Characterizing the Elastic Properties of Naïve, Activated, and Re-Activated CD8+ T-Cells

Sulchek Lab

Morgan Labrie

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Abstract

CD8+ T-cells are a crucial part of the adaptive immune system, which helps restore the body back to health when harmful pathogens such as bacteria or viruses are identified in the body. Once a pathogen is identified, CD8+ T-cells are activated and undergo a conformational change, then migrate through the body until identifying a specific antigen. The CD8+ T-cells then undergo a second activation and conformational change in order to begin fighting the pathogen. Not much is known about the mechanical properties of CD8+ T-cells during these conformational changes. This study looks at the stiffness of these cells during naïve, activated, and re-activated states through measurements taken by Atomic Force Microscopy. Evaluation of results showed that after the first activation, CD8+ T-cells become softer, and after re-activation, become more stiff, which aligns with the properties that may aid these cells in their migration and attack of pathogens. These results show promise for characterizing the relationship between stiffness and the lifecycle of CD8+ T-cells and could have future implications for potential treatments involving the immune system.

Introduction

The Immune System

The immune system, responsible for protecting the human body against potentially harmful foreign entities, is an important part of survival. One of the main tasks of the immune system is to recognize pathogens, bacteria, or other harmful molecules that have entered the body and disable their ability to be harmful by killing

them, removing them, or neutralizing them. Additionally, the immune system protects against harmful substances in the environment that could interact with the body. The third main purpose of the immune system is being able to recognize when an individual's own cells have changed or mutated in ways that are harmful to the body and enact a defense mechanism to halt proliferation or induce apoptosis to these specific cells. When an individual is healthy, the functioning of immune system is not apparent. However, when the body encounters a pathogen, or some type of injury, the immune system is responsible for restoring the body back to health.[1]

The immune system can be divided into two subsystems: the innate immune system and the adaptive immune system. The innate immune system involves a variety of immune cells, and relies heavily on phagocytosis and recognition of common pathogen associated molecular patterns (PAMPs) through toll-like receptors (TLRs) on immune cells. Once a foreign pathogen is recognized, the innate immune system triggers a defense response which occurs during the first and most critical hours of exposure. As bacterial cells have fast replication times, it is important for the innate immune system to react quickly upon recognition of these pathogens in order to minimize the possibility of spreading throughout the body. Many of these cells have different features such as glycoproteins, Celia, or peptide strands that all work together to protect the body. For example, endothelial cells are riddled with *defensins* which are microbial peptides that aid in halting pathogen proliferation[2]. However, the innate immune system is not able to differentiate between specific types of pathogens or adapt to encountering substances the body has never been exposed to. This paves the way for the other subset of the immune system - the adaptive immune system.

The adaptive immune system primarily comprises of B-cells, T-cells, and dendritic cells (DCs). B-cells develop from stem cells in bone marrow, and are responsible for developing and secreting antibodies, which then locate pathogens using pathogen-specific receptors[3]. DCs are responsible for identifying the antigen-presenting cells and triggering the immune system response[4]. T-cells, however, comprise a larger population of differentiated immune cells.

CD8+ T- Cells

T-cells are a specific type of white blood cells that circulate in the blood. They are created in the bone marrow of adult humans[1], and upon leaving the bone marrow, travel to the Thymus gland. There, they mature and differentiate into different types of T-cells including CD8+ T+cells[5]. CD8+ T-cells, often called 'killer T-cells', go through two stages of activation. Once the immune system recognizes a pathogen, CD8+ T-cells are activated, and migrate through the blood until identifying a specific antigen. Once they reach the antigen, they go through a second activation. They then induce apoptosis of the pathogen by the secretion of cytokines, the release of cytotoxic granules, and the destruction of infected cells through Fas/Fas L Interactions.[6] There is a lot of information on the biology underlying the conformational changes that CD8+ T-cells undergo; however, the mechanical properties that change during these phases have never been studied.

The present study focuses on the lifespan of CD8+ cells, and the differing of mechanical properties, specifically stiffness, between the naive, activated, and reactivated phases.

Atomic Force Microscopy

Atomic Force Microscopy (AFM) could potentially serve as a powerful tool for characterizing the specific properties of the various groups of immune cells. AFM is able to analyze properties including stiffness, rigidity and viscosity, as well as provide clear images and real-time video of individual cells undergoing mechanical stress loads. This information can be used to advance diagnostic techniques as well as provide insight on the effects that conformational changes cause to cellular properties.

