

MECHANOREGULATION OF CYTOTOXIC T LYMPHOCYTE KILLING

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MECHANOREGULATION OF CYTOTOXIC T LYMPHOCYTE KILLING

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Immune cell-cell communication is canonically presented as an interlinked network of biochemical pathways through which immune cells process information. However, this characterization fails to consider the physical methods that immune cells employ in order to interact with their local environment and enhance their cellular function. The conversion of mechanical stimuli into biochemical responses is called mechanotransduction. Cytotoxic T lymphocytes (CTLs) are an ideal system for studying these techniques because they are both physically active and immunologically communicative. Additionally, they are biomedically important, playing crucial roles in homeostatic and therapeutically induced immune responses against both foreign pathogens and cancer, thus making them therapeutically urgent and interesting to study.

CTLs exert physical force against target cells, thereby straining target cell membrane and mechano-potentiating the pore-forming activity of perforin and overall effector response. However, it remained unclear what structures T cells formed in order to exert physical force, and what proteins or signaling molecules could be involved in this phenomenon. We found that T cells utilized actin-rich protrusions at the cell-cell interface against the target cell to distort the target cell membrane. The formation of protrusions were dependent on the actin nucleation promoting factor Wiskott-Aldrich Syndrome protein (WASP), and were necessary for physical distortions in the target cell membrane that amplified T cell cytotoxicity. These results mechanistically clarified how T cells

exert physical force against their targets to amplify the pore-forming effects of perforin oligomerization in the target cell surface. However, it remained to be seen how T cells could coordinate these two arms of cytotoxic signaling for optimized cytotoxicity.

Given that higher levels of T cell mechanical force exertion were correlated with higher levels of killing, we were curious to know if T cells used mechanical cues at the synapse to determine where to secrete their lytic granules into the intracellular space, thereby optimizing the release of the cytotoxic payload. Such a phenomenon could also reconcile how T cells avoid killing healthy bystander cells in a crowded cellular environment, by requiring physical engagement from antigen-presenting cells as a signal of cellular adjacency and safety. We found that T cells specifically degranulated near the engaged integrin receptor LFA-1 (lymphocyte function-associated antigen 1) that formed upon the detection of antigen. Fascinatingly, this relationship was dependent on the mechano-transductive behavior of LFA-1, as ablation of its force-generating capacity via talin inhibited LFA-1 force exertion and degranulation completely. The ability to convert mechanical stimuli into a degranulation signal was also broadly generalizable to other integrins. These results, in their totality, identify how T cells are able to secrete lytic granules near areas of force exertion, and clarify how T cells are able to clear diseased cells with minimal bystander killing.

Our studies describe the sophisticated mechanisms used by the T cell to mechanically optimize its cytotoxicity. We identified WASP-driven actin-rich protrusions as a major driver of force exertion against the target cell membrane to prime it for perforin insertion and pore formation. Additionally, we identified a new mechanical role for the integrin LFA-1 (and broadly, all other integrins) in establishing sites of cell-cell proximity and therefore permissive lytic granule

release in order to safely and efficiently deposit the cytotoxic payload against the target cell. Altogether, these studies further clarify the mechanical pathways that CTLs utilize to engage with and interpret their immune environment, ultimately resulting in efficient clearance of infected and tumor cells.

BIOGRAPHICAL SKETCH

Mitchell was born in 1993 in Palo Alto, California and came of age in the suburbs of Philadelphia, Pennsylvania. After his high school education, he attended New York University, where he studied for his Bachelor of Arts degree as a double major in both biology and economics. During his time at NYU, he studied Alzheimer's disease in the laboratory of Dr. Yueming Li at Memorial Sloan Kettering Cancer Center. Upon graduating in 2015 and with an interest in the biomedical sciences, he entered the Pharmacology program at Weill Cornell Graduate School of Medical Sciences and joined the laboratory of Dr. Morgan Huse at Memorial Sloan Kettering Cancer Center for his thesis work, where he has since studied the principles of mechanical force exertion in T cells and their contribution to cellular cytotoxicity.

Mitchell's best times away from the lab were spent in the warmth of Jacob Riis beach, in the colorful company of his friends he met in New York City, and behind the lens of his digital and film cameras.

for Mama, Baba, and Nick

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LIST OF ABBREVIATIONS

ADAP	Adhesion- and degran.-promoting adaptor protein
Akt	Protein Kinase B
APC	Allophycocyanin
APC	Antigen presenting cell
Arp2/3	Actin related protein-2/-3 complex
Bak	BCL2 antagonist/killer 1
Bax	BCL2 associated X
BID	BH3 interacting-domain death agonist
Ca^{2+}	Calcium ion
CAR	Chimeric antigen receptor
CD3 ζ	Cluster of differentiation 3 zeta-chain
CD11a	Cluster of differentiation 11a
CD28	Cluster of differentiation 28
CD45	Cluster of differentiation 45
CD69	Cluster of differentiation 69
CD107a	Cluster of differentiation 107a, see Lamp-1
Cdc42	Cell division control protein 42 homolog
CLP	Common lymphoid progenitor cells
CRAC	Calcium release-activated channel
CRISPR	Clustered regularly interspaced short palindromic repeats
cSMAC	Central supramolecular activation cluster
CTL	Cytotoxic T lymphocyte
DAG	Diacylglycerol
DMSO	Dimethylsulfoxide
dSMAC	Distal supramolecular activation cluster
Endoplasmic reticulum	ER
ERK	Mitogen-activated protein kinase
F-actin	Filamentous actin
FA	Focal adhesion
FITC	Fluorescein isothiocyanate
G-actin	Globular actin
GEF	Guanine nucleotide exchange factor
GFP	Green fluorescent protein
gRNA	Guide RNA for CRISPR
GTPase	Guanosine triphosphatase
Gzmb	Granzyme B
H2Kb-OVA	OVA presented by the class I MHC protein
HSC	Hemaetopoietic stem cells
ICAM-1	Intercellular adhesion molecule 1
IFN γ	Interferon gamma

I κ B	Inhibitor of kappa B
IL-2	Interleukin-2
ILP	Invadosome-like protrusion
<i>IP</i> ₃	inositol 1,4,5-trisphosphate
InsP3R	Inositol trisphosphate receptor
iRFP670	Infrared fluorescent protein 670
IS	Immunological synapse
ITAM	Immunoreceptor tyrosine-based activation motif
Lamp1	Lysosomal-associated membrane protein 1
LAT	Linker for activation of T cells
Lck	Lymphocyte-specific protein tyrosine kinase
LDH	Lactate dehydrogenase
LFA-1	Lymphocyte function-associated antigen 1
LG	Lytic granules
MAPK	Mitogen-activated protein kinase
MHC	Major Histocompatibility Complex
MTOC	Microtubule organizing center
mTOR	The mammalian target of rapamycin
MTP	Membrane tension probe
Nck	Non-catalytic region of tyrosine kinase protein
NF κ B	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer
NPF	Nucleation promoting factor
NT	Non-targeting
OT-1	TCR specific for the OVA peptide
OVA	Ovalbumin 257-264 peptide (sequence: SIINFEKL)
PAMP	Pathogen-associated molecular pattern
PD-1	Programmed cell death protein 1
PDMS	Polydimethylsiloxane
PFA	Paraformaldehyde
pHluorin	pH-sensitive GFP
PI3K	Phosphoinositide 3-kinase
<i>PIP</i> ₂	Phosphatidylinositol-4,5-bisphosphate
<i>PIP</i> ₃	Phosphatidylinositol-3,4,5-trisphosphate
PKC θ	Protein kinase C theta
PLC γ	Phospholipase gamma
PMA	Phorbol 12-myristate 13-acetate
PMAi	Phorbol 12-myristate 13-acetate and ionomycin
pMHC	Peptide-MHC, see MHC
pN	Piconewton
pSMAC	Peripheral supramolecular activation cluster
RAG	Recombination-activating gene
Ras	Rat sarcoma virus protein

RFP	Red fluorescent protein
SA	Streptavidin
STIM1	Stromal interaction molecule 1
TCR	T cell receptor
TIRF	Total internal reflection microscopy
Vav1	Vav Guanine Nucleotide Exchange Factor 1
WASP	Wiskott-Aldrich syndrome protein
WAVE2	WASP-verprolin homolog 2
WT	Wild-type
YFP	Yellow fluorescent protein

CHAPTER 1

INTRODUCTION AND BACKGROUND

1.1 The immune system and T cells

The majority of organisms have an immune system, defined as a biological network of processes that defend the host organism against foreign pathogens and disease, that is vital for maintaining homeostasis. The earliest manifestation of an immune system first arose hundreds of millions of years ago in jawless fish in the form of variable lymphocyte receptors that recombine using a large panel of leucine-rich-receptors, creating the earliest lymphoid lineages [1]. For mammalian immune systems, this network is broadly broken into two major arms: the innate immune system and the adaptive immune system [2]. The innate immune system responds initially to pathogens but is limited in the molecular scope of the threats it responds to, as it uses pathogen-associated molecular patterns (PAMPs) to trigger its activity, which are generally conserved. Therefore the innate immune system responds faster but generically to pathogens - which, while useful to the host for clearing the majority of infectious agents, means the innate immune system can be overwhelmed by pathogens that may have evolved to evade innate immune system processes [2]. It is this circumstance that creates the need for an adaptive immune system.

The adaptive immune system responds much more slowly than the innate immune system, using this time to harvest and collect antigen and begin upramping of its antigen-specific molecular processes. The adaptive immune system is composed of two major types of cells (called lymphocytes): B cells and T cells. B cells (named for originating from the bone marrow) produce antibodies that

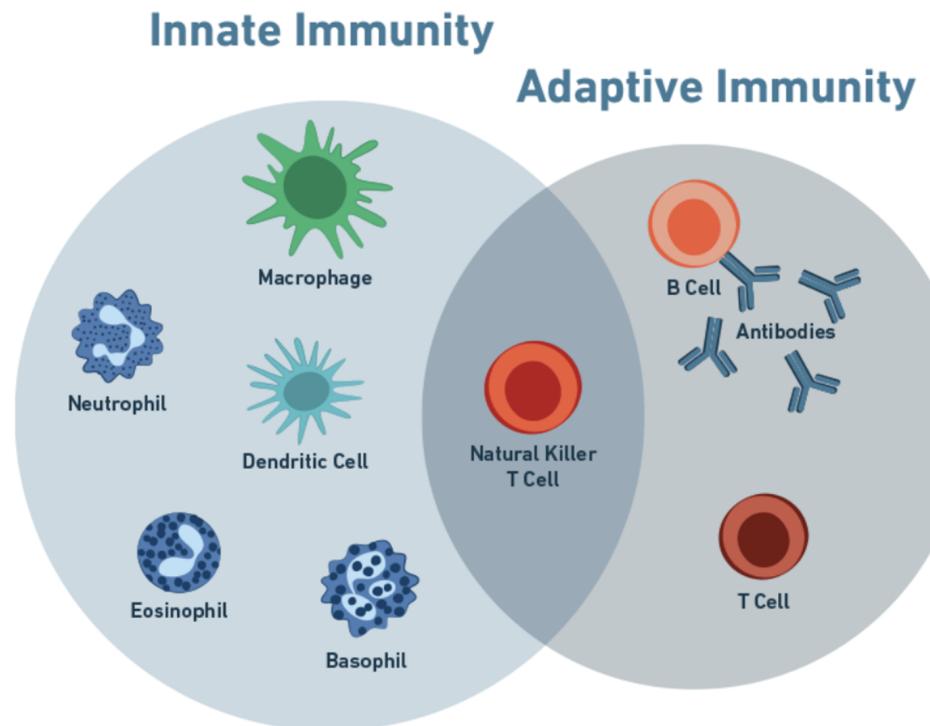


Figure 1.1: Cell types of the innate vs. the adaptive immune system

A schematic Venn diagram of the immune system. The typical conception of the vertebrate immune system is typically two-armed, broken into a faster-but-general responding innate immune system (left), and a slower-but-more-specific adaptive immune system (right), of which T cells are a part of. Almost all cell types that modern immunology research is concerned with falls somewhere within these two circles or their overlap - some immunology research is interested in characterizing certain effector or support immune cell types that fall in between innate and adaptive immunity (or can transfer their skills and interface between the two, calling into question the ontics of "innate" and "adaptive" immune systems) [3]. This figure is adapted from [4].

specifically bind to antigens and identify their bound partners as foreign entities meant for destruction. T cells on the other hand (named for originating in the thymus) directly manage the cytotoxic activity of the adaptive immune system. T cells are composed of two main functional subtypes: CD8+ killer T cells (the primary focus of this thesis and heretofore referred to as any of the following:

cytotoxic T lymphocytes (CTLs), cytotoxic T cells, CD8+ T cells) and CD4+ helper T cells.

1.2 T cell development

T cells as a whole are defined by their expression of the T cell receptor (TCR), the receptor responsible for recognizing foreign antigenic peptide presented by the target cell via its Class I or Class II Major Histocompatibility Complex (MHC, or pMHC). The TCR is multi-subunit receptor that is composed of varying combinations of α -, β -, γ -, δ -, ϵ -, and ζ -chains [5], whose specific combination defines the unique sub-identity of the T cell, although the vast majority of T cells (95%+) express the α and β chains and are thus called $\alpha\beta$ T cells. There are a number of additional co-receptors that bind (either directly or indirectly) to the TCR. The most relevant to $\alpha\beta$ T cells are the CD8 or CD4 co-receptors, mentioned above. These co-receptors transduce differing signals within the specific T cell and additionally define the specificity of the $\alpha\beta$ T cell to the two different classes of MHC: CD8+ T cells bind to MHC Class I, while CD4+ T cells bind to MHC Class II.

The ability of T cells to properly recognize and engage only with foreign antigen while ignoring self-peptides is tightly regulated and begins at a very early stage in immune system development. T cells derive from hematopoietic stem cells (HSCs) that originate in the bone marrow. HSCs then ultimately differentiate into common lymphoid progenitor cells (CLPs), which migrate to the thymus to ultimately differentiate into natural killer (NK), B, or T cells.

The process of differentiating into T cells is primarily motivated by the need to create a functional TCR that does not react to self-antigen but does react to foreign antigen. As TCRs are made up of alpha and beta chains that are evolved to react to a wide range of possible antigens that an organism may encounter in its lifespan, T cell differentiation has been characterized in a stepwise manner that first begins with TCR-beta chain selection. T cells at this stage express an invariant pre-alpha chain called pre-T α that the varying beta chains (generated by VDJ recombination of the TCR-beta locus) attempt to form a stable binding partner with. Once an appropriate TCR-beta chain is identified as capable of stable binding to pre-T, the same process begins on the TCR- α chain against the now mature TCR- β chain, generating a stable (but not necessarily functional) TCR.

Once a stable TCR heterodimer has been formed, the T cells must undergo a process of selection in order to build a functional repertoire of TCRs, called positive and negative selection. Positive selection involves presenting the T cells with self-antigen presented on MHC with the selection criteria of being able to bind with this complex. T cells that cannot bind either MHC Class I or Class II via their TCR will die (called "death by neglect" - this removes cells with TCRs that are unable to bind self-MHC which is functionally useless to the organism, or may be able to bind non-self MHCs, which is also functionally useless), while those T cells that can will survive. Those T cells that are able to bind to an MHC complex presenting self-antigen receive a survival signal. Therefore, the body positively filters/selects for T cells with TCRs that can recognize MHC molecules leaving those that cannot recognize MHCs to die off [6].

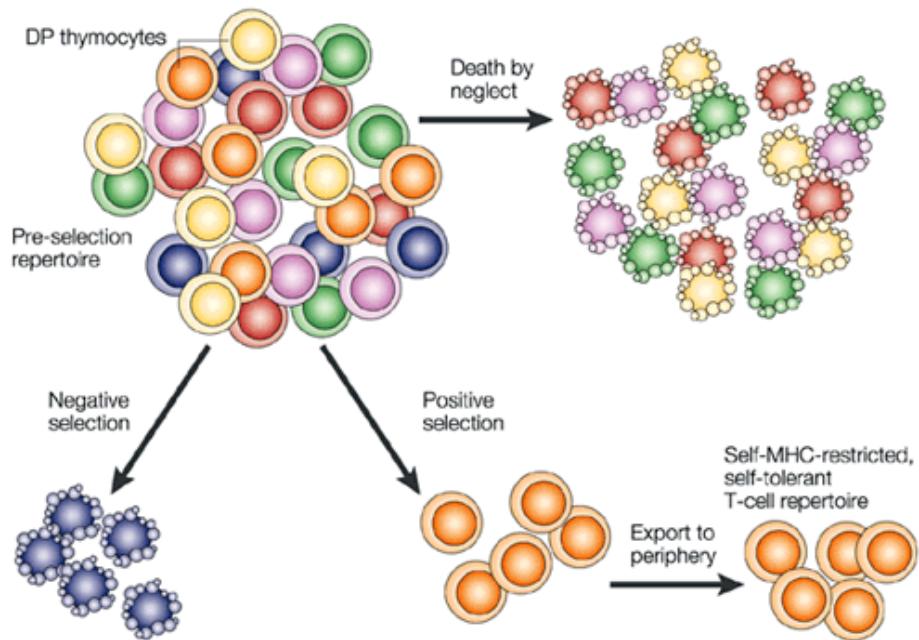


Figure 1.2: Positive and negative selection

Thymocytes that are double positive (DP) for CD4+ and CD8+ begin with an unselected repertoire of TCRs. The process of positive selection ensures that DP thymocytes that do not bind MHC Class I or II die ("death by neglect"), while those that do bind MHC will receive a signal to survive. Negative selection filters out TCRs that are too reactive to self-antigen, which would be intolerable for a functioning immune system. These selection processes results in the generation of T cells that are self-MHC restricted, self-antigen tolerant T cells that are ready to fight potential infection. This figure is adapted from [6].

After obtaining T cells that are capable of binding to MHC molecules, the next step is to select for T cells that do not auto-react to self-antigen, called negative selection. This ensures that T cells are tolerant of self-antigen and prevents auto-immunity conditions in the organism. In this stage of selection, T cells that bind to MHC presenting self-antigen and activate receive an apoptotic signal that leads to cell death. Negative selection therefore prunes the T cell population for T cells that can not only bind to MHC but do not react to self-antigen. The vast majority of thymocytes (98%+) fail to pass positive and negative selection. Following positive and negative selection, the resulting T cell set can therefore

bind to any antigen (presumably belonging to any foreign pathogen) so long that the antigen is distinct from the body's self-antigens. After these stages of T cell development, these T cells (called naïve T cells) then exit the thymus and begin to circulate in the host, where they will spend their time surveying the host for disease and foreign antigens.

1.3 TCR activation and downstream signaling

If a CD8+ T cell engages an infected cell presenting foreign peptide, the T cell will initiate a cascade of antigenic-specific intracellular signaling events. Upon ligation of the TCR to the pMHC complex, the CD3 proteins (CD3 $\epsilon\gamma$ and CD3 $\epsilon\delta$ heterodimers and a CD3 ζ homodimer) bearing ITAM (immunoreceptor tyrosine-based activation motif) also bind to the TCR. The ITAMs on the CD3 ζ get phosphorylated, so that cytosolic signaling proteins can bind to phosphorylated ITAMs and propagate the signal from the triggered TCR further downstream into the T cell. CD3 ζ contains three ITAMs, while CD3 δ , CD3 γ , and CD3 ϵ all only contain one ITAM. The CD3 chains are needed as the cytosolic tail of the TCR is extremely short and structurally do not support significant adaptor features for signal amplification molecules. This modularity also allows for an increased range of TCR signaling for fine-tuning key features of the T cell effector response (e.g. killing, proliferation, signaling, cytokine secretion) in response to a number of diverse inputs such as antigen strength, lifetime of interaction, and number of bound-TCRs at the IS.

