Characterizing the mechanical changes of activated and re-activated CD8+ T-cells

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Abstract

The adaptive immune system is comprised of many types of white blood cells that clear pathogens from the body by promoting an inflammatory response. CD8+ T cells are an integral part of this process, as they exclusively induce apoptosis in infected cells and pathogens that invade the body. Much is known about the changes that accompany a CD8+ T cell when it is initially activated and re-activated by antigens. However, the mechanical changes that occur during these stages of activation have not yet been investigated. In this study, CD8+ T cells were activated using anti-CD3 particles and Dynabeads and later re-activated with the same stimulant. Mechanical changes were assessed with Atomic Force Microscopy (AFM). It was hypothesized that activation would cause the T cells to become softer, and that re-activation would cause the cells to become stiffer. It was also hypothesized that Dynabeads would cause a more complete activation of the cells as compared to the anti-CD3 particles. Results revealed that activation causes CD8+ T cells to soften (CD3: p=3.14e-17, Dyna: p=9.99e-14, but only confirmed that the T cells became stiffer after re-activation with anti-CD3 particles (p= 0.002). Additionally, it was observed that anti-CD3 particles cause a larger mechanical change after activation than stimulation with Dynabeads (p=0.00983).

Introduction

The human immune system is a crucial component of human survival, and is responsible for eradicating harmful cells and pathogens that can upset the natural balance of a healthy individual’s body. It is comprised of two processes that operate synergistically in the immune system to eradicate any pathogen(s) that invades the body. The first process, named the innate
immunity, is present at birth. It 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. However, this system has its drawbacks, namely that it cannot eradicate larger, more invasive, and more aggressive foreign pathogens that infect the body. To compensate for this deficiency, the second process, named the adaptive immunity, develops over time to eliminate these pathogens that evade the innate immune system.\textsuperscript{1,2}

The adaptive immune system primarily comprises of B-cells, T-cells, and dendritic cells (DCs). B-cells are generally responsible for creating mobile immunoglobulins, called antibodies, that attach onto the receptors of pathogens and mark them for phagocytosis by other immune cells. Dendritic cells are antigen-presenting cells (APCs) that act as phagocytes for foreign invaders, and alert immune cells to target the pathogen they have encountered. T-cells, however, have many sub-populations that accomplish different tasks during an immune response, including helper T-cells, memory T-cells, and arguably the most important T-cell, the cytotoxic t-lymphocyte (CTL) cell. CTL cells are primarily responsible for promoting apoptosis in foreign cells that are infected with viruses through the development of surface immunoglobulins called T-cell receptors (TCRs), and the recognition of specific antigens present on these infected cells.\textsuperscript{2,3,4}

The lifecycle of a CTL cell requires many conformational changes to prepare each cell for activation and successful targeting of harmful cells. Undifferentiated T-cells originate in the bone marrow of adult humans, and upon leaving the bone marrow, travel to the thymus gland to mature into naive T-cells. After maturation, these cells circulate throughout the bloodstream
and are periodically drawn into the thymus by a combination of surface cells, chemokines, and cytokines.\textsuperscript{2,5} Throughout this circulation, these naive cells are “double positive”, meaning they possess both a CD4 and a CD8 co-stimulatory receptor. Each of these receptors assists in responding to different APCs through interaction with major histocompatibility complexes (MHCs).\textsuperscript{6,7,8} MHCs develop on the surface of APCs after they encounter an antigen or are infected and bind with either CD4 or CD8 receptor on naïve T cells. When a naïve T-cell interacts with one of these two MHC complexes, a process called positive selection occurs, which causes the T-cells to change and only present CD8 or CD4 for interaction with the MHC complex.\textsuperscript{1,2}

After positive selection, CD8+ T-cells become CTL cells. After selection, activation of CTLs occurs through two signals. The first signal involves the interaction of the T-cell Receptor (TCR) and MHC class I surface molecules, with the additional stabilization of the CD8 surface molecule. The second signal comes from the stimulation of the CD28 surface receptor by CD80 or CD86 (sometimes called B7-1 and B7-2). After activation has occurred, the CD8+ T cell develops specific antigen receptors that recognize the antigen that caused activation. They then undergo clonal expansion to elevate the response to a specific antigen, and begin to circulate within the bloodstream.\textsuperscript{1,2,4}

Another conformational change occurs in CD8+ (CTL) T cells when they encounter an antigen that binds to the developed surface receptors. When exposed to these APCs, CD8+ cells bind and secrete cytotoxins such as perforin, granzymes, and granulysin. Perforin creates holes in the bound APC, while the granzymes and granulysin promote apoptosis (programmed cell
death) for the APC. The CTL cell then moves on and re-enters the bloodstream, waiting to encounter the same antigen on another cell.\textsuperscript{2,9}

