studies either did not assess whole-cell deformability (Lichtman 1970) or used an indirect measurement such as cellular transit time through capillary pores via microfiltration (Hallows and Frank 1992, Sharma 1993). As recent research has shown, leukemic blast cells and endothelial cells may indeed interact dynamically leading to adhesion and transmigration (De Rossi, et al 1994, Mustjoki, et al 2001, Stucki, et al 2001). However, these studies utilized qualitative assays and the relative contributions each interaction may have to the overall pathophysiology remains unknown. In addition, few definitive studies have been reported that compare the biomechanical and adhesive properties of myeloid blast cells versus lymphoid blast cells, and no studies have been reported that assess the propensity for leukemic cells to adhere each other, which may be a vital component of leukostasis. Most importantly, no published study to date has investigated whether biophysical differences exist between leukemic cells taken from patients with leukostasis versus leukemic cells taken from asymptomatic patients. These data are necessary to determine if cell mechanical measurements can be used for the diagnosis and/or prevention of leukostasis in acute leukemia.

Currently, the mainstay of treatment for leukostasis is prompt leukocytoreduction, or removal of leukemic cells from the circulation, via exchange transfusion or leukapheresis, in addition to aggressive intravenous hydration, which prevents renal failure from leukemic breakdown products. Although extremely effective and rapid in
reducing the number of leukemic cells, these techniques are only temporizing and delay the initiation of chemotherapy, the definitive therapy for leukemia. In addition, other disadvantages include: invasive placement of deep intravenous lines (which may be difficult in small children), the need for anticoagulation, and limited availability in some hospitals. The most appropriate and optimal use of these techniques remains unclear, and the few trials reported show no significant decrease in mortality when leukocytoreduction is compared to hydration alone (Porcu, et al 2002). Leukapheresis and exchange transfusion do not address the issue of leukemic cell adhesion leading to endothelial damage, which may be the reason why these techniques have not shown to improve survival in leukostasis. Therefore, a more complete and clarified view of the pathophysiology of leukostasis is needed in order to guide the most effective therapy when presented with this life-threatening emergency.

The goal of this work has been the application and adaptation of new technologies in biophysics and bioengineering to further our understanding of leukostasis pathophysiology with the ultimate aim of developing new assays for the prediction, diagnosis, and therapeutic efficacy of leukostasis in acute leukemia. In Chapters 2 and 3 of this dissertation, the atomic force microscopy and microfluidic methodologies we used to quantify leukemia cell deformability are discussed. Chapter 4 discusses the alterations of leukemia cell mechanics in leukostasis and compares the differences in leukemia cell deformability between leukostasis leukemia patients and asymptomatic leukemia patients. Chapter 5 discusses the biophysical changes leukemia cells undergo when exposed to chemotherapy. Finally, Chapter 6 concludes this dissertation by discussing how the tools we have developed and data we have collected may be translated to
predicting, diagnosing, and treating leukostasis. In addition, this chapter also presents the future outlook of this line of research by describing how, using the tools and techniques we have developed in this work, the drug pentoxifylline may be a potential treatment or prophylaxis for leukostasis by modulating leukemia cell stiffness and adhesiveness.


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2.1. **Abstract**

Atomic force microscopy (AFM) has become an important tool for quantifying mechanical properties of biological materials ranging from single molecules to cells and tissues. Current AFM techniques for measuring elastic and viscoelastic properties of whole cells are based on indentation of cells firmly adhered to a substrate, but these techniques are not appropriate for probing non-adherent cells, such as passive human leukocytes, due to a lateral instability of the cells under load. Here we present a method for characterizing non-adherent cells with AFM by mechanically immobilizing them in microfabricated wells. We apply this technique to compare the deformability of human myeloid and lymphoid leukemia cells and neutrophils at low deformation rates, and we find that the cells are well described by an elastic model based on Hertzian mechanics. Myeloid (HL60) cells were measured to be a factor of 18 stiffer than lymphoid (Jurkat) cells and 6 times stiffer than human neutrophils on average ($E_\infty = 855 \pm 670$ Pa for HL60 cells, $E_\infty = 48 \pm 35$ Pa for Jurkat cells, $E_\infty = 156 \pm 87$ for neutrophils, mean $\pm$ SD). This work demonstrates a simple method for extending AFM mechanical property measurements to non-adherent cells and characterizes properties of human leukemia cells that may contribute to leukostasis, a complication associated with acute leukemia.

2.2. **Introduction**

In some diseases, the mechanical properties of individual cells are altered. For example, osteoarthritic chondrocytes (cartilage-producing cells) have been shown to be less stiff than normal chondrocytes, and malignant hepatocytes (liver cells) have been
shown to be more stiff than normal hepatocytes (Alexopoulos, et al 2003, Costa 2003, Wu, et al 2000). In diseases of the blood, changes in cell mechanical properties can have profound effects on the cells’ ability to flow normally through the vasculature, since increased stiffness impedes progress of cells through small capillaries (Somer and Meiselman 1993). The reduced deformability of erythrocytes infected with *Plasmodium falciparum*, the parasite that causes malaria, has been shown to hinder the cells’ ability to flow through microfabricated channels (Shelby, et al 2003). Recent research on diabetes mellitus suggests that some complications of the condition can be attributed to increased lymphocyte stiffness (Perrault, et al 2004). In acute leukemia, immature blood cells of the myeloid or lymphoid lineages, called myeloblasts and lymphoblasts respectively, proliferate uncontrollably. Decreased deformability of these cells, as well as increased adhesion and transmigration, is thought to be linked to leukostasis, a poorly understood condition in which cells aggregate in the vasculature (Lichtman 1973a). This condition often results in intracranial hemorrhage and respiratory failure that rapidly leads to death, and current therapies based on removal of leukemia cells from the circulation have not proven to decrease mortality (Porcu, et al 2002). Better knowledge of biophysical changes in leukemia cells such as deformability is necessary for improved understanding of the disease, but no widely accepted method or model exists for quantifying the mechanical properties of leukemia cells relevant to leukostasis.

Atomic force microscopy (AFM), first developed as a surface imaging tool (Binnig, et al 1986), can also be used to measure the stiffness of cells firmly adhered to a substrate (Radmacher, et al 1996). The primary method of measuring stiffness is indenting the cell with a flexible cantilever driven at a constant extension rate (piezo
extension rate) with respect to the sample. The deflection of the cantilever as it indents
the cell, which is linearly related to loading force for small deflections, is recorded by
reflecting a laser off the cantilever into a split photodiode. A linear elastic model of the
cell based on Hertzian mechanics (Hertz 1882) is commonly used with AFM deflection
data to determine cell elasticity (Radmacher 2002). In addition to constant piezo
extension rate measurements, elastic and viscoelastic properties of cells can be obtained
from creep experiments (Wu, et al 1998) and oscillating indentations (Alcaraz, et al

Non-adhesive cells, such as normal and malignant leukocytes prior to activation
of the inflammatory response, pose a challenge for AFM because they tend to slip from
under the cantilever tip under an applied load. While non-adhesive cells can occasionally
be probed using AFM without slippage (Wu, et al 1998), the experimental throughput is
low and may favor those cells which have some level of adherence to the surface. One
solution is to coat the substrate surface with polypeptides, making it sticky to the cell.
Attaching a non-adherent cell to a surface coated with fibronectin or poly-lysine will
prohibit it from moving while probed but can change its morphology and mechanical
properties, similar to the changes in cell properties seen upon neutrophil activation (Frank
1990). Non-adherent cells trapped within Millipore filters have been used for AFM
imaging (Kasas and Ikai 1995), but the pressure used to trap the cells is likely to
significantly deform the cells and may change their elastic response.

Micropipette aspiration has been used extensively to determine mechanical
properties of fully differentiated leukocytes such as neutrophils (Dong, et al 1988, Evans
1991, Tsai, et al 1993). During micropipette aspiration, a section of membrane and cytoplasm of the cell are drawn into the pipette by a pressure differential. This technique can be used to describe an apparent membrane tension and cytoplasmic viscosity using the liquid droplet model, in which the cell is assumed to be a viscous fluid-filled bag with a constant surface tension. Since aspiration of a cell draws in cytoplasm more readily than nucleus, application of this technique to leukemia cells may overestimate their deformability due to characteristically high nucleus to cytoplasm ratio (Loffler and Gassmann 1994). Whole cell aspiration is potentially more appropriate for leukemia cell deformation and has been used to describe HL60 mechanical behavior (Tsai, et al 1996a, Tsai, et al 1996b). However, previous analysis makes use of a power-law fluid model, which is not able to describe a static deformation such as when a cell plugs a capillary, as is believed to occur in leukostasis. A simple method for characterizing and comparing the deformability of leukemia cells at low deformation rates is needed.

We used microfabricated wells (Figure 2-1) to mechanically immobilize and study the deformability of HL60 and Jurkat cells, prominent acute myeloid leukemia (AML) and acute lymphoid leukemia (ALL) cell lines, and human neutrophils. AML has been associated with leukostasis at a significantly lower cell concentration than ALL, but the reason for this clinical observation is not known. Comparative measurements of deformability will help in understanding the pathophysiology of the disease. We analyzed our experimental data with two models – a Hertzian mechanics and a liquid droplet model, and determined that the Hertz model is a more appropriate description of these cell lines at low deformation rates. HL60s were found to be significantly stiffer
than Jurkat cells and neutrophils, consistent with a model of leukostasis in which stiffness contributes to vessel blockage.

Figure 2-1. Microfabricated wells for force microscopy of non-adherent cells. (A) Schematic diagram of the microwells showing SU-8 photoresist structures on a glass wafer in which non-adherent cells sit. Cells resting inside the microwells are mechanically immobilized for force microscopy with an AFM cantilever. (B) Scanning Electron Micrograph (SEM) of microwells fabricated in 8 x 8 arrays. Scalebar is 50 µm. (C) SEM of a single microwell showing the vertical sidewalls of the SU-8. Scalebar is 2 µm.

2.3. Materials and Methods

2.3.1. Cell Culture

In this study, the HL60 and Jurkat cell lines (American Type Culture Collection, Manassas, VA, ATCC numbers TIB-152 and CCL-240, respectively) were used as models for AML and ALL, respectively. Cells were cultured in RPMI 1640 containing 10% fetal bovine serum (Gibco, Carlsbad, CA), and maintained in a 5% CO₂ humidified atmosphere at 37°C. Before force microscopy measurements were made, cells were incubated in RPMI 1640 without fetal bovine serum for 24 hours at 37°C and 5% CO₂ in

To isolate neutrophils, whole blood was drawn from healthy donors and collected in heparin. Histopaque 1077 (Sigma-Aldrich, St. Louis, MO) was layered atop Histopaque 1119 to create a dual density gradient. Whole blood was then layered atop the upper gradient and the solution was centrifuged at 700g for 30 minutes, which isolated the neutrophil layer between the two histopaque layers. All layers above the neutrophils were discarded and the neutrophils were collected and pelleted. The cell pellet was then resuspended in sterile distilled water to lyse any red blood cell contaminants within the solution. After 10 seconds 1 part of 10x Hanks Buffered Salt Solution was added. Cells were pelleted and resuspended twice in RPMI to eliminate red blood cell ghosts.

For cell diameter and nucleus-to-cytoplasm ratio measurements, a Zeiss Axiovert 200 microscope (Carl Zeiss, Thornwood, NY) with a Zeiss 100X 1.3 NA oil immersion phase objective was used. Nuclei were fluorescently stained with Hoechst 33342 (Molecular Probes, Eugene, OR) for visualization, and cell morphology was imaged with phase microscopy. Metamorph software (Molecular Devices, Downingtown, PA) was used to quantify the cross sectional areas of the nuclei and whole cells.

HL60, Jurkat, and neutrophils cells were labeled with Alexa Fluor 546 phalloidin (Molecular Probes) following the standard protocol in order to image the actin cytoskeleton. Images were taken with a Zeiss LSM 510 Meta confocal microscope (Carl Zeiss).
2.3.2. Microwells

To create microfabricated wells, or microwells, for mechanical immobilization of the cells (Figure 2-1), the photocurable epoxy SU-8 5 (Microchem, Newton, MA) was spun onto piranha-cleaned Borofloat glass wafers (Precision Glass and Optics, Santa Ana, CA) and pre-baked (2 min at 65°C and 5 min at 95°C). The wafer was then exposed through a mask with a mercury arc lamp (175 mJ/cm²), post-exposure baked (1 min at 65°C and 2 min at 95°C), and developed in SU-8 developer (Microchem) for 3 min. Well depth was controlled by spin speed during SU-8 application and measured to be 11.0 µm with an Alpha-Step IQ Surface Profiler (KLA-Tencor, San Jose, CA). Wells were patterned in arrays with diameters ranging from 8 to 20 µm, though for the experiments in this paper microwells with diameters of 13.6 ± 0.3 µm (mean ± S.D., n = 10) were used for HL60 and Jurkat indentation and microwells with diameters of 10.8 µm ± 0.6 µm (mean ± S.D., n = 18) were used for neutrophil indentation. No differences in cell morphology or viability were observed between cells incubated on SU-8 and glass surfaces.

2.3.3. AFM experiments

All force microscopy measurements were obtained on a modified commercial AFM. A Bioscope AFM (Veeco, Santa Barbara, CA) mounted onto a Zeiss Axiovert 25 held the fluid-cell-mounted cantilever (Microlevers, Veeco). For deformability measurements, we used a closed-loop single-axis 50 µm range, 0.7 nm accuracy piezoelectric positioning platform (“piezo”, Mad City Labs, Madison, WI) instead of the piezoelectric tube on the Bioscope AFM head. The piezo platform and photodiode signal were controlled by a RHK SPM 7 controller and RHK SPM32 software (RHK
Technology, Troy, MI). V-shaped gold-coated silicon nitride cantilevers (Veeco) with a spring constant of 9-11 pN/nm (calibrated by the thermal noise method (Hutter and Bechhoefer 1993)) were used in all experiments.

Cells were pipetted onto the wafer and allowed to settle into the microwells. Some cells were moved into wells for force microscopy by gently herding them with the cantilever tip. This movement did not cause any measurable change in cell modulus when analyzed with a two way ANOVA with cell type (HL60 versus Jurkat) and microwell status (cells moved into microwells versus cells already in microwells) as the independent variables and cell stiffness as the dependent variable ($n_{HL60} = 15$, $n_{Jurkat} = 16$). Cells pushed into the microwells with the AFM cantilever and cells that had fallen into the microwells on their own did not differ in stiffness ($p = 0.93$). Also, there was no cell type versus microwell status interaction ($p = 0.63$), indicating that the difference in stiffness between HL60s and Jurkats was the same for cells that were moved into the microwells as for cells that had fallen into the microwells.

All data was taken at 25°C. Though temperature likely affects the material properties of the cells studied (Lichtman and Kearney 1976), this study is primarily comparative. Experiments were performed within one hour after cells were removed from the incubator. HL60 apparent stiffness increased slightly over that one hour period ($R^2 = 0.10$, $p = 0.01$). Jurkat and neutrophil apparent stiffness showed no significant increase over time ($R^2 < 0.001$, $p = 0.92$ and $R^2 = 0.06$, $p = 0.24$, respectively), indicating that the null hypothesis of no correlation cannot be rejected.

A microwell-trapped cell was moved underneath the cantilever tip with a two axis translation stage. Mechanical properties were determined by extending the piezo
platform at a constant rate, deflecting the cantilever upon contact with the cell until approximately 800 pN of force was applied or the cell was indented 3 µm (approximately 25% of cell diameter). Substrate effects may influence stiffness values at indentations more than 10% of cell diameter for the Hertz model (Dimitriadis, et al 2002), though we observed no deviation in the model fit over the entire indentation range. Furthermore, substrate effects would not be expected to affect comparative studies of leukemia cell properties. Only the loading curve was used in analysis of the data.