AFM has been used in multiple studies to look at mechanical characteristics of different types of T-cells. One study by Zhang et al. used AFM in order to characterize the properties of T-lymphoctyes during interactions with human umbilical vein endothelial cells[7]. Another study by Puech looked at the complexes involved in the recognition by TCR at the surface of a T-cell through force measurements taken with AFM.[8]

While AFM has not been used to look at how mechanical properties of CD8+ T-cells may differ in the naive state, activated state, and reactivated state, previous research involving T-cells does indicate that AFM could lead to characterizations of these properties.

The current study aims to characterize how stiffness properties may change in CD8+ T-cells when comparing naïve, activated, and reactivated cell groups. Both anti-

CD3 particles and Dynabeads will be used as two ways of stimulation, and it is hypothesized that the cells that are activated by the Dynabeads will exhibit softer properties after the first activation than those stimulated by CD3. This is hypothesized because Dynabeads are widely used for T-cell activation, and CD3 is expected to be a moderate activator. It is also hypothesized that after the first activation, both stimulated comparison groups will get softer, and then stiffen again after the second activation. This would align with the biology behind CD8+ T-cell migration after their initial activation, and stationary behavior once reaching the antigen and re-activating.

Methods

Isolation and Seeding of CD8+ T-cells

T-cells were isolated from a whole blood sample via negative MACS bead selection. From this T cell population, CD8+ T-cells were isolated utilizing an additional MACS bead negative selection. CD8+ T-Cells were frozen in a DMSO solution in 1 ml at -80° C at an approximate density of 100,000 cells/ml.

Activation of CD8+ T-cells

Cd8+ T-cells (1 ml aliquot) was thawed into 5 ml of a media solution containing supplemented RPMI (10% FBS), and then transferred to a T-25 flask. The CD8+ T-cells were allowed to rest for a day to allow them to regain regular functionality. After 24 hours, 1 ml of the suspended culture was removed for AFM analysis. After this removal, the cells were counted, and two samples of 45,000 cells were removed and placed into two 12-wells. In the first T-12 well, Dynabeads were added at a 1:1 ratio and placed

back in the incubator for 1 hour. After 1 hour, the Dynabeads were removed from the sample using a magnet. 200ul of the solution was then centrifuged and re-suspended in 1ml of un-supplemented RPMI media in order to be used for AFM analysis. The remaining solution was returned to the incubator (37° C) for 72 hours, and then was re-stimulated and analyzed in the same manner as described earlier. In the second 12-well, anti-CD3 particles were added in a 1:1 ratio and placed back in the incubator for 1 hour. After 1 hour, 200 μl of the solution was removed for centrifuging and resuspension in 1ml of un-supplemented RPMI medium in order to be used for AFM analysis. The remaining solution was returned to the incubator for 72 hours, and was then re-stimulated and analyzed in the same manner as described.

Adherence of T-Cells to Dish

Due to the nature of CD8+ T-cells, they require a suspension culture to remain viable and to proliferate. Because of this, these cells must be adhered to a dish or coverslip before analysis through AFM. To accomplish this, the surface area of dish/coverslip was first calculated, and used to determine the amount of Cell Tak needed. Cell Tak is deposited at 3.5 μg/cm^2 into two 12-wells. The calculated amount of Cell Tak was then added into an appropriate amount of PBS to cover the dish or coverslip. The dish/coverslip was then treated with this mixture and incubated for 20 minutes in a standard incubator. The remaining liquid was aspirated upon removal from the incubator. In one 12-well, 1ml of DynaBeads-stimulated CD8+ T cell-solution was added. In the other 12-well, 1ml of CD3-stimulated CD8+ T-cell solution was added. The 12-well plate was centrifuged and the coverslip was then removed and placed into a

Fluorodish. 1ml of un-supplemented media was added to the Fluorodish in order to perform AFM analysis.

Atomic Force Microscopy Measurement

The AFM instrument was calibrated according to standard protocol, and data was collected utilizing Asylum Software. The preset “BioContact” program was used, and integral grain (0.6) and z voltage were adjusted prior to data collection to minimize interfering noise and oscillation of the cantilever while engaged. Approximately 30 lymphatic cells were measured per experiment, according to standard protocol. Three force curves were collected for each cell and used to determine the Young’s Modulus of each cell being measured. The “D” cantilever was used, and the force distance was set to 4.18μm.

