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Force Based Comparison Between Naïve and Activated CD4+ T Cells during Interaction with Dendritic Cells

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Force Based Comparison Between Naïve and Activated CD4+ T Cells during Interaction with Dendritic Cells

by

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Abstract

Antigen recognition requires the communication between T cells and antigen presenting cells (APC) such as dendritic cells (DC). Although there is progress in understanding molecular components at the intercellular contact, the specific role of the physical cross-junctional adhesion between CD4+ T cells and DCs is not fully understood. In this study, we investigate cell-to-cell contact mediated by adhesion molecules between these cells in order to elicit the mechanism involved. Lymphocyte function-associated antigen-1 (LFA-1) adhesion molecule on the surface of T cells interacts with intracellular adhesion molecule-1 (ICAM-1) on the APC. This adhesion pair has been shown to be essential for the contact between the cells. The role of this adhesion pair and its expression and regulation on naïve CD4+ T cells has not been compared to its role on activated T cells. Using atomic force microscopy (AFM) we investigated the increase in interaction forces between naïve CD4+ T cells and DCs. These forces were compared to those involving activated T cells to see the difference between naïve and activated T cells in their adhesion properties with DCs, which involves LFA-1 integrin molecule. Understanding the role of LFA-1 interaction with ICAM-1 provides further insight into therapies using CD4+ T cells.

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Dedication

I lovingly dedicate this thesis to my family for always believing in me. I give my deepest expression of love and appreciation for all they have done to help and support me throughout my master's journey.

To all my friends, thank you for your understanding and encouragement in my many moments of crisis. I cannot list all names here, but you are always on my mind.

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Table of Contents

List of Figures and Illustrations

List of Symbols, Abbreviations and Nomenclature

Symbol	Definition
AFM	Atomic force microscope
AIDS	Acquired Immunodeficiency Syndrome
ANOVA	Analysis of variance (one-way)
APC	Antigen-presenting cell
β I-like domain	Beta inserted-like domain
CCM	Cell culture media (RPMI + 10%FBS)
CD	Cluster differentiation
CO_2	Carbon dioxide
cSMAC	Center of supramolecular activation clusters
Cyto-D	Cytochalasin D
DC	Dendritic cell
DC2.4	An immortalized murine dendritic cell line
ddH_2O	Double-distilled water
DOPA	Dopamine hydrochloride
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorting
HIV-1	Human immunodeficiency virus-1
IFN	Interferon
ICAM-1	Intracellular adhesion molecule-1
IL	Interleukin

KO	Knockout
LAD	Leukocyte adhesion deficiency
LFA-1	Lymphocyte function-associated antigen-1
M17.4	Anti-LFA-1 antibody
MBCD	Methyl β cyclodextrin
MHC	Major histocompatibility complex
mM	millimolar (mmol/L)
nN	nano Newton
OTII	Transgenic T cell receptor specific for OVA 323-339
OVA	Ovalbumin
PBS	Phosphate buffer saline
PFA	Paraformaldehyde
PMA	Phorbol myristate acetate
pMHC	Peptide bound MHC
pSMAC	Peripheral of supramolecular activation clusters
RSV	Respiratory syncytial virus
SMAC	Supramolecular activation clusters
TCR	T cell receptor
TNF-α	Tumor necrosis factor-α
Treg	Regulatory T cell
YN1	Anti-ICAM-1 antibody

Chapter One: Introduction and Background

1.1 CD4+ T cells and their role in the immune system

The immune system is responsible for protecting against diseases and potential damages. To provide this protection, the immune system relies on various types of cells that are in charge of antigen recognition and destruction, for instance, T cells and B cells. These cells form a dynamic network, and the immune system is therefore capable of defending against invading pathogens. [1, 2].

Multiple mechanisms are involved in the immune response. They can be mostly categorized into two collaborative systems, innate and adaptive immunity. Innate immunity is considered the first line of defense against infections [3]. It is a highly effective system and able to non-specifically recognize invading antigens to eliminate them within hours of encounter [3]. In addition, innate immunity is required to induce a specific adaptive immune response, the second line of defense, which is initiated by antigens or antigen presenting cells (APCs), mostly dendritic cells (DCs) [4]. When an APC takes up antigen and is stimulated by "danger" factors associated with the infection, it starts its maturation process and proceeds to migrate to the secondary lymphoid tissues (lymph nodes, spleen and mucosal lymphoid tissues) [4].

APCs express major histocompatibility complex I and II (MHC I and II) on their surfaces. The most potent APCs are dendritic cells [5, 6]. DCs are specialized APCs that have the highest ability to present costimulatory signals to naïve T cells in the context of antigen presentation in order to activate them to start an adaptive immune response [5].

T lymphocytes are white blood cells that play a crucial role in the immune system. There are two major subclasses of T cells, CD4+ and CD8+ T cells, characterized by their surface expression of the said co-receptors. Among CD4+ T cells, a subgroup that expresses CD25 and FoxP3 on the surface and intracellularly, respectively, are regulatory T cells that mediate immune suppression. All T cells have a unique surface molecule known as the T cell receptor (TCR). The TCR is a heterodimer consisting of alpha (α) and beta (β) chains linked by disulfide bonds [7]. Each chain is composed of variable and constant domains [7, 8]. T cells originate in the bone marrow and mature in the thymus, which gives its name. In the thymus, T cell maturation is controlled by TCR-MHC interactions [6]. Thymic positive selection generates single-positive T cells, either CD8+ T cells or CD4+ T cells [9]. CD4+ T cells are selected on MHC II while CD8+ T cells are selected on MHC I. During negative selection, the cells that bind to self-peptides presented on MHC molecule very strongly are removed [9]. Mature CD4+ T cells remain in the secondary lymphoid organs as naïve or resting cells until they are activated by incoming APCs [10]. Mature T cells that have not yet encountered their specific antigen are known as naïve T cells. Activated T cells, on the other hand, are the ones that have met their specific antigens presented by APCs.

In general, both the maturation of T cells in thymus and the activation of mature T cells in the periphery are influenced by the involvement of MHC molecule on the surface of antigen presenting cells [11]. To generate an adaptive immune response, either humoral or cell-mediated, activation of CD4+ T cells is needed. CD4+ T cells activation is initiated via the primary interaction between TCR/CD3 complex and antigen peptides presented by MHC II on APCs. T cell activation also requires the interaction between

multiple membrane markers including adhesion and co-stimulatory molecules [12]. These interactions initiate a cascade of signaling events that induce naïve CD4+ T cells to undergo repeated cell division. At the same time, these signals trigger the production of IL-2 gene and IL-2 receptor (CD25) [13]. Secretion of IL-2 and the binding between CD4+ T cells and APCs cause T cell proliferation and differentiation into memory and effector T cells populations. Memory T cells are antigen-generated long-lived cells that express many of the same cell surface markers as effector T cells [14]. Effector T cells carry out specialized function as a central role in immune protection. Examples of the main functions of CD4+ T cells are helping B cells make antibodies, enhancing cytotoxic killing activity by CD8+ T cells and recruiting different cells, such as neutrophils, to site of infection and inflammation [15].

Effector cells are short-lived cells that express more of the activated cell membrane molecules. According to the different panels of cytokines secreted by CD4+ T cells, they can be differentiated into several subpopulations; the main two subpopulations are T helper 1 (Th1) and T helper 2 (Th2) [16]. Th1 secretes cytokines such as IL-2, IFN-γ and TNF-β, which makes it responsible for cell-mediated functions [16]. On the other hand, Th2 produces IL-4, IL-5, IL-6 and IL-10 and functions as a helper for B cell activation [16].

Cytotoxic T cells or CD8+ T cells have lytic ability, which makes it critical in the regulation and elimination of altered-self cells [17]. CD8+ T cells are MHC I restricted cells that is activated as a result of their recognition of antigen peptides presented by MHC I molecule on the surface of APCs. It has been proven that APCs in this case must acquire the ability to activate CD8+ T cells by the prior interaction with CD4+ T cells in

a process called licensing [18]. Licensing takes place through the interaction between CD4+ T cells and antigen peptides bound to MHC II [18]. Once licensing, APC interacts with CD8+ T cells through MHC I to cause its activation [18]. However, IL-2 produced by CD4+ T cells is important for CD8+ T cells activation, as it has been shown in IL-2 knockout mice that the absence of IL-2 abolishes CTL-mediated cytotoxicity [18].

Moreover, activation of B cells requires two signals; the first one requires the recognition of antigen by B cell receptor (BCR) [19]. The other signal is initiated by CD4+ T cells recognition of cognate antigen after the interaction between B cell and activated CD4+ T cells [19]. Once CD4+ T cells recognizes the processed antigen presented by MHC II on the membrane of B cell, the two cells interact to form a conjugate that involves CD40 and CD40L up regulation and engagement on both cells [19]. In addition, B cell requires cytokine signal from CD4+ T cells to perform its function.

Clearly, CD4+ T cell has a unique and crucial role in initiating an adaptive immune response to pathogens. It also can modulate the functions of the innate immune cells through their cytokine profile and cell surface markers. Obviously, CD4+ T cells are vital for defending against various diseases and for survival.

In addition to the role in pathogen defense, the importance of CD4+ T cells has been shown in antitumor immunity. CD4+ T cells accomplish this partly via secretion of different cytokines that fine tune the immune response against tumors. [20]. In addition, extensive research over the past decades has increased our knowledge of the mechanism underlying infection by human immunodeficiency virus-1 (HIV-1) and how CD4+ T cells are involved. Related to our work discussed later, Pantaleo *et al* highlighted the

contribution of lymphocyte function-associated antigen-1 (LFA-1) molecule on the surface of CD4+ T cells to the depletion of HIV-infected CD4+ T cells, where the expression of LFA-1 is necessary for cell fusion and syncytia formation in the infected cells [21]. Therefore, new findings on the mechanisms of CD4 T cells are continuing to provide a knowledge base that can be applied to the medical field in order to generate treatments for various immune related diseases.

1.2 Defining CD4+ T cells markers and immune synapse formation

On the surface of T cells, there are invariant chains of CD3, which include δε and γε heterodimers. The association of CD3 dimers with the T cell receptor (TCR) and ςς homodimer leads to the initiation of a signaling cascade that results in T cell activation and cytokine release [22]. In addition to the interaction between TCR and pMHC, the engagement of other surface molecules is also required for T cell activation [23]. It has been proven that blocking of costimulatory and adhesion molecules on the cell surface results in reduced TCR recognition and requires more pMHC for a given degree of T cell activation than the control [24].

Immune synapse formation is essential for T cell activation as it organizes the required dynamic cell surface framework to better engage the target cell [25]. Mature immune synapse is defined as the bull's eye arrangement of supramolecular activation clusters (SMACs) [26]. Using fluorescence microscopy, it has been shown that the center of the bull's eye or cSMAC is for the interaction between TCR and pMHC; whereas the outside ring of the bull's eye or peripheral SMAC (pSMAC) contains the integrin LFA-1 and its ligand intracellular adhesion molecule-1 (ICAM-1) [26]. It has been proposed by

Lee *et al* that cSMAC is involved in sorting of ubiquitinated TCR complexes for degradation [27, 28]. The final stage of immune synapse formation is the stabilization of the cSMAC by the engagement of TCR to an adequate number of pMHC complexes allowing the activation of T cell to begin [29]. T cell stimulation requires a two-signal mechanism [30, 31]. Such an arrangement reflects the requirement of two signals for T cell activation: an antigen-specific signal that is mediated by the interaction between TCR and pMHC, and a second signal that is delivered by co-stimulatory/adhesion molecules present through pSMAC [30, 31].

Even though extensive studies have been undertaken to elucidate the different mechanisms involved during the formation of immune synapse, much remains unknown. Horgan and colleagues reported that naïve T cells require additional co-stimuli in order to get activated [32]. It is known that activated T cells have faster kinetics of immune response than naïve T cells upon antigen specific stimulation [33]. Izzie *et al* were able to determine the duration of antigen exposure that is required for both naïve and effector T cells [33]. They have shown that naïve cells need prolonged antigen stimulation for approximately 20 hours to become activated, while effector T cells can become committed in approximately one hour. In fact, an extended antigenic trigger induces cell death in activated T cells [33]. Moreover, it has been concluded by Croft *et al* that there is a decrease in the dependency on co-stimulation in effector T cells, as the cells in this stage are able to respond to TCR signals alone [34]. It also has been proven that effector T cells are more sensitive to antigen concentration than naïve cells [35]. While effector T cells need low concentration of antigen, naïve T cells require high antigen concentration in order to get activated [35]. Kimachi and colleagues analyzed how naïve and effector

CD4+ T cells differ in the ligand affinity by comparing the two cells' subsets with respect to their ability to be activated by low-affinity ligands for the TCR [36]. They found that previously primed CD4+ T cells are able to be activated by low-affinity ligands, whereas naïve CD4+ T cells cannot [36]. Hence, all of the previous studies are suggestive that there is a difference between naïve and activated CD4+ T cells in the way they respond to a foreign antigen. Specifically, naïve and activated T cells differ from each other with respect to the necessity of co-stimulatory and adhesion molecules. However, what causes these differences remains unknown. How these differences are related to immune synapse formation is also unknown.

1.3 Potential molecules involved in direct cell contact

Several molecules have been implicated in the T cell's ability to function through direct cell contact. The main surface markers that form immune synapse on T cells include: CD3/TCR, LFA-1 (CD18/CD11a), CD28 and CD40L (CD154) [37]. The ligands for these molecules on DCs are as follows: MHC, ICAM-1, CD80/CD86 and CD40, in the same order [37].

The adhesion interactions required for T cells to become activated and perform their function are mediated by three families of interacting molecules: the immunoglobulin superfamily, which includes the ligation between TCR and pMHC; the integrin family that is necessary for the regulation of cell adhesion and migration; and finally the selectins, which are important in the interaction between T cells and endothelia [38]. The presence of integrins is essential for T cells to perform their functions. T cells need integrins to migrate in and out of lymph nodes and to enter into other tissues [39].

Notably, integrins are also important for the formation of the immune synapse between T cells and APCs [39]. Therefore, these integrins have to be tightly controlled.

1.4 LFA-1 and its role in the immune system

The most abundant and widespread integrin is Lymphocyte function-associated antigen-1 (LFA-1) [39]. It is also known as α_L β_2 integrin or (CD11/CD18), which belongs to β_2 integrin family of adhesion molecules and consists of α and β chains that are linked non-covalently [40]. LFA-1 is constitutively expressed in an inactive state on all lymphocytes and binds to its ligand intracellular adhesion molecule-1 (ICAM-1), which is a member of the immunoglobulin superfamily [38, 41]. LFA-1 plays a crucial role in many phases of immune cell activation and host defense, such as T and B cell regulation, T cell mediated killing, natural killer cell activity, etc. [40]. The importance of LFA-1 is made evident in leukocyte adhesion deficiency (LAD) disorder, which is developed as a result of the failure to activate integrins [42]. LAD occurs as a result of the deficiency in LFA-1 because of some mutations in the β_2 subunit [38]. It has been demonstrated by Bachmann and colleagues that LFA-1 sustains the adhesion between T cells and antigen presenting cells, which facilitates T cell activation [43]. In addition, LFA-1 deficient mice exhibit reduced lymph node cellularity and splenomegaly, which indicates that LFA-1 has a major role in lymphocyte recirculation and homing [44]. It has been documented by Grakoui *et al* that in vitro, APC can be mimicked by a lipid bilayer containing pMHC and ICAM-1, which is sufficient for immune synapse formation between T cells and APCs [26]. This demonstrates the central role of LFA-1 in the interactions between T cells and their cognate ligands.