To determine the role of deformation rate and cell viscosity on deformation response, the piezo extension rate was varied between 24 nm/s and 8643 nm/s. The viscosity of the media had negligible effects on cantilever deflection during probing. This was determined by acquiring indentation data on a hard glass surface at all rates and finding no difference in the loading curves. The cells were probed in a random order of rates to avoid measurement bias. A population of HL60 (n = 60), Jurkat (n = 37), and neutrophil (n = 26) cells were indented to determine the average elasticity of the cells in the limit of low deformation rates (apparent equilibrium Young’s modulus, E∞). For this experiment, the platform moved at 415 nm/s and cells were indented five times each. Student’s t-test was used to determine if deformability of the cell types statistically differed.

Experiments were conducted with both the pyramid AFM tip and a 10 µm diameter sphere indenter. The pyramid silicon nitride tip is 3 µm in height with a 35° half-angle. To attach a sphere to the cantilever tip, cantilever chips were mounted on a manual three axis micromanipulator and visualized with light microscopy. The cantilever of interest was lowered onto a glass slide containing 5-minute epoxy (Devcon, Danvers,
MA) and the end of the cantilever was wet with the adhesive. The cantilever tip was then lowered onto a glass slide with dried 10 µm polystyrene beads and adhered to an individual bead. Bonding between the cantilever and the bead was confirmed with light microscopy.

2.3.4. Modeling and Analysis

Numerous mechanical models have been used to characterize cell deformability, including the liquid droplet (Evans and Yeung 1989), Hertzian mechanics (linear elastic) (Radmacher, et al 1996), Maxwell fluid with constant surface tension (Dong, et al 1988), standard linear solid (Sung, et al 1988), power law fluid (Tsai, et al 1996a), compound drop (Tran-Son-Tay, et al 1998), and variations of these (Drury and Dembo 2001, Herant, et al 2003). In this paper we consider two representative models in the limit of low deformation rate: the liquid droplet and Hertzian mechanics models. The Hertzian mechanics model was selected because it accounts for probe geometry and has been extensively used with AFM (Radmacher 2002, Radmacher, et al 1996, Wojcikiewicz, et al 2004). Furthermore, at low deformation rates, the standard linear solid model reduces to a linear elastic model. The liquid droplet model was selected because of its extensive use with leukocytes (Hochmuth 2000). For low deformation rates, the Maxwell fluid with constant surface tension model reduces to the liquid droplet model.

Hertzian Mechanics Model

Most AFM mechanical property measurements are made by acquiring cantilever deflection versus sample height with a constant piezo extension rate. These curves can be analyzed by a Hertzian mechanics equation, first derived for two spherical lenses in
contact by Hertz (Hertz 1882). For a pyramid punch (Bilodeau 1992), indentation $\delta$ is related to punch load $F$ by:

$$\delta^2 = \frac{4F(1-\nu^2)}{3E\tan \alpha}$$

(1)

and piezo position $z$ is related to cantilever deflection $d$ by:

$$z = z_0 - (d - d_0) - \left( \frac{4k(1-\nu^2)(d - d_0)}{3E\tan \alpha} \right)^{1/2}$$

(2)

where, $z$ is platform position (with negative movement being in the upwards direction), $z_0$ and $d_0$ are the contact point, $k$ is the cantilever spring constant, $\nu$ is the Poisson ratio (assumed to be 0.5), $E$ is the apparent Young’s modulus, and $\alpha$ is the cantilever tip half-angle. This form of the Hertzian mechanics model rests on several assumptions: (i) the material is homogeneous, isotropic, and semi-infinite, (ii) the material is a linear-elastic solid, and (iii) the material undergoes infinitesimally small strains. The validity of these assumptions is examined in the results section. For further discussions of AFM and Hertzian mechanics see Costa et al. (Costa and Yin 1999), Dimitriadis et al. (Dimitriadis, et al 2002), and Radmacher (Radmacher 2002). Custom Matlab (Mathworks, Natick, MA) and Igor (Wavemetrics, Lake Oswego, OR) scripts were used to fit the Hertzian mechanics model to experimental data. The contact point $(z_0, d_0)$ and $E$ were fit to the data using a non-linear least squares optimization method.
**Liquid Droplet Model**

The liquid droplet model with a constant cortical tension has been used extensively to describe neutrophils (Evans and Yeung 1989, Hochmuth 2000). This model has been used predominantly with micropipette experiments where membrane tension is determined by aspirating part of the cell into the pipette. Lomakina et al. derived the liquid droplet model for the indentation of a cell with a sphere (Lomakina, et al 2004), and we extend the derivation for a pyramid indenter appropriate for our AFM measurements.

This model is derived for the static case in which the cell is in static equilibrium with the indenter (Figure 2-2). First, an arbitrary section of the cell is considered to satisfy a force balance between the ambient fluid pressure, $P_0$, and the internal cell pressure, $P_c$:

\[ P_0 (\pi R_0^2) + T \sin(2\pi R_0) = P_c (\pi R_0^2) \]  \hspace{1cm} (3)

where $R_0$ is the radius of the projected cross-sectional area, $T$ is the cortical tension, and $\theta$ is the half-angle of the section arc. By relating $R_0$ to cell radius $R_c$ and $\theta$, Eq. 3 simplifies to:

\[ P_c - P_0 = \frac{2T}{R_c} \]  \hspace{1cm} (4)
Figure 2-2. Geometry of a pyramid-tipped cantilever indenting a cell. (A) Schematic diagram of a cell sitting in a microwell (A). $P_t$, $P_o$, and $P_c$ are the pressures of the cell, surrounding fluid, and cantilever tip, respectively. \( T \) is cortical tension. The projected contact area between the cell and the cantilever tip is the square defined by \( S \). \( R_c \) is the cell radius. \( R_o \) is the radius of the projected cross-sectional area of an arbitrary cell section taken away from the microwell walls and the cantilever tip. \( \theta \) is the half-angle of the arc of this arbitrary cell shell section. (B) Expanded view of the tip-cell interaction showing that $\delta_t$, the penetration depth of the pyramidal tip into the cell, is the sum of $\delta_c$, the indentation of the tip beyond the plane described by $S$, and $\delta_o$, the indentation described by the distance between the sphere shell and the projected contact area. $\alpha$ is the cantilever tip half angle.

Next the force balance between the pyramid indenter and the cell is examined. The pressure applied by the tip and the ambient fluid, $P_t + P_o$, over the projected contact area described by the half-length of the contact edge, $S$, is related to \( T \) and $P_c$ by the force balance:
\[ P_t(4S^2) + T \cos \alpha (8S) = (P_t + P_0)(4S^2) \quad (5) \]

where \( \alpha \) is the cantilever tip half-angle. \( P_t \) can then be related to \( R_c, S, \) and \( \alpha \) by combining Eq. 4 and Eq. 5:

\[ P_t = 2T \left( \frac{1}{R_c} + \frac{\cos \alpha}{S} \right) \quad (6) \]

This pressure difference can then be linked to cantilever deflection, \( d - d_0 \), using Hooke’s law:

\[ d - d_0 = \frac{8TS^2}{k} \left( \frac{1}{R_c} + \frac{\cos \alpha}{S} \right) \quad (7) \]

Tip, or cantilever, indentation \( \delta \) is equal to the sum of \( \delta_t \), the indentation of the tip beyond the plane described by \( S \), and \( \delta_c \), the indentation described by the distance between the sphere shell and the projected contact area:

\[ \delta = \delta_t + \delta_c \quad (8) \]

\( S \) can be related to \( \delta \) by:

\[ \delta = S \cot \alpha + R_c - (R_c^2 - S^2)^{1/2} \quad (9) \]
Lastly, \( z \) is related to \( d \) and \( \delta \) by:

\[
z = z_0 - (d - d_0) + \delta
\]

The liquid droplet model described here rests on several assumptions: (i) the internal contents of the cell are a homogeneous viscous liquid, (ii) the cortical tension is constant around the cell, (iii) the indenter is moving at a rate slow enough so the viscosity of the contents and the membrane are inconsequential, (iv) the cortical shell conformally deforms around the tip during indentation, and (v) that the radius of the cell remains constant during indentation. The validity of these assumptions is examined in the discussion section. A custom Igor script was written to determine cortical tension and contact point using a non-linear least squares optimization method. Recently, Sen et al. developed a theoretical model for indenting a red blood cell adhered to a surface with a cone-shaped AFM tip (Sen, et al 2005). This model could be modified for a spherical cell not adhered to a surface to avoid some of the assumptions used here, but there is no simple analytical solution for it.

2.4. Results

We considered three questions. First, at what deformation rates are any viscous contributions from the cytoplasm to the apparent cell elasticity minimized? Second, which model (Hertzian mechanics vs. liquid droplet) more accurately describes the deformability of the cells? Third, how different are the mechanical properties of leukemia cells from myeloid and lymphoid lines when compared to each other and to
normal neutrophils? Answers to the first two questions are described here in detail for the myeloid (HL60) cell line but were also obtained for the lymphoid (Jurkat) cell line and neutrophils. The three cell types are quantitatively compared to answer the third question and provide biophysical insight into clinical complications associated with acute leukemia.

2.4.1. Cell Deformability Measurements

A sample curve from an indentation experiment into a HL60 cell is shown in Figure 2-3. When the piezo position is positive, the cantilever tip has not yet come into contact with the cell. As the tip contacts the cell, the cantilever begins to deflect and the curve increases non-linearly. The influence of deformation rate, and hence cell viscosity, on mechanical property measurements obtained with the AFM was evaluated by conducting experiments at piezo extension rates from 24 to 8643 nm/s. An examination of one specific HL60 cell shows a viscous response at increasing piezo extension rates, since deflection curves increase in slope as rate increases (Figure 2-4A). This trend is seen clearly when using a Hertzian mechanics model to determine apparent stiffness (Figure 2-4B). Apparent stiffness remains relatively constant at and below 415 nm/s but increases monotonically at higher piezo extension rates. The constant apparent stiffness at low deformation rates is seen more clearly in Figure 2-4C, where HL60 cells show no increase in apparent stiffness when piezo extension rate is increased from 24 nm/s to 415 nm/s (n = 8), indicating that deformability measurements in this range of piezo extension rates are not significantly influenced by viscosity. For this analysis, apparent stiffness for each cell within the sample was normalized due to the variance of stiffness across cells. This domain of low deformation rates, in which we denote the apparent stiffness $E_\infty$,
simplifies the models to which the experimental data is compared and is likely most relevant for the case of a cell plugging a capillary in leukostasis.

Figure 2-3. A typical deflection-position curve of a cantilever indenting a HL60 cell in a microwell. Indentation is in the direction of the arrow, and negative piezo position indicates extension after contact with the cell. Contact point is denoted by the circle. Piezo extension rate in this experiment was 1506 nm/s. Deflection of the cantilever is small compared to the indentation of the cell due to the greater stiffness of the cantilever when compared to the cell. Inset: illustration of the relationship between piezo movement $z$, indentation $\delta$, and deflection $d$. The deflected cantilever is solid, and the undeflected cantilever is dashed.
Figure 2-4. Effect of piezo extension rate on apparent stiffness of HL60 cells. (A) At increasing piezo extension rates, the viscosity of the HL60 causes increased cantilever deflection for the same piezo position. All legend values are in nm/s. At rates up to 415 nm/s, the deflection curves overlay each other. This indicates that the indentation rate was slow enough for viscosity not to be a factor. (B) HL60 apparent stiffness determined by Hertzian mechanics remains constant at low rates. Data in (B) is from the same cell as (A). In (C), the apparent stiffnesses of 8 cells at low piezo extension rates were normalized and averaged. Normalization was performed by averaging the stiffness of each cell across the experimental extension rates and then dividing the stiffness at each rate by this average. At rates of 415 nm/s and below, apparent stiffness remained constant. Error bars represent the standard deviation.
This same viscous response at higher piezo extension rates was seen in an identical rate analysis performed on Jurkat cells and neutrophils. Both cell types had similar plateaus of apparent stiffness at low piezo extension rates with apparent stiffness increasing monotonically with increasing piezo extension rate. The apparent stiffness of neutrophils was level at 501 nm/s and below while that for Jurkat cells was level at 948 nm/s and below. Please refer to the Supplemental Material on for more details of these experiments.

The microwell walls are not expected to constrain cells during mechanical property measurements due to size differences between the well and cell diameter. HL60 cell diameter was 12.4 ± 1.2 µm (n = 51) and Jurkat cell diameter was 11.5 ± 1.5 µm (n = 44) while microwell diameter was 13.6 ± 0.3 µm (n = 10). Smaller wells (10.8 ± 0.6 µm, n = 18) held the similarly smaller neutrophils (8.3 ± 0.6 µm, n = 49) for indentation experiments. Cells were selected to be smaller than the wells in which they fell or were placed. For a sample of Jurkat and HL60 cells (n_{HL60} = 13, n_{Jurkat} = 8), there was a near significant correlation between cell diameter and modulus ($R^2 = 0.16$, $p = 0.06$). However, this finding should be interpreted in light of the demonstrated effect of cell type on stiffness. HL60 cells, which are shown to be significantly stiffer than Jurkat cells, also in general are larger. A partial correlation between cell size and stiffness, controlling for cell type, revealed no significant relationship ($p = 0.83$). This indicates that once the hypothesized relationship between cell type and stiffness is accounted for, cell size is no longer related to stiffness.
2.4.2. Comparison of Hertzian Mechanics and Liquid Droplet Models

The Hertzian mechanics and liquid droplet models described previously were fit to the experimental indentation curves for each cell type. An example fit for a HL60 cell indentation is seen in Figure 2-5A. Example fits for Jurkat and neutrophil data can be seen in Figure 2-10 in Supplemental Material. The Hertz model was used to determine apparent stiffness \( E_\infty \) and contact point \( (z_0, d_0) \), and the liquid droplet model was fit for membrane tension \( T \) and the contact point \( (z_0, d_0) \). For each cell type, the Hertz model had a lower mean square of the error (MSE) than the liquid droplet model (Table 2-1). Paired Student’s t-test comparing the MSE showed this difference was statistically significant for each cell type (\( \alpha = 0.05 \), \( p_{HL60} < 0.001 \), \( p_{Jurkat} = 0.01 \), \( p_{Neutrophil} = 0.003 \)).

Much of the observed error in the liquid droplet model arose from non-normally distributed residuals near the contact point, which may be due in part to assumptions of the liquid droplet model used here. Based on these fits, we decided to use the Hertzian mechanics model to compare the stiffness of the different cell types.
Figure 2-5. Comparison of mechanical models with cell deformation data. (A) A HL60 cell was indented at 24 nm/s. The Hertzian mechanics model (dashed line) fits the data (gray line) better than the liquid droplet model (dotted line). Contact point is denoted by the circle. (B) A sphere shaped indenter with a diameter of 10 µm was attached to the end of the cantilever and pushed into a different cell at 415 nm/s. The Hertzian mechanics (dashed line) and liquid droplet (dotted line) models were modified for a spherical punch and were fit to the data (gray line). Again, the Hertz model was a better fit to the data.
Table 2-1. Summary of parameters determined for each cell type. Values are mean ± standard deviation. Hertzian fit MSE and liquid droplet fit MSE refer to the mean squared error (MSE) of the Hertzian mechanics model and the liquid droplet model to the data. Sample sizes for determining stiffness and tension and the respective MSEs are 60, 37, and 26 for HL60, Jurkat, and neutrophil cells, respectively. Sample sizes for cell diameter measurements are 51, 44, and 49 for HL60, Jurkat, and neutrophils cells, respectively. Sample sizes for nucleus to whole cell ratio measurements are 40, 47, and 49 for HL60, Jurkat, and neutrophil cells, respectively.