The phosphorylated CD3 ITAMs recruit the protein tyrosine kinase ZAP70 that binds to the phosphorylated tyrosine motifs via its SH2 domain. This brings

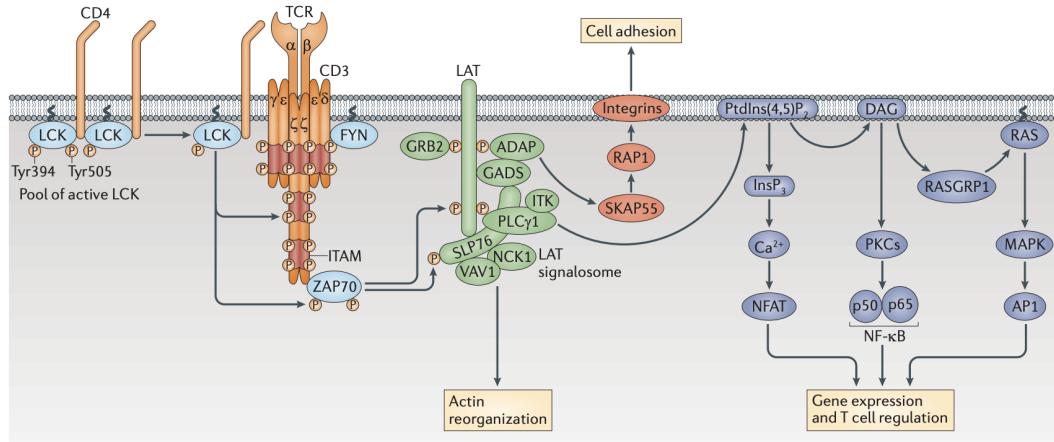


Figure 1.3: The T cell receptor and its signaling partners

An overview of TCR signalling. Signaling through the TCR commences upon recognition of cognate pMHC on the target surface. The assembly of the CD3 γ , δ , and ζ -chains recruits Lck, which phosphorylates the ITAMs of the CD3 γ , δ , and ζ chains, recruiting ZAP70. Activated ZAP70 primes the LAT signalosome to coordinate critical signaling molecules such as PLC γ , GRB2, SLP76, ADAP, ITK, Nck1, and VAV1. The LAT signalosome is a node for three major signaling pathways: the Ca^{2+} , the MAPK, and the NF- κ B pathway, which initiate critical signaling pathways for the expansion, differentiation, and activation of T cells. These signals also modify integrin signaling, which strongly influences cytoskeletal dynamics and therefore adhesive and migratory behavior. The figure is adapted from [7].

ZAP70 closer to CD8-bound Lck, which further phosphorylates ZAP70, activating ZAP70. Upon Lck-mediated phosphorylation of ZAP70, ZAP70 itself phosphorylates and activates the transmembrane protein called linker for activation of T cells (LAT). LAT acts as a scaffold, providing binding sites for a number of signaling molecules (via its own phosphorylated sites) that link these signaling molecules, including the lymphocyte cytosolic protein 2 (SLP76) which provides even further additional binding sites on the LAT signaling complex, SOS, GRB2, ITK, Vav, Nck1, and FYB (or ADAP), in space and time.

One of the most crucial signaling molecules that binds to LAT is the protein phospholipase $C\gamma 1$ (PLC γ), which generates key lipid secondary messenger

molecules. Phosphatidylinositol 4,5-bisphosphate (PIP_2) is an input to PLC γ function. Once phosphorylated and activated by Itk, PLC γ 's enzymatic activity (using PIP_2 as a substrate) generates two products: diacylglycerol (DAG) and the soluble inositol 1,4,5-trisphosphate (IP_3). Diacylglycerol accumulates in the IS, which prompts recruitment of the microtubule motor protein dynein and drives polarization of the microtubule-organizing center (MTOC) [8] and the recruitment of lytic granules to the immune synapse (further discussed in the T cell degranulation and target cell death introduction section, section 1.5 [9]). IP_3 is soluble and diffuses throughout the cytosol, eventually binding to its receptor inositol trisphosphate receptor (InsP3R), located on the endoplasmic reticulum (ER). The binding of IP_3 to InsP3R triggers release of calcium (Ca^{2+}) ions into the cytosol, initiating calcium-dependent transcription programming. These include the NFAT, NF- κ B, and AP1 signaling pathways, all of which assist in fully activating the T cell. Another notable lipid secondary messenger is phosphatidylinositol (3,4,5)-trisphosphate (PIP_3). PIP_2 is phosphorylated by the phosphoinositide 3-kinase (PI-3K) to give PIP_3 , which further activates downstream signaling proteins, most notably Akt, which is highly involved in T cell metabolism.

When calcium depletion is detected in the cytosol by the STIM1 protein, calcium release-activated channels such as Orai1 are activated to replenish cytosolic calcium. This calcium flux is a required step for lymphocyte degranulation [10].

The generation of spatial gradients and patterns of lipid secondary messenger molecules at the plasma membrane is highly consequential, resulting in the formation of a necessary killing and major signaling structure called the immune synapse.

1.4 The immunological synapse and the T cell cytoskeleton

The immunological synapse (also called the immune synapse) is the highly organized, structurally stereotyped interface that T cells form against an APC. It is classically characterized as a concentric, annular structure that bears a "bulls-eye" center surrounded by a ring of filamentous actin (see Figure 1.4, left). The immune synapse is a major T cell signaling and killing megastructure, and robust formation of the immune synapse is essential for the T cell to achieve its maximal cytotoxic efficacy, in particular when concerned with lytic granule secretion (see 1.5) [11].

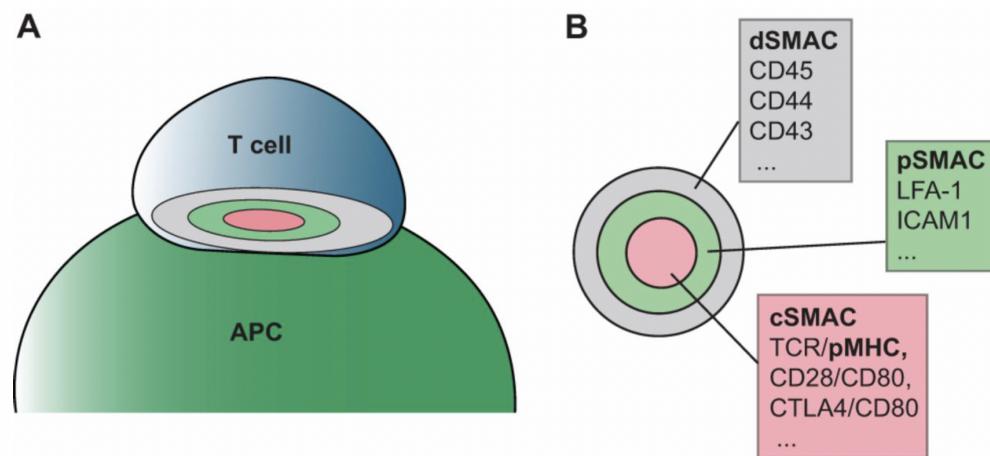


Figure 1.4: The immunological synapse

A: A schematic of the canonical model of the immune synapse. The T cell (blue, above) is in contact with the APC (green, below). The IS is shown as a series of interfacial concentric rings. **B:** The IS is classically divided into three regions, extending from the center outwards: the cSMAC, the pSMAC, and the dSMAC. The proper formation of the IS results in functionally distinct and biochemically patterned regions. This figure is adapted from [12].

Upon TCR activation, the actin cytoskeleton rapidly polymerizes and organizes itself to achieve an annular arrangement, forming the IS. Existing work in the field highlights the necessity of PIP_3 accumulation at the synapse for step-

wise formation of actin structures [13]. PIP_3 recruits the guanine exchange factor Dock2, which locally activates the Rho family GTPase Rac. This activated Rac then signals to the Wiskott-Aldrich syndrome protein family member 2 (WAVE2), generating lamellipodial growth [13, 14]. Lipid gradients also control Cdc42, another Rho family GTPase. Together, PIP_2 and Cdc42 (activated by Vav1 [15]) activate the Wiskott-Aldrich syndrome protein (WASP), generating filopodia [16].

WASP and WAVE2 are actin nucleation promoting factors that activate nucleation factors such as Arp2/3, which themselves resist the inhibition of spontaneous actin polymerization (by proteins such as profilin) by acting as actin nucleation seeds [17]. Actin nucleation (-promoting) factors are essential for proper cytoskeletal remodeling, and the role of WASP and WAVE2 on immune synapse dynamics is the subject of Chapter 2, where their function proves to be critical for cytotoxicity.

From a macromolecular perspective, the IS is segregated into three supramolecular activation clusters (SMACs), called the central, distal, and peripheral supramolecular activation clusters (cSMAC, pSMAC, and dSMAC, respectively, and named in their order of concentricity starting from the center. See Figure 1.4). The cSMAC contains the TCR signaling complex, CD28, and PKC θ [18, 19]. The pSMAC contains the lymphocyte function-associated antigen 1 (LFA-1) and talin [18]. The role of these proteins in lytic granule fusion and cytotoxicity is explored deeply as the subject of Chapter 3. Notably, the CD45 phosphatase is initially located in the pSMAC, but then is sterically excluded from the pSMAC due to its size to the dSMAC [20]. The dSMAC of course includes the filamentous-actin ring, as well as CD43 and CD44, proteins that are

linked to adhesion and phenotypically dovetail with stronger T cell-target cell contact [12].

It is important to note that most of the work that has been done to investigate the localization of proteins to different regions of the immune synapse as described above has been performed in the lipid bilayer experimental system using TIRF microscopy. In this context, T cells reliably form "bulls-eye" immune synapses. However, this neglects the 3D environment that the T cell-target cell interface actually is, and does not address significant amounts of evidence that immune synapses can take on additional organizations, such as TCR microvilli [21] or multiple synapses against multiple targets [22]. Other strategies have been developed to characterize immune synapses in more physically relevant environments [23], which should prove fruitful in truly understanding IS structure and dynamics.

Broadly, disrupted synapse formation (ranging widely from organization in space, organization in time, and presence/absence of certain molecular players) can dramatically affect T cell cytotoxicity. This highlights the functional importance of the immune synapse.

1.5 T cell degranulation and target cell death

The killing mechanism of CTLs is fundamental to robust anticancer and antiviral responses. CTLs achieve their cytotoxic effects by managing a two-pronged approach 1. specifically destroying only infected or oncogenic target cells (recognized via ligation between the TCR and its specific pMHC interaction) and 2. preserving the integrity of the surrounding healthy bystander tissue.

Spatially, these aims are realized through the precise signaling mechanisms of the immune synapse.

The T cell has evolved countless mechanisms in order to balance this dual mandate. The most prevalent mechanism of T cell-mediated killing of target cells involves the secretion of the hydrophobic protein perforin and granzyme proteases through the immune synapse, called degranulation [24, 25]. When released, perforin oligomerizes in a calcium dependent manner [26] and forms pores on the target cell surface, (around 16nm in diameter) [27], inducing significant membrane damage. The target cell responds to this traumatic event at the plasma membrane using a mechanism that permits granzymes entry to the cytoplasm, where they cleave apoptotic substrates that induce apoptosis [28]. Because all cell types are capable of being killed and cleared in this way (that is to say, both infected and healthy cells), specialized mechanisms have evolved over time in immune cells to ensure that the effects of perforin and granzyme are constrained to the target cell alone.

Cytotoxic T lymphocytes store their perforin and granzymes in specialized secretory lysosomes called lytic granules (0.5 to 2 μm) [29], whose acidic pH environment quenches the lytic and apoptotic activity of both proteins [30, 28]. Following mere minutes of target cell recognition, the lytic granules are trafficked along microtubules to the immunological synapse (IS). At this site, they fuse with the plasma membrane, releasing their contents into the intercellular space.

At the same time, F-actin and the associated actin nucleation promoting factors (NPFs) at the IS undergo significant spatiotemporal remodeling. This results in a highly complex landscape of highly dynamic actin sheets and protru-

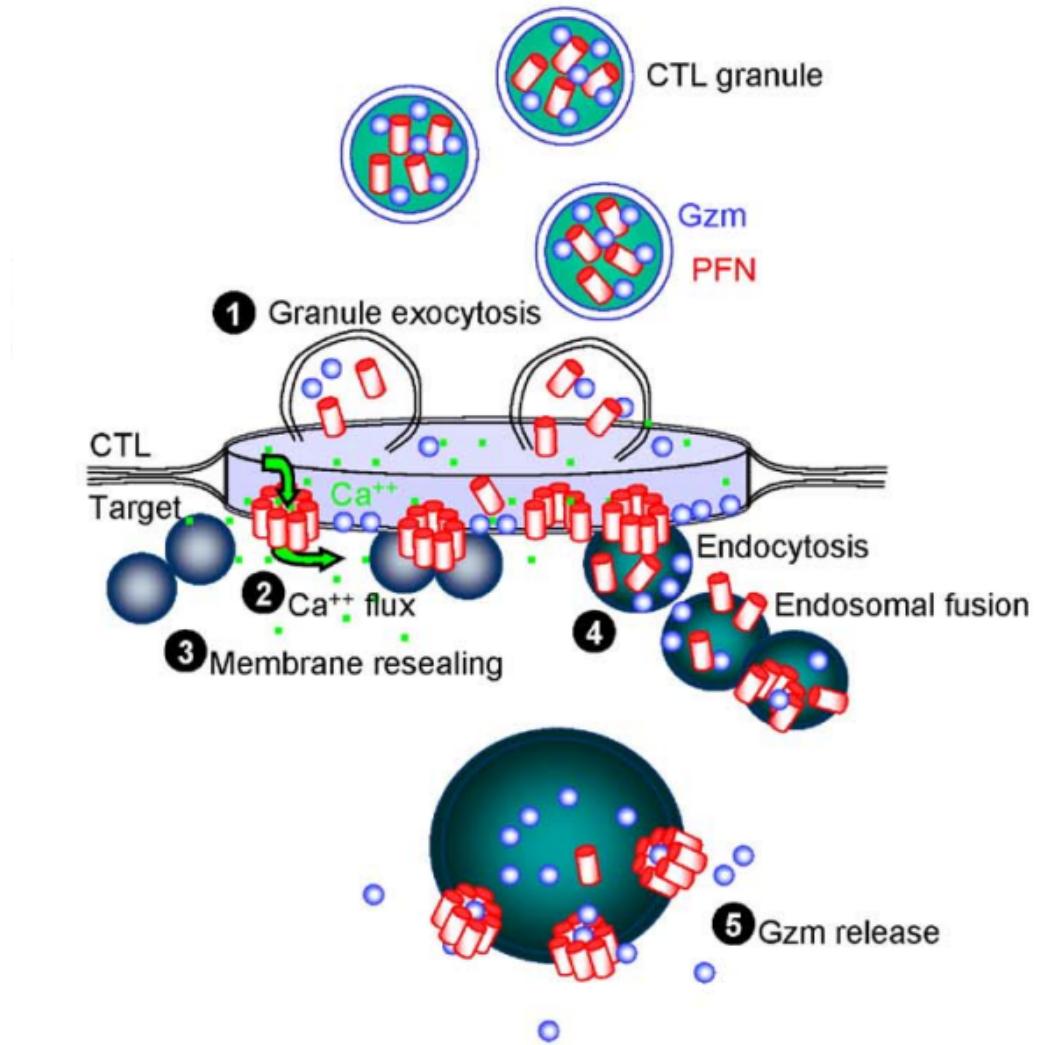


Figure 1.5: Lytic granule secretion and perforin pore formation

A schematic of lytic granule secretion and entry of perforin and granzyme into the target cell. Lytic granules are coordinated to the immune synapse and selectively fuse at the synapse, emptying their cytotoxic contents, perforin (red) and granzymes (blue), into the intercellular space. Perforin requires the presence of calcium ions (Ca^{2+} , green) to pierce the target cell membrane. and permit entry of granzyme proteases. Target cell membrane repair programs begin to repair the damaged membrane. The resealing of the target membrane results in endocytosis of intercellular granzyme and perforin (as well as Ca^{2+} ions), which again re-pierce the endocytic vesicular membrane and allow for true granzyme entry into the cytosol. where granzymes cleave apoptotic effectors. This figure is adapted from [31].

sions [11]. Canonically, actin clearance from the center of the immune synapse (cSMAC) is required for fusion of lytic granules to the center of the synapse [11].

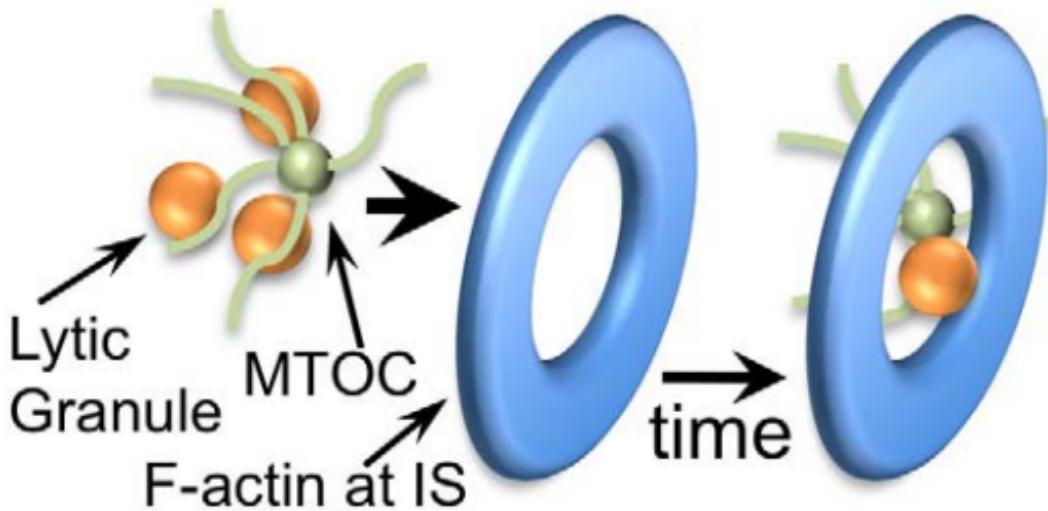


Figure 1.6: Canonical model of actin clearance as a necessary condition for lytic granule secretion

Model of central actin clearing for MTOC polarization and lytic granule fusion. Upon TCR activation, MTOC polarizes towards the immune synapse with the assistance of the motor protein dynein, bringing lytic granules towards the IS and docking them in preparation for degranulation. The actin meshwork clears from the center of the IS, forming a mature dSMAC actin-ring (as well as the associated pSMAC and cSMAC) and exposing available synaptic membrane for degranulation. This figure is adapted from [32].

Newer, more recent models of degranulation highlight the need for transient actin polymerization at the site of degranulation to potentiate the pore forming effects of perforin [33]. This is related to T cell mechanical force exertion against the target, which significantly amplifies T cell effector function [33].

1.6 T cell mechanical force exertion

Immune cell-immune cell interactions are typically described as a series of interrelated biochemical receptor-ligand signaling processes (and this introduction has done the same). While this characterization is not incorrect, it ignores the mechanical dimension of immune cell interactions, which have been identified as significant hubs of cellular decision making. Immune cells have been demonstrated to have a number of biophysical sensing functions, including cancer cell surveillance and immune cell function and cytotoxicity.

The IS is a physically dynamic structure exerts mechanical force [11, 34]. Naïve T cells exert mechanical force on polydimethylsiloxane (PDMS) pillars coated with activating proteins, as observed via traction force microscopy [34]. It is a reasonable guess that T cells have evolved mechanisms to use this force information as a method of sensing the mechanical properties of surfaces that they come in to contact with. This hypothesis is supported by the finding that T cells are more strongly activated stiffer, rather than softer, PDMS surfaces, as measured by cytokine production and proliferation [35]. Evidently, avenues of mechanical communication inform the T cell's actions, and almost certainly vice-versa.

Recent studies demonstrate that the actin-rich structures formed during T cell adhesion and degranulation are actually involved in boosting the lytic activity of perforin by dynamically applying mechanical force against the target cell [36, 33] and sensitizing the target cell to perforin insertion, which must overcome the hydrophobic interior of the plasma membrane in order to pierce the target cell plasma membrane and trigger cell lysis (see Figure 1.7). This phenomenon is an

example of *mechanopotentiation*, or the boosting of biochemical functions through mechanical means. This theme is extensively explored throughout this thesis in Chapters 2 and 3.

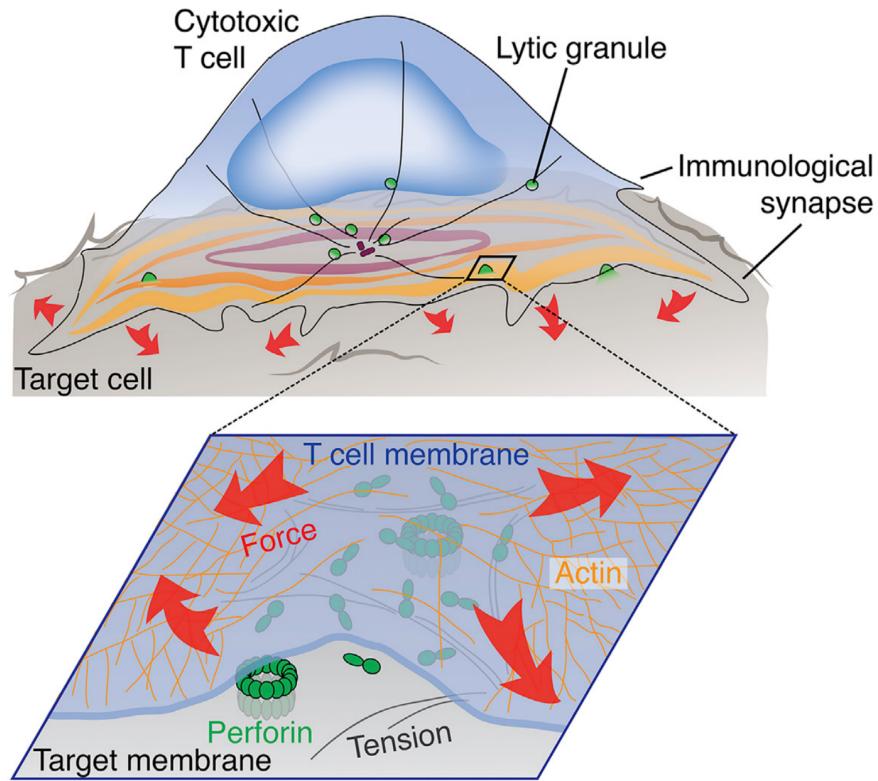


Figure 1.7: T cells exert mechanical force against target cells

Illustration of T cell force exertion against the target cell. T cells use biophysical force (via cytoskeletal proteins such as actin) in concert with biochemical signaling axes to optimize their own activation and cytotoxic programming. This force focuses the secretion of lytic granules and the pore-forming effect of perforin. This figure is adapted from [32].