LFA-1 is expressed on the surface of T cells in an inactive state that is not optimally functional, and must be activated in order to bind to its ligand strongly [40]. It has been demonstrated by Muller *et al* that TCR stimulation by agonist pMHC induces LFA-1 dependent T cell adhesion to ICAM-1 [45]. Activation of LFA-1 can be achieved in two ways: inside-out activation and outside-in activation [45]. In the process of inside-out activation, LFA-1 activation in the form of conformational changes results from the ligation of cell-surface receptors [40]. For instance, the interaction between the T cell receptor and pMHC generates intracellular signals that initiate LFA-1 activation [39]. Outside-in activation, on the other hand, happens when activating antibodies, for example, bind to the extracellular part of LFA-1, which can cause the conformational changes also known as the activation of LFA-1 [39]. Integrins are usually present on the surface of the cells in the inactive state, with their α and β subunits in a bent form [42]. However, when they are activated, they extend into an open form [42]. In fact, LFA-1 exists in three forms on the cell membrane according to its binding strength: low, intermediate, and high affinity conformational states (figure 1.1) [42]. In the low affinity state, or the bent state, LFA-1 α and β chains are linked in the transmembrane domain by membrane clasps. In the intermediate affinity state, LFA-1 flips open into an extended conformation while breaking the inner membrane clasp and maintaining the headpiece, contains β inserted-like (I-like) domain, hidden. Opposing forces further activate LFA-1 causing the final membrane clasp to break, spreading the cytoplasmic tails away from one another and exposing the head containing the β I-like domain, leading to LFA-1 ligand binding [42]. Although, its conformational states are known, regulation of LFA-1, its affinity states, and lateral movement in the membrane are complex and less understood.

Currently, research in the field of human LFA-1 has shed light on the stimulatory factors that can convert LFA-1 from its bent shape to its activated form. Stewart *et al* have demonstrated that PMA stimulation and TCR/CD3 stimulation of T cells do not only induce high affinity LFA-1 but also increase adhesion and cell spreading [46]. Therefore, it seems that the transformation of LFA-1 into its high affinity state is not merely a structural change but is also dependent on the involvement of the cytoskeleton [46]. The cytoskeleton is a cellular network of structural, adaptor and signalling molecules that regulate most cellular functions that are related to the immune response, including migration, extravasation, antigen recognition, activation and phagocytosis by different subsets of leukocyte [47]. Interaction of the cytoplasmic domains with the cytoskeleton is regulated during adhesion and their relationship may be the key in controlling the activation state of LFA-1 and consequently its function. As a universal rule, the adhesive properties of integrins are tightly regulated to prevent inappropriate adhesion and to maintain efficient migration from the blood to tissues. Otherwise, disruption in this regulation can lead to inefficient T cell activation, migration, and function [38, 42].

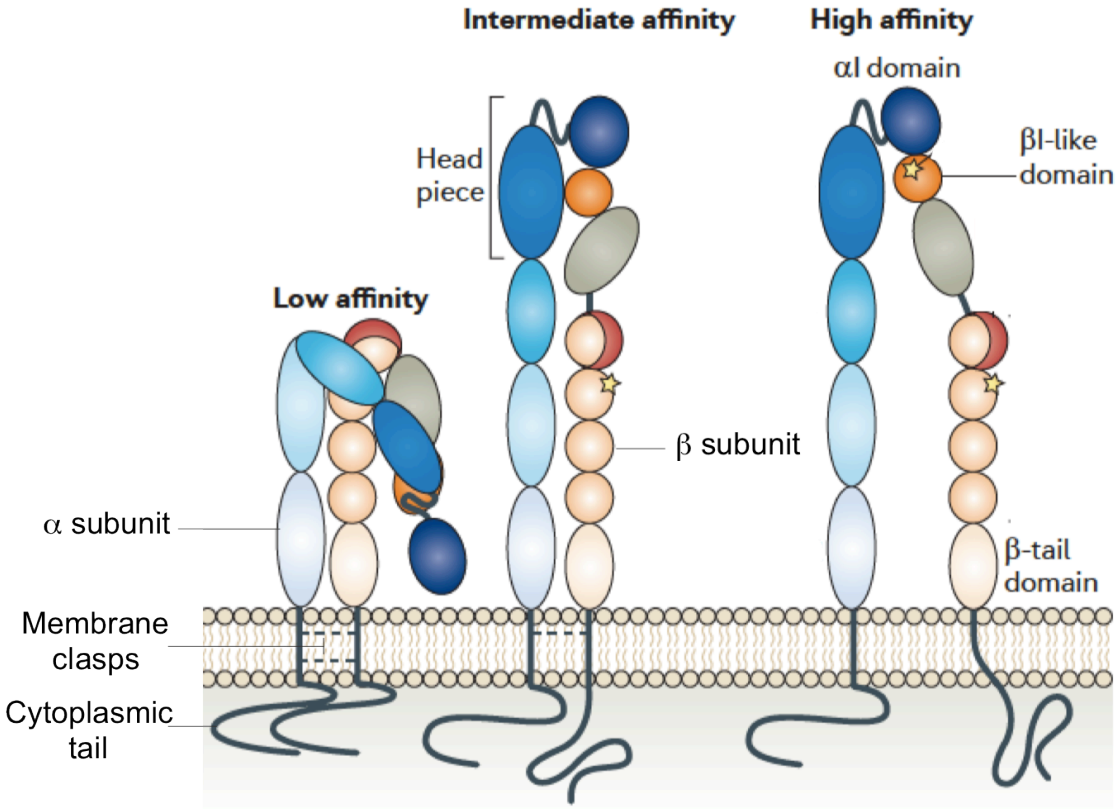


Figure 1.1. Different conformational states of LFA-1 [42].

There are three distinct conformations of LFA-1 classified according to their binding affinity for ICAM-1. In the low affinity form of LFA1, the extracellular regions of the α- and β-subunits are acutely bent. Higher affinity forms of LFA1 are extended. When fully activated, the headpiece opens and the βI-like domain exposed.
α and β subunits are closely held together by outer and inner membrane clasps. The inner membrane clasp is disrupted in the intermediate affinity form of LFA1, and in the high affinity LFA1 conformation both clasps are disrupted. [42].

1.5 Regulation of LFA-1 surface molecule

There might be multiple mechanisms involved in regulating and inducing different affinities and avidities of LFA-1. An increase in ligand binding activity can be induced by changes in lateral movement of the integrin allowing clustering to occur and consequently increase binding intensity and probability of contact. In addition to changes in avidity, induction of different conformational states will also increase the receptor's affinity for its ligand [38, 42]. Avidity and affinity changes have been linked to reorganization of the actin cytoskeleton. It is known that associations with the cytoskeleton contribute greatly to the function of surface adhesion molecules in general and this area of research is beginning to take hold. Indeed, deletions or mutations of the cytoplasmic tails of integrins can result in an active conformation and has revealed links to the cytoskeleton [48].

Molecules known to disrupt actin assembly have been studied in order to examine its importance in integrin regulation. High dosages of cytochalasin D (CytoD) treatment disrupts the actin cytoskeleton and abolished LFA-1 mediated adhesion, as previously mentioned by Lub *et al* and Hogg [40, 49]. Recently, Leitinger and Hogg have continued the search by examining the role of the cytoskeleton and the association of LFA-1 with lipid rafts on the cell surface [50]. Lipid rafts are subdomains of the plasma membrane that contain high concentrations of cholesterol and sphingolipids [50]. Treatment of Jurkat cells with lipid rafts activation antibody caused the formation of enlarged patches of lipid rafts on the cell surface. Both activated and non-activated forms (high affinity and low affinity, respectively) of LFA-1 were localized to these patches highlighting the importance of the lipid rafts in regulating the lateral movement of LFA-1 within the

membrane. Therefore, the cytoskeletal connection to LFA-1 appeared to maintain and regulate LFA-1 adhesion on the cell surface [40, 50, 51]. Cytoskeleton associated cytoplasmic proteins, such as talin and alpha-actinin, are also known to interact with the β chains of integrins and link them to the cytoskeleton. Linkages with the cytoskeleton can regulate cell adhesion by regulating integrin clustering. Indeed, talin co-localizes with integrin clusters and is vital to the formation of focal adhesions [52]. In addition, Stewart, McDowall and Hogg speculated the activation of a calpain-like enzyme was responsible for the increase in adhesive contact [51]. They proposed that stimulation of T cells causes Ca^{2+} fluxes and induces LFA-1 clustering by regulation of calpain activity, suggesting that the clustering of LFA-1 mediated this increase in adhesiveness [51]. However, research on calpain activity has been controversial as to how it affects adhesive properties of cells. Therefore, completing the current knowledge of LFA-1 affinity/avidity may indeed help us understand the complex interactions between a T cell and an APC.

Transmembrane proteins are proteins that are permanently attached to the cellular membrane and span from one side to the other passing through a lipid bilayer [53]. Their function is to permit the transportation of substances across the membrane [53]. In integrins, each α and β subunits has transmembrane protein that is a single α-helix [53]. α-helix is a spiral conformation of secondary structure of proteins which has amino acid side chains that donate a hydrogen bond to the backbone of carbonyl group of another amino acid that is four positions away [54]. The transmembrane helices have a conserved pattern of hydrophobicity that is lipid-embedded helps in the formation of well defined secondary structure and therefore transmission of activation signals [55].

Studies have shown that these transmembrane proteins play an important role in regulating integrin activation [56]. O'Toole and colleagues have shown that the association of transmembrane with cytoplasmic domains of α and β subunits of integrins regulates integrins' signaling that leads to their activation [48]. As mentioned before, integrins' transmembrane domains are held by inner membrane clasp (IMC) and outer membrane clasp (OMC) [42]. Talin binds to the tail of β chain of integrins with high affinity, which stabilizes the helical structure of the integrin tail [57]. Thus, talin in complex with other cytoplasmic domains form a salt bridge that potentially disrupt the membrane clasps spreading α and β subunits away from each other [57]. This structure causes the formation of phospholipid patches that allow talin to alter the tilt angle of β subunit transmembrane domain, leading to full activation of integrins [57].

Recent years have seen a significant increase in the use of atomic force microscopy (AFM) in biological studies. Single-cell force spectroscopy by AFM has been established an important tool for the study of cell adhesion [58]. This technique allows the analysis of adhesion forces under physiological conditions. AFM has shown its value in the quantification of binding forces between single cells at Newton (N) scale.

In addition, AFM made it possible to study single-molecule force spectroscopy of soluble proteins and cell bound ones. For instance, it was used in a variety of research to characterize the actual mechanics involved in integrin-ligand interactions. In our research, we successfully measured the dynamics of contact forces in CD4+ T cell/DC interactions by AFM.

To represent the protein behavior in AFM experimental process, single-cell force spectroscopy method can be used to stretch and unfold the α helical protein. During force

measurements, pulling forces are applied on the surface proteins causing the α-helix to unfold by breaking the hydrogen bonds that stabilize the helical structure [59, 60]. This unfolding process increases the affinity strength, which includes energy changes [61, 62]. Each α-helix is tightly bound and therefore stores energy in its bonds. In order for α-helix to unwind, outside forces must break these energy barriers [61]. In general, molecular structures tend toward the lowest energy configuration [61, 62]. In our AFM experiments, the minimum energy state is represented by the minimum point on the force curve (figure 2.1b), which corresponds to folded state of the α-helix. When a pulling force is applied to the system, the bond value potentially increases, which interprets that the hydrogen bonds of the alpha helix are broken. Immediately after that, the structure reaches a new equilibrium state. Indeed, this is the structural and energetic features illustrated in each force curve we get from AFM force measurements (figure 2.1b).

1.6 Preliminary observation and hypothesis

While much is known about various molecular and cellular interactions between T cells and antigen presenting cells that are crucial for successful outcome needed for host defense, the specific events and mechanisms by which immunological synapses form remain not fully understood. In order to understand how the surface molecules contribute to the interaction in different CD4+ T cell activation states, it is important to know how CD4+ T cells physically interact with antigen presenting cells, dendritic cells specifically, in terms of developing binding forces. We planed to approach the subject at a single cell level by using atomic force microscopy to provide a clear explanation of the cellular contact. Therefore, we investigated cell-to-cell contact mediated by adhesion molecules between CD4+ T cells and DCs.

Our initial results indicated that naïve CD4+ T cells interact with dendritic cells with stronger force in comparison to activated CD4+ T cells. This high force of binding may correlate to the mechanism in which surface molecules interact with each other. This made us suspect that the increase in the force involves the integrin LFA-1. The interactions between LFA-1 and ICAM-1 and the role of these molecules have been studied on naïve T cells. However, that has not been fully compared to the interaction of these adhesion molecules on activated T cells. Thus, here we describe and discuss the biophysical data that we have generated in an effort to obtain a better understanding of the role played by the common accessory molecules LFA-1 and ICAM-1 during interaction between DC's and naïve vs. activated T cells.

Our data show that unlike the interaction between activated CD4+ T cells and DCs, LFA-1 is required for the strong adhesion forces in the interaction between naïve

CD4+ T cells and DCs. We believe that the difference in adhesion can be unveiled by examining the differences in expression and/or regulation of LFA-1 on naïve vs. activated CD4+ T cells.

Therefore, we hypothesize that LFA-1 is the main mediator of the adhesion force between naïve CD4+ T cells and DCs, where the ensuing tight contact permits activation signals to be transduced. Moreover, we suspect that LFA-1 cell surface dynamics are regulated differently in naïve and activated CD4+ T cells, which provides naïve CD4+ T cells with stronger adhesive properties.

Chapter Two: Materials and methods

2.1 Mice, cell lines and reagents

2.1.1 Mice

OTII mice were purchased from Jackson Laboratory and bred onsite. OTII mouse was used for antigen specific responses, as this strain is transgenic for a TCR specific for ovalbumin 323-339, in context of major histocompatibility class II molecule (MHC II) I-A^b [63]. All mice were handled according to the University of Calgary animal care guidelines.

2.1.2 Cell lines

DC2.4 cell line was donated by Kenneth Rock of the University of Massachusetts Medical School. DC2.4 is an immortal dendritic cell line. DC2.4 has the ability to present exogenous OVA antigen on both MHC II and I [64]. DC2.4 was cultured in RPMI culture media containing 5% calf bovine serum at 37°C and 5% carbon dioxide (CO_2). This cell line has the ability to attach to a glass disk, which is helpful during AFM experiments.

OTII cells were obtained from OTII splenocytes and grown in RPMI media with 10% fetal bovine serum (FBS), incubated at 37°C and 5% CO_2.

For T cells activation, OTII T cells were isolated from OTII mice and pre-exposed to 100 µg/mL of soluble OVA for 48 hours, then washed three times and rested for another 48 hours to be used afterword.