Data Analytics

After obtaining the Young’s modulus, adjustments were made to ensure the proper cantilever tip geometry was being used for calculations. The radius was set to 4.67 μm, and the material was set to fused silica. Next, each graph was fit using the Asylum Software and exported as a .jpg file. Young’s Moduli were calculated through the use of a best fit line as seen in **Figure 1** . For each CD8+ T Cell, a Young’s Modulus

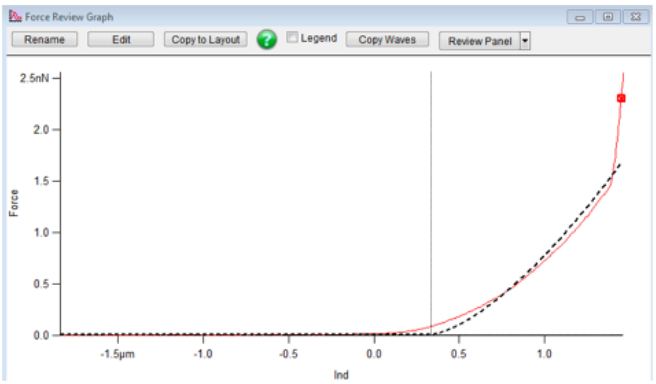

Figure 1. Graph output from Atomic Force Microscopy. Visual Representation of the force curve being analyzed through the Asylum software by using a best fit line. This process determines the Young’s Modulus of the cell.

was calculated for each of the three force curves. These Young's Moduli were compared between naïve (control) , activated (Dynabeads and CD3), and re-activated CD8+ T-cells using One-Way ANOVA tests in order to test for statistically significant differences in stiffness throughout the study (**α**=0.05).

Results

Initial Activation

During the initial activation study, the Young's Moduli of the control group, CD3-activated group, and Dynabead-activated group were all compared. The control group had an average Young's Modulus of 224.9 kPa. The CD3 activated group had an average Young's Modulus of 3.9 kPa. The Dynabead-activated group had an average Young's Modulus of 39.8 kPa. One-Way ANOVA tests were performed between the control group and the CD3 group (p-value = 3.14 x 10^-17), the control group and Dynabeads group (p-value = 9.99 x 10^-14), and the CD3 group and the Dynabeads group (p-value = 0.01). This indicates that both CD8+ T-cells activation groups were statistically significantly softer than the control comparison group. A visual representation of all Young Modulus measurements collected during the first activation can be seen in **Figure 2**, and shows the trend that both the CD3 group and Dynabeads group became softer after activation**.**

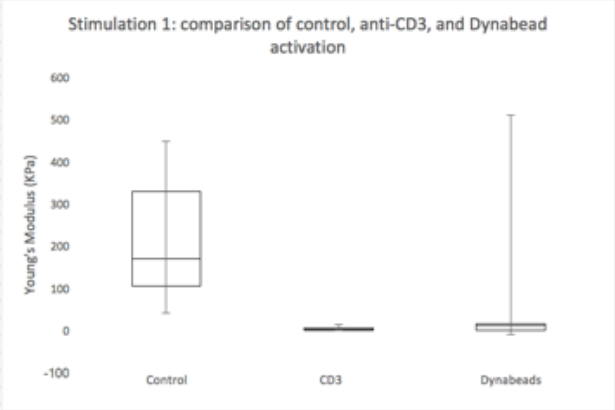

Figure 2. Box-and-Whisker Plot showing results from the first stimulation. The CD3 group (p-value = 3.14 x 10^-17) and the Dynabead group (p-value = 9.99 x 10^-14) are both significantly softer than the control group.

Re-Activation

After 72 hours, the cells were re-activated. During the re-activation study, the Young's Moduli of the CD3-activated group and Dynabead-activated group were compared to each other, and additionally each of these groups was compared to their own results from 72 hours prior. The re-activated CD3 group had an average Young's Modulus of 64.1 kPa. The re-activated Dynabead group had an average Young's Modulus of 50.5 kPa. One-Way ANOVA tests were performed to compare the re-activated CD3 group and the re-activated Dynabead group (p-value = 0.53), the CD3 results from the initial activation to re-activation (p-value = 0.002), and the Dynabead results from initial activation and re-activation (p-value = 0.34). This indicates that the only significant difference observed was the increase in stiffness of the CD3 group after the second activation. After plotting the data (**Figure 3**) from the initial activation and the re-activation, it can be seen that the Young's Moduli trend in the direction of increased stiffness once activated a second time for both groups, even though the only statistically significant increase in stiffness was seen in the CD3 group.