We can also look more closely at force application across the immune synapse, at a molecular scale. The exertion of force against a resisting target or surface can also be actualized through a specific receptor-ligand interaction. Typically, the resistance through receptor-ligand interaction amplifies T cell signaling and function [37]. The TCR itself is mechanosensitive. Recent work has demonstrated

that the TCR uses mechanical force to distinguish single amino acid differences in peptides. This work demonstrates that the TCR is a mechanosensor that uses force transduced directly orthogonal to the IS to induce conformational changes in the ζ -chain of the TCR, in order to expose phosphorylation sites in its ITAM domains [38]. Additional work has emphasized the importance of this force-dependent function for the induction of calcium signaling [39]. In totality, these studies suggest that force is necessary to expose ITAM domains on the TCR, which leads to the induction of TCR and membrane proximal signaling, as described in 1.3.

Furthermore, the TCR-pMHC interaction actually functions as a catch bond [39]. A catch bond is a particular type of bond in which application of a force increases the lifetime (to an extent) of the interaction, as opposed to a slip bond, whose lifetime decreases as more force is applied. The TCR's capacity to form a catch bond plays a critical mediating role in the TCR's ability to discriminate between high and low affinity agonists [39], as well as in responding differentially between them (i.e. in a graded, not a binary, fashion). A recent report emphasizes the fine-tuning of TCR strength for tumor-specific T cells, in which T cells that received either too high or too low of a signal strength both upregulated inhibitory receptors (such as PD-1) and failed to improve tumor clearance [40], suggesting a molecular convergence. However, while $signal^{hi}$ tumor-specific T cells lost effector function (suggesting an exhausted state), $signal_{low}$ tumor-specific T cells achieved a functionally inert state, defying expectations of molecular convergence [40]. A complete understanding of the mechanical signaling of T cell-specific receptors remains at large, and will require many years of study to disentangle their mechanical activity from their biochemical, which remains

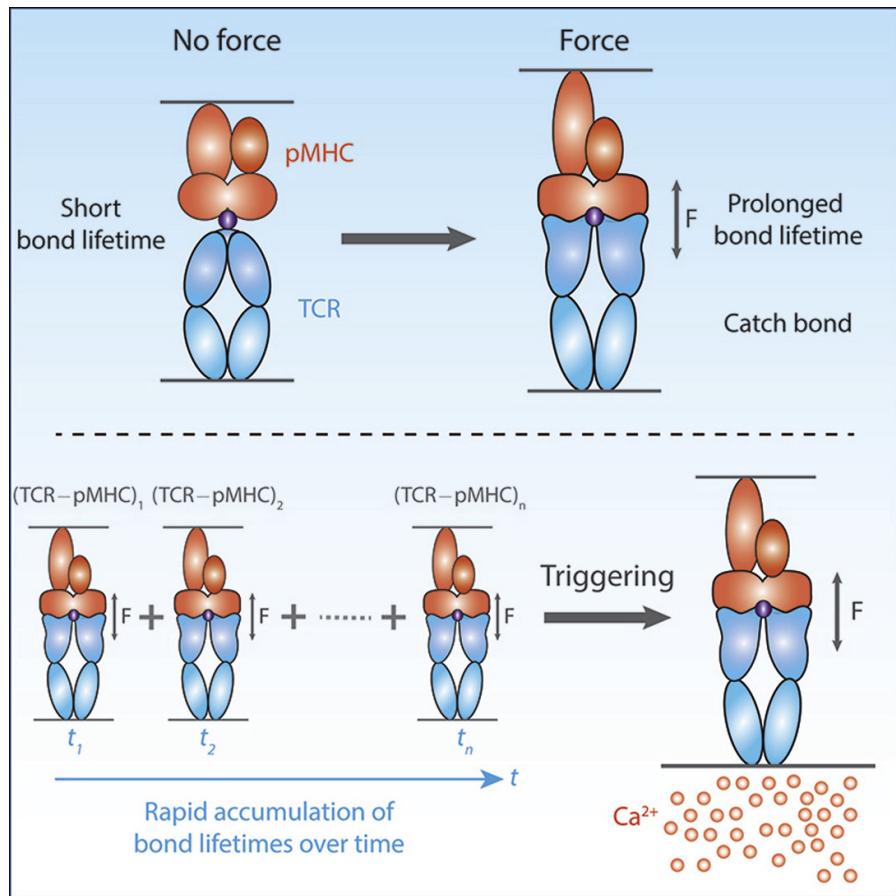


Figure 1.8: Mechanosensitive receptors of the immune synapse

An illustration of the principle of mechanosensitive receptors. The lifetime of a receptor-ligand interaction in response to applied force across the bond can either significantly increase the lifetime of the bond (a catch bond) or rapidly decrease (slip bond). The TCR is an example of a mechanosensitive receptor - it demonstrates catch bond behavior with applied force. If a sufficient accumulation of signaling bonds accrue (here, the TCR) then TCR activation is triggered (e.g. flux of Ca^{2+} ions, see 1.3) and highly potentiated by the increased avidity and number of active TCR-pMHC bonds. This figure is adapted from [39].

ever the challenge in mechanobiology. Together these studies demonstrate the important role of mechanobiology in T cell activation and signaling.

Other receptors are also mechanosensitive, including ion channels, G-protein coupled receptors (GPCRs), and integrins [41, 42, 43]. Integrins are of particular interest to this thesis - see subsection 3.1.3 for further details. Further research

is needed to understand the precise role of surface tension on T cell activation. Yet, these studies are intriguing and support the concept that signaling and cell biology are not merely biochemical reactions. These observations show that mechanical properties of biology are important factors that have been largely overlooked in immunology until recently.

1.7 Thesis Aims

The further successful development of immune cell therapies thus far has been and will continue to be dependent on a deeper understanding of basic T cell effector function. To this end, this thesis is chiefly focused on a molecular approach to studying the mechanotransduction of T cell effector function and the dynamic relationships between its biophysical and biochemical dimensions. The results of this study are broken across two chapters, whose contents are summarized below.

While it was known that the CTLs use physical force to amplify the pore-forming effects of perforin and resulting positive correlation between force exertion and target cell death, it was unclear what cellular structures T cells form in order to actualize this force against the target cell membrane and manipulate this relationship. It was also unclear what proteins or molecules were involved in this membrane perturbation/distortion process. Chapter 2 of this thesis will address the results of the experiments testing these outstanding questions through the use of pharmacological and genetic perturbations against the actin nucleation promoting factors (NPFs) Wiskott–Aldrich Syndrome protein (WASP) and WAVE2 (WASP-verprolin homolog 2) that directly affected T cell force

exertion, and studying their resulting cytotoxic capabilities and dynamics via co-culture assays and imaging.

Another outstanding question arose from the observation that T cells degranulate specifically near areas of synaptic force exertion. While this dovetails with the observation that force exertion amplifies the pore-forming effects of perforin (as discussed in the Introduction and Chapter 2), it was unknown what types of forces or interactions coordinated these two phenomena of degranulation and synaptic force exertion so that they could be unified in space and time. It was also unknown what force-sensitive molecules could be mediating this intercellular communication. Chapter 3 addresses the study conducted to address these questions, which also used pharmacological and genetic perturbations against integrins (primarily lymphocyte function-associated antigen 1, or LFA-1) on T cells in order to modify the force output of these T cells and change their degranulation and cytotoxic behavior as observed through co-culture assays and unique and creative imaging experiments. A discussion of the implications of the results of Chapters 2 and 3 and possible future directions can be found in the Chapter 4. Altogether, this thesis aims to clarify the specifics of the T cell killing event (encompassing T cell force exertion and degranulation) in order to better inform future T cell studies and therapy design.

CHAPTER 2

INTERFACIAL ACTIN PROTRUSIONS MECHANICALLY ENHANCE KILLING BY CYTOTOXIC T CELLS

Results presented in this chapter have been published as: Tamzalit, F., Wang, M. S., Jin, W., Tello-Lafoz, M., Boyko, V., Heddleston, J. M., Black, C. T., Kam, L. C., and Huse, M. *Interfacial actin protrusions mechanically enhance killing by cytotoxic T cells*. Science Immunology. 2019 Mar 22;4(33).

2.1 Background

Dynamic cell-cell interactions coordinate bidirectional information transfer in the immune system and control the potency and the scope of effector responses [44]. One of the most important of these interactions is the immunological synapse (IS) formed between a cytotoxic T lymphocyte (CTL) and the infected or transformed target cell it aims to destroy [24, 25]. IS formation is rapidly induced by recognition of cognate peptide-major histocompatibility complex (MHC) on the target cell by T cell antigen receptors (TCRs) on the CTL. Once firm contact is established, the CTL secretes a toxic mixture of granzyme proteases and the hydrophobic protein perforin into the intercellular space. Perforin forms pores in the target cell membrane that stimulate the uptake of granzymes into the cytoplasm, where they induce apoptosis by cleaving specific substrates [30]. Perforin- and granzyme- mediated killing is the most prevalent mode of lymphocyte cytotoxicity, and it likely plays an important role in cellular immunotherapy approaches against cancer [45].

IS formation is accompanied by dramatic reorganization of both microtubules and filamentous actin (F-actin) [46]. Within minutes of TCR stimulation, the centrosome (also called the microtubule-organizing center) moves to a position just beneath the IS. The centrosome is closely associated with lytic granules, the secretory lysosomes that store perforin and granzyme, and its reorientation positions these granules next to the synaptic membrane [25]. This promotes the directional secretion of granule contents into the intercellular space, enhancing both the potency and the specificity of killing. Whether (and how) F-actin remodeling contributes to cytotoxicity is, by comparison, less clear. Our current conception of synaptic F-actin is strongly influenced by imaging studies in which the target cell is replaced by a glass surface or a supported bilayer containing stimulatory TCR ligands. In this context, T cells form radially symmetric synapses characterized by intense F-actin accumulation at the periphery and depletion from the center [47, 13, 48, 49]. This annular configuration is thought to encourage lytic granule fusion at the center of the IS by clearing F-actin from the plasma membrane in this zone [25, 9, 11]. Although this model is conceptually appealing, it is unclear how well it applies to granule release in bona fide CTL–target cell conjugates, where synaptic F-actin rings are less apparent and, when observed, often quite transient. Synaptic F-actin is also highly dynamic, forming protrusions and lamellipodial sheets that exhibit both centripetal retrograde flow and radial anterograde movement [49, 11, 50, 51, 52]. These dynamics enable T cells to impart mechanical force across the IS [53, 34]. In CTLs, the capacity to exert synaptic force is notably correlated with cytotoxic potential [36]. Biophysical and imaging experiments suggest that force enhances cytotoxicity by increasing the membrane tension of the target cell, which in turn promotes the pore-forming activity of secreted perforin. Here, we applied microfabrication and high-resolution live imaging to

investigate how CTLs mechanically potentiate the chemical activity of perforin, a process we refer to as mechanopotentiation. Using stimulatory micropillar arrays that trigger IS formation in three dimensions, we have found that lytic granule release occurs at the base of F-actin-rich synaptic protrusions that extend into the antigen-presenting surface. These protrusions, which are generated by the Wiskott-Aldrich syndrome protein (WASP) and the actin related protein (Arp)2/3 actin nucleation complex, are required for synaptic force exertion and cytotoxic efficiency. Our results provide insight into how cytotoxic lymphocytes organize mechanical output and demonstrate how three-dimensional architecture influences the functionality of communicative interfaces in the immune system.

2.1.1 Force exertion through the actin cytoskeleton

The actin cytoskeleton is a dynamic network of actin polymers and associated actin binding proteins. It is the primary means by which the T cell achieves its shape, and its migratory and interactive capabilities against the target cell. Central actin depletion is reported as necessary for granule fusion at the immune synapse, a reminder of the intimate link between the actin cytoskeletal dynamics and the killing functions of the T cell. Accordingly, the T cell uses actin-mediated mechanical force against the target cell in order to achieve its full cytotoxicity (see section 2.1.2 [36]. Perturbations of PTEN (an antagonist of PI3K-dependent F-actin remodeling via Dock2, see section 1.4) enhanced synapse formation, strongly increased force exertion, and markedly increased killing [13, 36]. This perturbation did not affect granule organization or release, suggesting that actin-mediated force is at least partially responsible for this increased cytotoxic

effect [36]. The T cell has also been reported to form protrusions of varying function - some groups highlight the ability of these protrusions to facilitate antigen recognition [50], enriched T cell activation[54], and trogocytosis [21]. In totality, these findings highlight an intimate link between proper IS formation and optimal cytotoxicity that is mechanical in nature.

2.1.2 Modifying target cell sensitivity to perforin

The insertion of perforin into the target cell membrane requires overcoming an energetic barrier that is imposed by the features of the plasma membrane, namely the hydrophobic inner leaflet and the tensile state of the target cell surface. Membrane tension is a measure of the cost of increasing the membrane area, as opposed to cortical tension [55]. Although intimately related to membrane tension (see Figure 2.1), cortical tension is strictly defined the tensile forces involved in cytoskeletal cortex [55]. Membrane tension can be affected in many ways, particularly in cell surfaces as compared to pure lipid vesicles, because of the following factors: the presence of transmembrane proteins, membrane-cytoskeletal interactions within the cell itself, and potential external forces imparted by interacting cells [55].

T cells manipulate the membrane tension of target cells in order to sensitize the target cell to perforin [36]. Perforin clearly has mechanisms to naturally accomplish this [26] even without the application of physical force by T cells, as evidenced by experiments in which recombinant perforin is added to tumor cells and is capable of lysing target cells [36]. Therefore, it follows that increased membrane tension has been found to be a defining factor in overcoming the energy

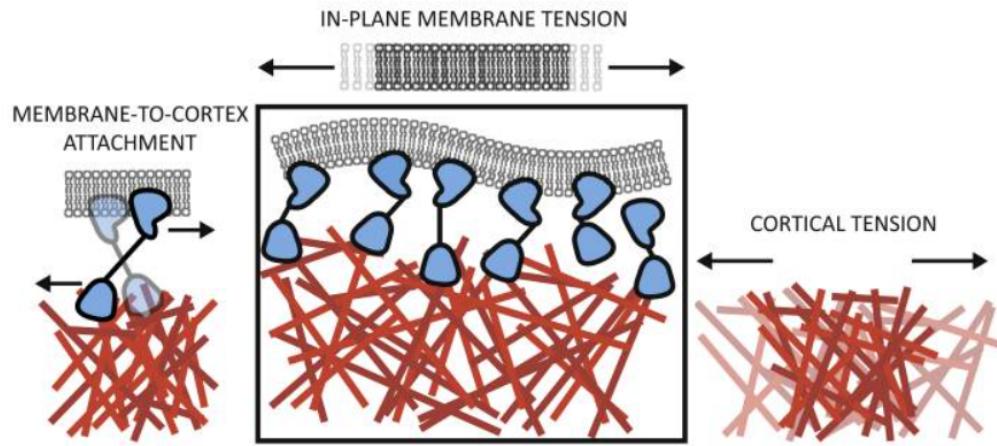


Figure 2.1: Mechanics of the plasma membrane and the underlying actomyosin cortex.

Both the plasma membrane (gray) and the cortex (red) are under tension, a measure of the energetic cost of increasing their area. In cells, membrane tension arises from both in-plane tension (final distance between lipids exaggerated for clarity) and membrane-to-cortex-attachment (MCA, blue), which is related to cortical tension (right). This figure is adapted from [55].

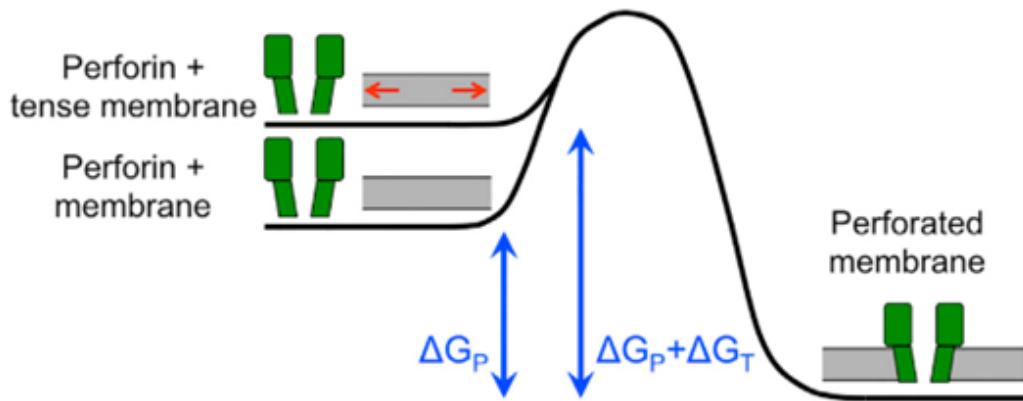


Figure 2.2: Perforin entry into target cell membrane reaction coordinate.

Schematic diagram of the reaction coordinate for perforin pore formation in a membrane, illustrating how increased membrane tension can effectively reduce the activation barrier of the process. ΔG_P = free energy of pore formation, ΔG_T = free energy of tension release.

This figure is adapted from [36].

barrier imposed by the plasma membrane (Figure 2.2), effectively sensitizing the target cell to perforin. Tumor cells grown on stiffer hydrogels (resulting in higher levels of membrane stiffness as confirmed by AFM) were more sensitive to recombinant perforin lysis in a T cell-free system, indicating that the manipulation of target cell membrane tension is one avenue by which T cells may potentiate their killing function [36]. However, the nature of the exact cellular structures that T cells formed in order to physically manipulate the target and what molecular pathways may regulate them remained unknown, and was the primary interest of the study detailed in this chapter.

2.2 Results

2.2.1 CTLs form actin-rich protrusions on stimulatory micropillars

Synaptic force exertion can be measured by imaging T cells on arrays of flexible polydimethylsiloxane (PDMS) micropillars bearing immobilized TCR ligands and adhesion proteins (Figure 2.3) [34, 36]. T cells form IS-like contacts with these arrays and induce pillar deflections that can be converted into force vectors based on the known dimensions and composition of the pillars. Using this approach, we previously found that lytic granule release tends to occur in regions of active pillar deflection [36]. This result raised the possibility that there might be specific structures within the IS that mechanopotentiate perforin function by imparting force in close proximity to granule secretion.

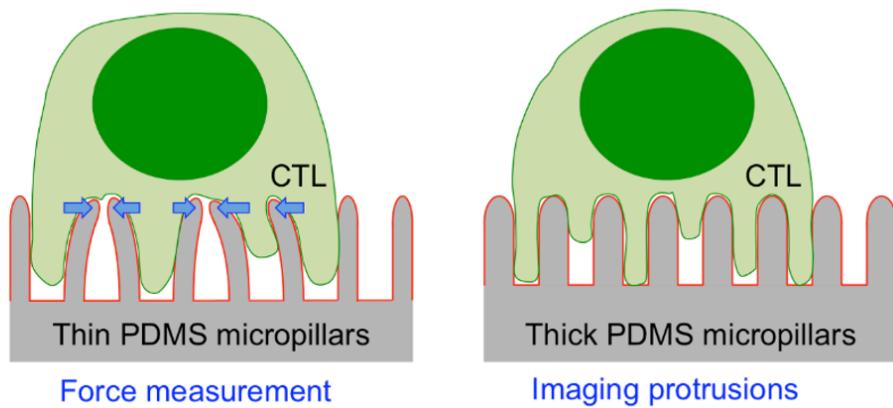


Figure 2.3: Using PDMS micropillars as 3D environment to observe and measure CTL cytoskeletal structures.

Schematic diagram of thin micropillars used for force measurements (left) and thicker micropillars used for imaging protrusions (right).

To identify candidate structures that could be involved in this mechanopotentiation, we closely examined the dynamic architecture of CTL synapses on micropillar arrays. For these experiments, we used primary CTLs expressing the OT1 TCR, which is specific for the ovalbumin 257–264 peptide presented by the class I MHC protein H2-K^b [H2-K^b-ovalbumin (OVA)]. OT1 CTLs were retrovirally transduced with Lifeact-green fluorescent protein (GFP), a fluorescent probe for F-actin, and imaged by confocal microscopy on micropillars coated with H2-Kb-OVA and ICAM1 (intercellular adhesion molecule 1), a ligand for the $\alpha L\beta 2$ integrin LFA1. The pillars in these arrays (1 to 1.5 μm in diameter and 4 to 5 μm tall) were thicker, shorter, and therefore more rigid than the pillars used for T cell force measurements (0.7 μm in diameter and 6 μm tall) (Figure 2.3). These thicker pillars are not substantially deflected by CTLs and function as a regularly crenulated stimulatory surface that facilitates quantitative assessment of IS growth in three dimensions.