2.1.3 Reagents

The antibodies used were as follows. Rat anti-mouse LFA-1 antibody (clone: M17.4, isotype: IgG2a κ), used to block LFA-1 adhesion molecule on T cells, was purchased from BioLegend. Rat anti-mouse ICAM-1 antibody (clone YN1/1.7.4) was used to block ICAM-1 adhesion molecule on dendritic cells (BioLegend). Mouse anti-human CD4 (Invitrogen). Rat IgG2a κ was used as isotype control for anti-mouse LFA-1 antibody; it was purchased from BD Pharmingen. Mouse IL-2 ELISA kit was purchased from eBioscience. Ovalbumin (OVA) was purchased from Molecular Probes.

2.2 Atomic Force Microscopy

JPK Nanowizard II AFM was used in the CellHesion mode to conduct all AFM experiments in this research. CellHesion mode allows single cell interactions to be measured in order to give force curves (figure 2.1 a and b).

Atomic force microscopy, first introduced to immunological research by our lab, has been instrumental in our work on APC activation in response to immune adjuvants [64]. In AFM force spectroscopy experiments, individual cells are glued to a cantilever. The cantilevers used were tipless non-coated cantilevers (ARROW-TL1) with silicon nitrate substrate purchased from Nanoworld. T cells were glued on this cantilever using dopamine hydrochloride (DOPA) purchased from Sigma [65]. DOPA was made by preparing 4mg/mL of dopamine hydrochloride in 10 mM Tris buffer, pH: 8.5. After that, the cantilever was incubated for one hour in DOPA solution then washed with ddH_2O to be ready for use (figure 2.2).

AFM has motors that allow us to move the cantilever around within the well to come in contact with the cell of interest. Our cantilever with DOPA was then pressed gently against the desired cell, which is a T cell in this case. The cantilever with the cell was lowered to an empty section of the glass disk surface to be calibrated in CCM, culture media. The calibration is needed to give a quantitative force value in Newton (N). In calibration, there are two measurements that are required. First one is to determine the sensitivity, which is the measurement of the deflection of the cantilever in nanometers (nm). The second measurement is calibration of the spring constant of the cantilever, so that the nanometers deflection of the cantilever will be converted into actual force values (N). Spring constant measurement is done by measuring the thermal noise, which is the background thermal fluctuation frequency of the cantilever [66-70].

Once the cantilever is calibrated, the glued cell was used to measure the forces between this T cell and a DC that is attached to a glass disk, as DCs efficiently adhere to the glass disks [71, 72]. Developing the forces between the cells was done by positioning the cantilever with the T cell over the desired DC and lowering the cantilever until there was an interaction between the cells. When the T cell was in contact with the DC, it was left for 15 seconds and then retracted. This interaction is referred to as the tapping mode. When the cantilever is pulled away from the surface, adhesion forces can be measured by measuring the deflection of laser shone on the cantilever (figure 2.1a). This deflection is then translated into force curves blotted over time where they give the maximum force required to separate the cells after binding to each other. This value is represented as the minimum point in the curve (figure 2.1b). The force curves were analyzed using JPK NanoWizard II software.

The parameters used in all AFM experiments are:

IP gain: 5 Hz; IG gain: 0.0002 Hz; Set point: 0.5 nN; Z length: 55 nm; Z end: 45 nm; contact time: 15 seconds.

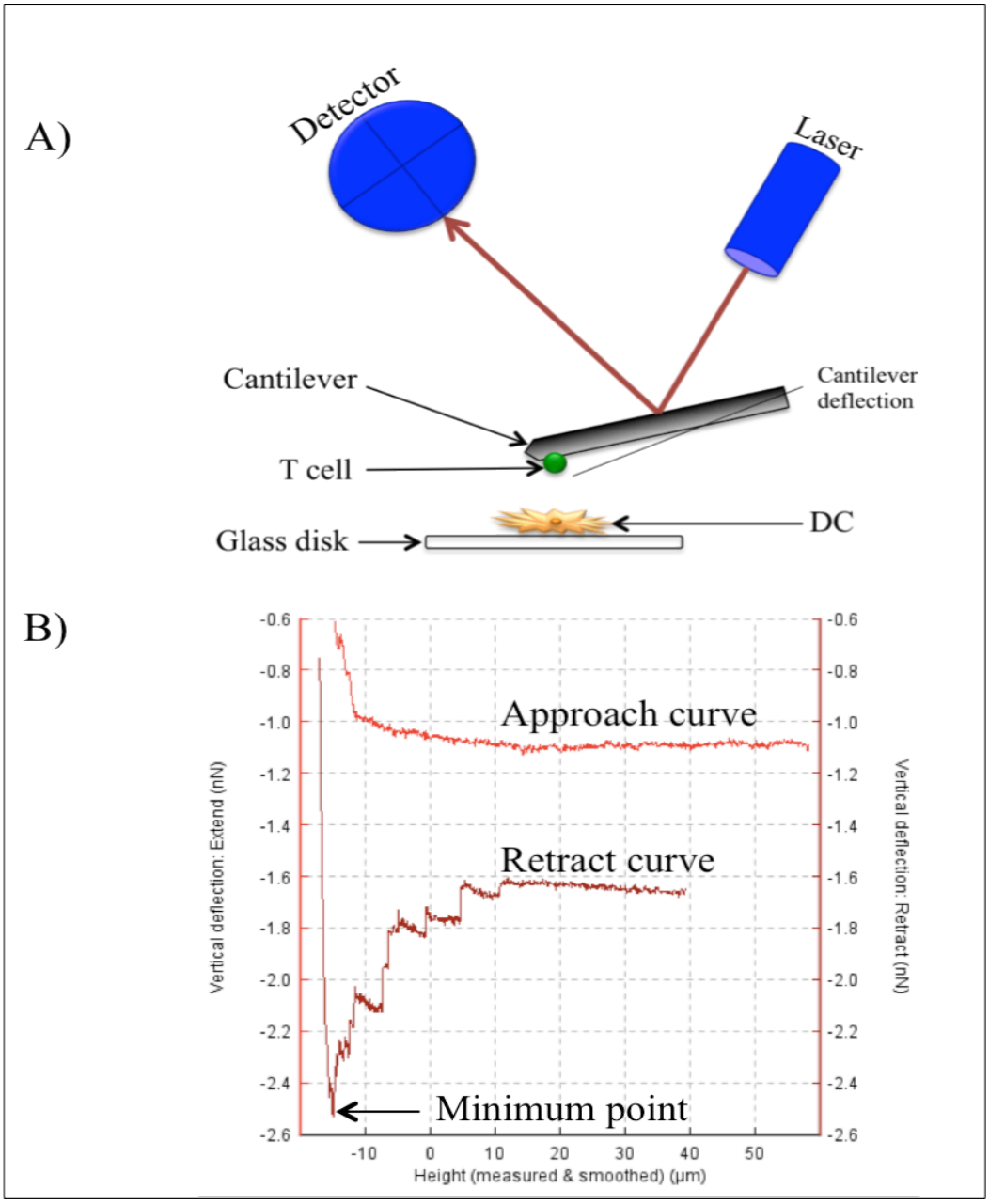


Figure 2.1. Schematic of the atomic force microscope.

A) The illustration represents a typical AFM experiment setup. A cell is adhered to the tip of a cantilever, which is controlled by a set of motors. The stage holds the disk coated with the cells of interest. The detector detects changes in the angle of deflection when the cantilever is deflected. A force curve is generated and illustrated in B.

B) The approach curve reflects the cantilever moving toward the cell on the stage. The retract curve is the cantilever moving back to its normal position away from the cell of interest. The minimum point of the force curve is what is measured and plotted over time.

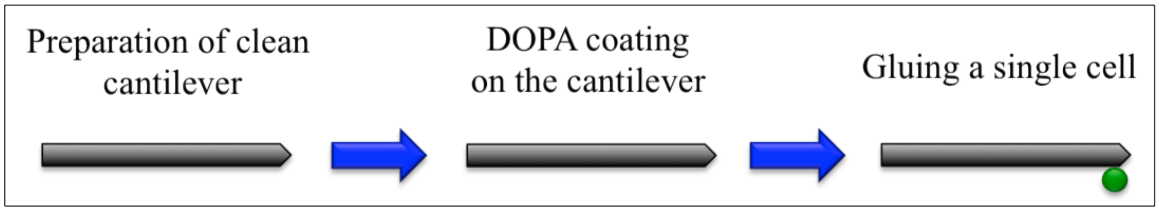


Figure 2.2. DOPA gluing method.

To glue a cell, preparation of a clean cantilever is needed for coating with DOPA.

2.2.1 Force spectroscopy interaction of naïve and activated T cells with DCs in the presence and absence of OVA antigen

To conduct these experiments, the interaction forces were examined between T cells and dendritic cells. The cells adhered to the cantilever were OTII T cells that were obtained from OTII mice. DC2.4 cells were cultured on glass disks in the presence and absence of 100 μg/mL OVA.

2.2.2 Force spectroscopy interaction of naïve and activated T cells with DCs after blocking of LFA-1

The same cells that were used to examine the interaction forces between a T cell and a dendritic cell were also used here after adding 25 μg/mL of M17.4 antibody for 10-15 minutes to block LFA-1 on T cells.

2.3 Enzyme-linked immunosorbent assay (ELISA)

Interleukin-2 (IL-2) quantitative detection was done by ELISA using cell culture supernatants. DC2.4 cells were plated for 24 hours in advance and naïve and activated OTII T cells were obtained from OTII splenocytes and added to the DC2.4. 100 μg/mL of OVA, 25 μg/mL of M17.4 and YN1, and 25 μg/mL of rat IgG2a κ isotype control were added to the appropriate wells. IL-2 ELISA kit was used to measure IL-2 concentration.

2.4 Flow Cytometry

Cells were washed and stained with the appropriate antibodies for 30 minutes at 4°C. The cells were then washed with 3 mL of PBS, centrifuged, and supernatant

removed. If secondary antibody staining is needed, the process was repeated. The cells were then fixed with 100 μL of 1% paraformaldehyde (PFA). Stained cells were analyzed using FACScan flow cytometers and the data was analyzed using Flowjo.

2.4.1 LFA-1 cell surface expression

LFA-1 expression was examined on CD4+ T cells using M17.4 anti-LFA-1 antibody. The cells were isolated, centrifuged at 1,500 RPM and co-stained for LFA-1 and CD4 for 30 minutes at 4°C. The cells were then washed in 3 mL PBS, centrifuged, and fixed with 100 μL of 1% PFA. The samples were then read on the FACScan and analyzed using Flowjo software.

2.5 Statistical analysis

Student T-tests were used to compare two samples. ANOVA was used to compare the means of 3 or more groups of observations. However, an ANOVA p-value indicates significance but does not inform where the significance lies. Therefore, the multiple comparison test, Tukey's Test, was performed to determine which groups were significantly different from one another [73].

Chapter Three: Results

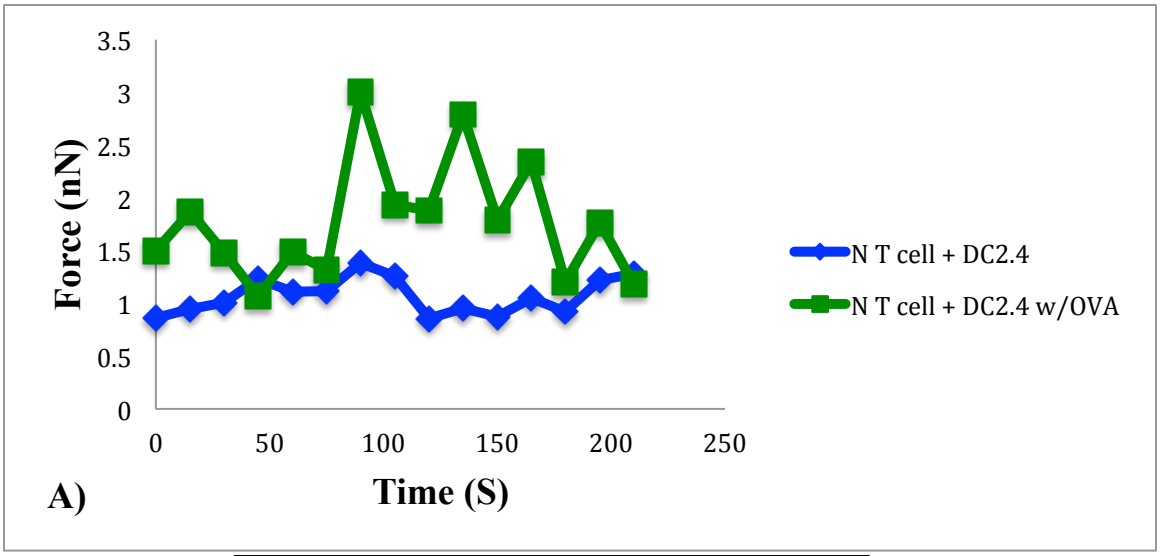
3.1 The force of interaction between naïve OTII T cells and DC2.4 is increased in the presence of OVA antigen

The atomic force microscope is a novel technology utilized to examine cell-to-cell interactions [72, 74, 75]. Cellhesion, a mode on the atomic force microscope, enables single cell interactions to be directly examined through the generation and analysis of force curves. It allows a working distance (z axis) in which the cell adhered to the cantilever can be completely separated from the cell or substrate of interest on the slide. Currently, the AFM utilized for experiments is placed in an environment similar to that of an incubator. The temperature is set at 37°C and at 5% CO_2.

The AFM consists of a cantilever on which a cell can be glued using biologically inert glue (DOPA). Once calibrated, the cantilever with the attached naïve or activated OTII T cell is positioned over a DC2.4 and is lowered until an interaction between the two surfaces is detected. The contact between the cells remains for 15 seconds and is then retracted. The deflection of the cantilever is then recorded by the detector and is measured by the change in the angle of reflection. This change is then translated into a force curve, which is then analyzed using JPK image processing software.

We first observed cell-to-cell interactions using the AFM. As illustrated in figure 3.1a-b, naïve OTII T cells interacted with an incresed force of interaction with DC2.4. The force seen in the presence of antigen was significantly greater than the force in the absence of OVA antigen. As shown in figure 3.2a-b, naïve OTII T cells were then replaced with activated OTII T cells. The force seen before and after adding antigen to

activated OTII T cells was similar; however, there was a significant difference between naïve OTII T cells and activated ones when interacting with DC2.4 in the presence of OVA antigen as shown in figure 3.4a-b. These results indicate that naïve OTII T cells interact with a higher physical force with DC2.4.



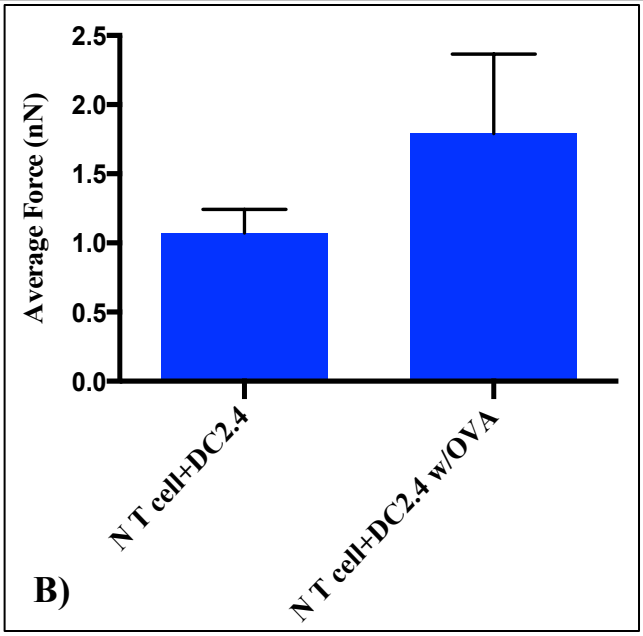


Figure 3.1. Atomic force microscopy shows increase in the force of interaction between a naïve OTII T cell and a dendritic cell in the presence of antigen.