<table>
<thead>
<tr>
<th></th>
<th>HL60</th>
<th>Jurkat</th>
<th>Neutrophil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stiffness (Pa)</td>
<td>855 ± 670</td>
<td>48 ± 35</td>
<td>156 ± 87</td>
</tr>
<tr>
<td>Hertzian fit MSE (nm²)</td>
<td>2.8 ± 1.6</td>
<td>1.2 ± 0.6</td>
<td>1.7 ± 0.7</td>
</tr>
<tr>
<td>Cortical tension (pN/µm)</td>
<td>155 ± 81</td>
<td>21 ± 13</td>
<td>48 ± 20</td>
</tr>
<tr>
<td>Liquid droplet fit MSE (nm²)</td>
<td>8.0 ± 4.4</td>
<td>1.6 ± 1.2</td>
<td>2.6 ± 1.7</td>
</tr>
<tr>
<td>Cell diameter (µm)</td>
<td>12.4 ± 1.2</td>
<td>11.5 ± 1.5</td>
<td>8.3 ± 0.6</td>
</tr>
<tr>
<td>Nucleus:cell ratio (%)</td>
<td>49 ± 6</td>
<td>55 ± 8</td>
<td>39 ± 11</td>
</tr>
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</table>

2.4.3. Comparison of HL60, Jurkat, and Neutrophil Cell Deformability

Myeloid and lymphoid leukemia cells as well as human neutrophils were indented under low deformation rates to determine $E_\infty$. Indenting at a piezo rate of 415 nm/s was determined to be in the equilibrium regime, where apparent stiffness is not affected by changes in rate, for all cells indented. We report HL60, Jurkat, and neutrophil cells to have an apparent stiffness of $855 \pm 670$ Pa, $48 \pm 35$ Pa and $156 \pm 87$ (mean ± SD, n = 60, 37, and 26 respectively) (Figure 2-6, Table 1). Effect of cell type on stiffness is significant ($\alpha = 0.05$, $p < 0.001$) when using a one way ANOVA analysis. Using Bonferroni t-tests for post-hoc pairwise analyses, we found HL60 cells to be significantly stiffer than Jurkat and neutrophil cells ($p < 0.001$) and neutrophil cells to be significantly stiffer than Jurkat cells ($p < 0.001$).
Figure 2-6. Comparison of myeloid and lymphoid cell line and neutrophil stiffness at low piezo extension rates. With a piezo rate of 415 nm/s, HL60 cells have an average apparent stiffness of 855 Pa with a standard deviation of 670 Pa (n = 60) while Jurkat cells are significantly softer (p < 0.001) with an average apparent stiffness of 48 Pa and a standard deviation of 35 Pa (n = 37). Neutrophils have an average apparent stiffness of 156 Pa ± 87 Pa (n = 26, mean ± standard deviation), significantly softer than HL60 cells and significantly stiffer than Jurkat cells (p < 0.001 for both).

2.5. Discussion

2.5.1. Modeling

For the three cell types tested, the Hertz model was found to fit the data significantly better than the liquid droplet model, based on paired t-tests described in results. The mechanical models used here rest on assumptions that affect the determined parameters for stiffness and tension. We examined how the pyramidal indenter model pushing into a liquid droplet could contribute to the observed inaccuracy. First, the model assumes that the cell deforms conformally around the pyramid during indentation. This assumption might not be correct, given previous data which showed that for aspiration experiments with micropipettes smaller than 1 µm radius, the simple liquid droplet model was not valid due to the bending modulus of the membrane and cortex (Zhelev, et al 1994). To test this hypothesis, we indented HL60 cells with a 10 µm diameter spherical indenter to reduce the effect of the bending modulus. We fit the
Hertzian mechanics model for two spheres in contact and fit the liquid droplet model for a spherical punch to the data (Figure 2-5B) and found that the MSE of the fits were similar to the pyramid punch (MSE$_{\text{Hertz}} = 1.1$ nm$^2$ versus MSE$_{\text{droplet}} = 11.3$ nm$^2$), indicating that cell deformation around the indenter tip is not the primary reason the liquid droplet model does not fit the data as well at the Hertzian mechanics model.

Second, the liquid droplet model assumes the indenter and cell are in static equilibrium at all points during indentation. The repeatability of indentation curves shown in Figure 2-4A as well as the constant apparent stiffness at slow deformation rates in Figure 2-4C supports this assumption. Time constants reported by Lomakina et al. are also consistent with the assumption of static equilibrium (Lomakina, et al. 2004).

While the Hertzian mechanics model fits well to the data, errors in the absolute value of elasticity are expected when using this model to estimate the apparent stiffness at low deformation rates, $E_\infty$. The assumption that a cell is homogeneous and isotropic is clearly incorrect—leukocytes, like other cells, have a cytoskeleton, organelles, and nucleus that make them inhomogeneous (Schmid-Schonbein, et al. 1980). To quantify the influence of local inhomogeneities on our measurements, we compared the standard deviation of $E_\infty$ of a population of HL60 cells indented with the 10 µm diameter sphere versus the pyramid tip. We expected the standard deviation for $E_\infty$ to be reduced because any inhomogeneities would be averaged out over the larger contact area, and indeed it was. The standard deviation for $E_\infty$ was reduced from 78% of the mean to 53% of the mean (data not shown). Using such a large indenter, however, caused the semi-infinite solid assumption for Hertzian mechanics to be less valid – a model from Dimitriadis et al. (Dimitriadis, et al 2002) showed increased deviation for the Hertz model as indenter tip
radius increases for a sample with finite thickness. For example, when indenting a sample with a modulus of 855 Pa with a 4 µm diameter tip, the apparent modulus is overestimated by 12%. For a 10 µm diameter tip, the apparent modulus is overestimated by 85%. Usage of the sphere indenter requires the contact radius to be no more than 10% of the indenter radius for the results to be reliable (Yoffe 1984). However, this is difficult when probing soft leukocytes with commercial AFM cantilevers since the cantilevers do not deflect enough when compared to noise in the system to extract adequate data.

The pyramid tip also has limitations when used with the Hertzian mechanics model if the tip (35° half-angle) creates local strains that exceed the linear-elastic assumption. Dimitriadis *et al.* found this geometry overestimated Young’s modulus when compared to that found with a sphere indenter by 60% (Dimitriadis, *et al* 2002). The smaller tip does allow for smaller total strain, reducing the effect of the hard glass surface below the cell which would tend to overestimate the Young’s modulus. Non-ideal tip geometry could also create errors in estimation of stiffness. For instance, using a blunter tip (one with 5° larger half angle) while still modeling with the expected 35° tip half angle would result in an overestimation of the apparent stiffness by 20%. Similarly, using a 5° half angle sharper tip would result in an underestimation of apparent stiffness by 18%.

All simple analytical models have limitations when used to describe constitutive mechanical behavior of cells. While the Hertzian mechanics model is no exception, it is able to quantify differences in deformability between cell types in a simple and effective manner, which is of interest in the application of cell mechanics to clinical problems where comparisons among cell types are necessary.
2.5.2. Deformability Comparison

Cell deformability is thought to play a role in the pathophysiology of leukostasis, as stiffer cells have a higher tendency to mechanically obstruct the vasculature (Lichtman, *et al* 1987, Porcu, *et al* 2002). Leukostasis has been found to occur at a much higher frequency in AML than ALL at the same cell concentration (Porcu, *et al* 2002). Clinicians commonly accept that AML cells are more rigid than ALL cells and normal neutrophils (Bast, *et al* 2000, Kelly and Lange 1997). However, there is scant data that supports this assertion. We found HL60 myeloblasts to have a mean $E_\infty$ of 855 Pa and Jurkat lymphoblasts to have an $E_\infty$ of 48 Pa (Figure 2-6). This difference is significantly greater than found in filtration experiments which found ALL cells and AML cells equally deformable (Lichtman 1973a, Lichtman and Kearney 1976) and implies that leukemic cell deformability may play a larger role in leukostasis than previously thought. As described in results, Jurkat lymphoblasts and neutrophils were found to be significantly less stiff than HL60 myeloblasts. This is consistent with clinical findings that ALL and chronic myeloid leukemia (a leukemia of more mature myeloid cells) rarely go into leukostasis.

Comparisons of leukemia cell deformability were initially performed in the 1970’s. With nucleopore filtration experiments, it was reported that ALL cells were more likely to pass through 8 µm pores than AML cells (Lichtman and Kearney 1976). When accounting for cell diameter, however, these cells were reported to be equally filterable. Micropipette experiments showed that myeloblasts were less deformable than leukemic lymphocytes from patients with chronic, or mature, lymphoid leukemia (Lichtman 1973a). However, because non-blastic lymphocytes rarely cause leukostasis,
these findings are not very revealing. While our measurements directly compared myeloblast and lymphoblast properties, the cells used were from leukemic cell lines and may not reflect the properties of leukemia cells in vivo. Primary leukemia cell measurements must be performed before any definitive conclusions can be made.

Our data is in the same range for Jurkat $E_\infty$ determined previously with micropipette aspiration using a standard linear solid model (Yao, et al 2002). While $E_\infty$ of HL60 cells has not been measured previously, we can compare these measurements to stiffness measurements of other cell types. Wojcikiewicz et al. reported the 3A9 cell, a T-Cell hybridoma, has an elasticity of 1.4 kPa when indented at 5000 nm/s (Wojcikiewicz, et al 2003), on the order of our findings. Domke et al. found stiffness of adherent osteoblasts away from the stress fibers to be around 500 Pa.

Our values for deformability of neutrophils are also in the range found previously by others. When using the liquid droplet model, we found neutrophils to have a cortical tension of 48 ± 20 pN/µm (mean ± SD). This is comparable to the findings from micropipette aspiration experiments of Evans and Yeung (35 pN/µm (Evans and Yeung 1989)), Needham and Hochmuth (24 pN/µm (Needham and Hochmuth 1992)), and Tsai et al. (27 pN/µm (Tsai, et al 1993)). Our value is slightly higher than the cortical tension found by Lomakina et al. (16-24 pN/µm) when they collided neutrophils into spheres via fluid flow (Lomakina, et al 2004).

### 2.5.3. Biophysical Mechanism for Stiffness Difference

Nuclei have been documented to be substantially larger in most types of leukemias and leukemia cell lines when compared to normal leukocytes (Figure 2-7) (Tkachuk, et al 2002) and have been previously thought to dominate leukemia cell
deformability behavior (Lichtman 1973a). We found that HL60 cell nucleus is $49\% \pm 6\%$ (mean ± SD, n = 40) of total cross section, Jurkat cell nucleus is $55\% \pm 8\%$ (mean ± SD, n = 47) of total cross section, and neutrophil nucleus is $39\% \pm 11\%$ of total cross section (mean ± SD, n = 49), agreeing with previous measurements for AML, ALL, and neutrophils (Ochiai and Eguchi 1987, Schmid-Schonbein, et al. 1980). When analyzed with a one way ANOVA, there was a significant effect of cell type on nucleus:whole cell ratio, with significant differences between all ratios in a Newman-Keuls post-hoc pairwise analysis ($p_{\text{HL-60-Jurkat}} = 0.002$, $p_{\text{HL-60-neutrophil}} < 0.001$, $p_{\text{Jurkat-neutrophil}} < 0.001$). While nuclei of some cells have been shown to be significantly stiffer than the whole cell (Dong, et al. 1991, Guilak, et al. 2000), our data shows that simply having a larger nucleus does not mean that a cell is stiffer, as Jurkat cells were found to be of similar stiffness to the neutrophils even though their nuclei are significantly larger.
Figure 2-7. As seen with Wright-stained cells, the nucleus to cytoplasm ratio in HL60 cells (A) and Jurkat cells (C) is larger than it is for normal neutrophils (E). The cortical actin cytoskeleton density of HL60 (B), Jurkat (D), and neutrophil (F) cells does not appear significantly different. Scalebar is 5 µm.

To determine if the actin cortex was substantially thicker or denser in the HL60 cells than in the Jurkat or neutrophil cells, we fluorescently labeled the actin cytoskeleton and imaged the cells with confocal microscopy (Figure 2-7). Based on those images, we could not conclude that there were any differences in the actin cortex of the leukemia cells that would lead to the difference in stiffness. This raises the interesting question of what governs the mechanical properties of these diseased cells. Whether the stiffness difference observed in our measurements lies in cytoskeletal filament networks (actin, microtubules, or lamins), cytoplasm, nuclear or cell membranes, or a combination of these remains unclear. AFM is an ideal tool for further work on this question.
2.6. Conclusions

In this paper, we developed microwells to immobilize non-adherent cells for force microscopy and applied this technique to myeloid and lymphoid leukemia cell lines as well as neutrophils. We determined the Hertzian mechanics model fits the data well and yielded an apparent equilibrium stiffness for the HL60 that is 18 times higher than that of Jurkat cells and 6 times higher than that of neutrophils. These findings are consistent with the finding that myeloid leukemias are involved in leukostasis at a higher rate than lymphoid leukemias. Comparing leukemia cell deformability will improve the understanding of leukostasis in acute leukemia. Further studies will be needed to investigate the role of deformability with other hypothesized factors involved in leukostasis, including adhesion and transmigration.

2.7. Acknowledgements

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2.8. Supplementary Material
Figure 2-8. Effect of piezo extension rate on apparent stiffness for Jurkat cells. This figure is similar to Figure 4 in the paper but for Jurkat cells. (A) When piezo rate increases, viscosity of the Jurkat cell causes increased cantilever deflection for the same piezo position at higher rates. The legend values correspond to piezo rate and are in nm/s. When using a Herztian mechanics model to determine apparent stiffness (B), rate appears to have little effect on apparent stiffness below 981 nm/s. Data from (B) is from the same cell as (A). In (C), apparent stiffnesses of 4 cells at low piezo extension rates were normalized and averaged. At rates of 501 nm/s and below, apparent stiffness remained relatively constant. Bars represent standard deviation.
Figure 2-9. Effect of piezo extension rate on apparent stiffness for neutrophils. This figure is similar to Figure 4 in the paper but for neutrophil cells. (A) When piezo rate increases, viscosity of the neutrophil cell causes increased cantilever deflection for the same piezo position at higher rates. The legend values correspond to piezo rate and are in nm/s. At a piezo rate of 501 nm/s and below, the apparent stiffness remains relatively level (B). Data from (B) is from the same cell as (A). In (C), apparent stiffnesses of 5 cells at low piezo extension rates were normalized and averaged. At rates of 501 nm/s and below, apparent stiffness remained relatively constant. Bars represent standard deviation.
Figure 2-10. Comparison of mechanical models with cell deformation data for (A) Jurkat and (B) neutrophil cells. In these representative examples, the Hertzian model (dashed line) fits the data better than the liquid droplet model (dotted line). For the Jurkat, the Hertzian model yielded a stiffness of 48 Pa (MSE = 1.2 nm²) and the liquid droplet yielded a cortical tension of 22 pN/μm (MSE = 2.2 nm²). For the neutrophil, the Hertzian model yielded a stiffness of 147 Pa (MSE = 1.7 nm²) and the liquid droplet yielded a cortical tension of 64 pN/μm (MSE = 2.6 nm²). Contact point (determined by the Hertzian model) is denoted by the circles. Cells were indented at 415 nm/s.
3. Measurement of leukemia cell mechanics:

biophysical flow cytometry
3.1. **Abstract**

Pathological processes in hematologic diseases originate at the single-cell level, often rendering measurements of individual cells more clinically relevant than population averages from bulk analysis. For this reason, flow cytometry has been an effective tool for single-cell analysis of properties associated with light scattering and fluorescence labeling. However, conventional flow cytometry cannot measure cell mechanical properties, alterations of which contribute to the pathophysiology of hematologic diseases such as sepsis, diabetic retinopathy, and sickle cell anemia. While several biophysical techniques, including AFM and micropipette aspiration, can be used to quantify mechanical properties of single cells, these techniques are too slow to provide clinically useful data for patient diagnosis and treatment. Here we present a high-throughput microfluidics-based ‘biophysical’ flow cytometry technique that measures single-cell transit times of blood cell populations passing through *in vitro* capillary networks. To demonstrate clinical applicability, we apply this technique to characterize biophysical changes in two model disease states in which mechanical properties of cells are thought to lead to microvascular obstruction: (i) sepsis, a process in which inflammatory mediators in the bloodstream activate neutrophils and (ii) leukostasis, an often fatal and poorly understood complication of acute leukemia. Using patient samples, we show that cell transit time through and occlusion of microfluidic channels is increased for both disease states compared to controls and that mechanical heterogeneity of blood cell populations is a better predictor of microvascular obstruction than average properties. We observe that inflammatory mediators involved in sepsis significantly affect the shape and magnitude of the neutrophil transit time population distribution. We find that altered
properties of leukemia cell subpopulations, rather than of the population as a whole, correlate with symptoms of leukostasis in patients – a new result that may be useful for guiding leukemia therapy. By treating cells with drugs that affect the cytoskeleton, we also demonstrate that their transit times could be significantly reduced. Biophysical flow cytometry offers a low-cost and high-throughput diagnostic and drug discovery platform for hematologic diseases that affect microcirculatory flow.