Within minutes of initial contact with the arrays, the CTLs formed F-actin-rich protrusions that invaded the spaces between adjacent pillars (Figure 2.3). Time-lapse experiments using both confocal and lattice light-sheet microscopy revealed that the F-actin in these protrusions was highly dynamic, coruscating up and down the length of each pillar (Figure 2.3). Periodically, F-actin-free gaps appeared at the base of the protrusions, in the regions around the pillar tops. During protrusion growth, F-actin accumulation was often strongest at the leading edge, implying a causative relationship between actin polymerization and the formation of these structures (Figure 2.5). Most of the microtubule cytoskeleton, by contrast, was constrained to the region above the pillars, although individual microtubules were observed to extend into a subset of protrusions (Figure 2.3). In most cells, the centrosome reoriented to a position in the plane of the pillar tops but did not proceed into the interpillar spaces. Hence, on micropillar arrays, CTLs form dynamic, F-actin-rich protrusions at the IS that exclude the centrosome.

During initial cell spreading, invasion into the micropillar zone was typically constrained to the periphery of the contact (Figure 2.4). However, once the radial size of the IS stabilized, after around 60s, protrusions formed in the more central regions of the interface. This was intriguing to us because previous studies had indicated that lytic granules accumulate beneath the more central IS domains [25, 9, 56, 57]. To investigate the spatial relationship between synaptic protrusions and lytic granules, we imaged CTLs expressing Lifeact-mApple together with a GFP-labeled form of the lysosomal-associated membrane protein 1 (Lamp1-GFP). Lytic granules appeared as a cluster of distinct compartments within the CTL cytoplasm. In the first 2 min of contact formation, the granule cluster moved downward, settling around 5 μ m from the cell front, roughly at the level of

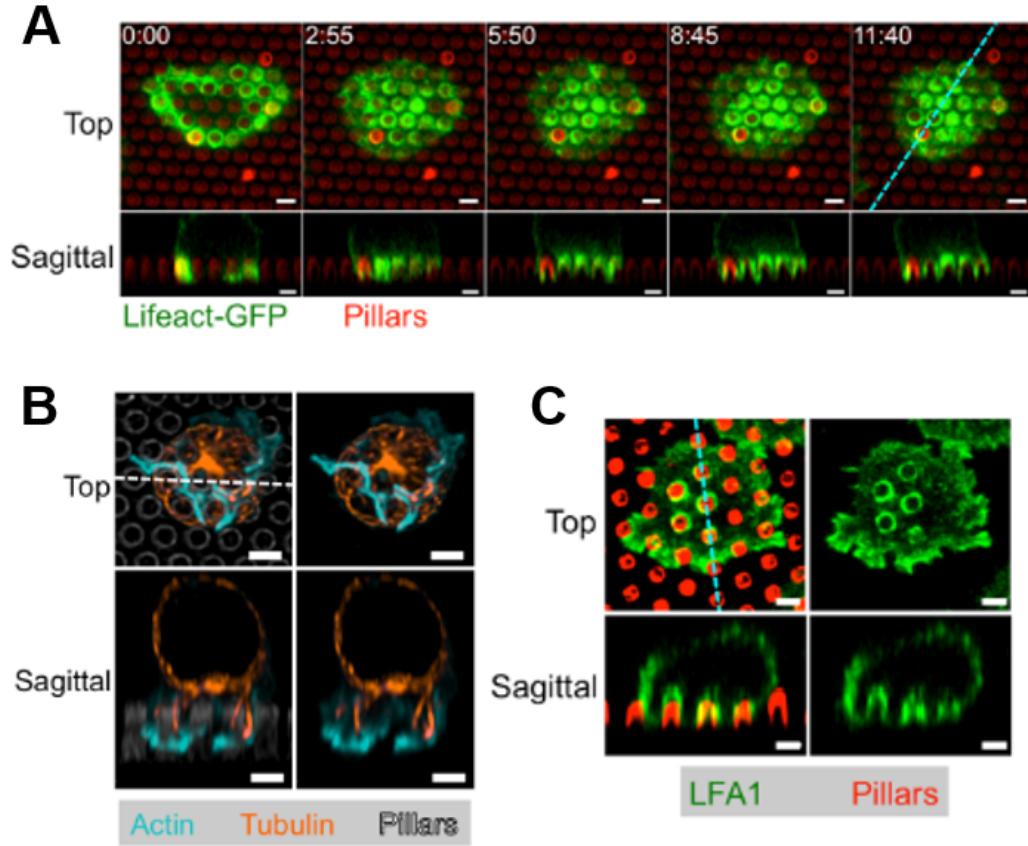


Figure 2.4: Characterizing the architecture of protrusions using micropillars.

(A-C): OT1 CTLs were imaged by confocal microscopy on micropillars bearing H2-K^b-OVA and ICAM1. z-projection images (top views) are shown above with sagittal views below. Dashed lines [cyan in (A) and (C); white in (B)] denote the slicing plane used for the sagittal images. **(A):** Time-lapse montage of a representative CTL expressing Lifeact-GFP, with micropillars shown in red. **(B):** Fixed image of a representative CTL stained with phalloidin (to visualize F-actin) and antitubulin antibodies. Micropillars are shown in gray in the left images. **(C):** Fixed image of a representative CTL stained with anti-LFA1 antibodies, with micropillars shown in red. All scale bars, 2 μ m. Mitchell Wang, Fella Tamzalit, and Morgan Huse performed these experiments.

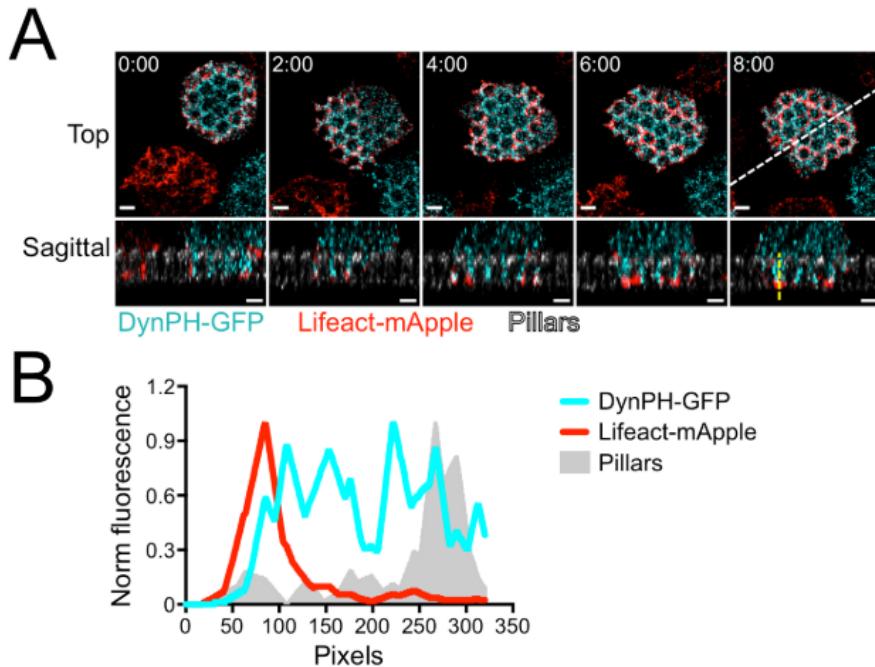


Figure 2.5: F-actin accumulates at the leading edges of synaptic protrusions.

OT1 CTLs expressing Lifeact-mApple and DynPH-GFP (a membrane marker) were imaged by confocal microscopy on fluorescent micropillars bearing H2-K_b-OVA and ICAM1. **(A)**: Time-lapse montage of a representative CTL, with z-projection images (top views) shown above and sagittal views below. The white dashed line denotes the slicing plane used for the sagittal images. Pillars (gray) appear in the sagittal images only. Time in M:SS is indicated in the upper left corner of each top view. Scale bars = 2 μ m. **(B)**: Linescan (derived from the dashed yellow line in **(A)**) showing normalized fluorescence intensity of DynPH-GFP, Lifeact-mApple, and the pillars. Mitchell Wang, Fella Tamzalit, and Morgan Huse performed these experiments.

the pillar tops (Figure 2.6). This behavior implied a close association between granules and the centrosome, as previously reported [9, 57] (21).

After orienting downward, the granules tended to occupy central locations within the IS, which we quantified by calculating the normalized proximity of granule fluorescence to the IS center of gravity (COG) (Figure 2.7). Analysis of this “centralization factor” revealed that the granules tended to be closer to the center of the IS than would be expected by chance (Figure 2.6).

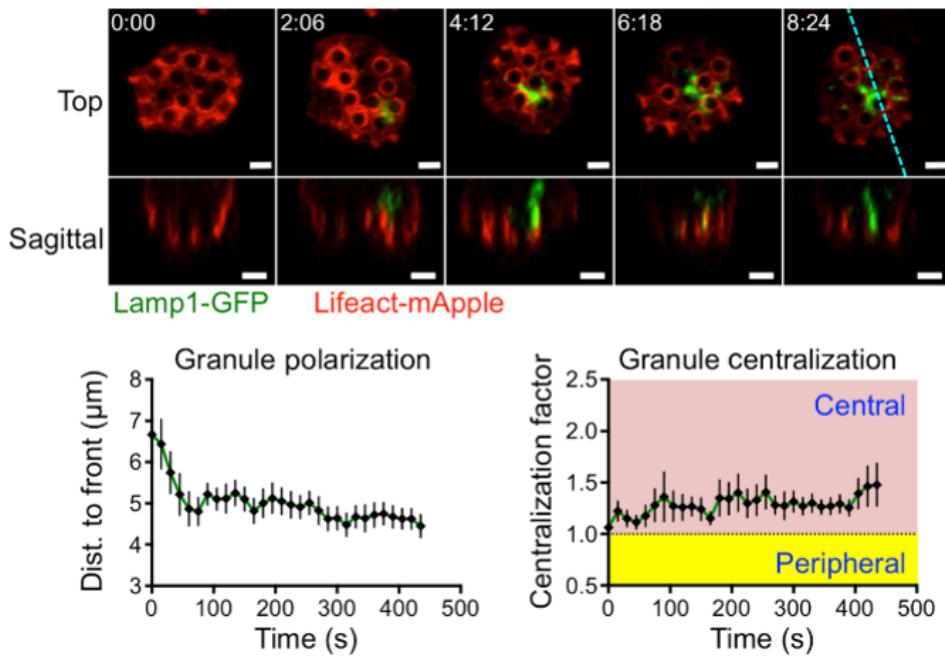


Figure 2.6: Granules polarize towards the center of the immune synapse.

Above, time-lapse montage of a representative CTL expressing Lifeact-mApple and Lamp1-GFP. Below left, mean distance between the lytic granule cloud and the cell front, graphed against time. Below right, centralization factor analysis of Lamp1-GFP. In both graphs, time 0 denotes initial contact with the pillars and error bars indicate SEM. n = 10.

Mitchell Wang, Fella Tamzalit, and Morgan Huse performed these experiments.

The proximity of lytic granules to the base of synaptic protrusions at the center of the IS raised the possibility that these structures might be involved in cytolytic mechanopotentiation. Synaptic protrusions were highly enriched in LFA1 (Figure 2.4), consistent with them being strongly adhesive and capable of exerting force. Previously, we found that mechanopotentiation requires phosphoinositide 3-kinase (PI3K) signaling and is enhanced by the depletion of phosphatase and tensin homolog (PTEN) that antagonized PI3K. Short hairpin RNA (shRNA)-mediated suppression of PTEN augmented F-actin accumulation in synaptic protrusions (Figure 2.8), further supporting the idea that these structures transmit forces that promote cytotoxicity.

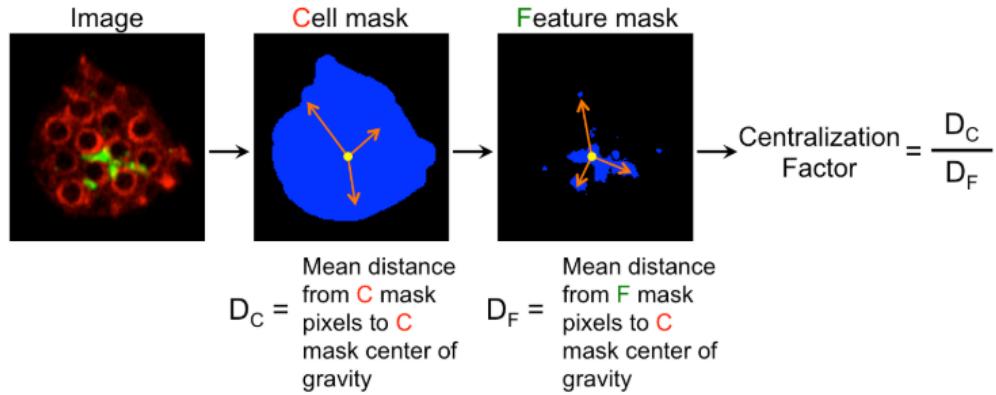


Figure 2.7: Centralization factor analysis.

The centralization factor compares the mean distance between a given feature within the IS (e.g. lytic granules) and the IS center of gravity (D_F) to the mean distance between all positions within the IS and the IS center of gravity (D_C). Mitchell Wang, Fella Tamzalit, and Morgan Huse performed these experiments.

2.2.2 Granule fusion occurs at the base of synaptic protrusions

Granule fusion events can be detected in single-cell imaging experiments with a fluorescent reporter containing a pH-sensitive GFP (pHluorin) fused to the granule-targeting domain of Lamp-1 [58]. Within lytic granules, the low pH environment quenches the fluorescence of pHluorin-Lamp1. Granule fusion with the plasma membrane, however, neutralizes the pH around the reporter, leading to a rapid increase in fluorescence. To explore the relationship between cytolytic secretion and synaptic protrusions, we imaged OT1 CTLs expressing pHluorin-Lamp1 by confocal and lattice light-sheet microscopy on fluorescent micropillars coated with H2-K^b-OVA and ICAM1I (Figure 2.9). Protrusions were visualized in these experiments either via Lifeact-mRuby2 or by staining with a fluorescent Fab against the surface marker CD45. After IS formation, fusion events appeared as sudden flashes of GFP fluorescence, which were often visible for only one time point. These events clustered close to the plane of the pillar

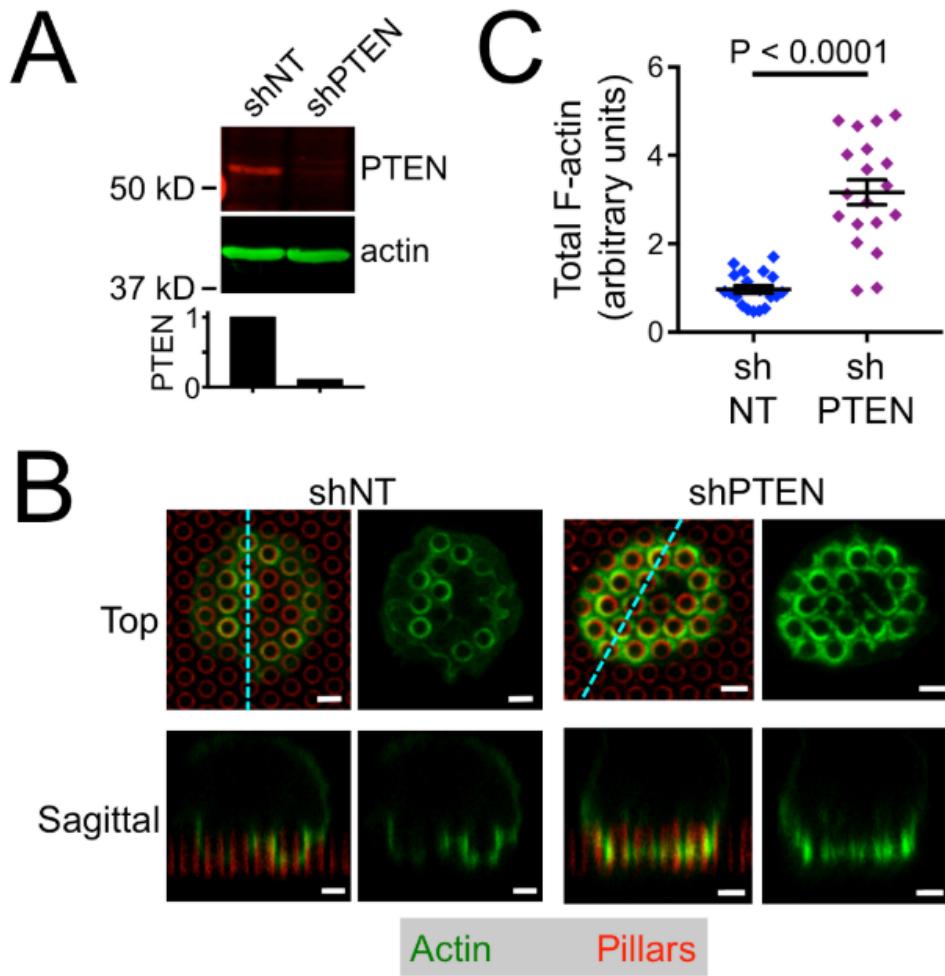


Figure 2.8: PTEN depletion enhances F-actin accumulation in protrusions.

OT1 CTLs expressing shRNA against PTEN (shPTEN) or a control nontargeting shRNA (shNT) were applied to fluorescent micropillars bearing H2-K^b-OVA and ICAM1, fixed, and stained with phalloidin. **(A)**: Immunoblot analysis of PTEN expression in shNT and shPTEN CTLs. Actin served as a loading control. Bars denote intensity of PTEN bands. **(B)**: Representative images of shNT and shPTEN CTLs, with z-projection images (top views) shown above and sagittal views below. Cyan dashed lines denote the slicing planes used for the sagittal images. Scale bars = 2 μ m. **(C)**: Quantification of sum F-actin intensity on micropillar arrays. Error bars denote SEM. N = 19 for each cell type. P calculated from two-tailed Student's T-test. Fella Tamzalit and Morgan Huse performed these experiments.

tops (Figure 2.9), the same vertical zone occupied by lytic granules and the centrosome. This position was well behind the leading edge of CTL protrusions, which extended about $5\text{ }\mu\text{m}$ into the interpillar space.

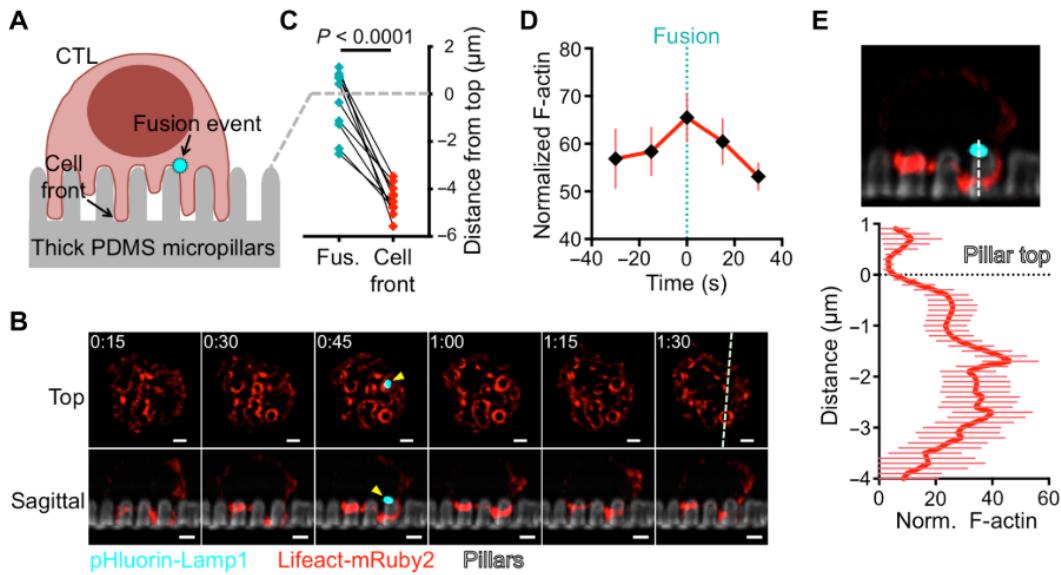


Figure 2.9: Granule fusion occurs at the base of synaptic protrusions.