OTII T cells were isolated from OTII mice and incubated overnight. The cells were then glued to the cantilever and touched DC2.4 cells. For measurements done in the presence of OVA, DC2.4 cells were cultured on a glass disk overnight and 100 µg/mL of soluble OVA was added 2-4 hours prior to the experiment.
A) Each series represents an individual naïve T cell (N T cell) touching a DC2.4 cell. Each point represents the maximum force required to separate the T cell from a DC over time, measured in seconds.
B) The bars represent the average forces (nN) obtained from the analyzed force curves. The error bars represent the standard error. (Naïve T cell + DC2.4 n= 4; Naïve T cell + DC2.4 w/OVA n= 4). Student T test p value=0.0002. The difference is statistically significant.

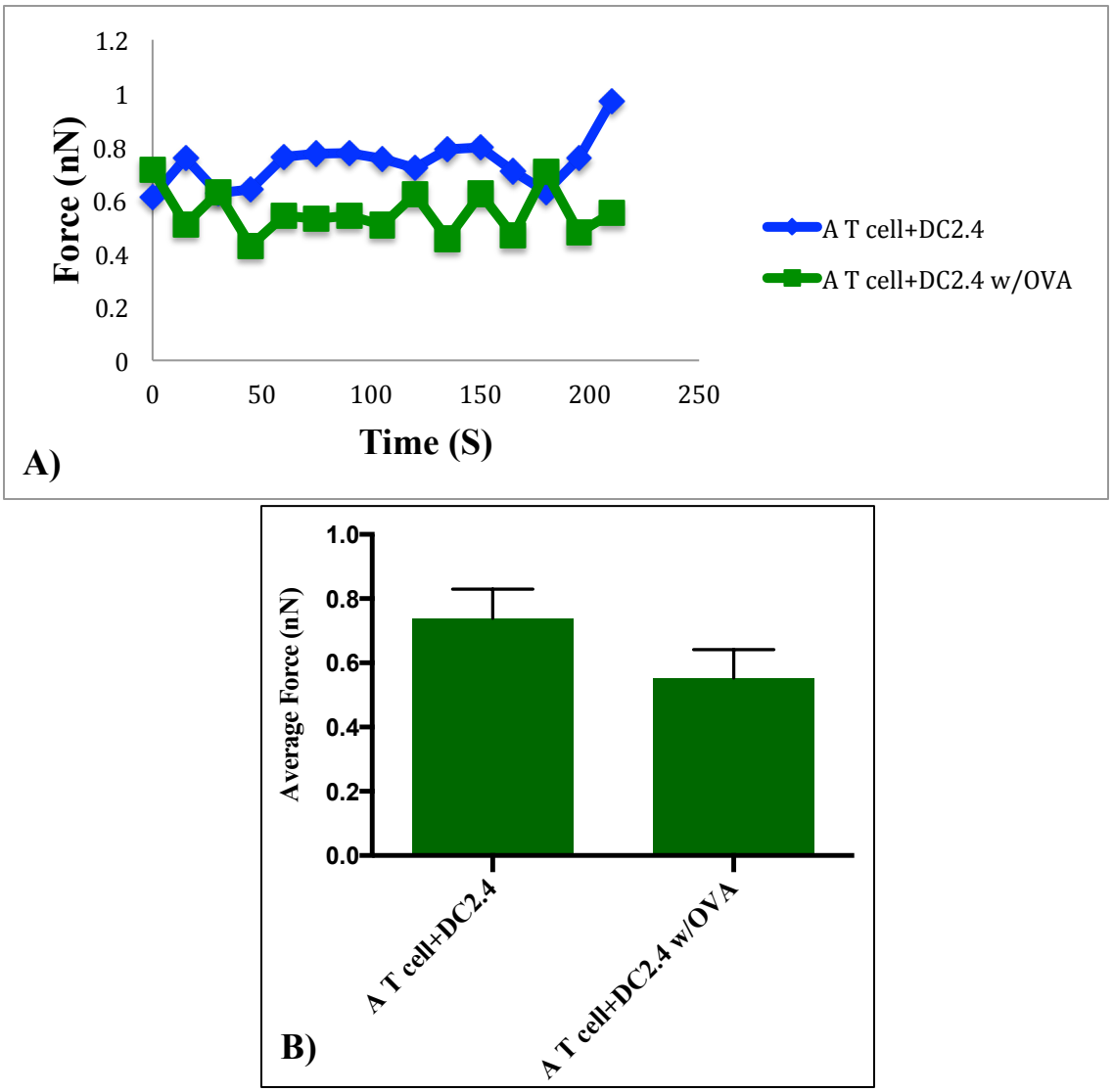
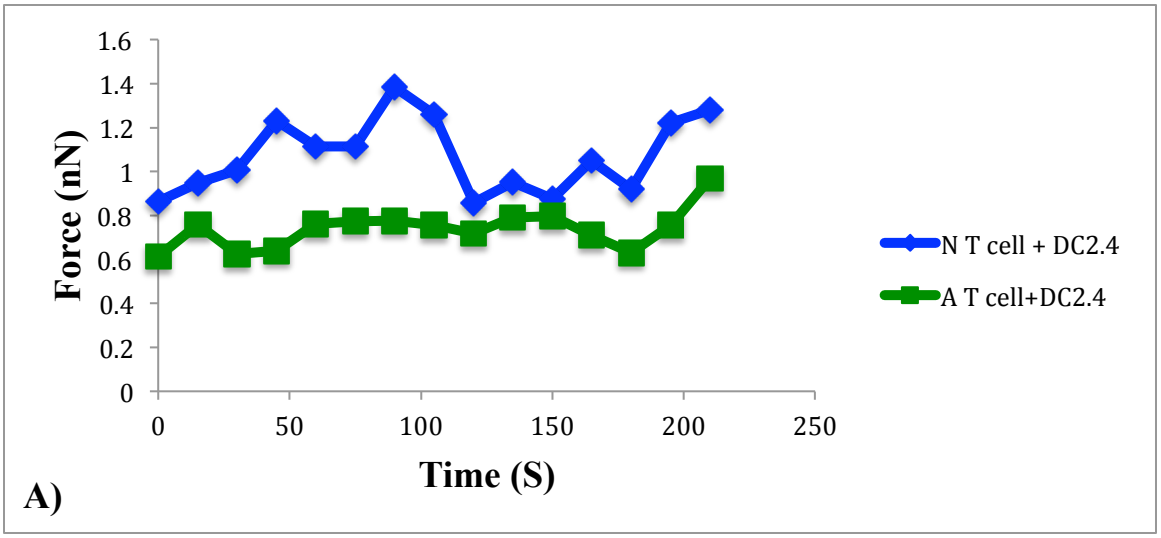


Figure 3.2. Atomic force microscopy shows no significant difference in the forces between activated OTII T cells before and after adding OVA when interacting with DC2.4.

OTII T cells were isolated from OTII mice and pre-exposed to 100 µg/mL of soluble OVA for 48 hours, then washed and rested for another 48 hours. The cells were then glued to the cantilever and touched to DC2.4 cells. For measurements done in the presence of OVA, DC2.4 cells were cultured on a glass disk overnight and 100 µg/mL of soluble OVA was added 2-4 hours prior to the experiment.
A) Each series represents an individual activated T cell (A T cell) touching a DC2.4 cell. Each point represents the maximum force required to separate the T cell from a DC over time, measured in seconds.
B) The bars represent the average forces (nN) obtained from the analyzed force curves. The error bars represent the standard error. (Activated T cell + DC2.4 n= 4; Activated T cell + DC2.4 w/OVA n= 4). Student T test p value=0.8961. The difference is statistically not significant.



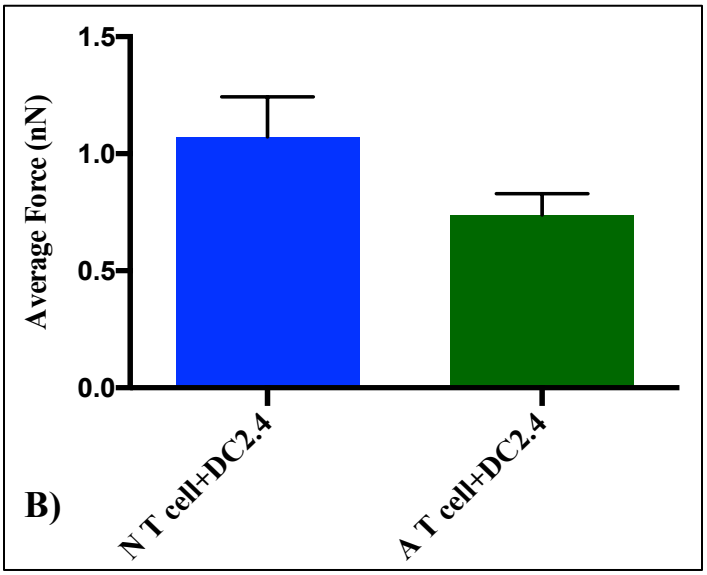


Figure 3.3. Atomic force microscopy indicates no significant difference in the forces between activated and naïve OTII T cells when interacting with DC2.4 in the absence of OVA.

OTII T cells were isolated from OTII mice, and to get the activated cells, the cells were pre-exposed to 100 µg/mL of soluble OVA for 48 hours, then washed and rested for another 48 hours. Both activated and naïve T cells were then glued to the cantilever and touched to a DC2.4 cell.
A) Each series represents an individual naïve or activated T cell (N T cell or A T cell, respectively) touching a DC2.4 cell. Each point represents the maximum force required to separate the T cell from a DC over time, measured in seconds.
B) The bars represent the average forces (nN) obtained from the analyzed force curves. The error bars represent the standard error. (Naïve T cell + DC2.4 n= 4; Activated T cell + DC2.4 n= 4). Student T test p value=0.0230. The difference is statistically not significant.

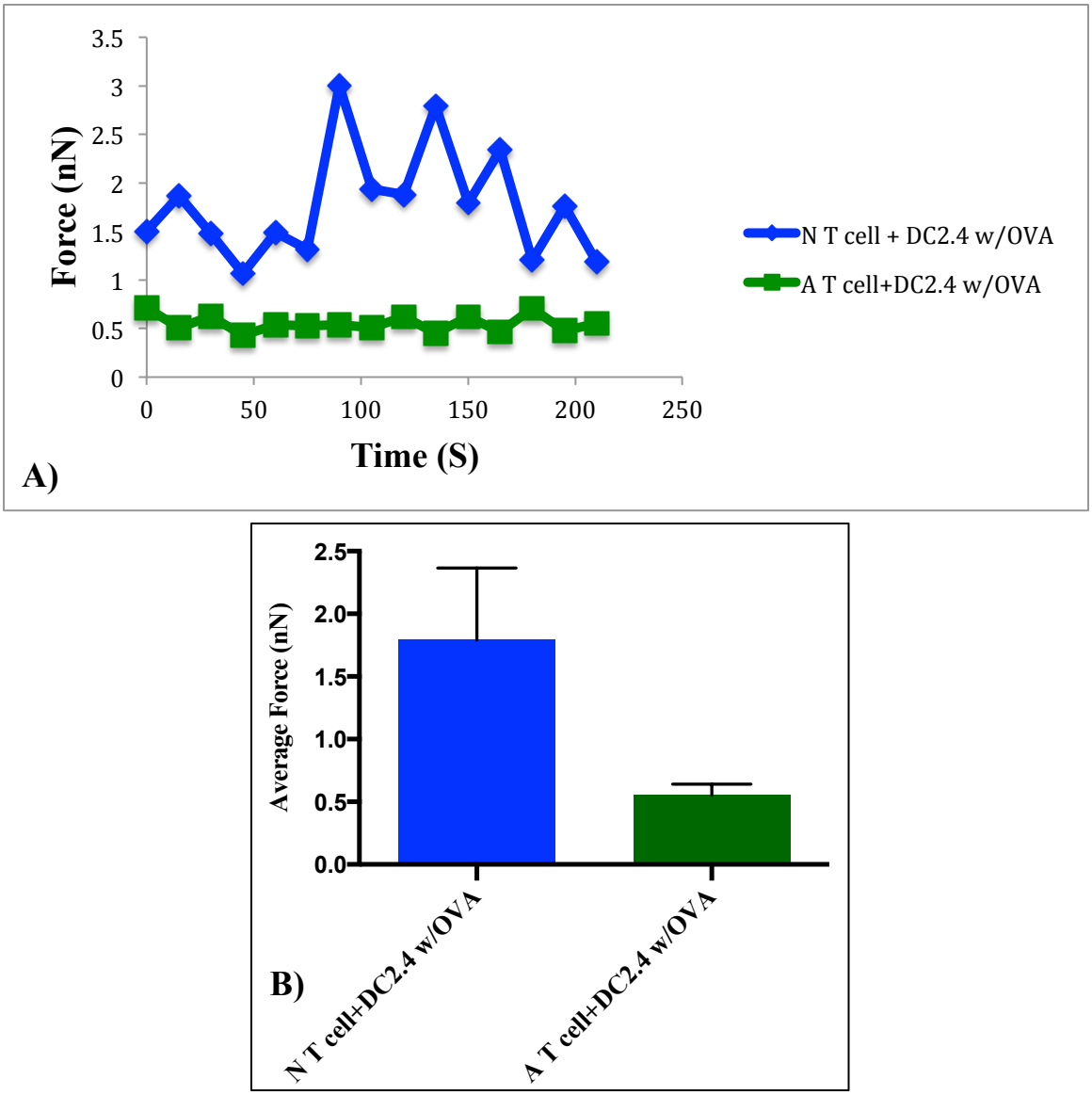


Figure 3.4. Atomic force microscopy indicates a significant difference in the forces between activated and naïve OTII T cells when interacting with DC2.4 in the presence of OVA.

OTII T cells were isolated from OTII mice, and to get the activated cells, the cells were pre-exposed to 100 µg/mL of soluble OVA for 48 hours, then washed and rested for another 48 hours. The cells were then glued to the cantilever and touched to a DC2.4 cell that were pre-exposed to 100 µg/mL of soluble OVA for 2-4 hours.
A) Each series represents an individual naïve or activated T cell (N T cell or A T cell, respectively) touching a DC2.4 cell. Each point represents the maximum force required to separate the T cell from a DC over time, measured in seconds.
B) The bars represent the average forces (nN) obtained from the analyzed force curves. The error bars represent the standard error. (Naïve T cell + DC2.4 w/OVA n= 4; Activated T cell + DC2.4 w/OVA n= 4). Student T test p value=0.0001. The difference is statistically significant.

3.2 LFA-1 is responsible for the high force of interaction between naïve OTII T cells and DCs

LFA-1, an essential component in T cell migration and immune synapse formation, binds to its ligand ICAM-1 on APCs [42]. LFA-1 is known for its adhesive properties and its importance for T cells functions. We regarded LFA-1 adhesive properties to be a potential target responsible for the force of interaction seen between CD4+ T cells and DCs. Using AFM, we wanted to examine how the force of interaction is affected when key adhesion molecules are eliminated from the T cell and/or DC.