Much is known about the conformational changes that aid CD8+ cells throughout their lifetimes, including fluctuating cytokine levels, changing surface receptors, and different binding affinities. However, little is known about the mechanical and overall structural differences that accompany these changes. One possible route to understanding the changing mechanical properties of CD8+ T cells may be in the use of Atomic Force Microscopy (AFM). These measurements allow for new diagnostics utilizing information from cells such as stiffness, viscosity and rigidity, providing insightful information about the structure and functionality of the cells. This method of analyzing cells has allowed researchers to collect mechanical and viscosity data for T cells. Previous studies have been conducted on T-cells utilizing AFM, showing promise for successful measurement of CD8+ T-cells in this study. Hu & Butte investigated the effects of delivering antigenic stimulation through a cantilever on the activation of T-cells, while Hu et al. utilized AFM to analyze the molecules that develop on the surface of CD4+ T-cells when they are activated.\textsuperscript{10,11} Finally, a study conducted by Huang et al. measured the changes of the T lymphocyte nanostructure (through AFM analysis) when treated with aminophylline.\textsuperscript{12}

The present study aims to analyze the mechanical changes that accompany activation and re-activation of CD8+ T cells through AFM analysis. The methodology employed for activation will be through anti-CD3 particles and Dynabeads. It is hypothesized that the T cells will become softer upon initial activation, and stiffer after a subsequent re-activation with the same antigen. This is based on the knowledge that CD8+ T cells leave the thymus to travel
through the body as they sample antigens, and later become stationary in order to induce apoptosis when they encounter the antigen that caused initial activation. It is also hypothesized that Dynabeads will provide a more thorough activation to the T cells than the anti-CD3 particles. This is because Dynabeads are seen as the standard for cell activation, and the anti-CD3 particles are not expected to have significant effect on the T cells.

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. The CD8+ T cells were then frozen at -80°C in a DMSO solution in 1 ml aliquots at an approximate density of 100,000 cells/ml.

Activation of CD8+ T cells

Cells (1 ml aliquot) were thawed into 5 ml of media solution containing a supplemented RPMI solution (glutamine+10% FBS), and then transferred to a T-25 flask. The CD8+ T cells were allowed to rest for a day at 37°C 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 containing 45,000 cells were removed and aliquoted into two separate 12-wells. To one 12-well, DynaBeads were added at a 1:1 ratio to activate the T cell population. After 1 hour, the DynaBeads were removed from the suspension via magnet and 200 μl was removed for centrifugation and re-suspended in a non-supplemented RPMI media
for plating for AFM analysis. The remaining cells were allowed to rest for 72 hours at 37° C and were then re-stimulated with Dynabeads and analyzed in the same way. To the second 12-well, anti-CD3 particles were added at a 1:1 ratio to activate the T cell population, and the cells were plated and analyzed in the same way as the Dynabeads population.

*Adherence of T-Cells to Dish (Cell Tak)*

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 by AFM. To accomplish this, the surface area of dish/cover slip was first calculated to determine the amount of Cell Tak needed. Cell Tak was deposited at 3.5 μg/cm². The calculated amount of Cell Tak was then added into an appropriate amount of RPMI + cell suspension to cover the dish or coverslip. The dish/cover slip was then treated with this mixture and centrifuged at 3500 Rpm for 10 minutes. The remaining liquid was removed by aspiration and the coverslip was placed on a fluorodish for 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 and z voltage were adjusted prior to data collection to minimize interfering noise and oscillation of the cantilever while engaged. During measurement, The D cantilever was utilized, with a 4.67 μm silica bead. Force distance was set at 4.18 μm. 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.

Data Analysis

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 made using the Asylum Software and exported as a .jpg file. Graphs for both the naïve, activated, and reactivated data were analyzed in for the 10-90% range of the curve (representing best fit). This can be seen in Figure 1. Young’s Moduli were calculated for each cell and compared. 3 Young’s Moduli were calculated for each force graph obtained, and were compared between naive, activated, and re-activated CD8+ T cells using one-way ANOVA statistical analysis (α=0.05).