(A): Schematic diagram showing lytic granule fusion (visualized by pHluorin-Lamp1) on micropillar arrays. **(B to E)**: OT1 CTLs expressing pHluorin-Lamp1 were imaged by confocal (**C**) or lattice light-sheet (**B, D, and E**) microscopy on micropillars bearing H2-K^b-OVA and ICAM1. **(B)**: Time-lapse montage of a representative CTL expressing Lifeact-mRuby2 and pHluorin-Lamp1, with micropillars shown in gray. z-projection images (top views) are shown above with sagittal views below. The white dashed line denotes the slicing plane used for the sagittal images. Yellow arrowheads indicate the fusion event. Time in minutes:seconds is indicated in the upper left corner of each top-view image. Scale bars, $2\text{ }\mu\text{m}$. **(C)**: Graph showing the vertical displacements of fusion events (Fus., cyan) relative to the plane of the pillar tops (dashed gray line) along with the corresponding position of the cell front (red, visualized with a fluorescent Fab fragment against CD45). $n = 11$ events. P values were calculated from two-tailed paired Student's t test. **(D)**: F-actin accumulation in the region of granule fusion in z-projection images of CTLs expressing Lifeact-mRuby2 and pHluorin-Lamp1. Graph shows the average Lifeact-mRuby2 intensity within a $1\text{-}\mu\text{m}$ -diameter circle centered on the fusion site, starting two time points before the fusion event and ending two time points after. **(E)**: Below, mean normalized (Norm.) Lifeact-mRuby2 intensity derived from linescans vertically bisecting the midpoint of the granule fusion site. The dotted black line denotes the plane of the pillar tops. Above, a representative image used for the analysis, with the linescan region indicated by the dashed white line. Error bars in **(D)** and **(E)** denote SEM. $n = 18$ events. Mitchell Wang, Fella Tamzalit, and Morgan Huse performed these experiments.

Granule fusion was not observed in zones of sustained F-actin depletion. Instead, it tended to occur in regions containing synaptic protrusions (Figure 2.9). To quantify this effect, we determined the Lifeact-mRuby2 intensity over time in the 1- μ m-diameter synaptic domain around each fusion site. F-actin accumulation within this domain actually increased modestly during granule fusion (Figure 2.9), implying that cytolytic secretion and protrusion growth could occur concurrently in the same region. Linescans of sagittal slice images demonstrated that F-actin did not overlap precisely with the fusion site. Instead, it tended to accumulate underneath it, closer to the bottoms of the pillars (Figure 2.9). We conclude that granule fusion on micropillar arrays occurs in small F-actin-free zones that form transiently at the base of active F-actin-rich protrusions.

2.2.3 WASP and WAVE2 control distinct subsets of protrusions

Having characterized the structure and dynamics of synaptic protrusions, we turned our attention to their molecular basis and biological function. Arp2/3 complex activity is controlled by regulators of the nucleation-promoting factor (NPF) family. Among NPFs, both WASP and WASP-verprolin homolog 2 (WAVE2) have been implicated in synaptic F-actin remodeling. WAVE2, which is activated by the guanosine triphosphate– bound form of the small guanosine triphosphatase (GTPase) Rac, is thought to promote cell spreading and adhesion during IS formation [13, 59, 60, 61]. WASP, for its part, functions downstream of the GTPase Cdc42 and the adaptor protein Nck, and it has been linked to IS stability and the formation of protrusive structures during diapedesis and antigen scanning [62, 63, 64, 65]. In humans, loss-of-function WASP mutations

cause WAS, a primary immunodeficiency associated with increased incidence of auto immunity and cancer [66, 67].

To investigate the role of WASP and WAVE2 in the formation of synaptic protrusions, we imaged OT1 CTLs expressing GFP-labeled forms of each protein on stimulatory micropillars. WAVE2-GFP accumulated strongly in the periphery of the IS during initial cell spreading (<1 min; Figure 2.10). In subsequent time points, transient bursts of WAVE2-GFP appeared in isolated peripheral domains (Figure 2.10, magenta arrowheads), often occurring concomitantly with lateral movement of the IS toward the same side. By contrast, WASP-GFP accumulated in annular structures that encircled individual pillars in central and intermediate synaptic domains (Figure 2.10). WASP-GFP exhibited a significantly higher mean centralization factor than WAVE2-GFP at all time points (Figure 2.10), confirming that WASP localized more centrally than WAVE2.

To assess the importance of WASP and WAVE2 for IS re-modeling, we used CRISPR (CR)-Cas9 to target the *Was* and *Wasf2* genes, respectively, in OT1 CTLs (Figure 2.11). WASP- or WAVE2-deficient CTLs prepared in this manner (WASP-CR and WAVE2-CR, respectively) were transduced with Lifeact-GFP and then imaged on micropillar arrays. Whereas control CTLs expressing nontargeting guide RNA (NT-CR) formed protrusions in both the center and the periphery of the IS, the protrusive activity of WASP-CR cells was largely constrained to the periphery (Figure 2.11). Centralization factor analysis revealed that the F-actin distributions of WAVE2-CR CTLs were more centralized than those of NT-CR controls, which were in turn more centralized than those of WASP-CR CTLs (Figure 2.11). Hence, WASP deficiency leads to a specific loss of central protrusions, whereas WAVE2 deficiency eliminates peripheral structures.

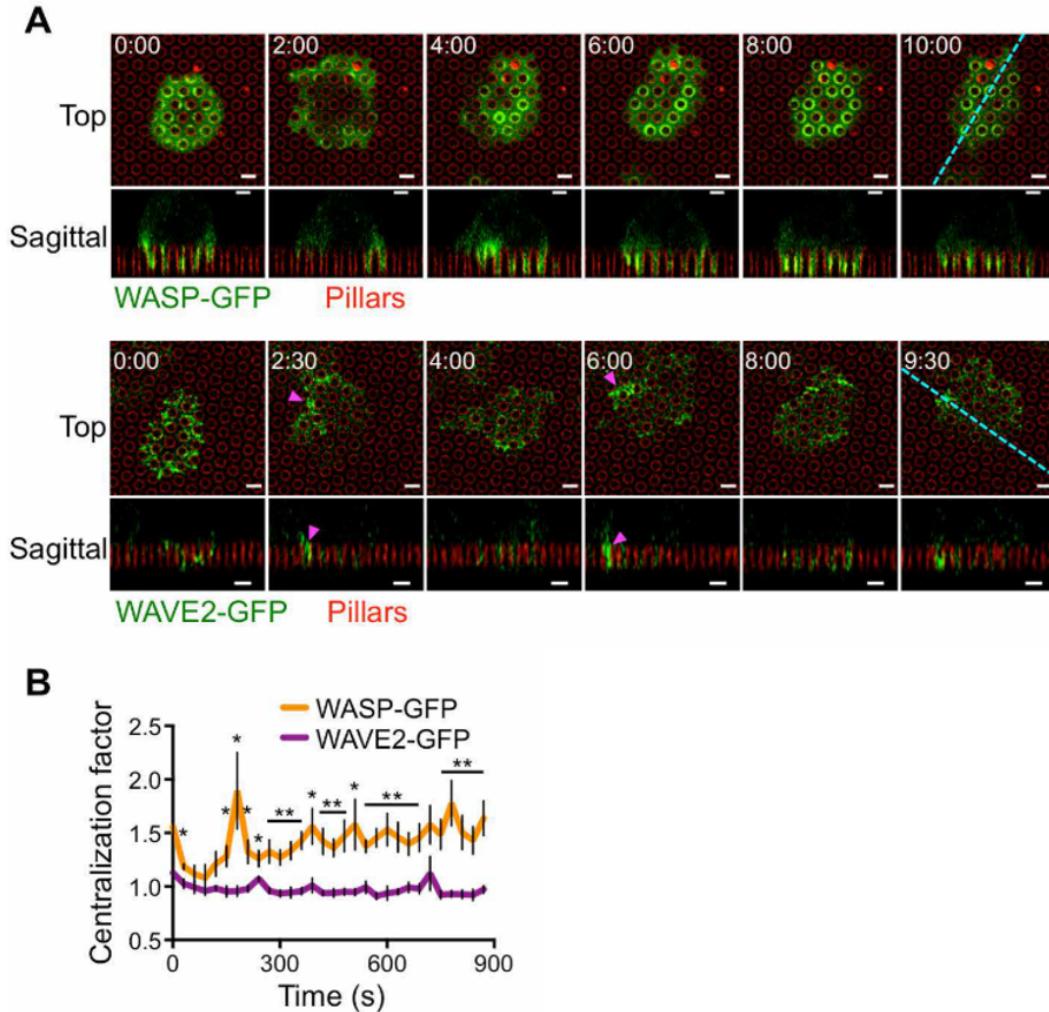


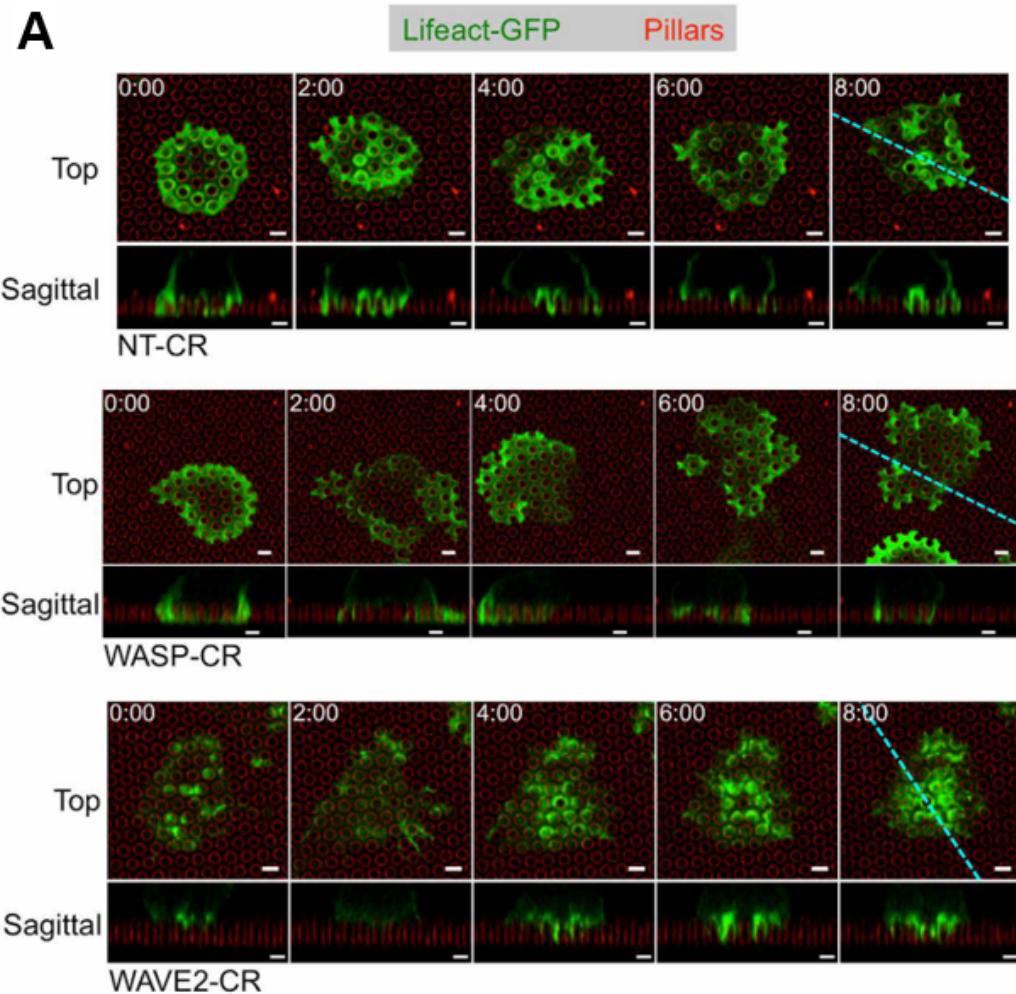
Figure 2.10: WASP and WAVE2 segregate at the synapse.

(A and B): OT1 CTLs expressing WASP-GFP or WAVE2-GFP were imaged by confocal microscopy on fluorescent micropillars bearing H2-K^b-OVA and ICAM1. **(A):** Time-lapse montages of representative CTLs, with micropillars shown in red. z-projection images (top views) are shown above with sagittal views below. Cyan dashed lines denote the slicing planes used for the sagittal images. Magenta arrowheads indicate representative lateral accumulations of WAVE2-GFP. **(B):** Centralization factor analysis of WASP-GFP and WAVE2-GFP, with time 0 denoting initial contact with the pillars. n = 6 for each cell type. In graphs, *P < 0.05 and **P < 0.01, calculated by two-tailed Student's t test comparing WASP-GFP to WAVE2-GFP **(B)**. Error bars denote SEM. Mitchell Wang, Fella Tamzalit, and Morgan Huse performed these experiments.

Collectively, these data indicate that WAVE2 controls peripheral F-actin growth involved in lateral motion, whereas WASP drives protrusion formation closer to the center of the IS.

Figure 2.11: WASP and WAVE2 control distinct subsets of protrusions.

(A: Time-lapse montages of representative CTLs, with micropillars shown in red. z-projection images (top views) are shown above with sagittal views below. Cyan dashed lines denote the slicing planes used for the sagittal images. **(B:** Centralization factor analysis of Lifeact-GFP in NT-CR, WASP-CR, and WAVE2-CR OT1 CTLs, with time 0 denoting initial contact with the pillars. n = 6 for each cell type. In all montages, time in minutes:seconds is indicated in the upper left corner of each top-view image. Scale bars, 2 μ m. In graphs, *P < 0.05 and **P < 0.01, calculated by two-tailed Student's t test WASP-CR (red) and WAVE2-CR (green) to NT-CR **(B)**. Error bars denote SEM. Mitchell Wang, Fella Tamzalit, and Morgan Huse performed these experiments.



B

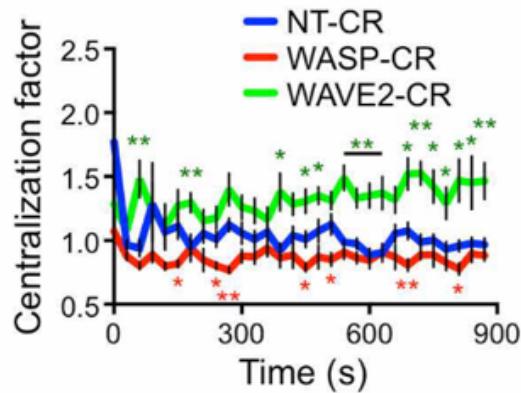


Figure 2.11 (Continued)

2.2.4 WASP and WAVE2 depletion induce distinct functional phenotypes

Next, we investigated the mechanical consequences of WASP and WAVE2 depletion by imaging NT-CR, WASP-CR, and WAVE2-CR CTLs on narrow micropillar arrays (Figure 2.3). WASP-CR CTLs avidly engaged the arrays and deformed them as quickly as did NT-CR controls. The overall magnitude of WASP-CR force exertion, however, was significantly reduced (Figure 2.12). By contrast, depletion of WAVE2 delayed the onset of force exertion but did not affect its overall magnitude (Figure 2.12). To assess the spatial patterns of these mechanical responses, we plotted the number of strongly deflected pillars as a function of radial distance from the IS COG (Figure 2.14). NT-CR and WAVE2-CR CTLs induced pillar deflections in both the central IS ($<3\text{ }\mu\text{m}$ from the COG) and the periphery ($>3\text{ }\mu\text{m}$ from the COG). By contrast, in WASP-CR CTLs, there was a marked absence of centrally localized events (Figure 2.13). Hence, the capacity to generate protrusions in the center of the IS was associated with force exertion in that domain.

Last, we examined the cytotoxic function of CTLs lacking WASP and WAVE2. WASP depletion induced a significant defect in killing, which we observed using both lymphocytic (RMA-s lymphoma) and adherent (MB49 urothelial carcinoma, B16 melanoma) target cells (Figure 2.13). This defect was most pronounced (around a 50% reduction) at low levels of antigen. At higher antigen concentrations, however, killing by NT-CR and WASP-CR CTLs was quite comparable. WASP-CR CTLs did not exhibit lower levels of lytic granule fusion (Figure 2.13), indicating that their reduced cytotoxicity could not be attributed to a defect in perforin and granzyme release. Depletion of WAVE2 led to a distinct and some-

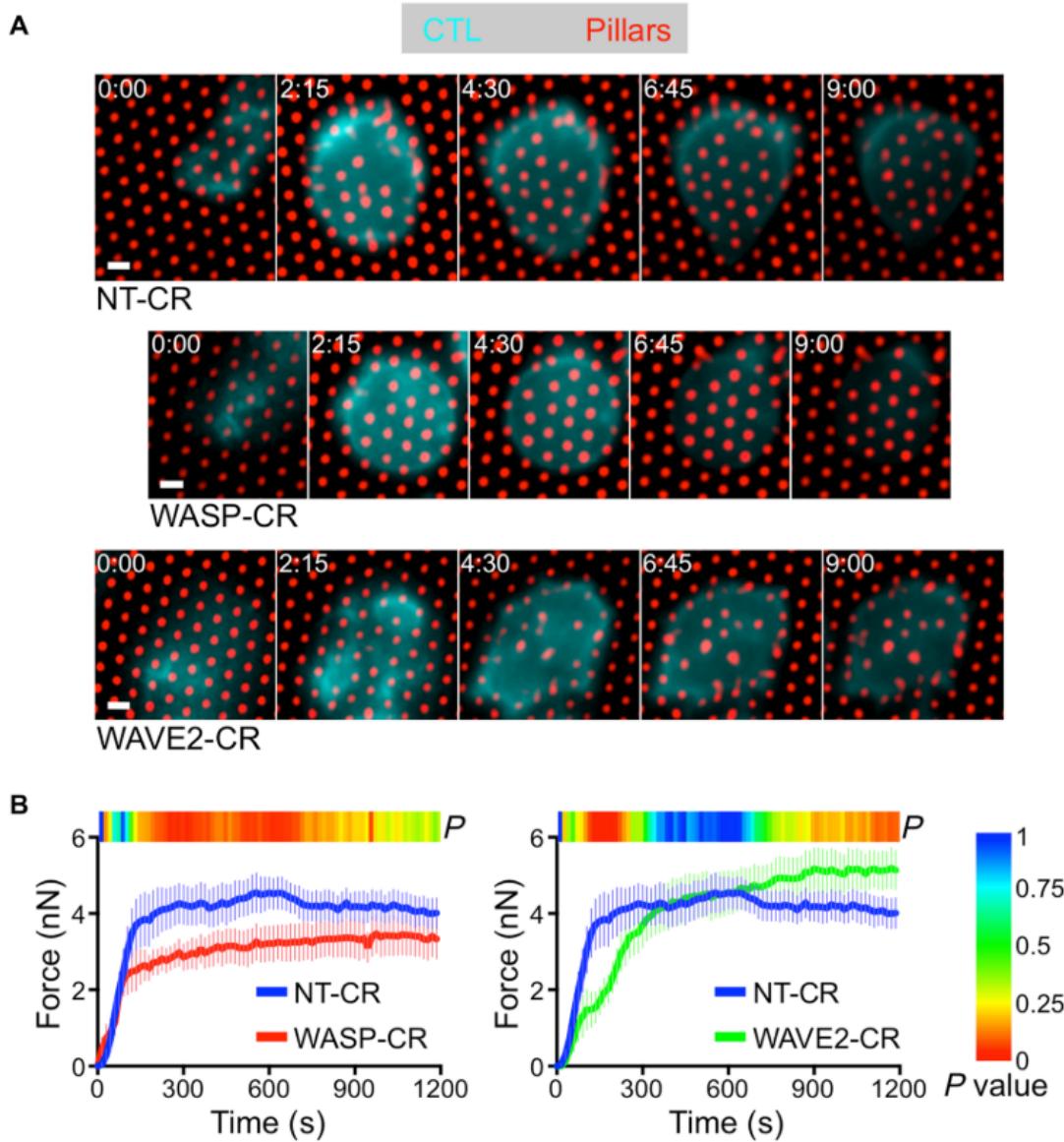


Figure 2.12: WASP and WAVE2 depletion induces spatially different patterns of force exertion.

(A and B): NT-CR, WASP-CR, and WAVE2-CR OT1 CTLs were labeled with a fluorescent anti-CD45 Fab and imaged on narrow fluorescent micropillars coated with H2-K^b-OVA and ICAM1. **(A):** Time-lapse montages of representative CTLs showing pillar deflection. Time in minutes:seconds is indicated in the upper left corner of each top-view image. Scale bars, 2 μ m. **(B):** Total force exertion against pillar arrays was graphed versus time. Color bar above each graph indicates the P value for each time point (two-tailed Student's t test). Mitchell Wang, Fella Tamzalit, Weiyang Jin, and Morgan Huse performed these experiments.

what variable cytotoxicity phenotype. In some experiments, we found little to no change in killing and granule fusion, whereas in others, we observed modest reductions that were most pronounced at high antigen concentrations (Figure 2.13 and 2.14). WAVE2-CR CTLs, but not their WASP-CR counterparts, exhibited significantly reduced conjugate formation (Figure 2.13), implying that WAVE2 promotes target cell adhesion. Consistent with this interpretation, depletion of WAVE2, but not WASP, impaired CTL adhesion to ICAM1-coated surfaces, both in the presence and in the absence of H2-K^b-OVA (Figure 2.14). We also examined indices of TCR signaling and found that WASP-CR and WAVE2-CR CTLs exhibited normal TCR-induced Ca^{2+} flux and activation of the MAPK, PI3K, and NF- κ B pathways (Figure 2.14). Hence, depletion of WASP or WAVE2 does not broadly disrupt early T cell activation.