After blocking of LFA-1 by using M17.4 anti-LFA-1 antibody, OTII T cells were unable to generate the strong force of interaction seen before blocking (figure 3.6a-b). Compared to naïve cells, activated OTII T cells showed no significant difference before and after blocking of LFA-1 as illustrated in figures 3.7a-b and 3.8a-b. In addition, an isotype control for LFA-1 blocking antibody was used to test whether anti-LFA-1 antibody had any other interference. Figures 3.9a-b and 3.10a-b show that M17.4 anti-LFA-1 antibody was directed against LFA-1 and did not have any cross-reactivity with FC γ receptors on dendritic cell. Therefore, LFA-1/ICAM-1 adhesion pair was responsible for the increase in the force of interaction between naïve OTII T cells and DCs in the presence of antigen.

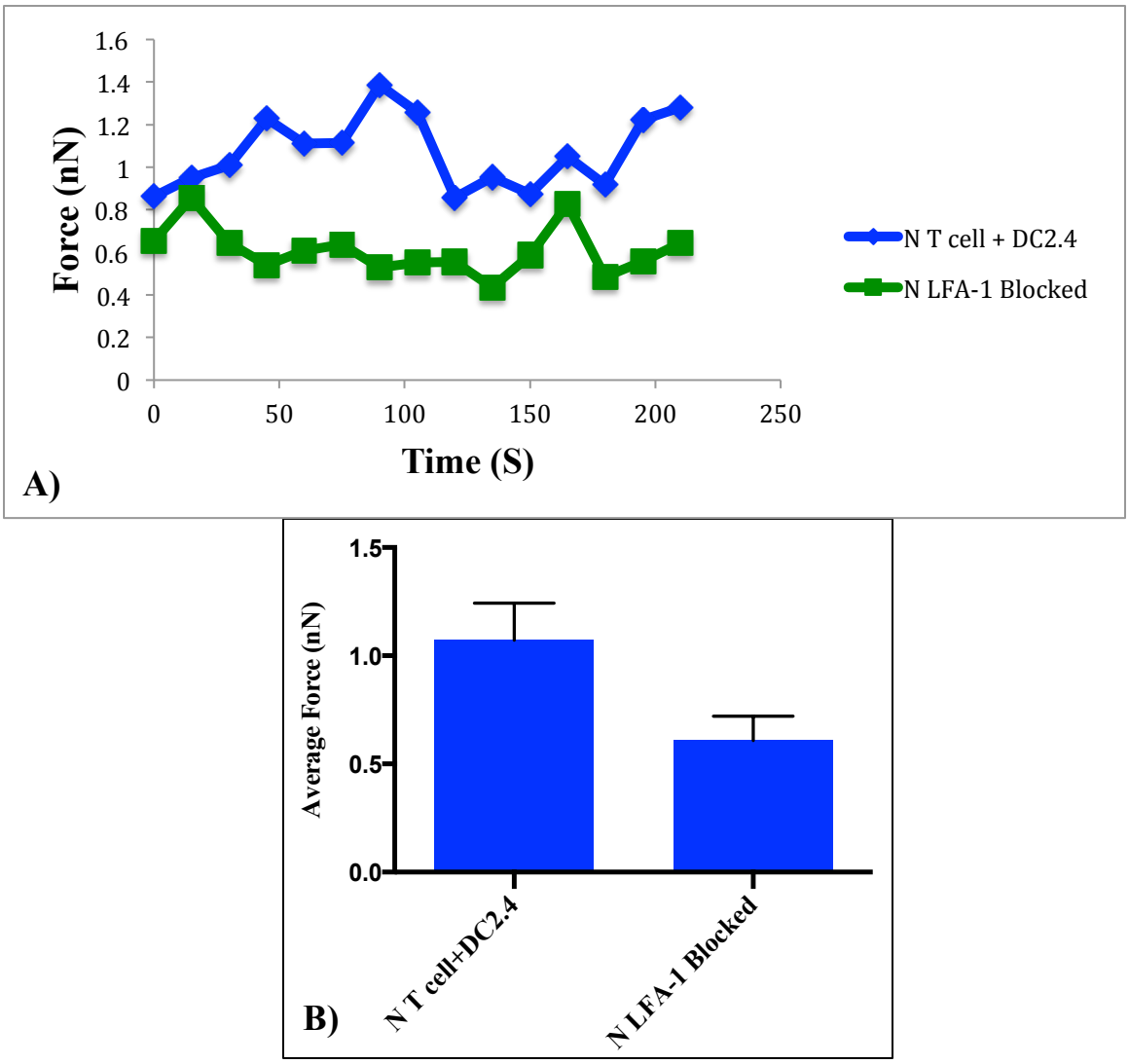


Figure 3.5. AFM shows no significant difference in the forces between the control (naïve OTII T cells and DC2.4), and the measurement in presence of LFA-1 blocking antibody.

OTII T cells were isolated from OTII mice and incubated overnight. The cells were then glued to the cantilever and touched DC2.4 cells in absence of OVA. For the measurement in the presence of M17.4 (N LFA-1 Blocked bar), DC2.4 cells were cultured on a glass disk overnight and 100 µg/mL of soluble OVA was added 2-4 hours prior to the experiment; 25 µg/mL of LFA-1 blocking antibody was added to OTII cells 10-15 minutes before experimentation.
A) Each series represents an individual naïve T cell (N T cell) touching a DC2.4 cell. Each point represents the maximum force required to separate the T cell from a DC over time, measured in seconds.
B) N T cell+DC2.4 bar represents the average force between naïve OTII T cell and DC2.4 with no OVA; and the bar on the right represents the average force between naïve OTII T cell and DC2.4 with OVA after adding M17.4 antibody. (Naïve T cell + DC2.4 n= 4; Naïve LFA-1 blocked T cell + DC2.4 w/OVA n= 4). Student T test p value=0.1317. The difference is statistically not significant.

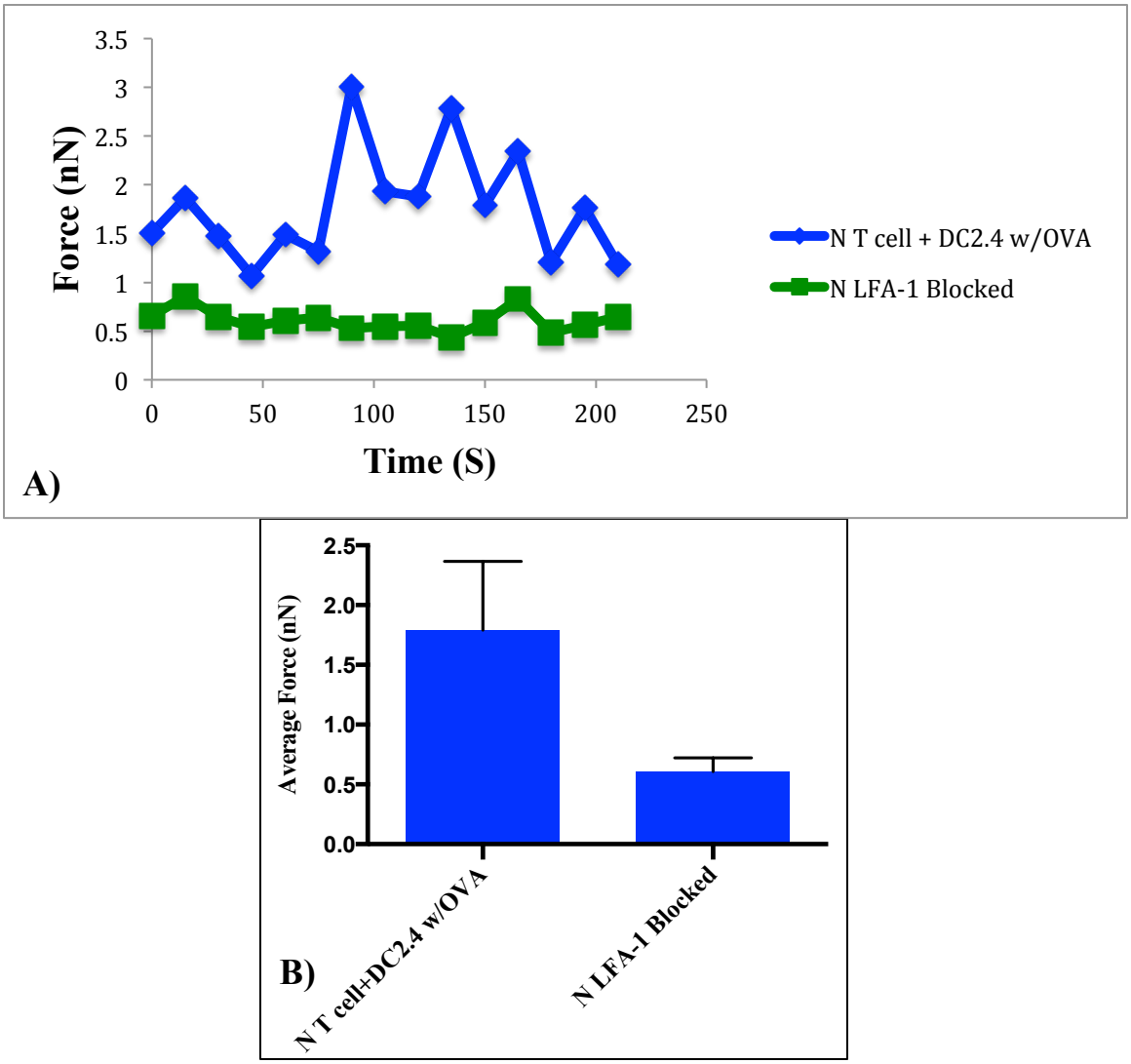


Figure 3.6. AFM results show a significant decrease in the forces after LFA-1 blocking on naïve OTII T cells when interacting with DC2.4 in the presence of OVA.

OTII T cells were isolated from OTII mice and incubated overnight. The cells were then glued to the cantilever and touched DC2.4 cells after adding OVA. For the measurement in the presence of M17.4 (N LFA-1 Blocked bar), DC2.4 cells were cultured on a glass disk overnight and 100 µg/mL of soluble OVA was added 2-4 hours prior to the experiment; 25 µg/mL of LFA-1 blocking antibody was added to OTII cells 10-15 minutes before experimentation.
A) Each series represents an individual naïve T cell (N T cell) touching a DC2.4 cell. Each point represents the maximum force required to separate the T cell from a DC over time, measured in seconds.
B) The bars represent the average forces (nN) obtained from the analyzed force curves. N T cell+DC2.4 w/OVA bar represents the average force between naïve OTII T cell and DC2.4 with OVA; and the bar on the right represents the average force between naïve OTII T cell and DC2.4 with OVA after adding M17.4 antibody. (Naïve T cell + DC2.4 w/OVA n= 4; Naïve LFA-1 blocked T cell + DC2.4 w/OVA n= 4). Student T test p value=0.0001. The difference is statistically significant.

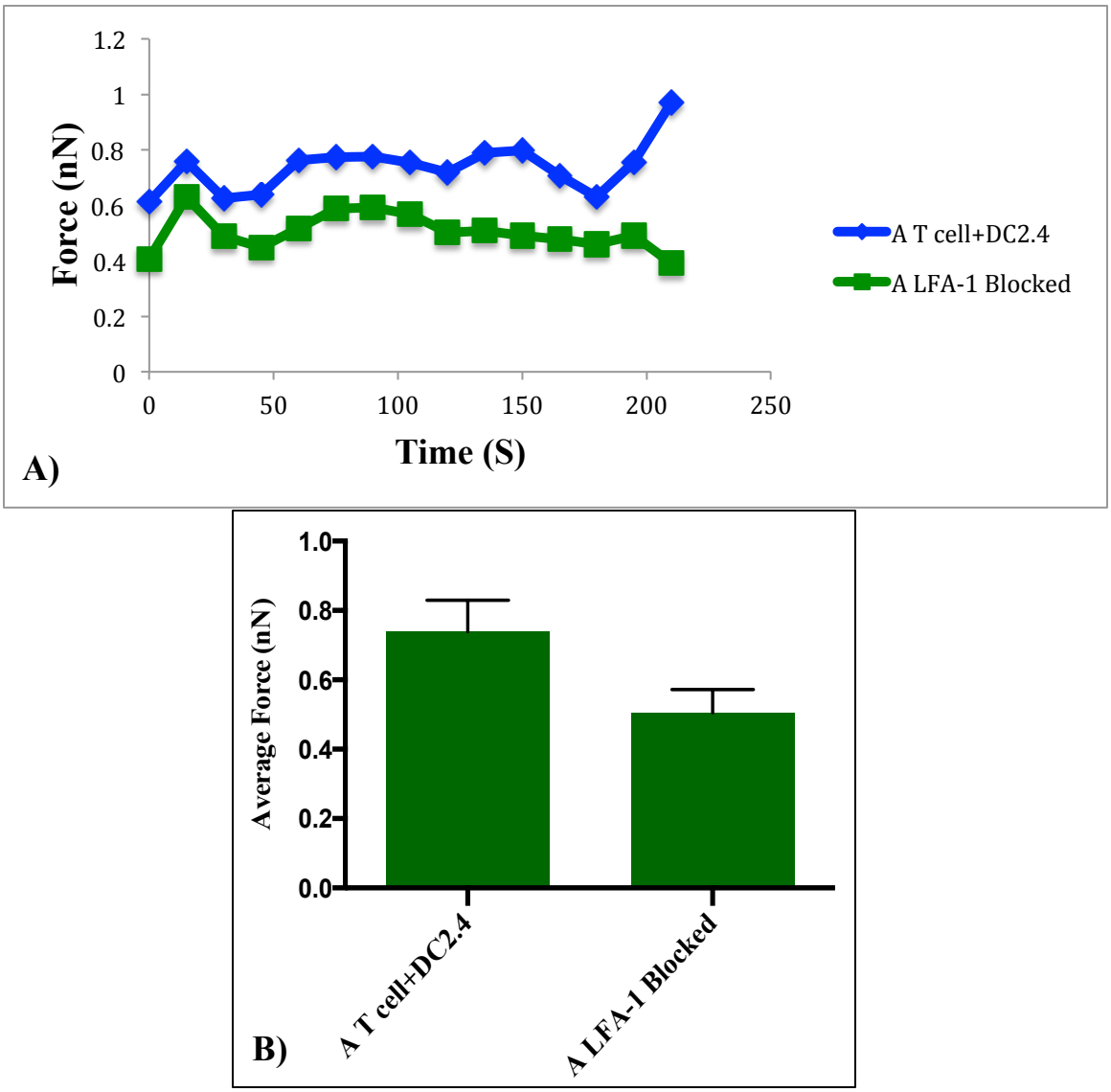


Figure 3.7. AFM indicates no significant difference in the forces between the control (activated OTII T cells and DC2.4), and the measurement in presence of LFA-1 blocking antibody.