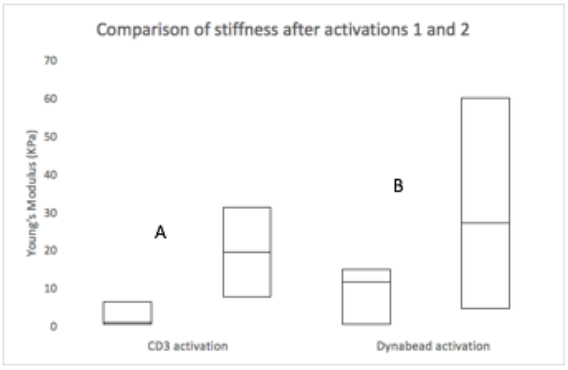

Figure 3. Visual representation of the increase in stiffness after the second activation for both CD3 and DynaBeads groups. There was a significant (p-value = 0.002) increase in stiffness from the first to the second activation in the CD3 group (A). While the DynaBeads group showed no statistical difference, it follows a trend of increase stiffness from the first activation to the second (B).

Discussion

Evaluation of the results support the hypothesis that the first activation will result in a softening of activated CD8+ T-cells in comparison to the control cells for both the CD3 an Dynaead groups. Additionally, the results support, specifically for the CD3 comparison group, the hypothesis that the second activation will result in increased stiffness when compared to the initial activation results. The trend indicates that the Dynabead comparison group also became more stiff between the first and second activation, though the results were not statistically significant. The cells were expected to first soften, and then stiffen upon the first and second activations due to the nature of their function in the body and the way T-cells are known to undergo conformational changes. When the CD8+ T-cells are first activated in the body, they migrate in order to find their target receptor, which could be aided by a decrease in structural stiffness. It is theorized that once the cell finds the target receptor and activates for a second time, stiffness would increase during the conformational change in order to aid the CD8+ T-cell in carrying out its function in a stationary location.

The results did not support the hypothesis that the DynaBeads would activate the CD8+ T-cells more than the CD3 during the first activation. While DynaBeads were thought to be more aggressive activators than CD3, the cells treated with CD3 were significantly softer than the cells treated with the Dynabeads. It is unclear why the CD3 cells were more strongly activated, and more research would need to be conducted to better understand the activation mechanism and properties of CD3.

The experimentation process could have been improved by having a higher level of magnification during the AFM analysis process. T-cells are incredibly small, with a diameter ranging from 2-7 μm (**Figure 4)**. This makes it more difficult to attain accurate measurements, as the cells are difficult to identify and occasionally move out from underneath the tip of the cantilever. Additionally, the bead on the tip of the cantilever used in this study was approximately the same size as a single cell, which made positioning the cantilever over the cell more difficult. The use of a smaller bead on a smaller cantilever may provide even more precise measurements.

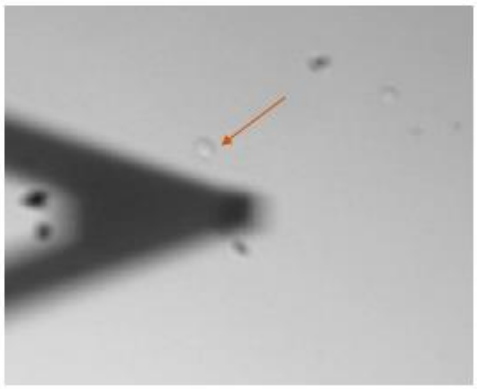

Figure 4. Picture showing the tip of the cantilever and its relative size in comparison to a T Cell. The orange arrow points to the T cell.

Conclusion

This study aims to gain a better understanding of the mechanical characteristics of CD8+ T-cells during their activation and reactivation by stimulating cells in vitro with Dynabeads and anti-CD3 particles and measuring the Young's Moduli. In order to further validate the results of this study, further research could include using real dendritic cells for activation to more accurately model the CD8+ T-cell activation in vivo. As CD8+ T-cells play a very important role in the immune system, knowing how the physical properties change throughout the activation and reactivation process could potentially have implications into future diagnostic or treatment methods. If a strong relationship was identified between stiffness and migration, there could possibly be a way to encourage CD8+ T-cells into migration, or help these cells remain stiff if they

need to remain in a specific location longer than their normal duration. While this research is in the preliminary stages, it does show promise that there may be a relationship between CD8+ T-cell activation and stiffness properties.

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