We conclude that WASP plays a more important role than WAVE2 in boosting cytotoxicity and that it does so in a manner independent of TCR signaling, conjugate formation, and granule release. The WASP-CR killing defect was strongest at low antigen concentrations, when granule release was lower and perforin levels were limiting, and it disappeared at high antigen concentrations, when perforin was abundant. Previous studies of CTLs derived from patients with WAS revealed a similar cytotoxicity phenotype: reduced killing (despite normal conjugate formation and granule release), which was rescued by strong TCR stimulation [64, 68]. This is precisely the pattern of results one would expect after blocking a mechanical process that boosts the per-molecule efficiency of perforin. Together with the imaging data described above, these results suggest a model in which centralized, WASP-dependent protrusions enhance target cell killing through cytolytic mechanopotentiation.

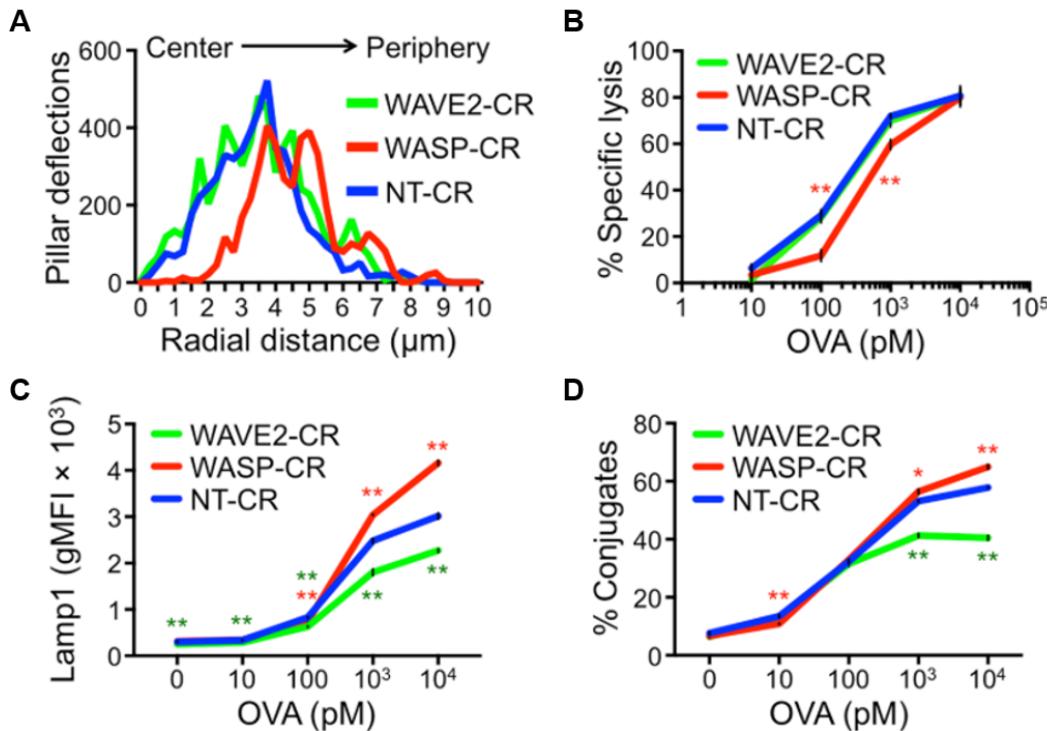


Figure 2.13: WASP and WAVE2 depletion induce distinct cytotoxic phenotypes.

(A): Histogram showing the distribution of strong deflections as a function of radial distance from the center of the IS. $n = 10$ for each cell type in Figure 2.12. (A to D): RMA-s target cells were loaded with increasing concentrations of OVA and mixed with NT-CR, WASP-CR, or WAVE2-CR OT1 CTLs. (B): Specific lysis of RMA-s cells. (C): Lytic granule fusion measured by surface exposure of Lamp1. (D): CTL-target cell conjugate formation measured by flow cytometry. All error bars denote SEM. In (A to D), * $P < 0.05$ and ** $P < 0.01$, calculated by two-tailed Student's t test comparing WASP-CR (red) and WAVE2-CR (green) to NT-CR. Mitchell Wang and Fella Tamzalit performed these experiments.

Figure 2.14: WASP and WAVE2 depletion induce distinct functional phenotypes.

(A): Immunoblot analysis of WASP and WAVE2 expression in NT-CR, WASP-CR, and WAVE2-CR CTLs. Actin served as a loading control. Bars denote intensity of WASP and WAVE2 bands. **(B):** Diagram schematizing the radial distance histogram analysis of pillar deflections shown in Figure 2.12. **(C):** Adherent MB49 (left) or B16 (right) cells were loaded with increasing concentrations of OVA and then mixed with NT-CR or WASP-CR OT1 CTLs. Target cell lysis was measured by LDH release. **(D):** RMA-s target cells were loaded with increasing concentrations of OVA and then mixed with NT-CR, WASP-CR, or WAVE2-CR OT1 CTLs. Specific lysis of RMA-s cells is shown. This experiment highlights the reduced cytotoxicity occasionally observed in WAVE2-CR CTLs at high antigen concentrations. **(E):** Adhesion of fluorescently labeled NT-CR, WASP-CR, and WAVE2-CR OT1 CTLs to wells coated with the indicated concentrations of ICAM1 in the absence (top) or presence (bottom) of H2-K^b-OVA (pMHC). **(F):** NT-CR, WASP-CR, and WAVE2-CR OT1 CTLs were loaded with Fura2-AM Ca^{2+} dye and then imaged on stimulatory glass surfaces coated with H2-K^b-OVA and ICAM1. Ca^{2+} signaling was analyzed by quantifying mean normalized Fura2 ratio over time. N ≥ 29 for each cell type. In **(C-F)**, * and ** indicate P < 0.05 and P < 0.01, respectively, calculated by two-tailed Student's T-test comparing WASP-CR (red) and WAVE2-CR (green) to NT-CR. **(G):** NT-CR, WASP-CR, and WAVE2-CR OT1 CTLs were stimulated with beads coated with H2-K^b-OVA and ICAM1 for the indicated times and then lysed. pErk1/2, pAKT, and I κ B levels were assessed by immunoblot using actin as a loading control. All error bars denote SEM. Mitchell Wang, Fella Tamzalit, and Morgan Huse performed these experiments.

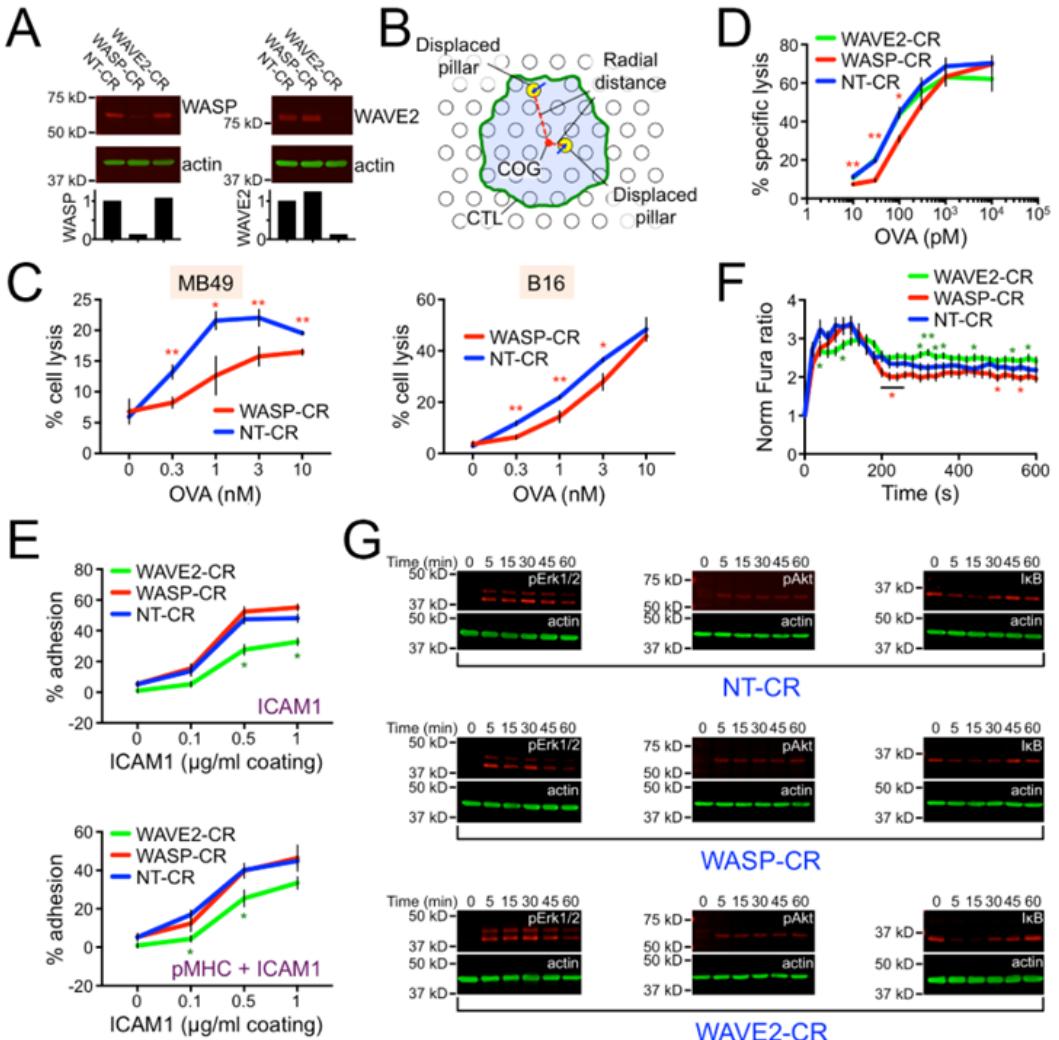


Figure 2.14 (Continued)

2.2.5 WASP controls target cell deformation at the IS

If synaptic protrusions mechanopotentiate perforin function, then they should be capable of physically deforming the target cell surface. To investigate this hypothesis, we performed live imaging experiments using H2-K^b murine endothelial cells as targets for OT1 CTLs [50, 62]. In culture, endothelial cells adopt a flat, stellate architecture that is stable over time. Hence, deviations in this

morphology within the IS can be attributed to the physical activity of the T cell (Figure 2.15). To facilitate imaging of cellular volume, we prepared endothelial cell lines that expressed mApple or infrared fluorescent protein 670 nm (iRFP670) uniformly in both the cytoplasm and the nucleus. These target cells were loaded with OVA, mixed with OT1 CTLs expressing fluorescently labeled Lifeact, and imaged by lattice light-sheet microscopy. Synapses formed readily and could be identified by their stability, as well as the strong accumulation of interfacial F-actin within the CTL. Within minutes of IS initiation, CTLs generated small, protrusive F-actin structures that invaded the space occupied by the target cell (Figure 2.15, yellow arrowheads). This was followed shortly thereafter by rapid displacement of the target surface, which was most obvious in conjugates where the CTL attacked from above (Figure 2.15). This displacement typically occurred before any obvious signs of target cell blebbing, suggesting that it was not part of the apoptotic cascade. CTLs lacking perforin also formed large holes in target cells (Figure 2.16), further supporting the idea that synaptic deformations result from a physical, rather than a chemical, process. TCR engagement was critical for these mechanical effects. In the absence of antigen, both the speed and the magnitude of target cell displacement diminished substantially (Figure 2.16), consistent with previous work [50]. Last, imaging of CTLs expressing Lamp1-GFP revealed that lytic granules accumulated close to areas of deformation (Figure 2.16), implying that physical manipulation of the target cell contributes to perforin- and granzyme-mediated killing.

Figure 2.15: WASP controls target cell deformation at the IS.

(A): Schematic diagram of a CTL deforming an adherent target cell. (B and C) NT-CR, WASP-CR, and WAVE2-CR OT1 CTLs expressing Lifeact-GFP were applied to cultures of OVA-loaded endothelial target cells expressing iRFP670 and imaged using lattice light-sheet microscopy. **(B):** Left: Time-lapse montages of representative “vertically” oriented synapses, with z-projection images (top views) shown above and sagittal views below. Cyan dashed lines denote the slicing planes used for the sagittal images. In z-projection images, target cells are visualized by surface representation. Two z-projections are shown for each time point; Lifeact-GFP is shown on the left and the outline of the CTL of interest is shown on the right. Time in minutes:seconds is indicated in the upper left corner of each sagittal image. Scale bars, 2 μm . Yellow arrowheads denote protrusive structures in the NT-CR CTL that invade the target cell space. Right: Target IS volume graphed against time, with time 0 denoting IS initiation. Each line corresponds to one CTL–target cell conjugate. **(C):** Graph of minimum target IS volume values achieved during the first 400 s of conjugate formation. $n = 7$ for each cell type. Error bars denote SEM. P value was calculated by two-tailed Student’s t test. Mitchell Wang, Fella Tamzalit, and Morgan Huse performed these experiments.

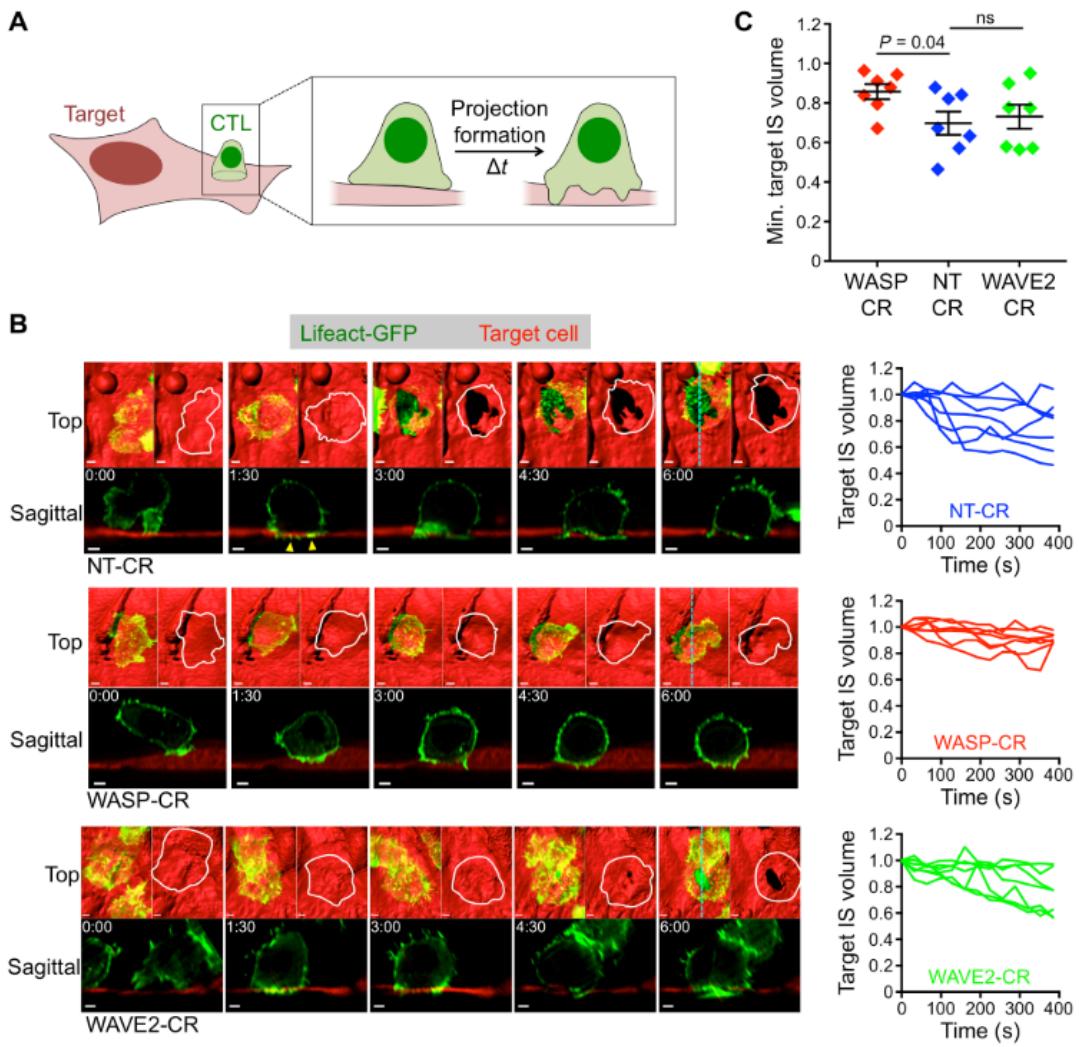


Figure 2.15 (Continued)

Next, we investigated the molecular basis of target cell displacement by comparing synapses formed by NT-CR, WASP-CR, and WAVE2-CR CTLs. Depletion of WASP markedly inhibited physical deformation of the target surface, despite the fact that robust synaptic F-actin accumulation still occurred (Figure 2.15). To quantify this result, we determined the volume beneath the CTL occupied by the target cell at a given time point and normalized this value to the volume occupied by the target cell in that same region before IS formation (Figure 2.17). Analysis of this “target IS volume” parameter confirmed that WASP depletion

significantly reduced the target cell displacement response (Figure 2.15). CTLs lacking WAVE2 exhibited a qualitatively distinct phenotype; although they were still capable of substantial deformation, their mechanical responses were somewhat delayed relative to those of NT-CR controls (Figure 2.15). Collectively, these results mirror the force exertion analysis of WASP-CR and WAVE2-CR CTLs (Figure 2.11, and they suggest that WASP-dependent synaptic protrusions play a particularly important role in the physical deformation of target cells.

Figure 2.16: CTLs physically manipulate the target cell surface.

(A-C): OT1 CTLs were applied to OVA-loaded endothelial target cells expressing mApple (A) or iRFP670 (B and C) and then imaged using lattice light-sheet microscopy. All time-lapse montages show “vertically” oriented synapses, with z-projection images (top views) shown above and sagittal views below. Target cells are visualized by surface representation in all z- projection images. Cyan dashed lines denote the slicing planes used for the sagittal images. Time in M:SS is indicated in the upper left corner of each sagittal image. Scale bars = 2 μ m. **(A)** A representative Prf1^{-/-} CTL, visualized using fluorescent Fab fragments against CD45, engaging a target cell. **(B)**: A representative CTL expressing Lifeact-GFP engaging a target cell in the absence of OVA. In A and B, two z-projections are shown for each time point; the fluorescent signal from the CTL appears on the left and is replaced with an outline of the CTL of interest on the right. **(C)**: A representative CTL expressing Lifeact-mApple and Lamp1-GFP, engaging a target cell beneath it. The montage above includes the Lifeact-mApple signal, whereas the montage below contains an outline of the CTL of interest. Mitchell Wang, Fella Tamzalit, and Morgan Huse performed these experiments.