OTII T cells were isolated from OTII mice and pre-exposed to 100 µg/mL of soluble OVA for 48 hours, then washed and rested for another 48 hours. The cells were then glued to the cantilever and touched to DC2.4 cells in absence of OVA. For the measurement in the blocked sample (A LFA-1 Blocked bar), 25 µg/mL of LFA-1 blocking antibody was added to OTII cells 10-15 minutes before experimentation; and 100 µg/mL of soluble OVA was added to DC2.4 2-4 hours prior to the measurement.
A) Each series represents an individual activated T cell (A T cell) touching a DC2.4 cell. Each point represents the maximum force required to separate a T cell from a DC over time (seconds). B) A T cell+DC2.4 bar represents the average force between activated OTII T cell and DC2.4 with no OVA; and the bar on the right represents the average force between activated OTII T cell and DC2.4 with OVA after adding M17.4 antibody. (Activated T cell + DC2.4 n= 4; LFA-1 blocked Activated T cell + DC2.4 w/OVA n= 4). Student T test p value=0.2789. The difference is statistically not significant.

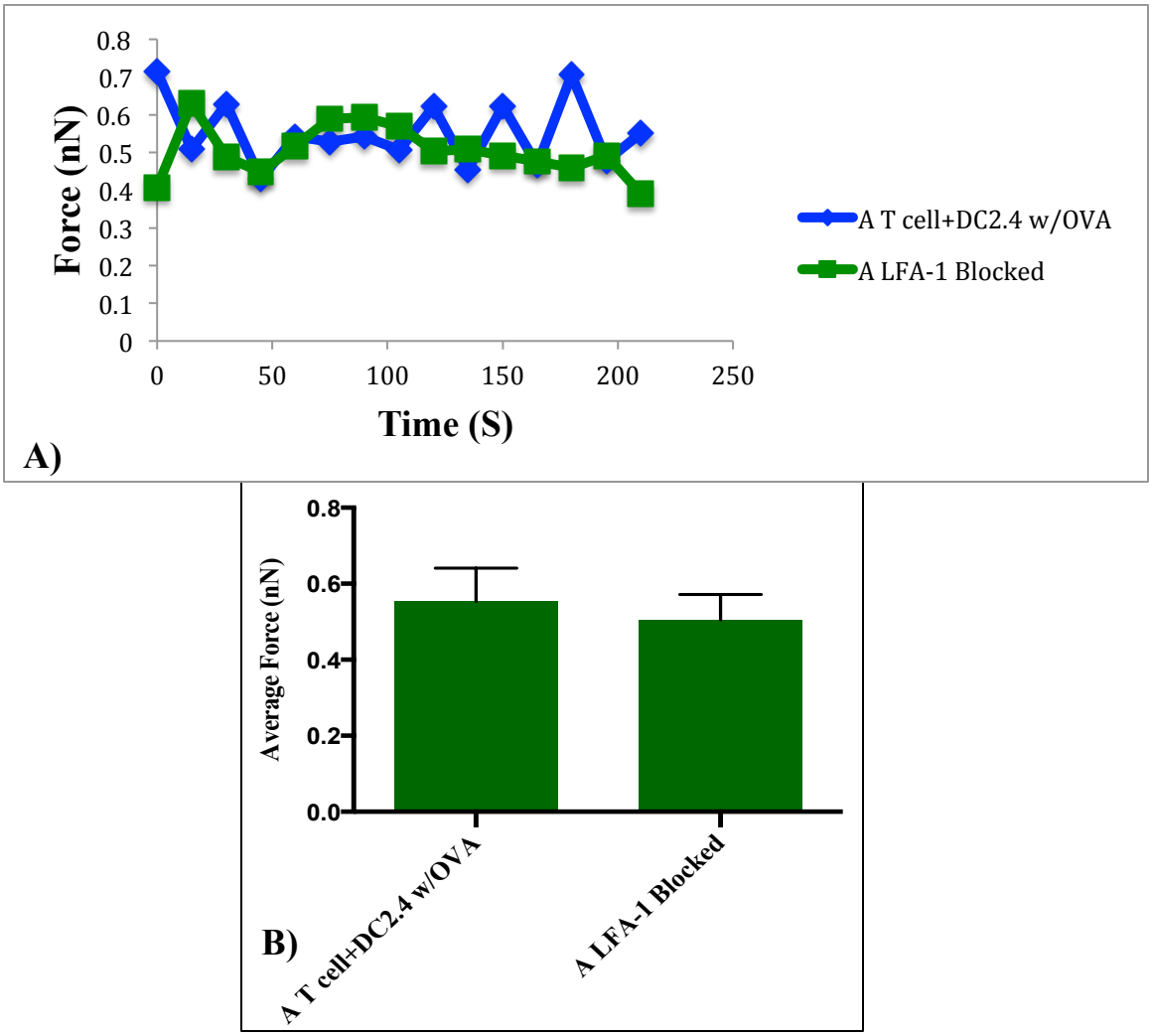


Figure 3.8. There is no significant difference in the forces between activated OTII T cells when interacting with DC2.4 in the presence of OVA before and after LFA-1 blocking.

OTII T cells were isolated from OTII mice and pre-exposed to 100 µg/mL of soluble OVA for 48 hours, then washed and rested for another 48 hours. The cells were then glued to the cantilever and touched to DC2.4 cells after adding OVA. For the measurement in the blocked sample (A LFA-1 Blocked bar), 25 µg/mL of LFA-1 blocking antibody was added to OTII cells 10-15 minutes before experimentation; and 100 µg/mL of soluble OVA was added to DC2.4 2-4 hours prior to the measurement.
A) Each series represents an individual activated T cell (A T cell) touching a DC2.4 cell. Each point represents the maximum force required to separate the T cell from a DC over time, measured in seconds.
B) The bars represent the average forces (nN). A T cell+DC2.4 w/OVA bar represents the average force between activated OTII T cell and DC2.4 with OVA; and the bar on the right represents the average force between activated OTII T cell and DC2.4 with OVA after adding M17.4 antibody. (Activated T cell + DC2.4 w/OVA n= 4; LFA-1 blocked Activated T cell + DC2.4 w/OVA n= 4). Student T test p value=0.3398. The difference is statistically not significant.

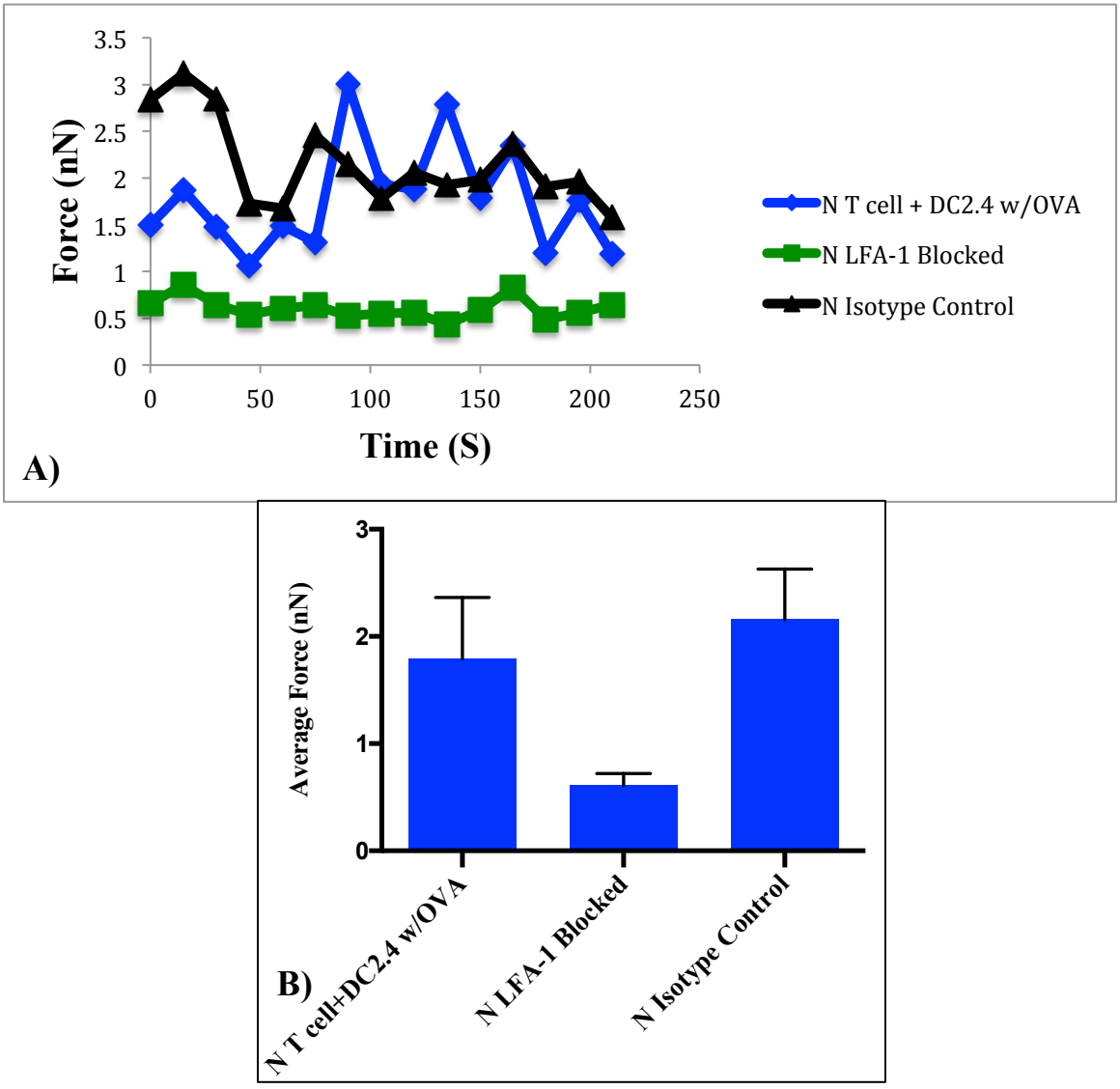


Figure 3.9. Atomic force microscopy indicates a significant difference in the forces between LFA-1 blocked naïve OTII T cells and isotype control.

In LFA-1 blocking measurement, 25 µg/mL of LFA-1 blocking antibody was added to the naïve OTII cells 10-15 minutes before experimentation. For the measurement of isotype control, 25µg/mL isotype control was added for the same time. 100 µg/mL of soluble OVA was added to DC2.4 2-4 hours prior to all the previous measurements.
A) Each series represents an individual naïve T cell (N T cell) touching a DC2.4 cell. Each point represents the maximum force required to separate the T cell from a DC over time, measured in seconds.
B) The bars represent the average forces (nN) obtained from the analyzed force curves. The error bars represent the standard error. (Naïve T cell + DC2.4 w/OVA n= 4; Naïve LFA-1 blocked T cell + DC2.4 w/OVA n= 4; Naïve T cell + DC2.4 w/OVA + isotype control =3). One-way ANOVA p value=0.0001. Tukey's Test indicates a significant difference between (N T cell + DC2.4 w/OVA vs. N LFA-1 blocked) and (N isotype control vs. N LFA-1 blocked).

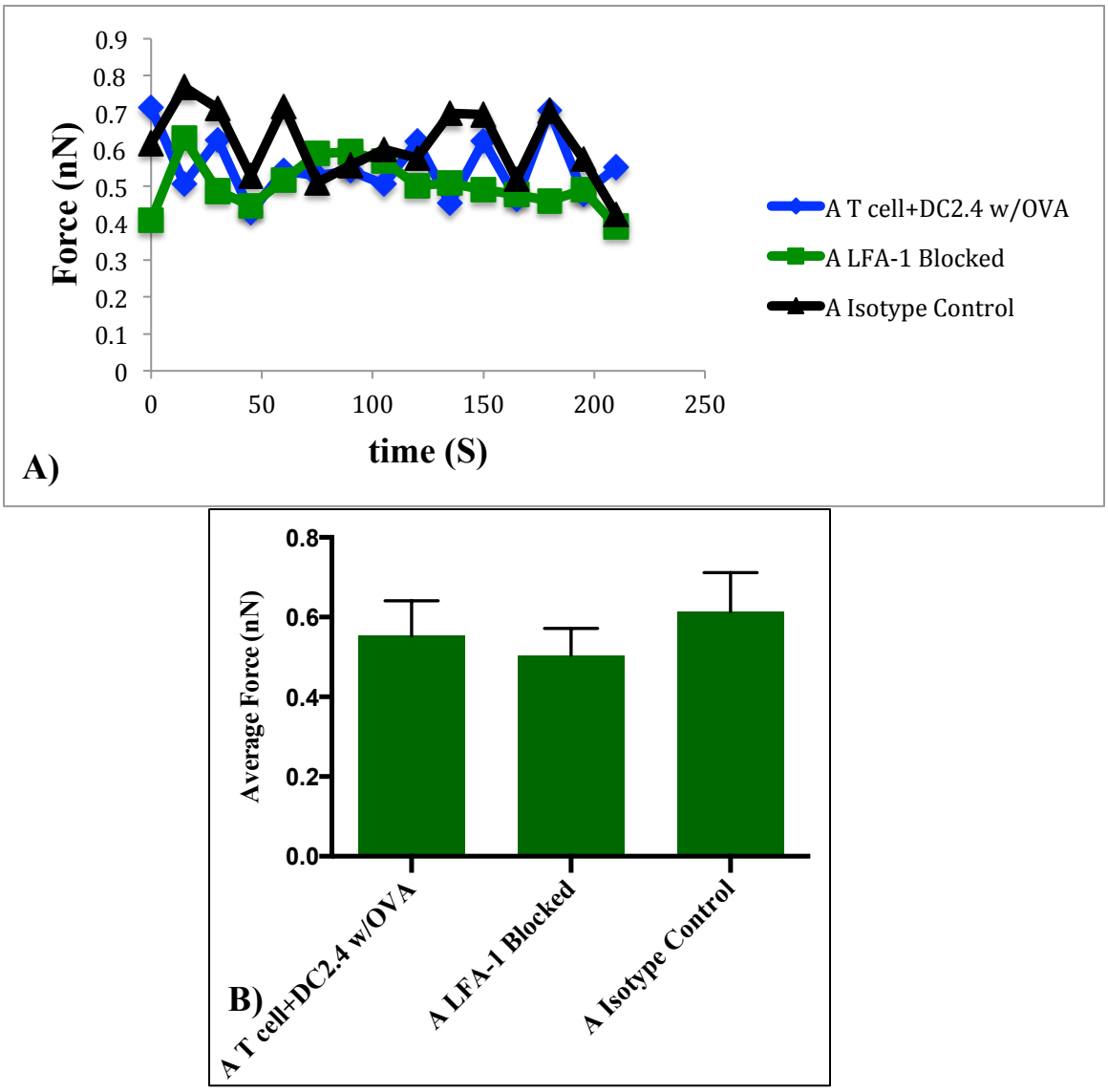


Figure 3.10. AFM shows no significant difference in the forces between LFA-1 blocked activated OTII T cells and isotype control.

In LFA-1 blocking measurement, 25 µg/mL of LFA-1 blocking antibody was added to the activated OTII cells 10-15 minutes before experimentation. For the measurement of isotype control, 25µg/mL isotype control was added for the same time. 100 µg/mL of soluble OVA was added to DC2.4 2-4 hours prior to all the previous measurements.
A) Each series represents an individual activated T cell (A T cell) touching a DC2.4 cell. Each point represents the maximum force required to separate the T cell from a DC over time, measured in seconds.
B) The bars represent the average forces (nN) obtained from the analyzed force curves. The error bars represent the standard error. (Activated T cell + DC2.4 w/OVA n= 4; Activated LFA-1 blocked T cell + DC2.4 w/OVA n= 4; Activated T cell + DC2.4 w/OVA + isotype control =3). One-way ANOVA p value=0.0046. Tukey's Test indicates no significant difference between the three samples.