3.2. Introduction

Flow cytometry has been an effective tool for high-throughput analysis of single cells and is widely used to quantify biological properties such as surface protein expression and signaling activity (Irish, et al 2006). By being able to fluorescently detect rare cells and distinct subpopulations that lead to disease (Davies, et al 2008, Sievers and Radich 2000), this tool has revolutionized medicine and has shown that measuring the properties of individual cells can be critical for effective health care.

Flow cytometry, however, cannot measure cellular mechanical properties, alterations of which contribute to the pathophysiology of various hematologic diseases, where occlusion of the microvasculature by blood cells can have profound effects on blood flow in the brain, lungs, and other vital organs (Eaton and Hofrichter 1995, Lipowsky 2005, Worthen, et al 1989). Furthermore, as relatively few mechanically-altered blood cells are theoretically sufficient to induce microvascular pathology (Aprelev, et al 2007, Bagge, et al 1980), symptoms associated with hematologic diseases may be attributable to the minority subpopulation of pathologic blood cells that would not be detected with a bulk assay that measures average properties. Therefore, a need
exists for high-throughput single-cell mechanical analysis to address basic questions in cell mechanics and provide diagnostic data for clinical hematology.

To address this need, several techniques have been developed to assess mechanical properties of blood cells. However, none have successfully demonstrated high-throughput analysis of single-cell mechanical properties in clinically-relevant conditions. The existing techniques can be divided into bulk and single cell approaches. Bulk microfiltration techniques have been used for several decades to study microcirculatory diseases by measuring changes in pressure or flow rate of blood cell solutions as milliliter-scale volumes of fluid are passed through micropore filters (Chien, et al 1983, Downey and Worthen 1988). Advances in this technique involve the coupling of microfabricated sieves with optical microscopy for direct cell visualization (Carlson, et al 1997, Nishino, et al 2005, Shevkoplyas, et al 2006), improved geometric modeling of the microvasculature (Shevkoplyas, et al 2006), and measurement of electrical impedance of the filter to measure transit time of fast moving cells (Drost, et al 1999, Frank and Hochmuth 1987, Koutsouris, et al 1988). While these studies have revealed strong evidence of altered mechanical behavior of cells in various disease states, they are only able to measure bulk or incomplete population blood cell behavior or endpoint results, such as occlusion, and they cannot attribute aberrations in flow behavior to shifts in properties of the whole population or smaller sub-populations.

Single-cell approaches such as micropipette aspiration and atomic force microscopy are able to measure changes in the mechanical properties of cells that are associated with disease (Costa 2003, Lam, et al 2007, Skoutelis, et al 2000a). While
precise, these approaches are limited by their inherent low throughput and high
equipment cost, making them unlikely candidates for clinical use and unable to
adequately describe cell populations. Microfluidics offers the promise of high-
throughput single-cell analysis of cell deformability for clinical applications. Using
2003, Tsukada, et al 2001), microfluidic-based systems have enabled observation of
differences between cell populations with single-cell resolution. Collectively, these
devices enable what we generally refer to as ‘biophysical’ flow cytometry, and like
standard flow cytometry, they are capable of high-throughput single-cell analysis.
Although studies of these techniques highlight device capabilities, the direct relevance of
these systems for diagnostic or therapeutic purposes remains unproven.

Here we show that a simple microfluidic device can be used to rapidly generate
clinically useful single-cell data that provides new insight into diseases states and new
motivation for the measurement of mechanical properties with microdevices. Our
biophysical flow cytometry system uses automated image analysis to track large numbers
of individual cells as they traverse a microfluidic capillary network and measures the
effect of cell deformability and cell size on cellular transit times. To demonstrate the
value of quantifying changes in physical properties of blood cells at the single cell level,
we used two conditions as model disease states: (i) sepsis, a process in which the
biophysical response of neutrophils to inflammatory mediators are well characterized
understood and often fatal complication of leukemia in which cell mechanical properties
of cancer cells are thought to lead to microvascular obstruction. We show three primary results with the device: (1) blood cell transit time into microchannels is not normally distributed, (2) inflammatory mediators and drugs that affect the cytoskeleton alter the shape and magnitude of the cell transit time population distribution, and 3) the distribution of transit time correlates with disease pathology and symptomology in leukostasis.

3.3. **Experiment**

3.3.1. **Microfluidic Device**

The main element of the device is the microfluidic capillary network. Using standard microfabrication techniques, a SU-8 master mold of the capillary network was patterned onto a silicon wafer. PDMS was then molded onto the SU-8 master at 60° C for at least 2.5 hours, removed, and bonded after exposure to oxygen plasma to a PDMS spin-coated glass slide to form the capillary network (Figure 3-1). The glass slide was spin-coated with PDMS to ensure a consistent surface material on all sides of the device. Two wide channels bypass the capillary network to maintain a relatively constant pressure drop across the network even when cells plug the smallest microchannels (Figure 3-1A).
Figure 3-1. Biophysical flow cytometer device. (A) Blood cells were loaded into a syringe and flowed into the device at a constant flow rate. The cytometry device trifurcates into two wide bypass channels and a network of bifurcating channels which split into 64 parallel capillary-like microchannels. Scalebar 1 mm. (B) Sixteen of these microchannels are shown here. Scalebar 100 μm. (C) A confocal image of fluorescein solution inside several of the microchannels.

The device was designed to be geometrically similar to capillary networks in vivo while creating a standard system for quantitative measurements. Capillaries in vivo range in diameter between 5-10 μm and length from 50-500 μm (Fung 1997a). Linear flow rate through capillaries has been measured to be 0.42-1.3 mm/s (Fung 1997a, Lipowsky and Zweifach 1977, Milnor 1982) with a pressure drop determined to be between 10-1000 Pa (Huang, et al 2001, Lipowsky and Zweifach 1977), depending on capillary length. Comparatively, the microchannels are 5.89 ± 0.08 μm wide (mean ± SD) wide by 13.3 um tall by 130 μm long. Flowing cell media at 1.0 μL/min, pressure dropped approximately 30 Pa across the capillaries, determined by Poiseuille flow equations (Beebe, et al 2002), which resulted in an average linear flow rate of 0.50 mm/s in the smallest channels. Because of the low Reynolds and Womersly numbers of the in vivo microcirculation (<<1), which relate the inertial and pulsatile effects to viscous effects, forces due to transient and convective acceleration of peristaltic flow are negligible (Fung
1997a), and hence, steady flow was used instead. Using constant flow with bypass channels instead of using pressure reservoirs makes the system less sensitive to air bubbles in the device and placement of the inlet and outlet holes to the device while also maintaining a high linear flow rate, which reduces the tendency of cells to settle onto the bottom of the device.

Clearly differences exist between the device microchannels and in vivo capillaries including rectangular instead of a circular cross section, microchannels in parallel without collateral vessels bridging them, stiffer vessel walls, and the lack of a living endothelium with the accompanying adhesion molecules. Because of these simplifications, our device serves as a first-order model to specifically study blood cell deformation under physiologic flow conditions in the absence of other factors.

3.3.2. Cells

Fresh patient leukemia cells used for experiments were obtained from newly diagnosed acute leukemia patients with detectable leukemia cells in their peripheral blood. Each sample was then immediately isolated via centrifugation with Histopaque 1077 (Sigma-Aldrich). Leukemic cells, which comprised >90% of the mononuclear fraction, were then either used immediately or cryopreserved for future use. Cell viability (trypan blue exclusion) was >95%. We have shown previously with atomic force microscopy (AFM) that the freeze-thaw cycle does not significantly alter cell stiffness.(Lam, et al 2007) To confirm this, we flowed a fresh acute myeloid leukemia sample through the device and later flowed cells from this same sample after it had been frozen and thawed and found no significant difference in cell transit time (Mann-Whitney, p=0.66).
Red blood cells (RBCs) and neutrophils were obtained from the peripheral blood of healthy adult volunteers using a dextran/Histopaque protocol published previously (Issekutz, et al 1999). Briefly, peripheral blood was drawn into a dextran-EDTA solution, and RBCs were purified from the leukocyte/plasma suspension via sedimentation at gravity. Neutrophils were further purified with Histopaque 1077 and 1119 (Sigma-Aldrich). Institutional review boards approved all experiments, and informed consent was obtained for each sample.

The HL60 myeloid leukemia cell line (ATCC) was used for drug experiments. Because HL60 cells are larger in diameter than patient samples, a device of the same geometry but with slightly larger microchannels (7.9 x 12.9 μm) was used, and the flow rate was decreased from 1.0 μL/min to 0.6 μL/min. In several experiments, cells were exposed to different drugs. Neutrophils were exposed to 10 ng/mL fMLP for 15 minutes, and HL60 cells were exposed to 2 μM cytochalasin D for 1 hour or 1 mM pentoxifylline for 3 hours.

### 3.3.3. Cytometry Experiment

After bonding, the channels were fouled for 30 minutes with either 5% autologous human plasma or 20 mg/mL bovine serum albumin (Sigma) when plasma was not available to block non-specific adhesion. Cells were diluted in RPMI cell culture media (Gibco) to a concentration of 3,000 cells/μL. Cells were loaded into a 250 μL heated glass syringe (Hamilton) (37° C) and pumped with a syringe pump (Harvard Apparatus) into the 37° C heated device at 1.0 μL/min (Figure 3-1). An additional syringe was used to flush the channels clear of cells between experiments. After passing through the
device, the cells drained to a waste container. Experiments were imaged with a 20x objective (Edmund Optics) and live images were streamed at 30 frames per second with a CCD camera (Watec) via a frame grabber (National Instruments) into Labview 7 (National Instruments) and saved as an AVI for future analysis. Between experiments, the device was flushed with media to clear residual cells from the microchannels.

Throughput for the device is approximately 50-100 cells/minute and is dependent on flow rate, number of microchannels in the device and in the field of view, and cell concentration. It also depends on the mechanical properties of the cells themselves, which determines how often channels become occluded and thus not available for measurement of other cells transiting through. Throughput could be raised by increasing the flow rate and therefore pressure drop, but then the cells would be subjected to non-physiologically relevant conditions. Increasing cell concentration could also increase throughput but would also increase cell-cell interactions, making it difficult to isolate the effects of single-cell properties on flow through the microchannels.

3.3.4. Automated transit time analysis

Essential to a high-throughput technique for quantifying single cell biophysical properties is a method to quantify the transit time of large numbers of individual cells through the microchannels. All analysis was performed with custom-written scripts in Matlab 7. To detect cell transit time, regions of interest (ROIs) were selected for each of the microchannels and the channels leading into them (Figure 3-2A-B). The standard deviation (SD) of pixel intensity was tracked for each ROI for each frame (Figure 3-2C). A cell’s presence increased the SD of the pixel intensity of the ROI due to light scattering. The SD of each ROI was thresholded to determine the presence of a cell.
Pertinent data such as transit time and blockage initiation was subsequently determined. Cells were included for analysis only if they did not come in contact with another cell while transiting through the microchannels. This technique effectively detects differences in transit time of cells passing through the cytometer.
Figure 3-2. Image analysis of cell transit time through microchannels. (A) Using video microscopy, cells are tracked as they pass into the smallest of the capillary channels. Sixteen of the 64 parallel microchannels are shown here. Scalebar 50 μm. Regions of interest (ROIs) are selected to measure cell transit time into the channels, which is defined as the amount of time it takes for a cell to enter ROI 1 and leave either ROI 4a or 4b (Bi). This neutrophil is visually tracked as it transits through ROI 1 (Bi), ROI 2 (Bii), ROI 3 (Biii), and ROI 4 (Biv). Scalebar 10 μm. Cells are tracked in each ROI by measuring the standard deviation of the pixel intensity in that region. If there is no cell present, the standard deviation is low. When a cell is present, the light scattering creates contrast in the ROI and standard deviation goes up. (C) Standard deviation of pixel intensity for each ROI is displayed versus time, with blue being low standard deviation and red being the highest standard deviation. Frames (Bi-iv) are highlighted by arrows. Two additional cells are shown passing through the ROIs at later times. A typical histogram of the distribution of neutrophil transit time is shown in (D). Cells that did not transit in less than 8 seconds are pooled into the “more” bin.
3.3.5. Data Analysis

To compare transit times of cell populations from different samples, a non-parametric ranked Mann-Whitney test was used to quantify differences with SPSS software. Cells that became stuck were given equal ranks in each population and included in analysis. All box plots represent the 25th, 50th, and 75th percentiles of the data. Chi-square analyses were used to determine significant differences in percent of cells that occluded vessels. Lastly, to determine differences in occlusion rate of capillaries a moderated linear regression was used.

No statistically significant differences in transit time between different microchannels were found, indicating consistency of flow through the parallel channels. Cell transit time was also consistent over the course of an experiment, which normally ran between 4-8 minutes, and there was no statistical correlation between cell transit time and elapsed experiment time. No significant differences in transit time were observed between different experiments of the same cell type.

3.4. Results and Discussion

3.4.1. Blood cell transit time through microchannels is not normally distributed

Statistical descriptions of cellular properties, such as deformability, in terms of a mean and standard deviation implicitly assume the property is normally distributed. We find that typical histograms of cellular transit times through the microchannels show distinct non-Gaussian distributions, indicating that blood contains biophysically heterogeneous cell populations (Figure 3-2D). Although other researchers have previously proposed multiple subpopulations of blood cell deformability (Evans, et al
2001, Nash, et al 1988), their conclusions were based on mathematical models of bulk microfiltration data. Live tracking of individual cells passing through the microchannels provides direct experimental evidence of blood cell mechanical heterogeneity in microcirculatory flow conditions and allows comparison among different populations of cells. Transit time distributions of all blood cell populations analyzed in this study are significantly positively skewed, with long right-sided tails (skewness range: 1.4 to 7.8, range of standard error of skewness: 0.012 to 0.21). Measurements of dispersion that ignore skewness, such as standard deviation or interquartile range, are consistent with previously published measurements of cell mechanical properties using comparable techniques (Armstrong, et al 1990, Inoue, et al 2006, Needham, et al 1989, Skoutelis, et al 2000b), suggesting that information about subpopulation properties is masked by a simple mean and dispersion about the mean. Thus bulk methods, which cannot assess the impact of outliers, or low throughput techniques, which do not yield large enough sample sizes, cannot accurately describe cell biophysical properties. The statistical power of biophysical flow cytometry, however, allows for robust comparisons of different blood cell types and biophysical effects of drugs and biological modifiers.