Results

Changes in the stiffness of CD8+ T cells were assessed and compared through differences in the Young’s Moduli between un-activated (control), CD3-activated and Dynabead-activated cells after stimulations one and two.
**Stimulation One**

After analysis of each force curve, the calculated average Young’s Modulus for the control, CD3, and Dynabead activated cells were 224.45 KPa, 3.94 KPa, and 39.77 KPa, respectively. After plotting the data in a box-and-whisker plot (Figure 2), the general trend noted was that activation causes CD8+ T cells to become drastically softer. This trend was evaluated statistically, and it was found that cells activated by CD3 or Dynabeads were softer to a statistically significant degree when compared to the control (CD3: p=3.14e-17, Dyna: p=9.99e-14). Additionally, it was concluded that activation with CD3 causes a larger mechanical change than activation with Dynabeads (p=0.00983).

![Figure 2. Comparison of the Young’s Moduli of the control, anti-CD3, and Dynabead cells after activation. Depicted above are box-and-whisker plots showing the Young’s Moduli for the control, CD3 and Dynabead activated CD8+ T cells. General trends to note are that activation results in a decrease of cell stiffness, and anti-CD3 particles provided a more intense stimulation.](image)

**Stimulation 2**

Following stimulation 1, the control group was excluded from stimulation 2 as it was not expected to undergo additional conformational changes. The

![Figure 3. Comparison of the Young’s Moduli of the anti-CD3, and Dynabead cells after activation and re-activation. Depicted above are box-and-whisker plots showing the Young’s Moduli for the CD3 and Dynabead activated and re-activated CD8+ T cells. General trends to note are that re-activation results in an average increase of cell stiffness.](image)
calculated average Young’s Modulus for the CD3 and Dynabead activated cells were 64.09 KPa, and 50.51 KPa, respectively. These were compared to each other statistically, and no difference was found (p=0.529). The data for CD3 and Dynabead-activated cells was then compared across both stimulations to evaluate changes in stiffness through box-and-whisker plots (Figure 3). The overarching trend noted was that activation causes CD8+ T cells to become soft, and re-activation causes them to become stiffer. The data for the CD3 and Dynabead activations was then statistically compared across stimulations 1 and 2. Statistical significance was found between activations for CD3 (p=0.002), but no significance was found for Dynabeads (p=0.344).

Discussion

Analysis of the results revealed several unexpected trends when comparing the changes that accompany activation of CD8+ T cells by anti-CD3 particles and Dynabeads. When examining the results from the first activation, the hypothesis that activation would cause CD8+ T cells to become softer was supported both through general trends and statistical analysis, as the stiffness of activated cells was noticeably lower than that of the control group. However, the hypothesis that Dynabeads would provide a more vigorous activation of the T cells was not supported, as CD3 activation caused the cells to become softer than cells activated by Dynabeads. This was a surprising conclusion, as Dynabeads are a known standard for T cell activation. It is unknown why CD3 activation would cause a more drastic conformational change. Finally, the hypothesis that CD8+ T cells would become softer after one activation, and subsequently become stiffer after a second activation was partially supported. Although general trends suggested that the cells followed this trend, the hypothesis was only supported
statistically by CD3-activated cells. Again, this was unexpected and suggests that CD3 may provide a stronger activation for CD8+ T cells than Dynabeads.

During experimentation, there were several obstacles encountered that may have skewed the data collected. First, the cells being measured are miniscule, typically ranging from 2-7 µm in size (Figure 4). This made it difficult to accurately place the beaded cantilever on a cell, and may have resulted in force curves that do not accurately represent the cells. Additionally, the bead used was 4.67 µm in size, placing it at around the same size as the cells being measured. This size similarity may have also caused inaccurate force curves if it was placed incorrectly.

Finally, the magnification shown in Figure 4 (20x) is the highest magnification available on the AFM machine. A higher magnification power may have allowed for better placement of the bead onto each cell, and resulted in more accurate force readings.

**Conclusion**
This study was conducted to gain a more robust understanding of the mechanical changes that accompany CD8+ T cells during activation and re-activation. It is difficult to draw final conclusions from the data obtained, as this experiment would need to be repeated to confirm the difference in the changes seen by CD3 and Dynabead activation. Additional related future studies could include utilizing actual dendritic cells in lieu of Dynabeads or testing the effects of softening and stiffening agents on the functionality of activated and re-activated CD8+ T cells. Overall, the findings of this study confirmed that CD8+ T cells undergo mechanical changes when activated and reactivated with anti-CD3 particles. This knowledge could provide an avenue for new treatments that create a concentrated and/or prolonged immune response in one specific area of the body, such as a tumor. Through forced cell migration caused by intentional stiffness alteration, treatments such as CAR-T therapy could become even more specific and have longer therapeutic effects.
Literature cited