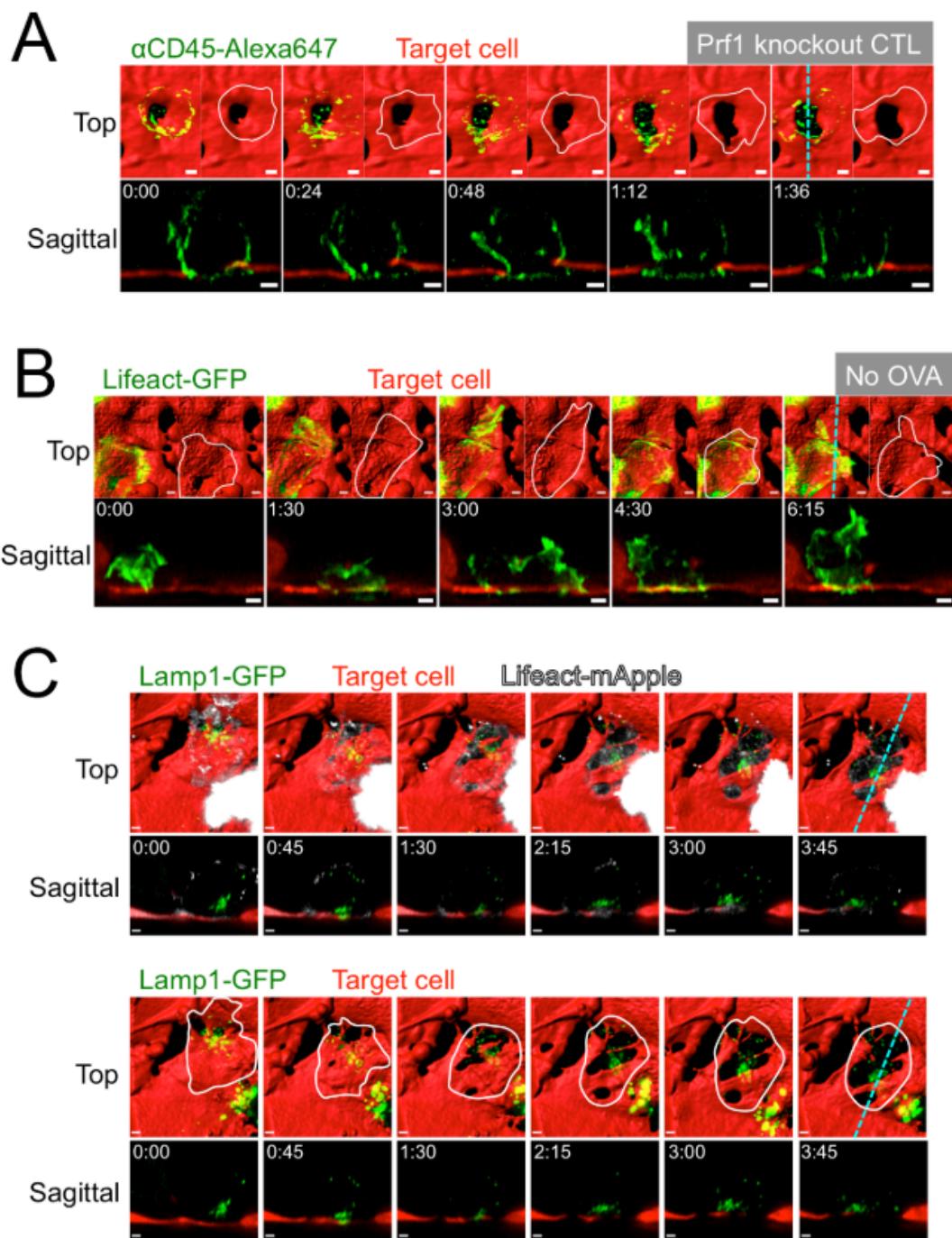


Figure 2.16 (Continued)

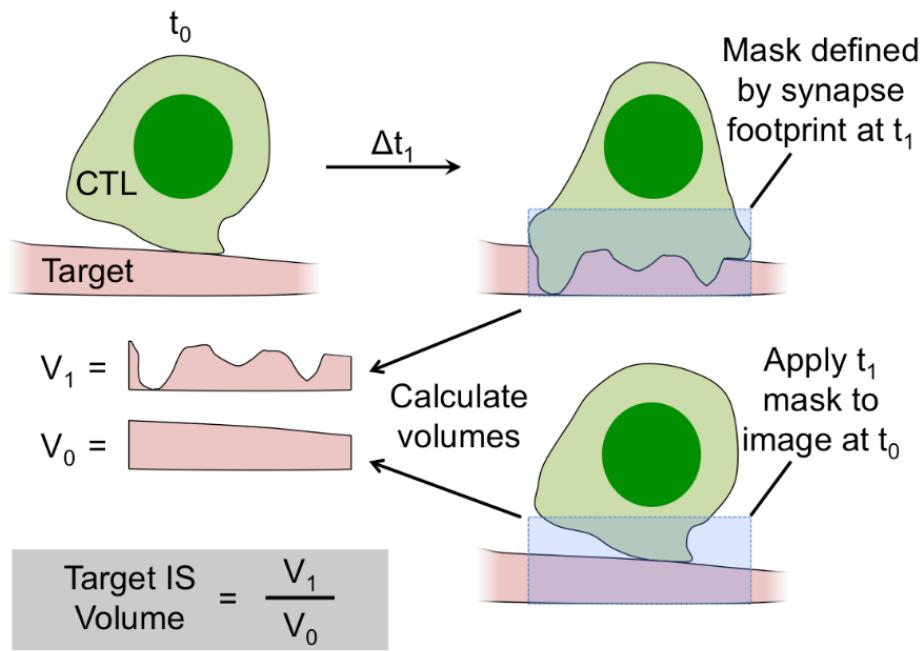


Figure 2.17: Target volume analysis.

Target IS volume compares the endothelial cell volume in the region beneath a CTL at a given time (V_1) with the volume occupied by the endothelial cell in that same region at time 0 (V_0). Analysis designed by Morgan Huse.

2.3 Discussion

The cytotoxic IS boosts perforin toxicity by spatially coordinating its secretion with the exertion of mechanical force. In the present study, we found that perforin release occurs at the base of WASP-dependent, F-actin-rich protrusions (Figure 2.18). These protrusions were necessary for synaptic force exertion, particularly in more central regions of the IS close to lytic granules. They were also required for physical deformation of target cells in bona fide cytolytic interactions. WASP-deficient CTLs exhibited a defect in killing that could not be explained by reduced granule release or conjugate formation. Together, these data identify synaptic protrusions as key components of a physical delivery system that enables CTLs

to kill target cells with high efficiency. In putting forth this model, we do not suggest that synaptic protrusions are a prerequisite for lytic granule release. Indeed, multiple groups have demonstrated that rigid stimulatory surfaces induce robust cytolytic secretion in the absence of protrusive activity [56, 32, 69, 70, 71]. However, the converse relationship may be worth considering, namely, that lytic granule docking and fusion might influence local F-actin architecture and IS mechanics.

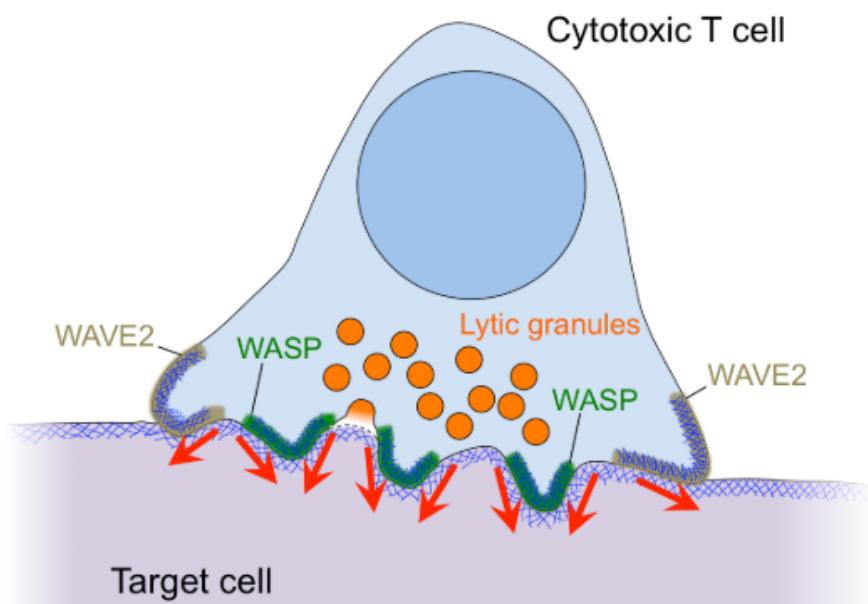


Figure 2.18: Cytolytic mechanopotentiation by WASP-dependent synaptic protrusions. Diagram of the cytolytic IS showing peripheral WAVE2-dependent protrusions and central WASP-dependent protrusions. Red arrows denote force exertion.

The marked concentration of granule fusion events at the base of synaptic protrusions implies that mechanisms exist for granule targeting to these domains. Previous studies have highlighted the importance of F-actin clearance for enabling granule access to the plasma membrane [9, 11, 32, 71]. This is consistent with our observation that, on micropillar arrays, granule fusion occurs

in transient F-actin–free regions at the base of synaptic protrusions. However, the presence of other F-actin hypodense areas in the CTL, which are not targeted by granules, implies that other factors contribute to the process. Lipid second messengers are known to influence exocytosis and membrane trafficking in a variety of cellular contexts (41). Among these, diacylglycerol is an interesting candidate because it tends to accumulate in central synaptic domains that experience F-actin depletion [72, 73, 74]. Granule delivery via microtubules is another possibility [75, 76]. We observed that a small subset of microtubules extends into synaptic protrusions, and it will be interesting to explore whether and how this subset contributes to granule trafficking from the centrosome toward fusion sites in the synaptic membrane. Last, it is possible that WASP itself plays a role. A recent super-resolution imaging study demonstrated that WASP promotes granule docking close to regions of integrin clustering [77]. T cells form a variety of protrusive structures at both interfacial and noninterfacial surfaces, which have been documented previously by electron microscopy and high-resolution fluorescence imaging [50, 51, 65, 78, 79]. Although the spatial distribution of these protrusions and their dynamics have implied roles in antigen scanning, signaling, and motility, their precise functions have, in most cases, remained undefined. Probably the best-studied are the invadosome-like protrusions (ILPs), which were first observed in T cells initiating diapedesis through endothelial monolayers [65]. Subsequently, ILPs were also found in antigen-induced synapses formed between T cells and endothelial cells, dendritic cells, or B cells [50]. ILPs are podosomal structures that are enriched in LFA1 and require WASP and Arp2/3 for their formation [62, 65]. The synaptic protrusions we have observed share these characteristics, and it is therefore tempting to speculate that they are a form of ILP. That CTLs might use the same structures to facilitate both diapedesis and

target cell killing highlights an underappreciated similarity between the two processes. Both rely on the physical deformation of other cells through direct contact, in the first case to facilitate transmigration and in the second to promote destruction.

The functional defects observed in T cells from patients with WAS and Wasp^{-/-} mice have been attributed to specific effects of WASP on TCR signaling [62, 64, 80, 81, 82]. WASP is expressed by all lymphoid and myeloid lineages [66, 67], however, and consequently, the phenotypes exhibited by any one immune subset in a WASP-deficient background result not only from the cell-intrinsic functions of the protein but also from the dysfunction of other cell types. Interpreting studies from patient samples is particularly complex because of the wide range of pathological WASP mutations, which vary in their penetrance and can therefore yield markedly distinct disease phenotypes [67]. In the present study, we circumvented this complexity by selectively deleting Wasp in CTLs using CRISPR-Cas9 targeting. The resulting cytotoxicity defect was not associated with reduced TCR signaling, Ca^{2+} flux, or granule release, but rather was caused by a failure to generate interfacial protrusions during the effector phase of the response. These data raise the possibility that WASP-dependent protrusions may contribute to interfacial effector pathways in other immune subsets. Previous studies have implicated WASP in macrophage phagocytosis [83, 84], T cell priming by dendritic cells [85, 86], and the induction of B cell class switching by follicular T helper cells [87]. It will be interesting to investigate how the protrusive activity of WASP influences these and other cell-cell interactions and, in turn, how defects in these interactions contribute to the complex etiology of WAS. In marked contrast to WASP, WAVE2 accumulated in peripheral synaptic protrusions, and CTLs lacking WAVE2 exhibited adhesion and conjugation

defects. These observations suggest a role in cell spreading and adhesion that is consistent with previous reports [59, 60, 61]. WAVE2 depletion only weakly affected synaptic force exertion and killing, indicating that the protein and the peripheral structures it generates are not involved in cytolytic mechanopotentiation. These results do not exclude the possibility that WAVE2 might promote cytotoxicity in other settings, particularly when target cells are more limiting and robust migration and adhesion are required for their destruction. What is clear from our data, however, is that the functionality of synaptic protrusions is partitioned both spatially (center versus periphery) and molecularly (WASP versus WAVE2). In vitro systems that collapse the IS into two dimensions have been invaluable for investigating its structure and function [88, 89]. The inherent constraints of these systems, however, have limited our understanding in significant ways. It was only by analyzing the IS in an oriented, three-dimensional environment that we were able to assess the formation of synaptic protrusions and to study the implications of these structures for IS mechanics and effector responses. Micropatterned reconstitution systems can reveal unexplored aspects of cellular architecture, and we anticipate that they will become increasingly important in future studies of complex immune cell biology.

2.4 Materials and Methods

2.4.1 Study design

The goal of this study was to understand how CTLs combine cytolytic secretion with force exertion at the IS. We used fluorescence imaging of mouse

CTLs, single-cell biophysical assays, and functional experiments. Micropatterned PDMS substrates were used both to visualize the growth of synaptic protrusions and to measure mechanical activity. To perturb protrusion formation and synaptic F-actin dynamics, we used CRISPR-Cas9 technology, shRNA, and the Arp2/3 inhibitor CK666. Experimental sample sizes were not pre-determined, and there were no predefined study end points. Experiments were not randomized, and investigators were not blinded during acquisition and data analysis. In general, experiments were performed at least twice (two biological replicates). Specific information about analysis methods can be found in the “Image analysis” section below.

2.4.2 Micropillar preparation

PDMS (Sylgard 184; Dow Corning) micropillar arrays were prepared as previously described [34]. Two types of pillars were used for this study: (i) 1 μm in diameter, 5 μm in height, and spaced hexagonally with a 2 μm center-to-center distance; and (ii) 0.7 μm in diameter, 6 μm in height, and spaced hexagonally with a 2- μm center-to-center distance. Micropillars were cast on chambered coverglass (Lab-Tek), washed with ethanol and phosphate-buffered saline (PBS), and stained with fluorescently labeled streptavidin (20 $\mu\text{g}/\text{ml}$) (Alexa Fluor 647 or Alexa Fluor 568, Thermo Fisher Scientific) for 2 hours at room temperature. After additional washing in PBS, the arrays were incubated with biotinylated H2-K^b-OVA and ICAM1 (10 $\mu\text{g}/\text{ml}$ each) overnight at 4°C (8). The pillars were then washed into RPMI containing 5% (v/v) fetal calf serum (FCS) and lacking phenol red for imaging.

2.4.3 Live imaging on micropillars

For force measurements, T cells were stained with Alexa Fluor 488–labeled anti-CD45.2 Fab (clone 104-2) and imaged on fluorescently labeled 0.7- μm -diameter pillars. Videomicroscopy was performed using an inverted fluorescence microscope (Olympus IX-81) fitted with a 100x objective lens and a mercury lamp for excitation. Images in the 488-nm (CTLs) and 568-nm (pillars) channels were collected every 15 s using MetaMorph software. Protrusion formation was imaged on 1- μm -diameter pillars stained with Alexa Fluor 647-labeled or Alexa Fluor 568-labeled streptavidin. Cells expressing fluorescent probes were added to the arrays and imaged using a confocal laser scanning microscope fitted with a 40x objective lens and 488-nm, 560-nm, and 642-nm lasers (Leica SP5 or Zeiss LSM 880).

2.4.4 Lattice light-sheet imaging

Lattice light-sheet microscopy was performed as previously described using 488-nm, 560-nm, and 642-nm lasers for illumination and a 25x water immersion objective [90]. Micropillar arrays were cast on 5-mm-diameter coverslips, which were coated with stimulatory proteins as described above and then mounted for imaging. Movies (3- to 20-s time-lapse intervals) were recorded immediately after addition of fluorescently labeled CTLs using two cameras. We collected 488-nm (30–60 mW laser power) and 560-nm (50 mW laser power) images on one camera and 642-nm (50 mW laser power) images on a second camera. For CTL-target cell imaging, CTLs were added to coverslips bearing 90% confluent monolayers of mApple- or iRFP670-labeled endothelial cells that had been in-

cubated overnight in 2- μ M OVA. Movies (15- to 20-s time-lapse intervals) were recorded immediately after addition of CTLs. We collected 488-nm (50 mW laser power), 560-nm (200 mW laser power), and 642-nm (200 mW laser power) images on one camera. Raw data were deskewed and deconvolved as previously described [90] using experimentally derived point spread functions. For two camera experiments, image alignment was performed in MATLAB using reference images of fluorescent beads.

2.4.5 Image analysis

Imaging data were analyzed using SlideBook (3i), Imaris (Bitplane), Excel (Microsoft), Prism (GraphPad), and MATLAB (MathWorks). Ca^{2+} responses were quantified by first normalizing the ratiometric Fura2 response of each cell to the last time point before the initial influx of Ca^{2+} and then by aligning and averaging all responses in the dataset based on this initial time point. To quantify F-actin intensity in fixed images (Figure 2.8, we determined the sum intensity of Alexa Fluor 546-labeled phalloidin in the region beneath the pillar tops for each cell after intensity thresholding. For protrusion enrichment, total Lifeact-GFP intensity in the region beneath the pillar tops was divided by the total Lifeact-GFP intensity in a region of identical volume beginning from the first z-section above the pillar tops and extending upward. Force exertion against 0.7 μ m-diameter pillars was calculated after extracting pillar displacements from the imaging data and then converting these displacements into force vectors using custom MATLAB scripts (17, 18). Radial distributions of pillar deflections (Figure 2.13) were generated by calculating the distances between strongly deflected pillars ($\geq 0.6 \mu$ m deflection) and the IS COG at each time point (Figure 2.14).

COG coordinates for the IS were generated from masks derived from the Alexa Fluor 488 (CTL) channel. To calculate the centralization factor (Figure 2.6 and Figure 2.10), we first generated a mask encompassing the entire IS (MC) and a mask containing only the features of interest (e.g., lytic granules) (MF) using xy-projection images. Then, the average distance between every pixel in MC and the COG of MC (DC) was determined and subsequently divided by the average distance between every pixel in MF and the COG of MC (DF) (Figure 2.7). Granule polarization (Figure 2.7) was quantified by determining the vertical distance between the centroid of a mask encompassing the lytic granules and the deepest CTL protrusion, using xz- or yz-projection images. To calculate target IS volume (Figure 2.15), we generated a three-dimensional mask at a time point of interest by tracing the edges of the IS and then propagating the resulting shape downward to encompass the sample lying directly beneath the CTL. The volume of this region occupied by the target cell was then divided by the volume of this same region occupied by the target cell at time 0 (Figure 2.17).

2.4.6 Killing assay, granule fusion assays, and conjugate formation

RMA-s target cells were labeled with carboxyfluorescein succinimidyl ester (CFSE) or the membrane dye PKH26, loaded with OVA and mixed in a 96-well V-bottomed plate with CellTrace Violet (CTV)-stained OT1 CTLs. To assess killing, we mixed cells at a 1:3 effector: target (E:T) ratio and incubated them for 4 hours at 37 °C. Specific lysis of CFSE+ target cells was determined by flow cytometry [91].

For granule fusion assays, the E:T ratio was 1:1, and cells were incubated at 37°C for 90 min in the presence of eFluor 660-labeled anti-Lamp1 (clone eBio1D4B; eBioscience). Lamp1 staining was then assessed by flow cytometry. To measure conjugate formation, we mixed CTLs and targets 1:1, lightly centrifuged (100g) the mixture to encourage cell contact, and incubated the mixture for 20 min at 37°C. Cells were then resuspended in the presence of 2% paraformaldehyde, washed in fluorescence- activated cell sorting buffer (PBS + 4% FCS), and analyzed by flow cytometry. Conjugate formation was quantified as (CFSE+ CTV+)/(CTV+). For killing of adherent target cells, MB49 or B16 cells were cultured overnight on fibronectin and then pulsed with OVA for 2 hours. OT1 CTLs were added at a 4:1 E:T ratio and incubated for 3 hours (MB49 cells) or 4 hours (B16) at 37°C in RPMI medium supplemented with interleukin-2 (30 IU/ml). Target cell death was quantified with an LDH (lactate dehydrogenase) cytotoxicity assay kit (Clontech) using the manufacturer's recommended protocol. All functional assays were performed in triplicate.

2.4.7 Statistical analysis

Figures show representative experiments. Analysis was carried out using either representative experiments or pooled data as indicated (n refers to the number of cells analyzed). Statistical analyses (unpaired or paired t tests) were carried out using GraphPad Prism or Microsoft Excel. All error bars denote SEM.

2.4.8 Constructs

Retroviral expression constructs for Lifeact-GFP, Lifeact-mRuby2, DynPH-GFP, and pHluorin-Lamp1 were previously described [13, 32]. The Lamp1-GFP, WASP- GFP, and WAVE2-GFP constructs were prepared by ligating the full-length coding sequences of mouse Lamp1, mouse WASP, and human WAVE2 into a pMSCV retroviral expression vector upstream of GFP [91]. The Lifeact-mApple fusion was prepared by PCR from an mApple-N1 template plasmid using oligos that encoded the Lifeact peptide N-terminal to mApple, followed by subcloning into pMSCV. shRNA constructs (shNT and shPTEN) [13] were subcloned into the miR30E vector using the following primers: miRE-Xho-fw (5'-TGAACTCGAGAAGGTATAT TGCTGTTGACAGTGAGCG-3') and miRE-EcoOligo-rev (5'-TCTCGAATTCT AGCCCCCTGAAGTCCGAGGCAGTAGGC -3'). gRNAs targeting WASP and WAVE2 were generated as previously described using the following oligos: NT gRNA: oligo-1 (5'- CACCGGGATACTGGGCC-GACTTTC -3') and oligo-2 (5'- AAACGAAAGTCGGCCCAGGTATCCC -3'). WASP gRNA: oligo-1 (5'- CACCGCTGGACCATGGAACACTGCG -3') and oligo-2 (5'- AAACCGCAGTGTCCATGGTCCAGC -3'). WAVE2 gRNA: oligo-1 (5'-CACCGAGCAAGGGAGTTACTCGGG-3') and oligo-2 (AAACCCCGAG-TAAACTCCCTGCTC -3'). Each gRNA was cloned into the LentiGuide-Puro vector and then amplified by PCR using the following primers: LMP BamHI F2 (5'- TTTTGATCCTAGTAGGAGGCTTGGTAG -3') and LMP EcoRI R2 (5'- TTTTGAATTCTGTCTACTATTCTTCCC -3'). The resulting fragments were digested with BamHI and EcoRI and ligated into miR30E previously digested with EcoRI and BglIII.