3.4.2. Inflammatory mediators change the shape and magnitude of cell transit time population distribution

The ability of neutrophils to successfully deform into capillaries much smaller than their diameter is essential for their circulation through the vascular system (Fung 1997b) Increased rigidity of neutrophils has been associated with their increased capillary retention leading to tissue ischemia seen in sepsis and acute respiratory distress syndrome (ARDS) (Drost, et al 1999, Nishino, et al 2005, Skoutelis, et al 2000a, Yodice,
Studies have shown that inflammatory mediators, such as N-formyl-methionyl-leucyl-phenylalanine (fMLP) or Tumor Necrosis Factor-α (TNF-α), are responsible for this stiffening behavior and can alone induce clogging of microfilters and increase neutrophil retention in the pulmonary capillaries (Drost, et al 1999, Olson, et al 2002, Worthen, et al 1989). As a control experiment to test the sensitivity of the microchannels in our device to changes in cell stiffness and also to address the question of how a population distribution of transit times will be affected by inflammatory mediators, we evaluated neutrophils exposed to inflammatory mediators with our device.

Populations of neutrophils with and without exposure to fMLP were flowed into the device (Figure 3-3). A typical control neutrophil (without fMLP exposure) neutrophil (Figure 3-3A) took less time to enter a microchannel than an fMLP exposed neutrophil (Figure 3-3B). As a population, the transit time of fMLP exposed neutrophils was significantly longer than control neutrophils (control n = 204, fMLP n = 182, p<0.001). The histogram of transit times of the control neutrophils shows a single peak at 0.24 s with a long tail having relatively few cells (C). A small fraction of the cells (5%) took longer than 8 s to enter into the channels. After addition of fMLP, the single peak in the histogram at short timescales drops considerably and nearly doubles to 0.40 s (Figure 3-3C). Interestingly, the most marked difference is in the increase of cells that take longer than 8 s to transit (21%), an indication that the tail of the population distribution has been extended. The population behavior can be summarized in a boxplot (Figure 3-3C inset) that shows the 25th, 50th, and 75th percentiles of the data shifting significantly upwards.
Figure 3-3. Exposure to the inflammatory mediator fMLP increases neutrophil transit time through microchannels. To determine sensitivity of the device to physiologically relevant conditions, neutrophils were passed through the device both with and without exposure to fMLP. (Ai-vi) A neutrophil not exposed to fMLP transits into and across the channel in less than 0.60 s. (Bi-vi) After exposure to fMLP, a neutrophil takes longer than 1.50 s to compress into the channel and travel across it. Cells appear as double cells in Ai, Aiv, and Bi due to interlacing. Scalebar 10 μm. (C) The histograms of the fMLP-exposed (red) and non-fMLP exposed (blue) neutrophil populations are distinctly different. After exposure to fMLP (red), the distribution of transit times increased significantly, with over 20% of neutrophils passing in greater than 8 s or clogging within the device. Median transit time of the population (inset) increased from 0.4 to 3.1 s (middle bar), and the 25th (bottom of box) and 75th (top of box) percentiles shifted up after FMLP exposure. n = 204 and 184 for control and fMLP exposed cells, respectively.

This data confirms previous findings that fMLP significantly increases neutrophil stiffness and retention in capillary-like structures (Bathe, et al 2002, Drost, et al 1999, Olson, et al 2002, Worthen, et al 1989), and it shows the ability to detect physiologically relevant changes in blood cell deformability with our cytometer. It also adds new information about the population response of white blood cells to inflammatory mediators, specifically that the response was not a linear shift in the control distribution.
but rather an increased skew of it. This suggests that obstruction of capillaries seen in the normal inflammatory response and in sepsis and ARDS may be due to a smaller number of outlying cells rather than due to an entire population of moderately stiffer cells.

3.4.3. Distribution of transit times correlates with hematologic disease pathology in leukostasis.

While the effects of altered deformability of neutrophils in sepsis have been well studied (Betticher, et al 1993, Buttrum, et al 1994, Drost, et al 1999, Worthen, et al 1989), cell deformability in some other disease pathologies have not been as thoroughly investigated. Leukostasis, a poorly understood and often fatal condition of acute leukemia in which leukemia cells aggregate in the vasculature, causes respiratory failure and brain hemorrhage (Porcu, et al 2000). Although leukostasis is known to occur more often in patients with acute myeloid leukemia (AML) than those with acute lymphoid leukemia (ALL) (Porcu, et al 2000), no effective methods currently exist to diagnose or predict leukostasis. Furthermore, chemotherapeutic treatments of leukemia cells have been shown to increase their stiffness by more than an order of magnitude (Lam, et al 2007), making monitoring of blood cell stiffness during treatment clinically important. Biophysical flow cytometry can determine if there are differences in transit time of the leukemia cells from leukostasis-symptomatic patients and whether those differences are due to a shift in the entire population or due to an increase in the number of outliers. Better knowledge of the biophysical differences among leukemia cells in leukostasis-symptomatic patients would be useful in developing assays to identify patients at risk for leukostasis and could significantly improve treatment options and reduce mortality.
To answer these questions, we analyzed samples from an AML patient with leukostasis symptoms (AML1), an AML patient without leukostasis symptoms (AML2), and two ALL patients without leukostasis symptoms, as well as neutrophils and RBCs from healthy volunteers. The leukostasis-symptomatic AML patient sample cells took significantly longer to deform into the microchannels than both the leukostasis-asymptomatic AML patient and the ALL patients, as well as the normal neutrophils and RBCs ($p < 0.001$, Mann-Whitney). The histograms of the leukemia samples show that the majority of the cells of all leukemia samples deformed into the microchannels in less than one second, with the majority of the populations forming bell curve distributions (Figure 3-4Ai-iv). Median transit times of all blood cells were remarkably similar, ranging between 0.13-0.50 s (Figure 3-4B). The leukostasis-symptomatic sample, however, had an increased distribution of slow transit time outliers. This behavior becomes more apparent when looking at both the number of cells that transited in greater than four seconds (Figure 3-4Aiv) and in the 75th percentiles of transit time (Figure 3-4B), which is over nine times higher in the leukostasis-symptomatic sample than the leukostasis-asymptomatic samples.
Figure 3-4. The distribution of transit time of cells from a leukostasis-symptomatic patient is distinct from cells of leukostasis-asymptomatic patient cells. (A) Four patient leukemia samples were flowed through the device to determine differences – two leukostasis-asymptomatic ALL samples (ALL1 and ALL2, Ai-ii, n = 239, 128 respectively), one leukostasis-asymptomatic AML sample (AML2, Aiii, n = 418), and one leukostasis-symptomatic AML sample (AML1, Aiv, n = 239). Distributions of the lower 50th percentile of the data are largely similar, but the upper 50th percentile substantially deviate. The leukostasis-symptomatic AML1 sample (Aiv) has a secondary subpopulation of cells with transit times ranging from 1-3 s. In addition, the fraction of cells that transit in greater than 4 s is substantially larger in the leukostasis-symptomatic AML1 (29%) than in the ALL and leukostasis-asymptomatic AML2 samples (9%, 8%, and 17%, respectively). (B) When looking at boxplots of the 25th, 50th, and 75th percentiles of the distributions, the differences in the population appear markedly different. While median transit times are substantially similar, the 75th percentile of the leukostasis-symptomatic AML1 sample is significantly higher than that of the leukostasis-asymptomatic samples and the RBCs and neutrophils.
Table 3-1. Summary of transit time and microchannel occlusion data in Figure 3-4 and Figure 3-5.

The fraction of cells from the leukostasis-symptomatic AML patient that were able to pass through the microchannels was also significantly lower than both the leukostasis-asymptomatic AML patient and the ALL patients, as well as the normal neutrophils and RBCs (Figure 5-3A) (Chi-square, $p<0.001$ for all comparisons with the leukostasis-symptomatic AML patient sample). A higher proportion of leukemia cells as compared to normal neutrophils and RBCs were not able to compress into the microchannels, with the leukostasis-asymptomatic AML sample having the second lowest fraction of cells passing through. This is consistent with clinical data showing myeloid leukemias having a higher propensity to cause leukostasis than lymphoid leukemias (Porcu, et al 2002).
Each microchannel that becomes occluded prevents upstream cells from flowing through. We tracked change in number of unblocked channels versus the quantity of cells that entered into the device (Figure 3-5B). While all leukemia cells created at least a small reduction in the number of unblocked microchannels during the experiments, the leukostasis-symptomatic AML patient sample occluded the microchannels at a significantly higher rate (moderated linear regression, \( p < 0.001 \) for all comparisons with the leukostasis-symptomatic AML patient sample), with only 10% of the original channels still open (3 of 31) at the end of the experiment. In acute leukemia, where leukemia cell concentrations can reach over 10 times normal white blood cell concentrations (5,000-10,000 cells/\( \mu \)L), this rate of occlusion could have profound effects.
on microvascular flow in the brain, lungs, and other vital organs. Taken together, this data shows that biophysical flow cytometry enables the detection of leukostasis in acute leukemia, even among patients with the same leukemia type, and offers a possible platform to predict and diagnose this potentially fatal complication.

Transit times of cells passing through small channels might be expected to be very sensitive to cell size, and it is not initially obvious whether variations in cell deformability or cell size are playing the most significant role in the measured transit time distributions. Similar to the multiple parameter measurements of standard flow cytometry, biophysical flow cytometry can simultaneously measure image-based parameters such as cell size with cell transit time. To investigate the impact of cell diameter on transit time, cell diameters of both the leukostasis-symptomatic and leukostasis-asymptomatic AML patient samples were measured before the cells deformed into the capillaries. Cells from the leukostasis-symptomatic AML patient sample were found to be larger than the leukostasis-asymptomatic AML patient sample (mean diameter = 9.6 μm vs 9.1 μm, respectively, p < 0.001), which would be expected to increase their transit time through microchannels. Interestingly, even when controlling for cell diameter using analysis of covariance, significant differences in transit time were still observed (p < 0.001). For both types, there was a significant but weak correlation between size and transit time (Fig 4B) (R²_{AML1} = 0.26, R²_{AML2} = 0.17). Taken together, these results indicate that although larger cells tend to transit in longer times, other factors such as deformability play more significant roles.
3.4.4. Drug treatment shifts the transit time distribution of leukemia cell populations

To test the use of biophysical flow cytometry as a platform to determine the effect of drugs on aberrant blood cell transit, HL60 cells exposed to drugs were flowed through the device. HL60 cells are an acute myeloid leukemia cell line that show a transit time distribution similar to the leukostasis-symptomatic AML sample, with a large number of long transit time outliers (Figure 3-6A-B) and a significant fraction of cells unable to deform into the channels (Figure 3-6C).
Figure 3-6. Drug treatment improved flow through microchannels. (A) HL60 cells, a model AML leukemia line with a histogram profile similar to the leukostasis-symptomatic AML1 sample, were flowed through the device without exposure to any drug (red, n = 146), after exposure to pentoxifylline (grey, n = 157), and after exposure to cytochalasin D (blue, n = 117). Substantial shifts in the histograms can be seen after exposure to drugs, with a large fraction shifting from > 4 s towards the median transit time. (B) The 25th, 50th, and 75th percentiles of the population all shifted downward after exposure to drugs, with cytochalasin D exposed cells experiencing more reduced transit time than pentoxifylline exposed cells. (C) The fraction of cells passing through the microchannels also significantly improved after exposure to cytochalasin D and pentoxifylline. (D) This resulted in a higher number of microchannels remaining open over time when drug-treated cells versus non-treated cells were flowed into the device.

Two drugs were used. The first, cytochalasin D, is a common cell biology tool for disrupting the actin cytoskeleton (Goddette and Frieden 1986). Though it cannot be used as a clinical treatment due to its systemic toxicity, cytochalasin D is known to significantly decrease cell stiffness and provides a positive control. The second drug, pentoxifylline, is a phosphodiesterase inhibitor that has been shown to reduce stiffness of TNF-α or fMLP exposed neutrophils while also reducing lung injury associated with hemorrhagic shock (Betticher, et al 1993, Deree, et al 2007, Ruef, et al 2004). We hypothesized that this drug could also attenuate stiffness of AML cells to decrease microvascular obstruction in patients with leukostasis.

We found that both drugs significantly reduced transit time of HL60s through the microchannels ($p<0.001$ for both drugs), and both drugs also created significant shifts in the distribution of transit times of HL60 cells. The non-treated cell population transit time was well spread with a low peak and a large number of slow-transiting cells (>4 s) (Figure 3-6A). Both cytochalasin D and pentoxifylline shifted the distributions towards shorter transit times and reduced the number of slow-transiting cells (Figure 3-6A). Median transit time was slightly reduced from 0.63 s to 0.23 for cytochalasin D and 0.37 for pentoxifylline, but most remarkably, the 75th percentile of transit time reduced from > 100 s (over 25% of the cells could not deform into the microchannels) to 0.70 s and 3.47 s for cytochalasin D and pentoxifylline, respectively. The fraction of cells that were able
to deform into the microchannels significantly increased from 73% to 97% and 89% for cytochalasin D and pentoxifylline, respectively, (Chi-square $p < 0.001$ for both drugs). Improved flow through the device was seen with both drugs due to reduced numbers of blocked channels (Figure 3-6D), indicating that reducing the outlying occluding cells significantly increases flow ($p < 0.001$). These results suggest that biophysical flow cytometry can be useful in identifying drugs that may be able to modulate the pathology of leukostasis.

### 3.5. Conclusions and Future Outlook

Many hematologic diseases are associated with reduced deformability of blood cells through the vasculature. We have shown that a simple microfluidic device and analysis system is able to quantify differences in blood cell deformability and that these differences are consistent with clinical outcomes. Specifically, we demonstrate here that we can detect distinctive differences between normal and aberrant blood cells that cause hematologic complications, such as microvascular occlusion. This occlusion is caused by an increased number of outliers rather than an overall shift in the cell population. In addition, we show that we can alter the distribution of transit time towards longer or shorter transit times by exposure to inflammatory mediators or drugs that affect the cytoskeleton, respectively.

Biophysical flow cytometry offers the potential to be a low-cost and straightforward tool to explore the mechanical properties of cell populations in a single-cell manner, highlighting that biophysical phenotype of individual cells may be more predictive of pathology than average population measurements. With our cytometer, we

Currently, there are no effective methods to predict or diagnose leukostasis in acute leukemia, and clinical deterioration is often rapid and irreversible, with most cases being fatal. In addition, the most effective treatment strategy for leukostasis remains unclear. Therefore a clear clinical need exists for improved techniques to identify patients at high-risk for leukostasis as well as to measure therapeutic efficacy. As measurements obtained with our cytometer were able to clearly show differences between cells taken from a leukostasis-symptomatic patient versus leukostasis-asymptomatic patients, biophysical flow cytometry may serve as a diagnostic and drug discovery tool to meet these needs.