3.3 LFA-1 surface molecule is required for IL-2 secretion in OTII T cells

In addition to the AFM, we wanted to examine the effect of LFA-1 blocking on naïve and activated OTII T cells when interacting with dendritic cells by using conventional immunological techniques. In figure 3.11, naïve OTII T cells were isolated and added to the appropriate wells containing DC2.4 cells. 100 μg/mL of soluble OVA was added to the appropriate wells and incubated for 2-4 hours. The wells were then washed and OT-II T cells were added. Supernatant was collected at 24 hours. IL-2 levels were measured in order to determine the dendritic cells' ability to stimulate CD4+ T cell activation. The same protocol was used to perform activated T cells ELISA. Both results show that naïve and activated OTII T cells were able to produce IL-2 cytokine when interacting with DC2.4 after stimulation with OVA antigen (figures 3.11 and 3.13).

Furthermore, we intended to investigate whether blocking of LFA-1 would interfere with the secretion of IL-2. Thus, as shown in figures 3.12 and 3.14, in the presence of M17.4 antibody, OTII T cells were not stimulated in the presence of antigen indicating that LFA-1 is an essential component for TCR-MHC II engagement and subsequent activation of CD4+ T cells.

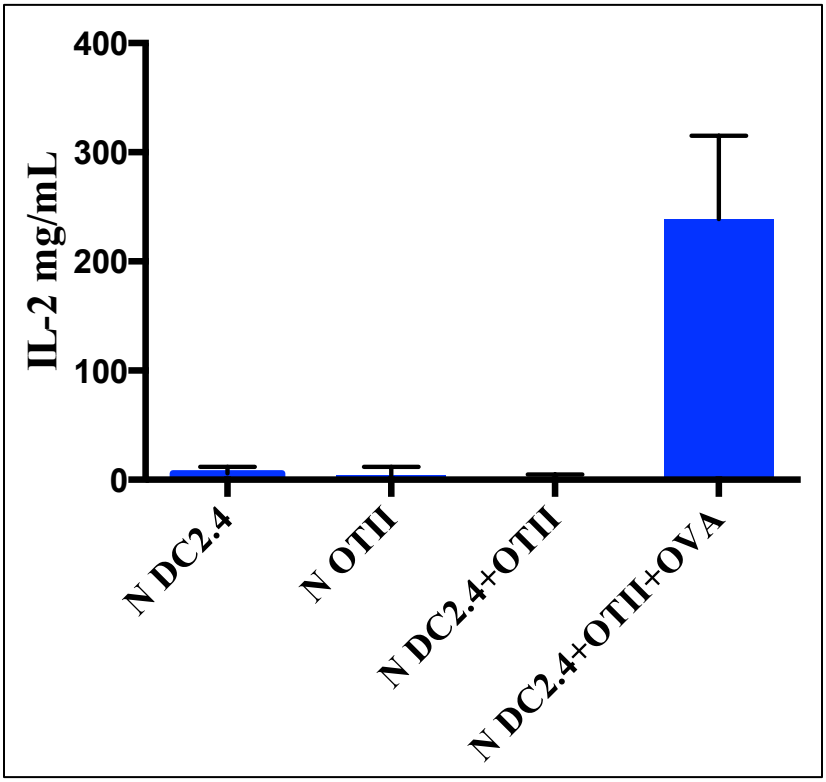


Figure 3.11. ELISA result shows that there is an increase in IL-2 production after adding OVA antigen to DC2.4 and naïve OTII T cells.

Naïve OTII T cells (N OTII) were isolated from OTII mice, and added to the appropriate wells containing DC2.4 cells. In the presence of antigen, 100 µg of soluble OVA was added to DC2.4 cells and incubated for 2-4 hours. Supernatant was collected at 24 hours (n=3).
One-way ANOVA p value=0.0001. Tukey's Test indicates a significant difference between (N DC2.4 vs. N DC2.4+OTII+OVA), (N OTII vs. N DC2.4+OTII+OVA) and (N DC2.4+OTII vs. N DC2.4+OTII+OVA).

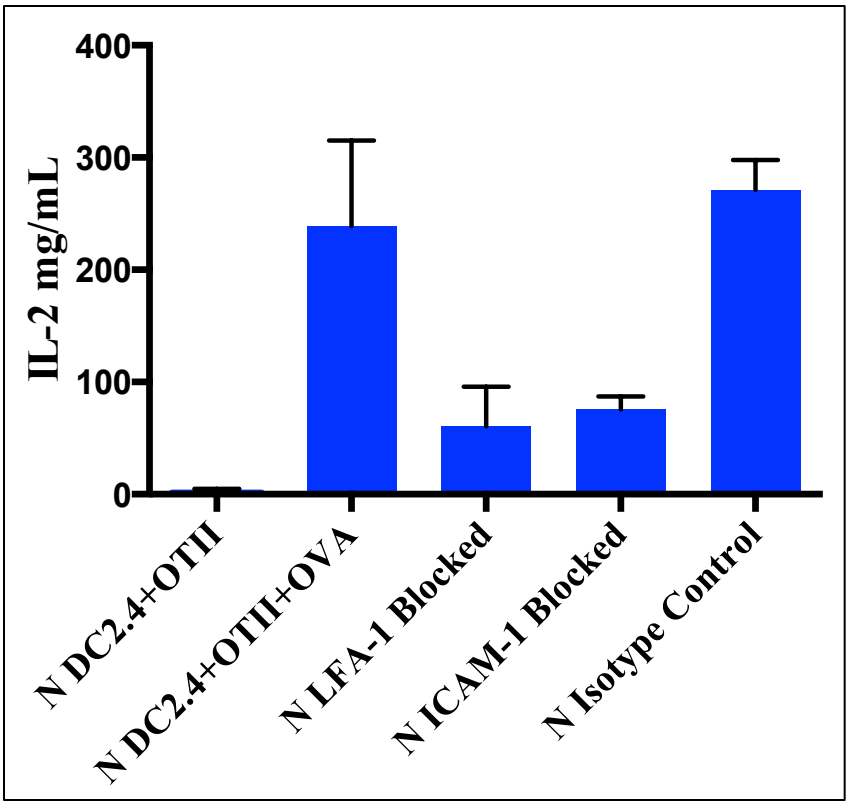


Figure 3.12. ELISA indicates that there is significant difference in IL-2 secretion after blocking of LFA-1 in naïve OTII T cells.

Naïve OTII T cells (N OTII) were isolated from OTII mice, and added to the appropriate wells containing DC2.4 cells. For antigen, 100 µg of soluble OVA was added to DC2.4 cells and incubated for 2-4 hours. 25 µg/mL of LFA-1 blocking antibody was added to the naïve OTII cells 10-15 minutes before experimentation. 25µg/mL isotype control was added for the same time. Supernatant was collected at 24 hours (n=3).
One-way ANOVA p value=0.0001. Tukey's Test indicates a significant difference between (N DC2.4+OTII vs. N DC2.4+OTII+OVA), (N DC2.4+OTII vs. N Isotype Control), (N DC2.4+OTII+OVA vs. N LFA-1 Blocked), (N DC2.4+OTII+OVA vs. N ICAM-1 Blocked), (N LFA-1 Blocked vs. N Isotype Control) and (N ICAM-1 Blocked vs. N Isotype Control).

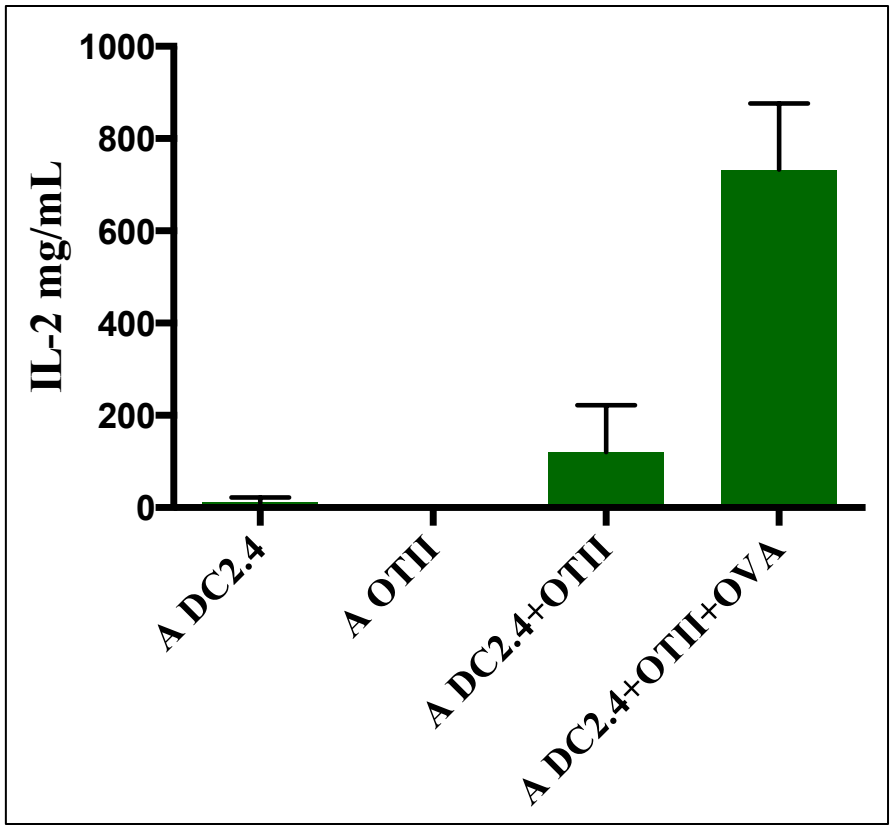


Figure 3.13. ELISA indicates that there is an increase in IL-2 production after adding OVA antigen to DC2.4 and activated OTII T cells.

Activated OTII T cells (A OTII) were isolated from OTII mice, then were pre-exposed to 100 µg/mL of soluble OVA for 48 hours, washed and rested for another 48 hours. The cells were added to the appropriate wells containing DC2.4 cells. In the presence of antigen, 100 µg of soluble OVA was added to DC2.4 cells and incubated for 2-4 hours. Supernatant was collected at 24 hours (n=3).
One-way ANOVA p value=0.0001. Tukey's Test indicates a significant difference between (A DC2.4 vs. A DC2.4+OTII+OVA), (A OTII vs. A DC2.4+OTII+OVA) and (A DC2.4+OTII vs. A DC2.4+OTII+OVA).

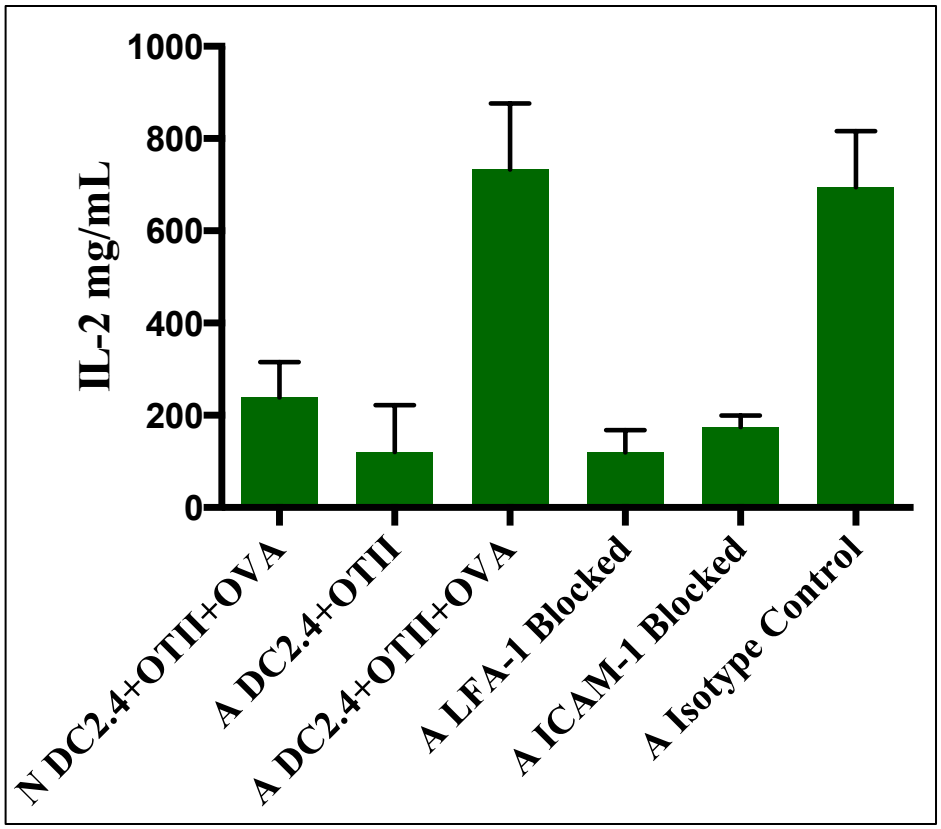


Figure 3.14. ELISA indicates that there are significant differences in IL-2 secretion after blocking of LFA-1 in activated OTII T cells.

Activated OTII T cells (A OTII) were isolated from OTII mice, and were pre-exposed to 100 µg/mL of soluble OVA for 48 hours, washed and rested for another 48 hours. The cells were then added to the appropriate wells containing DC2.4 cells. Activated OTII cells were pre-exposed to 100 µg/mL of soluble OVA for 48 hours, then washed and rested for another 48 hours. In the presence of antigen, 100 µg of soluble OVA was added to DC2.4 and incubated for 2-4 hours. 25 µg/mL of LFA-1 blocking antibody was added to OTII cells 10-15 minutes before experimentation. 25µg/mL isotype control was added for the same time. Supernatant was collected at 24 hours (n=3).
One-way ANOVA p value=0.0012. Tukey's Test indicates a significant difference between (A DC2.4+OTII vs. A DC2.4+OTII+OVA), (A DC2.4+OTII vs. A Isotype Control), (A DC2.4+OTII+OVA vs. A LFA-1 Blocked), (A DC2.4+OTII+OVA vs. A ICAM-1 Blocked), (A LFA-1 Blocked vs. A Isotype Control) and (A ICAM-1 Blocked vs. A Isotype Control).

3.4 Total LFA-1 expression on naïve CD4+ T cells is similar to that on activated CD4+ T cells

The strong force of interaction was correlated to the presence of adhesion molecule LFA-1. CD4+ T cells were examined for differences in LFA-1 cell surface expression on naïve and activated cells. The cells were then stained for LFA-1 using M17.4 antibody. The antibody M17.4 recognizes a particular epitope of the alpha chain of LFA-1. A previous report by Wang and colleagues indicated that M17.4 recognizes both high and low affinities of LFA-1 [76].

In figure 3.15, OTII T cells were isolated from OTII mice and were stained for LFA-1 cell surface expression. Interestingly, naïve CD4+ T cells did not result in a detectable increase in the expression of LFA-1 (figure 3.15). These findings implied that the increase in the force of interaction seen in naïve OTII T cells was not due to the upregulation of LFA-1 expression.

Our result of LFA-1 expression was confirmed in our lab by Ashley Mucsi. First, Ashley has shown that the expression of LFA-1 was similar on naïve CD4+ T cells and regulatory T cells. Then, she demonstrated that there was no difference in LFA-1 expression on untreated and IL-2 stimulated Tregs [77].