3.6. Acknowledgements

We would like to thank Erik Douglas for assistance with microfabrication and review of the manuscript, Ross Rounsevell for assistance with confocal microscopy, Tanner Nevill for help in readying the device for its close-up, and Nick Toriello and the
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4. Leukemia cell mechanical alterations in leukostasis
4.1. Increased leukemia cell stiffness is associated with symptoms of leukostasis in acute lymphoblastic leukemia

Leukostasis, a life-threatening complication of acute leukemia, occurs when leukemia cells accumulate in and damage the microvasculature of vital organs, with the brain and lungs being the most commonly affected. Neurological signs and symptoms range from mild visual disturbances to coma, while pulmonary manifestations range from cough and dyspnea to respiratory failure (Porcu, et al 2002). Although leukostasis predominantly occurs in patients with hyperleukocytosis and with acute myeloid leukemia rather than acute lymphoblastic leukemia (ALL), no reliable clinical predictor currently exists.

The pathophysiology of leukostasis is poorly understood but is thought to involve the biophysical properties of leukemia cells and their interactions with the microvascular environment. Previous work has shown that leukemic blasts are stiffer than their more mature counterparts and have suggested that cell deformability may play a significant role in leukostasis (Lichtman 1973b).

Atomic force microscopy (AFM) is a sensitive tool for measuring cellular mechanical properties on the nanometer scale and has been used extensively in cell biology to measure single cell deformability (Radmacher 2007). Although several groups have measured the stiffness of different leukemia cell types using other techniques (Lichtman 1973b, Sharma 1993), no comparisons have been reported between acute leukemia patients with and without leukostasis. Furthermore, as leukostasis is especially rare in pediatric ALL, more research is required to determine which specific patients are at risk and should receive preventive measures (i.e. leukapheresis). To determine if cell
stiffness is associated with leukostasis, we used AFM to measure the stiffness of individual leukemia cells taken from patients with and without symptoms consistent with leukostasis.

Blood was obtained, with informed consent, from fifteen pediatric ALL patients with >10% blasts in their peripheral blood. Leukemic blasts were then immediately isolated via density-gradient centrifugation and measured with AFM. Measurements were performed with the methodology previously described using a Hertzian mechanics model (Rosenbluth, et al 2006) (Figure 4-1). Cells were immobilized in microfabricated wells to prevent movement during AFM measurements. Media was constantly perfused into and out of the sample chamber, and measurements were taken at 37°C using a heated stage.
Leukemia cell stiffness, as measured with atomic force microscopy (AFM), is higher in pediatric acute lymphoblastic leukemia (ALL) patients with leukostasis symptoms than in asymptomatic patients. (A) Suspensions of ALL cells were pipetted into a flow chamber for AFM measurements. All stiffness measurements were taken on a heated stage held at 37°C. Cells were immobilized in microfabricated wells to prevent movement during experiments, and media was perfused into and out of the chamber. All reported stiffness values represent the average of 5 consecutive AFM measurements. N≥15 cells for all populations. (B) Typical AFM cantilever deflection vs. distance curves (raw data in grey). The cell stiffness can be determined using a Hertzian mechanics model (fits are colored lines). Shown here are the curves of an ALL cell with a stiffness of 1.1 kPa taken from a patient with leukostasis symptoms (red) and an ALL cell with a stiffness of 0.06 kPa taken from an asymptomatic patient (blue). (C) Stiffness of leukemia cell populations taken from the peripheral blood of patients with symptoms of leukostasis (red) and asymptomatic patients (blue). For each sample, 75%tile, median, and 25%tile stiffness values are represented by the top, middle, and bottom lines, respectively, of each bar. A population of normal lymphocytes (white, labeled “L”) taken from a healthy individual via the same isolation protocol were used as controls for comparison. Insert: Group median stiffness of the symptomatic (red) and asymptomatic (blue) patients were 0.72 ± 0.29 kPa and 0.13 ± 0.01 kPa, respectively (p<0.001, errors represent standard error of the mean) (D) Serial cell stiffness measurements, represented by 75%tile, median, and 25%tile values, taken daily from a fifteen-year-old boy with relapsed, refractory ALL (Patient 1) receiving palliative care during his last three days of life. Median leukemia cell stiffness significantly increased during that time from 0.07 kPa to 0.41 kPa (p<0.0001) to 0.69 kPa (p<0.01). Cell stiffness values taken at one day prior to Patient 1’s death are used in Figure 1C for this patient.
Table 4-1. Patient demographic and clinical information

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infection/sepsis (all patients were afebrile at the time of the blood draw) or renal dysfunction due to uric acid nephropathy and tumor lysis syndrome was seen in any of the patients studied.

** Of note, Patient 1 was diagnosed with aspergillous pneumonia (diagnosed via broncho-alveolar lavage) 6 months prior to his death. He was successfully treated with caspofungin and voriconazole, which he received continuously until his death. During the last two weeks of his life, he did not experience any respiratory symptoms or fever until the day of his death. Serial chest radiographs revealed no abnormality during that time. The only detectable change in his clinical status, aside from increase in leukemia cell stiffness, was an increasing number of circulating leukemia cells during his last week (leukemia cell concentration during his last three days of life: 10.92, 15.57, 32.0 x 10^9 cells/L, respectively). On the day he died, he remained afebrile but developed progressively worsening respiratory failure over several hours and hemoptysis the last hour of life. The correlation between cell concentration and cell stiffness was not statistically significant (p=0.23), and the leukemia cell concentration did not reach levels consistent with hyperleukocytosis.
As the central nervous system (CNS) and lungs are the major leukostasis target organs (Porcu, et al 2002), symptoms and signs of neurologic and pulmonary dysfunction were considered evidence of leukostasis only when the onset occurred at presentation and no other etiology could be identified, similar to previous studies on leukostasis (Novotny, et al 2005, Novotny, et al 2006). Out of the fifteen patients we studied with pediatric ALL and peripheral blasts, four had symptoms and/or abnormalities on physical exam or diagnostic imaging consistent with CNS or pulmonary leukostasis (Table 4-1). All patients were newly diagnosed with the exception of Patient 1, who had multiply-relapsed refractory disease and was receiving only end-of-life palliative care at the time of this study. This patient ultimately died of respiratory failure, and leukemia cell stiffness was measured daily during his last three days of life. Leukostasis symptoms resolved for the other three patients during induction chemotherapy.

Leukemia cell stiffness values were calculated for each patient sample (Figure 4-1C). Mann-Whitney analysis showed that the median stiffness values were higher in the four patients with symptoms consistent with CNS and respiratory leukostasis (group median: 0.72 ± 0.29 kPa) than in the eleven asymptomatic patients (group median: 0.13 ± 0.01 kPa, p=0.001, effect size = -0.74, errors represent standard errors of the mean). That such a considerable statistical significance and effect size, which indicates the magnitude of difference between groups, are obtained with a relatively small patient sample demonstrates that a substantial difference exists between these two patient populations. Furthermore, the 10%tile, 25%tile, 75%tile, and 90%tile cell stiffness values were higher in the symptomatic than asymptomatic patients (p range: 0.005-0.01), indicating that the observed differences in stiffness were consistent throughout entire cell populations.
Finally, the cell stiffness variance was more prominent in symptomatic patients, raising the possibility that a small proportion of stiff cells within the population may be sufficient to trigger leukostasis.

Although leukostasis tends to occur with hyperleukocytosis (Lowe, et al 2005), the exact relationship between leukostasis risk and WBC in pediatric ALL remains unclear. Our results showed no significant difference in WBC between symptomatic and asymptomatic patients ($p=0.40$) and leukemia cell stiffness ($R^2=0.004$, $p=0.84$) did not correlate with WBC, suggesting that increased cell stiffness may be an additional independent leukostasis risk factor.

Cell stiffness measurements taken daily during Patient 1’s last three days of life showed that the median leukemia cell stiffness significantly increased from 0.07 kPa to 0.41 kPa ($p<0.0001$) to 0.69 kPa ($p<0.01$). This increase preceded the development of respiratory failure, indicating that cell stiffness trends may possibly be used to predict the onset of leukostasis (Figure 4-1D, Table 4-1).

However, the underlying cause for the observed cellular stiffness increase is unclear. We have recently shown that chemotherapy-induced cell death increases leukemia cell stiffness (Lam, et al 2007). However, no patient in this study received chemotherapy around the time the sample was drawn. Elevated cytokines levels (i.e., TNF-α), which increase neutrophil stiffness in sepsis leading to microvascular occlusion (Skoutelis, et al 2000a), may alter cell stiffness in acute leukemia, but further studies are required to elucidate the etiology of leukemia cell stiffness and the exact role it plays in leukostasis pathophysiology.
Our results suggest that increased leukemia cell stiffness is associated with leukostasis in pediatric ALL. This is consistent with recent data showing that leukostasis-positive leukemia cells have longer transit times through capillary-sized channels than cells from asymptomatic leukemia patients (manuscript submitted). Though leukostasis is rare in pediatric ALL (Lowe, et al 2005), it is associated with high mortality, and a reliable clinical predictor would enable physicians to identify high-risk patients before irreversible damage occurs. Further research is required to determine whether increased leukemia cell stiffness, as well as other factors like adhesion or transmigration, can be used as an independent risk and/or prognostic factor for leukostasis. In addition, more patients, especially those with autopsy-confirmed leukostasis, must be enrolled to definitively determine the correlation, if any, between immunophenotype, cytogenetics, and cell stiffness.

4.2. Increased leukemia cell stiffness is associated with symptoms of leukostasis in acute myeloid leukemia

Although cell stiffness measurements have only been conducted on 4 AML patients, one of whom developed and ultimately died of neurologic leukostasis at presentation, we observe a trend similar to our ALL data (Figure 4-2). However, with AML patients, the differences in cell stiffness between the leukostasis positive patient and the asymptomatic patients are less prominent than in ALL. Although more clinical data is required before any definitive conclusion can be drawn it is likely that cell stiffness has less of a role in leukostasis in AML than ALL. It is apparent that the leukostasis pathophysiology is likely different for these two different leukemia types as leukostasis incidence is higher in AML than for ALL, and symptoms of leukostasis occur
at lower leukocyte concentrations in AML than in ALL (Porcu, et al 2002). As AML cells are noted to be more adhesion and adhesive molecules are more dynamic in AML cells, cell adhesion may be the dominant factor in leukostasis in AML.

**Figure 4-2. Leukemia cell stiffness of AML patients with and without leukostasis.** Stiffness of leukemia cell populations taken from the peripheral blood of a patient who died of CNS leukostasis (black) and asymptomatic patients (gray). For each sample, 75%tile, median, and 25%tile stiffness values are represented by the top, middle, and bottom lines, respectively, of each bar. $P < 0.05$ for all pair-wise comparisons between the leukostasis positive and asymptomatic patients.
5. Modulation of leukemia cell mechanics via pharmacological agents: chemotherapy

5.1. Abstract

Deformability of blood cells is known to influence vascular flow and contribute to vascular complications. Medications for hematologic diseases have the potential to modulate these complications if they alter blood cell deformability. Here we report the effect of chemotherapy on leukemia cell mechanical properties. Acute lymphoblastic and acute myeloid leukemia cells were incubated with standard induction chemotherapy, and individual cell stiffness was tracked with atomic force microscopy. When exposed to dexamethasone or daunorubicin, leukemia cell stiffness increased by nearly two orders of magnitude, which decreased their passage through microfluidic channels. This stiffness increase occurred before caspase activation and peaked after completion of cell death, and the rate of stiffness increase depended on chemotherapy type. Stiffening with cell death occurred for all cell types investigated and may be due to dynamic changes in the actin cytoskeleton. These observations suggest that chemotherapy itself may increase the risk of vascular complications in acute leukemia.

5.2. Introduction

Alterations of biophysical properties of blood cells contribute to the pathophysiology of hematologic diseases (Eaton and Hofrichter 1995, Lipowsky 2005, Worthen, et al 1989). While chemotherapy-induced cell death has been a mainstay of cancer treatment for decades and is well studied biochemically, little is known about the mechanical effects chemotherapy may have on leukemia cells. Furthermore, since
hyperleukocytosis accompanies some cases of acute leukemia, mechanical changes in leukemia cells due to chemotherapy could significantly alter the overall blood rheology.

In this work, we quantified the effect of standard induction chemotherapy on the stiffness of acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML) cells using atomic force microscopy (AFM), a tool for imaging and characterization of materials at the nanometer scale (Binnig, et al 1986). The high force sensitivity of AFM and its ability to measure properties of individual cells over long times makes the technique particularly appropriate for measuring dynamic changes in cell stiffness. We find that when exposed to chemotherapy, leukemia cell stiffness increased by nearly two orders of magnitude at a rate dependent on the type of chemotherapy employed.

5.3. **Study Design**

5.3.1. **Leukemia cell sources and reagents**

Leukemia cells were isolated via centrifugation from the blood of newly-diagnosed acute leukemia patients noted to have peripheral blast cells. Leukemic cell lines were purchased commercially (ATCC, Manassas, VA). UCSF and UC-Berkeley Institutional Review Boards approved all experimental procedures. Dexamethasone and daunorubicin (Sigma-Aldrich, St. Louis, MO) are mainstay induction chemotherapeutic agents for ALL and AML, respectively. Accordingly, lymphoid and myeloid leukemic cells were exposed to typical treatment doses of 1 µM dexamethasone and 1 µM daunorubicin respectively (Balis, et al 1987, Speth, et al 1987). Positive staining with
either 0.9 µM Propidium Iodide (PI) or 1 µM Sytox Green (Invitrogen, Carlsbad, CA), markers for loss of cell membrane integrity, indicated cell death or late apoptosis. To identify early apoptosis, we used cresyl violet conjugated to DEVD (Immunochemistry Technologies, Bloomington, MN), an indicator for early apoptotic caspase 3 and 7 activity.

5.3.2. Atomic force microscope measurements of cell stiffness

Force microscopy measurements were obtained on a modified commercial AFM. Details involving the use of AFM for biological applications, the modifications made to our system, and the analytic methodology used to calculate cell stiffness are given in Supplemental Information and were described previously (Rosenbluth, et al 2006). Briefly, a Bioscope AFM (Veeco, Santa Barbara, CA) mounted atop an epifluorescent microscope (Carl Zeiss, Germany) held the fluid-cell-mounted cantilever (Veeco, Santa Barbara, CA). Gold-coated silicon nitride cantilevers with a spring constant between 0.009 and 0.019 N/m, calibrated by the thermal noise method (Hutter and Bechhoefer 1993), were used in all experiments. Cells were mechanically immobilized in microfabricated wells(Rosenbluth, et al 2006) within a 37°C perfusion chamber for AFM indentation. The desired chemotherapeutic agents, along with cell death markers were then added. The AFM cantilever and nearby cells could be visualized simultaneously to determine whether the cells were alive, early apoptotic, or late apoptotic/dead.
5.3.3. Leukemia cell flow through microfluidic channels

Using standard photolithographic techniques (McDonald, et al. 2000), polydimethylsiloxane (PDMS) microfluidic channels were molded from a SU-8 photoresist master on a silicon wafer. Microchannels were designed to geometrically emulate a microvasculature network branching into 5 μm wide by 12 μm tall capillary-sized channels. ALL cells were incubated with dexamethasone for 6 hrs yielding ~10% cell death by trypan blue exclusion and then passed through the channels driven by a physiologic pressure difference of ~1 kPa typically observed across capillary beds (Fung 1997a). Microchannel obstruction by live and dead cells were visualized with brightfield and epifluorescence microscopy (Carl Zeiss, Germany).

5.3.4. Statistical analysis

All reported cell stiffness values represent the average of 5 consecutive AFM measurements. Stiffness levels in the different cell populations (n≥15 cells unless otherwise specified) were compared with analyses of variance using two-tailed significance tests. Errors are reported as standard errors of the mean.

5.4. Results and Discussion

5.4.1. Leukemia cells stiffen after exposure to chemotherapy

In the presence of chemotherapeutic agents, dexamethasone for ALL cells and daunorubicin for AML cells, leukemia cells exhibited a 14 to 91-fold increase in stiffness
as they underwent cell death (Figure 5-1A-C, Table 4-1 summarizes patient data). The average stiffness of both lymphoid and myeloid leukemia cells held at 37°C and exposed to chemotherapy (mean: 4.7 kPa) was significantly higher than the average stiffness of untreated control populations (mean: 0.2 kPa, \( p<0.05 \), Figure 5-1D-E). This observation was not, however, isolated to chemotherapy-induced cell death, as Fas-induced apoptotic cells and the rare dead cells in control populations were also noted to be significantly stiffer than live, untreated cells (data not shown). Although cell death is often coupled with a decrease in cell volume (Hessler, et al 2005, Lang, et al 2000), we found that cell shrinkage occurs after chemotherapy-induced cell stiffening (Figure 5-2). This suggests that the cause of stiffness increases is not simply increased density due to decreased volume.

Results from microchannel experiments using primary leukemia cells exposed to chemotherapy show that dead cells are more likely to obstruct capillary-sized channels and cause cell aggregation than live cells (Figure 5-1F-G). The fraction of cells initiating microchannel obstruction relative to those traversing the microchannel system was approximately seven times higher for dead cells than live cells.
Figure 5-1. Chemotherapy-induced cell death increases the stiffness of leukemia cell populations measured by AFM. (A) An illustration of the AFM setup (not to scale). A single cell sitting within a microwell is immobilized for force microscopy with an AFM cantilever. A polydimethylsiloxane (PDMS) collar is pressed upon the glass to create an open-air chamber. Tubes entering and exiting the chamber continually pass media through, keeping the media fresh over the long time scale of the experiments. The piezoelectric stage moves vertically, causing the cantilever to deflect against the cell. The stage is maintained at 37° C throughout the experiment. (B) An epifluorescence/brightfield overlay of a typical experiment. Seen here are an AFM cantilever tip and two dead K562 cells (PI positive, fluorescent), with the left cell immobilized in a microwell. An empty microwell is at the top. Scalebar is 20 µm. (C) Two typical cell indentation acquisitions. As the piezoelectric platform moves the cells up against the cantilever (in the direction of the arrow), the cantilever deflects. When the curves are fit to an elastic Hertzian model, the stiffness of the cells can be determined. The stiffness of a Pre-B ALL cell exposed to 1µM dexamethasone (red) was 4.3 kPa whereas the stiffness of a control (not exposed to chemotherapy) Pre-B ALL cell (green) from the same patient was 0.2 kPa. (D) Dead (red) lymphoid leukemic cells exposed to 1µM dexamethasone are significantly stiffer than untreated (green) cells. (E) Dead (red) myeloid leukemic cells exposed to 1µM daunorubicin are significantly stiffer than untreated (green) cells. Error bars represent standard error. (n>15, p<0.05 for all comparisons of dead/untreated populations). (F) Dual bright field/epifluorescence microscopy of dexamethasone-exposed Pre-B ALL cells that were passed, from left to right, through PDMS microfluidic channels modeling a branching microvasculature network. Dead (PI+) cells (red arrows) were more likely than live (unstained) cells (green arrows) to initiate obstruction and cause cell aggregation in the 5 µm wide by 12 µm tall capillary-sized channels. Frame from (G) was taken 15 sec after (F), illustrating the relative mobility of two live cells, one of which has left the field of view, compared to dead cells that remain fixed in place. Scalebar is 10 µm.
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<th>Blast cell count (%)</th>
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<td>9</td>
<td>M2 AML</td>
<td>M</td>
<td>78</td>
<td>22 x 10$^3$</td>
<td>82%</td>
<td>Peripheral blood</td>
</tr>
<tr>
<td>10</td>
<td>M4 AML</td>
<td>M</td>
<td>76</td>
<td>127 x 10$^3$</td>
<td>&gt;90% (marrow)</td>
<td>Bone marrow (cryopreserved)</td>
</tr>
<tr>
<td>11</td>
<td>M5b AML</td>
<td>M</td>
<td>8</td>
<td>104 x 10$^3$</td>
<td>&gt;90% (marrow)</td>
<td>Bone marrow (cryopreserved)</td>
</tr>
</tbody>
</table>

Table 5-1. Demographic and diagnostic data of patient samples. Measurements on Samples 1-6 and Samples 7-11 are represented in Figure 1-D and Figure 1-E respectively. Of note, two samples (Samples 10 and 11) comprised primary leukemia cells from the UCSF Hematopoietic Tissue Cell Bank that were thawed from cryostorage, a process which may alter cellular properties. However, control experiments with HL60, Jurkat, and primary Pre-B ALL cells showed no significant difference in stiffness before and after cryopreservation ($p=0.43$, $p=0.37$, $p=0.28$ for HL60, Jurkat, Pre-B ALL cells, respectively).
Figure 5-2. Cell size changes with cell death. (A) As the stiffness of a single HL60 cell (red circles) increases with exposure to 1µM daunorubicin, the average cell area of the cell population (green triangles, \( n = 8-38 \) cells/field of view) remains constant until cell death (transition from open to solid shapes). Time is also normalized to the point of cell death (grey line). (B) At the point of cell death, both HL60 (red triangles) and Jurkat (green circles) cell lines show a rapid decrease in cell volume. Jurkat cells lose on average 12% of their volume and HL60 cells lose 26% of their volume within 10 minutes (\( n=10 \) and \( n=7 \), respectively). Error bars represent standard error of the mean.

5.4.2. Cell death and stiffness kinetics are dependent on chemotherapy type

To determine the relationship between increasing cell stiffness and chemotherapy exposure time, serial single-cell stiffness measurements were taken over several hours after exposure. During stiffness measurements on each cell (\( n = 15-20 \)), the apoptotic state was tracked using dual fluorescent labeling with cresyl violet-DEVD and Sytox Green. Figure 5-3A shows the change in stiffness for a single leukemic cell taken from a patient newly diagnosed with M5 AML after it was exposed to daunorubicin. Cell
stiffness began increasing within an hour of exposure and increased most significantly after apoptosis was detected, peaking near the point of cell death. Interestingly, cell stiffness began to increase before peak caspase activation in apoptosis. Accordingly, for the same patient sample, the average apparent stiffness for populations of control cells, early apoptotic cells, and late apoptotic/dead cells exhibit significant increases with progression through the stages of chemotherapy-induced cell death (Figure 5-3B, \(p<0.05\)).

From serial single-cell stiffness measurements on several patient samples and cell lines, different chemotherapeutic agents were found to change cell stiffness at different rates (Figure 5-3C). In general, the stiffness of myeloid leukemia cells exposed to daunorubicin rapidly increased before the onset of cell death and then stabilized. Lymphoid leukemia cells exposed to dexamethasone exhibited a more gradual increase in cell stiffness as cell death occurred. Control cells from each sample remained PI negative throughout the experiments, and their stiffness remained close to the initial stiffness. To determine whether the difference in stiffening rate is due to the chemotherapeutic agent or to the cell type, populations of HL60 (myeloid) and Jurkat (lymphoid) cells were exposed to daunorubicin and dexamethasone separately, and cell deformability was tracked in four separate experiments. For both HL60 and Jurkat cell lines, cells exposed to daunorubicin exhibited an increase in cell stiffness and onset of cell death significantly earlier than cells exposed to dexamethasone, indicating chemotherapy type dominates the kinetics of cell stiffness, not leukemia cell type.
Figure 5.3. Stiffness of leukemic cells increases with progression of cell death and is attenuated by disruption of the actin cytoskeleton. (A) A typical stiffness trace of a single M5 AML cell exposed to 1µM daunorubicin (red circles). The apparent stiffness of a typical control cell remains relatively constant (green triangles) and does not undergo apoptosis or cell death during the course of the experiment. Error bars represent standard error. (B) From the same patient sample, the average apparent stiffness of a population of late apoptotic/dead AML cells was significantly stiffer than early apoptotic cells and controls (n=15, \( p < 0.05 \)). (C) Cell stiffness increases faster with 1µM daunorubicin (DNR, in red) than 1µM dexamethasone (DEX, in green). Solid and dotted lines represent myeloid and lymphoid leukemia cells, respectively. Transition from open to filled shapes represent onset of cell death (PI positive staining). (D) Exposure to 2 µM cytochalasin D, an actin polymerization inhibitor, reduces stiffening behavior in HL60 cells exposed to 1 µM daunorubicin. The cells represented by these three lines were exposed to daunorubicin at time 0. The cell represented by the green line was also exposed to cytochalasin D at time 0 (vertical green dashed line) and exhibited little stiffening behavior. The cell represented by the blue line was exposed to cytochalasin D after 45 minutes (vertical blue dashed line) and exhibited little stiffening behavior after exposure. As a positive control, the cell represented by the red line was not exposed to cytochalasin D. (E) HL60 and Jurkat cells were incubated with 1 µM daunorubicin and 2 µM cytochalasin D. The average stiffness of dead HL60 cells (n=15) exposed to daunorubicin and cytochalasin D (green) was 0.2 ± 0.05 kPa, whereas the average stiffness of dead HL60 cells exposed to daunorubicin alone (red) was 1.2 ± 0.3 kPa (\( p < 0.05 \)). Likewise, the average stiffness of dead Jurkat cells (n=15) exposed to daunorubicin and cytochalasin D (green) was 0.1 ± 0.03 kPa, whereas the average stiffness of dead Jurkat cells exposed to daunorubicin alone (red) was 0.5 ± 0.14 kPa (\( p < 0.05 \)).

5.4.3. The actin cytoskeleton contributes to cell stiffness increase in dying cells

cytoskeleton was involved in the observed chemotherapy-induced stiffness increase, the stiffness of single HL60 cells exposed to daunorubicin were tracked as 2 µM cytochalasin D, an inhibitor of actin polymerization, was added (Figure 5-3D). Cells exposed simultaneously to both cytochalasin D and daunorubicin exhibited almost no increase in cell stiffness as cell death progressed. When cytochalasin D was added 45 minutes after daunorubicin exposure, as cell stiffness was already increasing, cell stiffness ceased to increase within 15 minutes and declined to 40% of its maximum stiffness after 70 minutes. The average stiffness of dead HL60 and Jurkat cell populations exposed simultaneously to daunorubicin and cytochalasin D was found to be significantly less than the stiffness of cells exposed to only daunorubicin ($p<0.05$, Figure 5-3E). These decreases in stiffness due to cytochalasin D suggest that the stiffness increase with chemotherapy-induced cell death is at least partly due to dynamic changes in the actin cytoskeleton.

5.4.4. Clinical implications of chemotherapy-induced cell stiffening

Our results reveal that chemotherapy-induced cell death increases the stiffness of leukemia cells, which may influence vascular flow in the microcirculation. This observed link between cell death and increasing cell stiffness may have implications for acute leukemia patients with hyperleukocytosis. In some cases, clinical deterioration due to leukostasis paradoxically does not occur until after chemotherapy has been initiated (Lokich and Moloney 1972, Myers, et al 1983, Porcu, et al 2002, Wurthner, et al 1999), and alteration of leukemic cells’ biophysical properties by chemotherapeutic agents has
been hypothesized as a possible link (Lichtman and Rowe 1982, Lokich and Moloney 1972, Myers, et al 1983). Further research is needed to fully characterize the impact of decreased deformability of dying cells, as well as the role of other factors like leukemia cell-endothelial interactions and transmigration, on the pathophysiology of leukostasis in acute leukemia. With the capability to investigate these biophysical phenomena at the single-cell level, new research platforms like AFM and microfluidic assays may provide valuable insight into this and many other hematologic problems.

5.5. Supplemental Information

5.5.1. Atomic force microscope (AFM) measurements of cell stiffness

AFM uses a flexible cantilever to both measure and apply forces. As the cantilever interacts with a surface, its deflection can be measured to sub-nanometer precision by reflecting a laser onto the end of the cantilever and into a quadrant photodetector. The sample’s position relative to the cantilever can be controlled to nanometer precision via a piezoelectric positioning platform. Stiffness can be measured by indenting a cell with a cantilever at a constant speed with respect to the sample. Deflection of the cantilever as it indents the sample is directly proportional to the applied force, since force is linearly related to loading force for small deflections. Cells were typically indented 2-3 μm. An elastic model (Hertz 1882) can then be fit to the force versus indentation data to determine cell stiffness. The Hertzian mechanics model was selected because of its extensive use with AFM (Radmacher 2002, Radmacher, et al 1996, Wojcikiewicz, et al 2004) and previous analysis by the authors. For further
discussions on the appropriateness of this model for leukocytes and leukemia cells, see Rosenbluth et al (Rosenbluth, et al 2006).

A perfusion chamber was built to maintain fresh media during the experiments by molding a ring of PDMS and mounting it onto the microwell substrate. Media was perfused into the chamber at 0.6 mL/hr and out of the chamber at 0.5 mL/hr via syringe pumps (Harvard Apparatus, Holliston, MA and Cole Parmer, Vernon Hills, IL) to account for evaporative loss. Because temperature was noted to affect cell stiffness of leukemic cells, all measurements were taken at 37° C. Closed-loop temperature control was maintained via resistive heaters (Caddock, Riverside, CA) and a thermistor mounted onto a custom-built aluminum sample platform controlled by a variable DC temperature controller (Harvard Apparatus).

Because non-activated leukocytes and leukemia cells are non-adherent, cells were immobilized in microwells (Rosenbluth, et al 2006). Fabrication of these wells is described in Rosenbluth et al (Rosenbluth, et al 2006). Briefly, photocurable epoxy (SU-8 2007, Microchem, Newton, MA) was spun onto piranha-cleaned Borofloat glass wafers (Precision Glass and Optics, Santa Ana, CA), prebaked, and exposed though a mask. After post-baking and development, wells between 8 and 20 µm diameter were left on the glass surface. Well depth (8 µm) was controlled by spin speed during SU-8 application. Cell suspensions of approximately 100,000 cells/mL were placed onto the microwells in the perfusion chamber.

Much of the analytical methodology was previously characterized with a similar experimental setup using leukemia cell lines and normal neutrophils (Rosenbluth, et al 2006). Cell viability and morphology were the same for cells incubated atop SU-8 as
those incubated atop glass. Cells were selected to be smaller than the wells in which they were placed. No significant correlation between cell size and stiffness was observed ($p = 0.83$). Some cells were pushed into the microwells by the AFM cantilever before probing. These cells did not differ in stiffness from those that fell into the microwells ($p = 0.93$). Cells were indented at rates from 25 nm/s to over 8000 nm/s. Viscous effects were seen at rates above 415 nm/s. At rates below 415 nm/s, there was no rate dependency on measured stiffness

### 5.5.2. Cell size measurements of cells exposed to chemotherapy

To correlate changes in cell size with cell stiffness after exposure to chemotherapy, images of the cells of interest were acquired with each cell stiffness measurement using the brightfield and fluorescence microscope coupled to the modified AFM. Because the refraction from the microwell walls prevented an accurate size measurement of the cell of interest, the average cell area of the entire cell population in each field of view was calculated for each measurement. Images were acquired with an Axiovert 25 Microscope, 60X 0.7 NA objective, (Nikon, Melville, NY), QColor3 camera (Olympus, Center Valley, PA) and ImageJ software (NIH, Bethesda, MD).