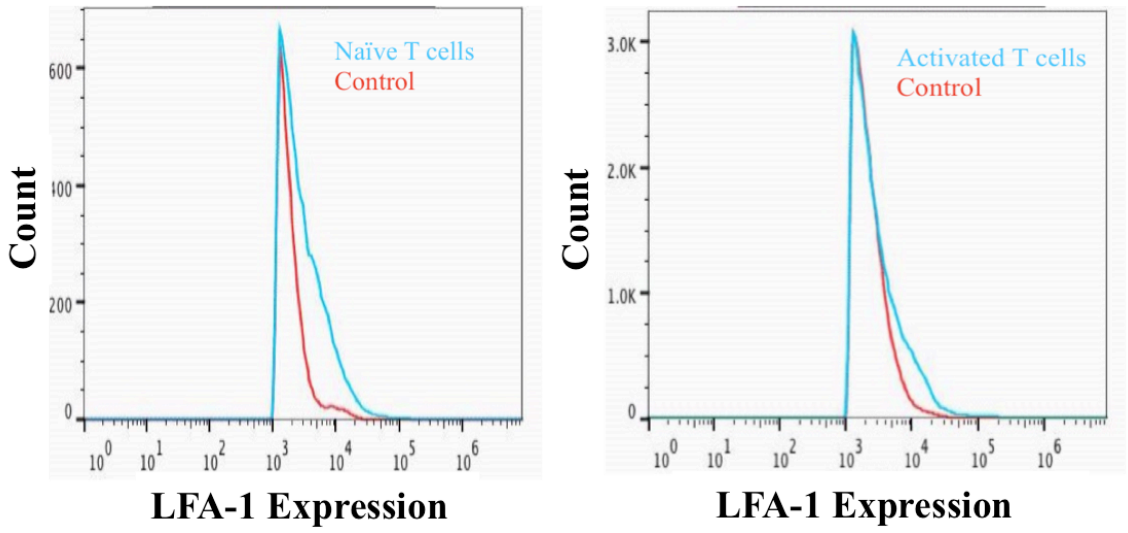


Figure 3.15. LFA-1 surface expression is similar on naïve and activated CD4+ T cells populations.

OTII T cells were isolated from OTII mice. In case of activated OTII T cells, the cells were pre-exposed to 100 µg/mL of soluble OVA for 48 hours, then washed and rested for another 48 hours. The T cells were then co-stained for CD4, and for LFA-1 using the M17.4 antibody. The cells were analyzed using flow cytometry. The data are displayed as histograms (n=1).

Chapter Four: Discussion

4.1 In comparison to activated CD4+ T cells, naïve CD4+ T cells display displays stronger force of interaction with DCs

The main role of the immune system is the defense against foreign invading organisms. Activated CD4+ T cells secrete a number of cytokines to control the participation of other immune cells and to produce an immune response. One of the main cytokines that is produced by CD4+ T cells is IL-2, which is crucial to the survival, development and differentiation of CD4+ T cells [12, 78]. Current research has implicated various mechanisms used by T cells for their activation, but failed to give a clear image about the involvement of cell surface molecules in the naïve and activated T cells. In immunology, it is well known that the initiation of T cell responses requires cell contact with APCs [79]. Using atomic force microscope, our study shows the difference between naïve and activated CD4+ T cells when interacting with dendritic cells. As illustrated in figures 3.1 - 3.10, there was a difference in the way naïve CD4+ T cells interact physically with DCs, in comparison to the contact between activated T cells and DCs. From these results we can conclude that naïve CD4+ T cells interact firmly with DCs and use LFA-1 as a main mediator of this interaction forces. On the other hand, activated CD4+ T cells do not rely on the binding force mediated by LFA-1/ICAM-1 per se between LFA-1 and ICAM-1 to perform its function.

As shown in figure 3.1, we observed that in the presence of antigen peptides, the interaction forces between a naïve CD4+ T cell and a DC increases; whereas in the absence of antigen the binding forces remain low. This increase suggests that the

strength of interaction forces depends on the maturation of the immune synapse between the cells. Moreover, our findings demonstrate that blocking of LFA-1 significantly reduces the interaction forces between naïve T cells and DCs, as shown in figure 3.6, which indicates that LFA-1 is a major adhesion-inducing component in the immune synapse formation. Hosseini and colleagues have demonstrated that LFA-1 is the main force mediator in the biophysical interaction between T cells and APCs after using LFA-1 molecule inhibitor and measuring the interaction forces by AFM [80]. Unfortunately, immune synapse formation and the physical interaction forces between activated T cells and APCs are not known, which makes it difficult to explain the exact reason causing the differences we see in our results between CD4+ T cells subsets when interacting with DCs.

4.2 The important role-played by LFA-1 in CD4+ T cell with DCs interactions

The novel use of the AFM has shed light into the forces involved in the interactions between T cells and APCs. The force of interaction, seen between naïve T cells and DCs in figure 3.1, has led us to ponder whether this force was important and what may be mediating this force. We also wanted to know if this strong force of interaction correlated to CD4+ T cells activity. The previous findings implicated the importance of direct cell contact with CD4+ T cell in order for these cells to conduct their job in the immune system.

Initially, we believed that the CD4+ T cells universally (naïve and activated) require the surface molecules for interaction and adhesion with DCs. Indeed, we further solidified the importance of LFA-1 in CD4+ T cell/DC interactions. As shown in figures

3.12 and 3.14, blocking of LFA-1 interaction with ICAM-1 prevented T cell activation in the presence of antigen, which decreased the production of IL-2 secretion. However, as illustrated in figure 3.6, blocking of LFA-1 significantly reduced the force of physical interaction between naïve T cells and DCs. It has been proven that LFA-1 needs to be activated in order to interact with high affinity with ICAM-1 [81, 82]. Using the atomic force microscope, we observed that the LFA-1/ICAM-1 adhesion pair is essential in generating the force of interaction seen between naïve T cells and DCs.

Although AFM data indicated an essential role of the LFA-1/ICAM-1 pair involved in the force of interaction, our results do not preclude other molecules that may contribute to the intensity of the interaction between the cells. Using AFM, Fc-ICAM-1 recombinant protein coated onto the glass disk was utilized before in our lab by Ashley Mucsi in order to determine whether the large forces seen between T cells (CD4+ and regulatory T cells) and DCs could be recreated while eliminating potential molecules and receptors on the DC that may contribute to the T cell/DC interactions [77]. The results have shown that the force increased when a CD4+ T cell interacted with Fc-ICAM-1 coated disk in comparison to a cell touching a non-coated glass disk [77]. Hence, The ability to replicate the increase in the force of interaction with the recombinant ICAM-1 coated disk emphasized the importance of the LFA-1/ICAM-1 adhesion molecule pair by eliminating other surface molecule interactions. In addition, Ashley Mucsi found that LFA-1 KO regulatory CD4+ T cells (Tregs) had low forces of interaction with the ICAM-1 coated slide, which demonstrates that the Treg/DC interaction was LFA-1 dependent [77].

Although LFA-1's role is evident, a question remains as to what is mediating the difference between naïve and activated CD4+ T cells during their interactions with DCs. However, whether the differences in the forces can be attributed to the number of molecules on the cell surface, the conformational state of the integrin, or clustering of the adhesion molecule remained to be elucidated. We aimed to determine what was causing the difference in binding intensity between the two cell types; therefore, we intended to examine LFA-1 expression and how it may differ between naïve and activated CD4+ T cells. As illustrated in figure 3.15, activation of CD4+ T cells does not result in a detectable difference in LFA-1 surface expression.

4.3 The role of membrane lipid rafts in LFA-1 adhesive properties

To examine the importance of lipid rafts and its possible role in LFA-1 clustering in the membrane, methyl β cyclodextrin (MBCD) can be used. MBCD removes cholesterol from the membrane [83], which consequently disrupts the formation of lipid rafts and renders the membrane fluid. Lipid rafts are found on the cell surface and are thought to cluster receptors and signaling molecules together thereby increasing the efficiency of signaling [84]. Cholesterol is an important component of lipid rafts thereby creating a signaling platform in T cells. A study that has been done in our lab by Ashley Mucsi explored the involvement of lipid rafts and their potential role in the force of interaction between regulatory CD4+ T cells and dendritic cells. It was found that upon initial treatment with MBCD (within 20 minutes), the force of interaction significantly increased between the treated Treg and ICAM-1 coated disk [77]. However, the force reverted to the normal range following the 20-minute treatment [77]. In contrast,

Leitinger and Hogg demonstrated that treatment of activated T cells with MBCD decreased adhesion to ICAM-1, implicating the dependence on lipid rafts for T cell adhesion to ICAM-1 [50]. Our lab findings are in conflict with Leitinger and Hogg's results even though it can be attributed to differences in experimentation and the duration of treatment, as Leitinger and Hogg examined the long-term effects of lipid raft integrity on binding [50]. Also, Ashley compared the effect of MBCD on a naïve T cell, while Leitinger examined the effect of MBCD on activated T cells [50]. Nevertheless, it is obvious in both cases that lipid rafts are important in adhesion between the cells.

4.4 Regulation of LFA-1 and its attachment to the cytoskeleton

Cytoplasmic proteins, such as talin and α-actinin, have been shown to interact with the cytoplasmic tail of the LFA-1 β-subunit [52, 85]. These proteins link LFA-1 to the cytoskeleton. Connections to the cytoskeleton may further stabilize the open conformational state of LFA-1, prolonging adhesive properties of the integrin. Talin association with the cytoplasmic domains of integrin has been previously shown by Tadokoro and colleagues to increase integrin affinity [85]. In addition, Franco's group observed that calpain activity played a role in regulating adhesion dynamics [74]. Franco *et al* transfected a mutant talin protein into a talin-deficient CHOK1 cell line, CHOK1 cells do not express the epidermal growth factor receptor. As a consequence of mutating talin, calpain was unable to cleave it thereby preventing disassembly of the focal adhesion complex and prolonging adhesive interactions [74]. Furthermore, Svenesson *et al* also observed that upon pharmacological inhibition of calpain, inefficient disassembly of LFA-1 adhesions occurred [86]. Reducing calpain activity on talin may cause LFA-1

to remain in an immobile state in the membrane leading to prolonged interaction with its ligand and further stabilizing its conformational state. Likely, naïve CD4+ T cells would regulate calpain activity differently in comparison to activated CD4+ T cells. Differences in calpain activity or regulation could translate into differences in LFA-1 avidity and localization in the membrane, which could potentially affect T cell adhesion properties.

4.5 Future experimentation to complete our understanding of the difference between the interaction of naïve and activated CD4+ T cell and dendritic cells

Our investigations have shed light into the importance of LFA-1 in the interaction between CD4+ T cells and DCs. Despite our findings, we have yet to discover the target activity that may be causing a difference in LFA-1 adhesive properties. Future studies must be performed to fully substantiate our findings and theories concerning the mechanism in which LFA-1 works on naïve and activated CD4+ T cells when interacting with dendritic cells. Our research illustrated that IL-2 is produced after activating the cells with OVA antigen, which influences their adhesive properties for DCs as we see in the AFM results. It is known that IL-2 signaling is crucial to the survival, development, and maintenance of the CD4+ T cell population [12, 78]. Unfortunately, IL-2 signaling and its effect on CD4+ T cells and DCs interactions and how it would contribute to the way LFA-1 works during these interactions has yet to be completely elucidated. IL-2 signaling has been implicated in causing phosphorylation of VAV-1 signaling molecule and was postulated to interact with talin, whether directly or indirectly through calpain activity [42, 87]. Correlating IL-2 signaling to calpain activity would complete our understanding of the interactions between these cells.

In addition to further investigating IL-2 signaling, focus centered on talin cleavage and its role in integrin attachment to the cytoskeleton would help elaborate and develop a deeper insight into the adhesion forces seen between a CD4+ T cell and a DC. Our observations and current research have led us to postulate that naïve CD4+ T cells interact more frequently with DCs in vitro. A deeper examination into naïve versus activated CD4+ T cell interactions with DCs in vivo would provide compelling evidence for how these affinity states of LFA-1 operate physiologically.

4.6 Potential therapeutic interventions

Manipulation of CD4+ T cells for effective medical treatment requires a detailed understanding of how different activation states of CD4+ T cells interact with APCs. Extensive studies of the role played by CD4+ T cells in the immune system provides a new therapeutic approaches to cancer, as T cells might be able to recognize and eliminate cancer cells [20]. Toes *et al* have demonstrated that tumor-specific CD4+ T cells conduct several effector functions to perform antitumor response [88]. It has been proven that the interaction between CD4+ T cells and APCs in the arterial wall leads to local T cell activation and production of proinflammatory cytokines [89]. These cytokines are interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α). The secretion of proinflammatory cytokines in this case promotes atherosclerosis by maintaining chronic inflammation [89]. This study showed the importance of the interaction between CD4+ T cells and APCs in sustaining chronic inflammation in atherosclerosis. Furthermore, HIV-1 is known to infect CD4+ T cells causing quantitative and qualitative defects in CD4+ T cells [90]. It was demonstrated that in Acquired Immunodeficiency Syndrome (AIDS)

patients, HIV-1 cause a decline in number and function of CD4+ T cells, which shows that these cells are target for HIV-1 [91]. Thus, for CD4+ T cells to perform their functions and prevent potential damages, it is important to understand the specific events that occur during the interaction between APCs and CD4+ T cells. Most importantly, research should investigate how different subsets of CD4+ T cells form the immune synapse and use integrins, such as LFA-1, to carry out their functions.

LFA-1 is also a potential therapeutic target for regulating immunity. Understanding how LFA-1 is regulated in the CD4+ T cell subsets may be crucial in rendering anti-LFA-1 treatment more successful due to increased specificity. Indeed, transient treatment targeted against LFA-1 and ICAM-1 prolonged tolerance to cardiac allografts [92], and anti-LFA-1 monotherapy was efficacious in long-term pancreatic islet allograft tolerance [93]. Moreover, anti-LFA-1 treatment of CD8+ cytotoxic T lymphocytes reduced illness associated with primary respiratory syncytial virus (RSV) infection; although clearance of SRV was also reduced [94]. These findings suggest that extensive knowledge on the mechanisms of interaction can be helpful for medical treatment applications.

Our observations suggest that naïve CD4+ T cells regulate LFA-1 activity differently in comparison to activated CD4+ T cells, which may correlate with CD4+ T cells' adhesive properties and regulation of LFA-1. Future studies regarding LFA-1 adhesion in CD4+ T cells can enhance our understanding and suggest potential therapies. By understanding the role which LFA-1 plays in regards to CD4+ T cells, prevention of tumor, HIV, inflammations and infections may be suggested.

4.7 Conclusion

In conclusion, we propose that naïve CD4+ T cells requires strong physical contact with dendritic cells, which is mostly dependent on the interaction between LFA-1 surface molecule on T cells and its ligand ICAM-1 on DCs. This force of interaction may be required for naïve CD4+ T cells to get activated and to perform their activities. In contrast, in previously activated CD4+ T cells, the physical binding between LFA-1 and ICAM-1 is not a key element for CD4+ T cells to perform their functions. CD4+ T cells are essential components of the immune system that is vital for fighting against diseases. Therefore, understanding the mechanisms of action is vital to future development and manipulation of medical treatments and interventions.

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