To track changes in cell volume after exposure to chemotherapy, the following procedure was used: Cells were incubated with 7 μM of the volume marker CellTracker Orange CMRA (Molecular Probes, Eugene, OR) for 30 minutes in serum-free RPMI 1640. Cells were then spun down and media was replaced with CO$_2$ Independent Medium. A concentration of 5 μM Sytox Green was added to determine cell death. Then
1 μM of daunorubicin for HL60 cells and 1 μM dexamethasone for Jurkat cells was added. Cells were then placed in a Molecular Cytomics (Boston, MA) LiveCell Array which allows for non-adhesive cells to remain immobile without fixation for tracking and imaging over long time periods. Images were acquired at one-minute time intervals with an Axiovert 200 Microscope, 40X 0.6 numerical aperture (NA) objective, (Carl Zeiss, Thornwood, NY), Cascade II camera (Photometrics, Tucson, AZ), and Metamorph software (Molecular Devices, Downingtown, PA).

5.5.3. Leukemic cells decrease in size when undergoing chemotherapeutic cell death

Cell death is often coupled with a decrease of cell size that (Hessler, et al 2005, Lang, et al 2000), if significant enough, may offset the associated increase in stiffness. To correlate in time changes in cell size with cell stiffness after exposure to chemotherapy, the average cell area of the cell population in each field of view was calculated for each cell stiffness measurement. During AFM measurements, cell stiffness was noted to markedly increase before the onset of cell shrinkage associated with cell death (Figure 5-2). As the stiffness of a single HL60 cell increased with exposure to daunorubicin exposure time, the average cell area of the cell population (n = 8 to 38 cells/field of view) remained constant until cell death. Within 31 minutes, the average cell area decreased 20% and remained constant thereafter.

To confirm cell volume change with chemotherapy-induced cell death, we estimated cell volume loss by tracking the average intensity of a cell volume tracker. HL60 cells began quickly losing volume several minutes before they positively stained
for Sytox Green (Figure 5-2). Jurkat cells also decreased in volume before Sytox Green positivity, but closer to the point of death. HL60 cells shrunk by near 30% prior to cell death marker staining, and then they gradually decreased in volume post cell death. Jurkat cells shrunk by 12% prior to cell death marker staining and then remained relatively static in volume for the next forty minutes. There was large variability in cell volume once the cells died. While some cells barely changed in volume, others decreased in volume by over 50%. From these findings we expect that the physiological effect of stiff dead cells in the microcirculation may be partially offset by cell shrinkage although the increase in cell stiffness appears to begin before cell size decrease.

5.6. **Acknowledgements**

The authors wish to thank Kevin Shannon, Benjamin Braun, Michelle Hermiston, Todd Sulchek, Sanjay Kumar, Sapun Parekh, Joshua Shaevitz and Martijn van Duijn for discussions and careful reading of the manuscript. Microfabrication was performed in the UC Berkeley Microlab. Several patient samples were obtained from the UCSF Hematopoietic Tissue Cell Bank courtesy of Mignon Loh.
The tools and techniques described in Chapters 2 and 3 have the potential to serve as drug discovery platforms for potential therapies in leukostasis. One such candidate therapeutic agent is the phosphodiesterase inhibitor pentoxifylline, which is known to decrease red cell deformability and has been used for several decades to improve microvascular blood flow in peripheral arterial disease. More recently, pentoxifylline has been shown to modulate inflammatory processes including the attenuation of leukocyte stiffening and adhesion that occur upon activation. Therefore, recent clinical studies have concluded that this drug may be an effective adjunctive therapy for acute lung injury and other disease states in which the inflammatory response is exaggerated and detrimental for the patient. However, no reported studies have investigated the biophysical effects of leukemia cells in the context of leukostasis. Here we describe preliminary results in which we measure the effect pentoxifylline has on leukemia cell stiffness and adhesion.

6.1. **Pharmacology of pentoxifylline**

Pentoxifylline, or 1-(5-oxohexyl)-3, 7-dimethylxanthine, is a xanthine derivative and has been clinically approved for the treatment of symptoms due to peripheral arterial disease resulting from obstructed arteries in the limbs, and vascular dementia, in which arterial insufficiency in the brain microvasculature affects neurologic and cognitive function (Samlaska and Winfield 1994). Pentoxifylline improves blood flow through blood vessels and therefore helps with blood circulation in the arms and legs (e.g. intermittent claudication). This drug has also been used for stroke and sickle cell disease, although clinical studies have had mixed results and there has not been definitively proven benefits in morbidity or mortality in those diseases (Ward and Clissold 1987).
Pentoxifylline’s exact mechanism of action remains unclear and is likely complex. However, it is known that pentoxifylline functions as a phosphodiesterase inhibitor, thereby increasing intracellular cAMP, which is a common intracellular second messenger in multiple cell signaling pathways. More recently, the drug has been shown to exert anti-inflammatory and anti-thrombotic effects on leukocytes, platelets, and endothelial cells (Lassiter 2000, Samlaska and Winfield 1994, Ward and Clissold 1987).

6.2. Effect of pentoxifylline on leukemia cell stiffness and adhesion


To determine if pentoxifylline alters leukemia cell mechanical properties, we exposed HL60 myeloid leukemia cells to pentoxifylline and found that leukemia cell transit time through microchannels decreased significantly, as described in Chapter 3 (Figure 3-6). In addition, we also used our previously published AFM protocols using microwells for cell immobilization and a flow chamber with heated stage to perform AFM measurements in an environment conducive for cell culture (Lam, et al 2007, Rosenbluth, et al 2006). HL-60 cells, a commonly-used promyelocytic leukemia cell line, and Jurkat cells, a commonly used T-cell leukemia cell line, were used as models for
AML and ALL cells, respectively. For each experiment, three cell subpopulations were compared: controls (no exposure to pentoxifylline), cells exposed to 1 mM pentoxifylline for 90 minutes, and cells exposed to 1 mM pentoxifylline for 180 minutes. Our data indeed showed a time-dependent change in stiffness for both cell lines when cells are exposed to pentoxifylline. Interestingly, the drug appeared to have opposite mechanical effects on the two cell lines. Stiffness for the AML cells decreased with pentoxifylline exposure over 3 hours, whereas it increased for ALL cells over the same time interval (Figure 6-1). Theses results indicate the pentoxifylline does in fact alter the mechanical properties of leukemia cells.

**Figure 6-1. Pentoxifylline alters leukemia cell stiffness in a time-dependent manner as shown by AFM.** (A) The stiffness of myeloid HL-60 cells decreases over time with exposure to pentoxifylline. Cells exposed to pentoxifylline for 90 minutes (n=15) showed a marginally significant decrease in stiffness compared to control cells (n=22, p=0.06), whereas a statistically significant decrease in stiffness was noted between cells exposed to pentoxifylline for 180 minutes (n=13, p=0.02) and controls. (B) In contrast, the stiffness of lymphoid Jurkat cells increases over time with pentoxifylline exposure. Cells exposed to pentoxifylline for 90 minutes (n=14) showed a significant increase in stiffness compared to control cells (n=15, p=0.03). Although stiffness continued to increase with further exposure to pentoxifylline, cell exposed to pentoxifylline for 180 minutes (n=11) only showed a marginal statistical difference when compared to the cells incubated for 90 minutes (p=0.075)

To investigate how pentoxifylline modulates the adhesive interactions between leukemia cells and human umbilical vein endothelial cells (HUVECs), a static adhesion assay was used in which pentoxifylline-exposed leukemia and endothelial cells were co-incubated, washed, and remaining adherent cells were quantified using brightfield microscopy.

Myeloid leukemia cells showed a statistically significant decrease in adhesion to endothelial cells (Figure 6-2). However, in the presence of the inflammatory cytokine TNF-α, pentoxifylline had no significant effect on altering the adherence of both HL-60 and primary AML cells to HUVECs. In addition, pentoxifylline exhibited no differential effect on leukemia cells taken from the leukostasis-positive patient or the asymptomatic patient.
Figure 6-2. Pentoxifylline decreases myeloid leukemia cell adhesion to human umbilical vein endothelial cells (HUVECs). HL-60 leukemia cells and primary AML cells taken from a patient who subsequently died of leukostasis and an asymptomatic were incubated with varying doses of pentoxifylline (with or without TNF-α) for 45 minutes, then co-incubated with HUVEC monolayers (with or without TNF-α) for another 45 minutes, and non-adherent cells were then washed away. Adherent cells were visualized and quantified with brightfield optical microscopy. Results are displayed as a percentage of remaining adherent cells relative to the corresponding control condition. Asterisks indicate statistically significant differences ($p<0.05$) between the designated and the corresponding control populations.

In contrast to the myeloid leukemia cells, lymphoid Jurkat leukemia cells increased adherence to HUVECs when exposed to pentoxifylline in the same experimental conditions. There was no increased adhesion, however, when cells were exposed to TNF-α in addition to pentoxifylline, implying that in lymphoblasts, pentoxifylline may trigger similar pathways as inflammatory cytokines.
Figure 6-3. Pentoxifylline increases lymphoid leukemia cell adhesion to human umbilical vein endothelial cells (HUVECs). Jurkat leukemia cells were incubated with varying doses of pentoxifylline (with and without TNF-α) for 45 minutes, then co-incubated with HUVEC monolayers (with and without TNF-α) for another 45 minutes, and non-adherent cells were then washed away. Adherent cells were visualized and quantified with brightfield optical microscopy. Results are displayed as a percentage of remaining adherent cells relative to the corresponding control condition.

As described in the previous chapters, leukostasis in acute leukemia is likely due to the biophysical alterations of leukemic cells leading to microvascular obstruction in vital organs. As pentoxifylline has been shown to soften neutrophils in both inactivated and activated states and decrease the adhesive leukocyte-endothelial interactions, this drug is a logical target for leukostasis treatment or prevention. Our data suggest that pentoxifylline does in fact affect leukemia cell mechanical properties, but interestingly, seems to have a differential effect between myeloid and lymphoid leukemia cells. Whereas pentoxifylline decreases the stiffness and endothelial cell adhesiveness of primary AML and HL-60 cells, the stiffness and adhesiveness is increased with lymphoid Jurkat T lymphoblasts. The underlying mechanisms for these effects are unclear and will require further study. In addition, more data with primary cells and other leukemia cell
lines need to be collected before any definitive conclusions can be drawn regarding the potential for using pentoxifylline to prevent or treat leukostasis. However, the mechanical changes we have documented do suggest that pentoxifylline may decrease microvascular occlusion due to AML cells and improve microcirculatory flow and justify more \textit{in vivo} patient-based research to determine if this drug has the potential to ameliorate leukostasis in AML. We will continue our current studies to further clarify the biophysical effects this drug may have on leukemia cells in the context of leukostasis.

Currently, there is no medication that successfully treats and prevents the life-threatening complication of leukostasis in acute leukemia. As pentoxifylline has been in use for several decades for the treatment of other diseases and is known to be a well-tolerated drug with minimal side effects, the risk-benefit ratio is significantly low and warrants a prospective clinical trial exploring the use of this drug for newly diagnosed patients with AML.
7. Clinical implications and conclusions
This dissertation describes the clinical relevance of the nascent field of cell mechanics and discusses how the techniques of this discipline can further our knowledge of hematologic diseases in which cell mechanical properties are altered. Specifically, this body of work uses the tools of cell mechanics to further our understanding of leukostasis, a complication of acute leukemia that is thought to be caused by alterations in leukemia cell mechanical properties, such as adhesion and cell stiffness. Interestingly, this biophysical theory of leukostasis has existed for decades, but never thoroughly investigated, likely due to the lack of necessary tools. It is only because of recent technological advances, namely with the advent of atomic force microscopy and microfabrication techniques in biological research, were we able to conduct our experiments to learn more about this life-threatening complication of leukemia.

As described in Chapter 2, our first goal was to characterize an atomic force microscopy technique to measure the stiffness of single leukemia cells. The first technical challenge we encountered was the necessity to immobilize leukemia cells for measurements while not activating them, which might lead to subsequent changes in mechanical properties and therefore lead to confounding data. To address this, we employed basic microfabrication techniques to create “microdivots” which were slightly larger than leukemia cells and provided lateral stabilization of the cell during AFM measurements. We established that this was a viable technique to measure cell stiffness and found that myeloid leukemia cells were stiffer than lymphoid leukemia cells, which is consistent with the clinical observation that leukostasis occurs more commonly in AML than ALL.
Although AFM is extremely sensitive, it suffers from low-throughput. Chapter 3 describes the use of a simple microfluidic device as a biophysical flow cytometer, capable of high-throughput, single cell mechanical analysis. Using this device we found that leukemia cells from patients with symptoms of leukostasis had significantly longer transit times than leukemia cells from asymptomatic patients. Although this does not prove that leukemia cell stiffening causes leukostasis, the data demonstrates that biophysical differences do exist between leukostasis-positive and leukostasis-negative leukemia cells. This data is extremely clinically relevant as it establishes that our biophysical flow cytometer has the sensitivity to potentially identify patients at risk for leukostasis. No existing assay has this capability and currently there is no reliable way to accurately predict leukostasis or identify high-risk patients.

As described in Chapter 4, AFM measurements taken from the leukemia cells of a larger group of newly diagnosed patients with and without leukostasis definitively show that the leukemia cell stiffness is higher in leukostasis-positive patients than in leukostasis-negative patients. This further illustrates the clinical utility of single-cell biophysical measurements and justify their potential use as a diagnostic marker for leukostasis.

Chapters 5 and 6 discuss how therapeutic agents may modulate leukemia cellular mechanical properties. Using AFM, we show that chemotherapy-induced cell death causes leukemia cell stiffening and that the rate of cell stiffness increase is dependent on the rate of cell death. This increase in cell stiffness is rapid and occurs before standard apoptosis markers are detectable. These results imply that the initiation of standard chemotherapeutic agents, especially those with a rapid onset of action, may paradoxically
put the patient at risk for leukostasis until the immune system can eliminate the dying stiff cells from circulation. In addition, this data also suggest that removal of circulating leukemic cells via leukapheresis may benefit certain at-risk patients before the initiation of chemotherapy, although more data is necessary to exactly define this patient demographic. Finally, in Chapter 6, I describe some preliminary work investigating the biophysical effects of the drug pentoxifylline on leukemia cells. The decrease in adhesiveness and cell stiffness of neutrophils deformability due to this phosphodiesterase inhibitor has been well characterized, but there has been no published work studying the effects of this drug on leukemia cells. Our studies show that whereas pentoxifylline decreases the myeloid leukemia cell stiffness and adherence to endothelial cells, this agent increases lymphoid cell stiffness and adherence to endothelial cells. Although more studies are obviously required to fully characterize the cell mechanical effects of pentoxifylline on leukemia cells, our work to date suggests that this agent may be a possible therapeutic modality to decrease leukostasis risk in newly diagnosed myeloid leukemia patients.

Although we have made some valuable contributions to the field of clinical hematology with our studies, leukostasis remains to be a poorly understood complication of acute leukemia and a multitude of questions remain. What is the role of adhesion molecules between leukemic and endothelial cells? How do cytokines and other soluble inflammatory molecules factor into these interactions? Out of the several biophysical parameters in question, which dominate and are the most significant causes of leukostasis? What role do platelets and coagulation have in leukostasis? We have only scratched the surface in our comprehension of leukostasis pathophysiology, but we have
now characterized several tools and techniques that will enable future leukostasis research to be conducted in a more efficient and informative manner. I and the rest of my colleagues will continue this line of work and hopefully, within a few years, this line of research will directly lead to therapies and new assays to diagnose or predict this dreaded complication and ultimately save lives.
8. References


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