2.4.9 Cells and small molecule inhibitors

The animal protocols used for this study were approved by the Institutional Animal Care and Use Committee of Memorial Sloan-Kettering Cancer Center. Primary CTL blasts were prepared by pulsing irradiated C57BL/6 splenocytes with 100 nM OVA and then mixing them with T cells from OT1 $\alpha\beta$ TCR transgenic mice in RPMI medium containing 10%(vol/vol) FCS. Cells were supplemented with 30 IU/ml IL2 after 24 h and were split as needed in RPMI medium containing 10% (vol/vol) FCS and IL2. RMA-s cells were maintained in RPMI containing 10% (vol/vol) FCS, while C57BL/6 murine cardiac microvascular endothelial cells (CellBiologics), MB49 cells, and B16 cells were maintained in DMEM medium containing 10% (vol/vol) FCS. To inhibit Arp2/3, CTLs were preincubated with CK666 (100-150 μ M, Sigma-Aldrich) for 10 min at 37°C, and CK666 was then maintained at the same concentration for the duration of the experiment.

2.4.10 Retroviral transduction

Phoenix E cells were transfected with expression vectors and packaging plasmids using the calcium phosphate method. Ecotropic viral supernatants were collected after 48 h at 37°C and added to 1.5×10^6 OT1 blasts 2 days after primary peptide stimulation. Mixtures were centrifuged at $1400 \times g$ in the presence of polybrene (4 μ g/ml) at 35°C, after which the cells were split 1:3 in RPMI medium containing 10% (vol/vol) FCS and 30 IU/ml IL2 and allowed to grow for an additional 4-6 days.

2.4.11 Fixed imaging

OT1 cells were incubated on stimulatory micropillars for 20 min at 37°C, fixed by adding 4% paraformaldehyde for 5 min, and washed with PBS. Samples were then blocked in PBS solution supplemented with 2% goat serum for 1 h at room temperature and stained overnight at 4°C with anti- β -tubulin (clone TUB 2.1; Sigma) or anti-LFA1 (clone M17/4; eBioscience). Actin was visualized using Alexa 546-labeled phalloidin (ThermoFisher Scientific). After washing, samples were incubated with the appropriate secondary antibody for 2 h at room temperature, washed and imaged using a Leica SP8 confocal laser scanning microscope fitted with a white light laser and a 40x objective lens.

2.4.12 Ca^{2+} imaging

CTLs were loaded with 5 μ g/ml Fura2-AM (ThermoFisher Scientific), washed, and then imaged on stimulatory glass surfaces coated with H2-K^b-OVA and ICAM-1 as previously described. Images were acquired using 340 nm and 380 nm excitation every 30 seconds for 30 min with a 20x objective lens (Olympus).

2.4.13 Adhesion assay

Integrin-mediated adhesion was measured in flat-bottomed 96-well plates bearing increasing densities of ICAM1. The plates were coated with 10 μ g/ml streptavidin in PBS followed by increasing concentrations of biotinylated ICAM1 in the presence or absence of 1 μ g/ml biotinylated H2-K^b-OVA in Hepes buffered saline (10 mM Hepes pH 7.5, 150 mM NaCl) with 2 % BSA. The total concentration

of biotinylated protein during coating was kept at 5 μ g/ml by the addition of nonspecific biotinylated MHC (H2- D^b). OT1 CTLs were fluorescently labeled with cell trace violet (CTV), resuspended in adhesion buffer (PBS with 0.5% BSA, 2 mM $MgCl_2$, 1 mM $CaCl_2$), and added to ICAM1-bearing wells in triplicate. After a 20 min incubation at 37°C, wells were washed with warmed adhesion buffer as described, and the bound cells quantified by fluorimetry.

2.4.14 Immunoblot

0.2-1 \times 10⁶ CTLs were lysed using cold cell lysis buffer containing 50 mM TrisHCl, 0.15 M NaCl, 1 mM EDTA, 1% NP-40 and 0.25% sodium deoxycholate. Suppression of PTEN, WASP, and WAVE2 was confirmed using the following antibodies: anti-PTEN monoclonal antibody (clone D4.3; Cell Signaling Technology), anti-WASP monoclonal antibody (clone B-9; Santa Cruz) and anti-WAVE2 monoclonal antibody (clone D2C8; Cell Signaling Technology). Actin served as a loading control (clone AC-15, Sigma). For signaling assays, serum and IL2 starved OT1 CTLs were incubated with streptavidin polystyrene beads (Spherotech) coated with H2- K^b -OVA and ICAM1 at a 1:1 ratio for various times at 37°C and immediately lysed in 2 \times cold lysis buffer containing phosphatase inhibitors (1 mM NaF and 0.1 mM Na3VO4) and protease inhibitors (cOmplete mini cocktail, EDTA-free, Roche). Activation of PI3K, MAP kinase and NF- κ B signaling was assessed by immunoblot for pAkt (Phospho-Akt (Ser473) Ab; Cell Signaling Technology), pErk1/2 (Phospho-Thr202 / Tyr204; clone D13.14.4E; Cell Signaling Technology), and I κ B (Cell Signaling Technology).

CHAPTER 3

MECHANICALLY ACTIVE INTEGRINS DIRECT CYTOTOXIC SECRETION AT THE IMMUNE SYNAPSE

Results presented in this chapter have been submitted as: Wang, M. S., Hu, Y., Sanchez, E. E., Xie, X., Roy, N. H., de Jesus, M., Jin, W., Lee, J. H., Hong, Y., Kam, L. C., Salaita, K., Huse M., as *Mechanically active integrins direct cytotoxic secretion at the immune synapse*.

3.1 Background

The secretory output of cell-cell interfaces must be tightly controlled in space and time to ensure functional efficacy. This is particularly true for the cytotoxic immune synapse (IS), the stereotyped junction formed between a cytotoxic lymphocyte and the infected or transformed target cell it aims to destroy [24]. Cytotoxic lymphocytes kill their targets by channeling a mixture of granzyme proteases and the pore forming protein perforin directly into the IS [25, 92]. The synaptic secretion of these toxic molecules constrains their deleterious effects to the target cell alone, thereby protecting innocent bystander cells in the surrounding tissue from collateral damage. Despite the importance of this process for immune specificity, the molecular and cellular mechanisms that establish secretory sites within the IS remain poorly understood. Here, we identified an essential role for integrin mechanotransduction in cytotoxic secretion using a combination of single cell biophysical measurements, ligand micropatterning, and functional assays. Upon ligand-binding, the α L β 2 integrin LFA-1 functioned as a spatial cue, attracting lytic granules containing perforin and granzyme and

inducing their fusion at closely adjacent sites within the synaptic membrane. LFA-1 molecules were subjected to pulling forces within these secretory domains, and genetic or pharmacological suppression of these forces abrogated cytotoxicity. We conclude that lymphocytes employ an integrin-dependent mechanical checkpoint to enhance both the potency and the security of their cytotoxic output.

3.1.1 Canonical centrosome-based model of degranulation

Given that degranulation is the lynchpin to effector T cell function, there has been much research into the precise mechanism of degranulation and how lytic granules arrive and fuse at the immune synapse for optimized killing. Effective target killing depends on the exclusive release of granule contents solely at the IS. This creates a local hyperconcentration of perforin and granzymes delivered to the target and minimizes collateral damage against bystander cells in the surrounding environment (see section 1.5 for further details).

Degranulation has long been thought to depend on the centrosome, a membraneless organelle that serves as the microtubule-organizing center (MTOC) [93, 94]. Microtubules extend outwards from the centrosome with their minus ends directed inward and their plus ends outward and direct f-actin polarity in lymphocytes [95]. A critical stage of proper IS formation is the movement of the centrosome to a position directly adjacent to the interface, driven by the generation of a local focus of diacylglycerol (DAG), recruited by the motor protein dynein [8]. The formation of this diacylglycerol gradient is required for T cell cytotoxicity [8]. Lytic granules tend to cluster around the centrosome in lymphocytes, so the reorientation of the centrosome to the IS positions the granules close

to the synaptic membrane [25]. The temporal synchronization between granule collection, centrosome polarization, and destruction of the target cells seemingly solidifies the centrosome's directorial role in delivering the granules to the IS for fusion, thereby heightening the potency and specificity of the response [25].

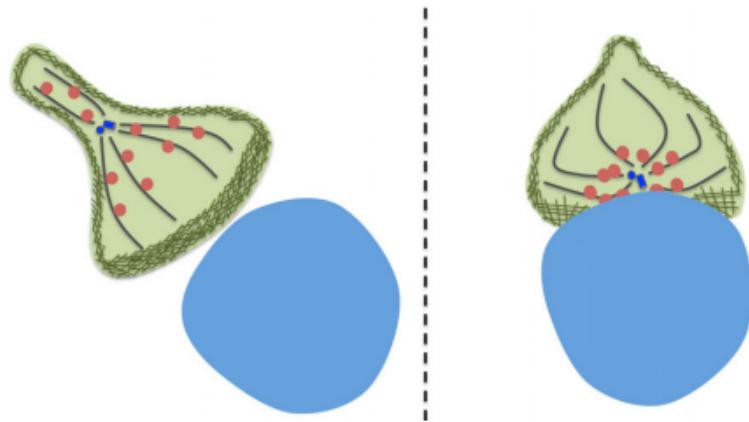


Figure 3.1: Canonical centrosome-based model of degranulation.

Canonically, upon recognition of the target cell (light blue), the centrosome (deep blue) within the T cell (green) polarizes towards the synapse, bringing lytic granules that cluster around the centrosome. The close proximity of the centrosome with the plasma membrane brings lytic granules next to the membrane at the synapse. Because actin cortical density (green) is low at the synapse, granules that come in close contact with the plasma membrane are able to fuse, releasing their contents toward the target cell for final target cell lysis. This figure is adapted from [96].

However, some studies have cast doubt on this centrosome-based model of degranulation, particularly in its role in directional secretion. Some studies have associated impaired centrosome reorientation and function with reduced cytotoxicity [8, 97, 98, 99] with one study going so far as even to delete the centrioles (cylindrical structures composed of microtubules that maintain centrosomal organization) [100]. Deletion of the centrioles resulted in T cells that degranulated less, but still degranulated directionally (i.e. through the immune synapse) [100], implying implies that non-centrosomal mechanisms of synaptic granule fusion exist. Further study of lytic granule polarization and synaptic homing

mechanisms will address how the granules directly arrive at the synapse for fusion.

3.1.2 Force-directed localization of synaptic granule fusion

Lytic granules are homed towards regions of synaptic force exertion, implying that T cells "aim" their lytic granules (see Figure 3.2) [36]. Such an arrangement brings the benefits of locally mechanopotentiating perforin pore formation and increases target cell lysis. This also conveniently piggybacks off of the effects of local increased TCR signaling activity as described in section 1.3 (although this relationship has yet to be further explored). What remains to be seen is how a physical, non-tangible phenomena (force) is transformed into a molecular query:response such as lytic granule fusion. Most likely, a mechanosensitive receptor that is associated with cytotoxicity with significant signaling capacity regulates this spatiotemporal relationship.

Such an arrangement could produce numerous benefits. An outstanding question in the T cell field is how T cells avoid the cytotoxic effects of their own perforin and achieve unidirectional killing [101]. Some studies search for particular shielding molecules expressed only on the T cell side of the synapse [102] or a broader T cell shielding method [103] from perforin. In lieu of a particular molecule, we would like to suggest that T cells use a particular step-wise logic in order to confirm that the queried target that they are engaged with is a *bona fide* target cell via physical force engagement (quite literally, hugging or embracing their target before applying *le baiser de la mort*, or the kiss of death). Once a safe contact against a *bona fide* target cell has been established, the T

cell can then secrete perforin into a hyperlocal, perforin-sensitive synaptic cleft environment for target cell destruction. Physical force is critical for mediating this hypothetical function.

In this study, we explored the integrin LFA-1 as a possible candidate molecule for synaptic force transduction to home lytic granules, given its capacity to mechanically signal (see section 3.1.3 for further information on integrins).

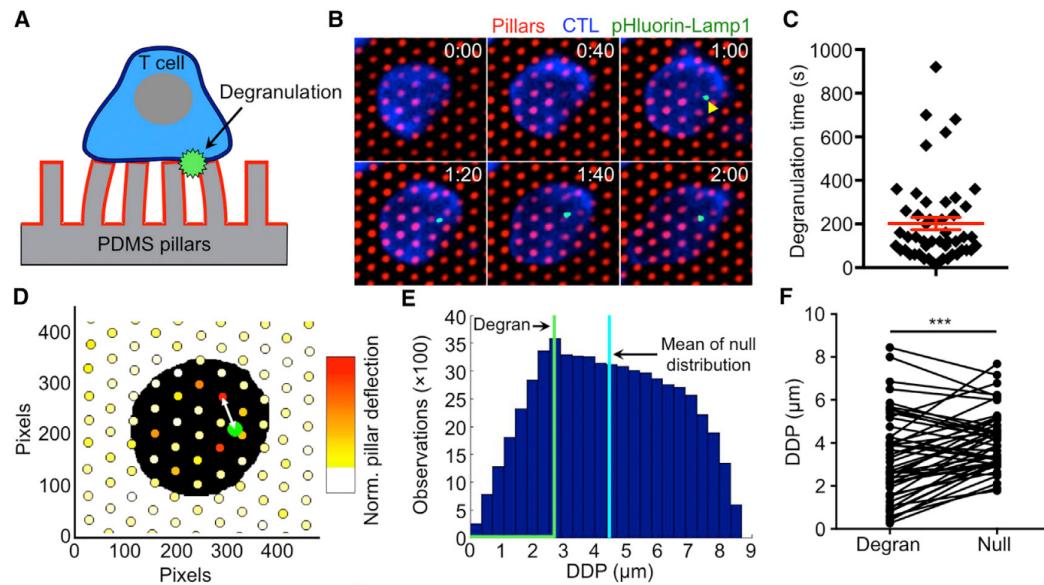


Figure 3.2: Degranulation is spatiotemporally correlated with force exertion at the IS.

(A): Diagram schematizing fluorescent detection of degranulation during a micropillar experiment. (B): Representative time-lapse montage showing pillar deflections during a degranulation event (indicated by the yellow arrowhead). Time is indicated in M:SS in the top right corner of each image. (C): Graph of the offset time between contact formation and degranulation (degranulation time). (D): Graphical representation of the pillar array in (B), with the degranulation position depicted as a green circle and the cell envelope at the moment of degranulation shown in black. Pillars are color-coded based on their average deflection during the degranulation. Warmer colors (e.g., orange, red) denote stronger deflections. The DDP for this degranulation is indicated by the double-headed white arrow. (E): Histogram plot derived from the experiment in (B) showing the DDP for each position in the CTL interface. The mean value for the distribution is denoted by the vertical cyan line. The vertical green line indicates the DDP for the degranulation itself. (F): DDPs of degranulation (Degran) were compared to the mean values of their paired null distributions. ***p < 0.001, calculated by two-tailed paired t test. This figure is adapted from [36].

3.1.3 Integrin/LFA-1 biology and mechanotransduction

Integrins are transmembrane obligate heterodimers that facilitate cell-cell adhesion. They are composed of an α chain and a β chain. Upon binding to their ligand, they greatly increase the adhesive capacity of the cell, and strongly upregulate signal transduction pathways as well as rearrange the actin cytoskeleton to increase force transduction across the synapse. Interestingly, integrins reach higher signaling capacities with the more force that is applied across their bond with their ligand, displaying a catch bond behavior [104] and more generally a functionally positive relationship with force application (as opposed to a slip bond). Integrins also fine-tune TCR signaling by restricting the flow of actin at the immune synapse [105], highlighting their signaling capacity as referenced in section 3.1.2. Here, we will specifically review the exact behavior of the integrin LFA-1, but the general principles of LFA-1 activation and binding to its ligand ICAM-1 apply broadly to integrin biology.

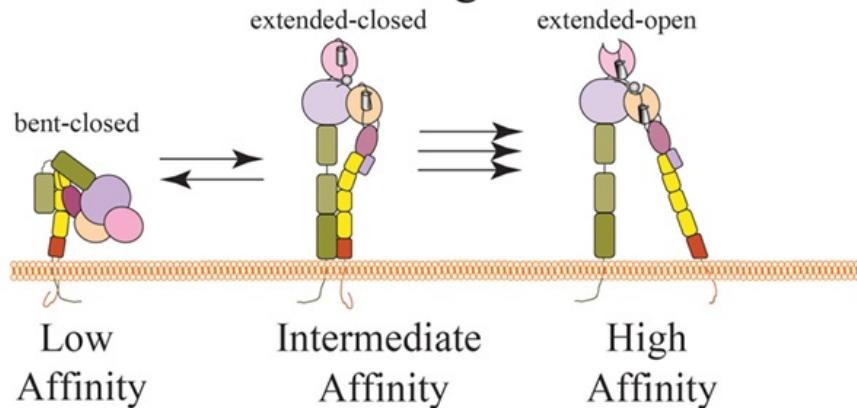
LFA-1 exists in an inactive, bent state of the surface of the T cell ("Low affinity", see Figure 3.3) [106]. Signaling events downstream of the TCR, trigger RAP1-GTPase recruits factors including RAPL to interact with the α subunit of LFA-1 to induce integrin activation (this is called inside-out signaling) [107]. Simultaneously, talin cleavage permits the FERM domain to interact with the NPxY motif of the β subunit cytosolic tail. This interaction induces a separation of the salt bridge inducing cytosolic tail separation ("Intermediate affinity"). Molecules such as RIAM, talin, paxillin, and vinculin then with the cytosolic tails to recruit additional actin binding molecules and bind to the ICAM-1 ligand, forming a molecular clutch against the target cell ("High affinity") [106]. This linkage promotes a mechanical scaffold to interact with actin and reinforce LFA-1

activity. The formation of this clutch affects: the effector response, including actin dynamics, migration [108], and outside in signaling. Arp2/3 will promote continued actin filament growth, and the interaction of LFA-1 with ICAM-1 and β -actin allows for force driven responses through the β subunit, promoting focal adhesion kinases (FAK) and other Rho family GTPases (Figure 3.3).

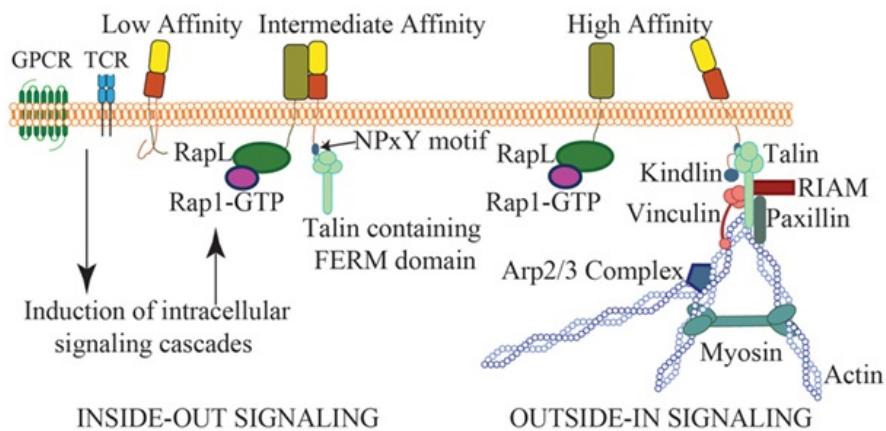
Figure 3.3: Multidimensional regulation of LFA-1 affinity and signaling.

(i): LFA-1 affinity for its ligand ICAM-1 changes according to LFA-1 conformation. In the low affinity state, the bent conformation obscures the ligand binding α L domain, preventing the α L domain from interacting with ICAM-1. In the intermediate affinity state, the extracellular leg domains are straightened upright (via Rap1-GTPase and the talin head domain), allowing for low affinity LFA-1/ICAM-1 interactions. In the final mature state of LFA-1, complete disruption of the salt bridge between the α L (CD11a) and β 2 (CD18) cytosolic tails results in conformational shift along the two domains, resulting in a high affinity LFA-1 molecule via the full exposure of the ligand-binding site. **(ii):** Upon TCR activation, the RAP1-GTPase recruits a number of factors to the α L domain(shown above) to induce integrin activation. The cleavage of talin and resulting interaction between the talin FERM domain and and the β 2 domain-located NPxY motif causes a dissociation of the inhibitory salt bridge, inducing cytosolic tail separation and further recruiting actin scaffolding proteins (such as paxillin, vinculin, and RIAM), resulting a LFA-1 molecule primed for mechanotransduction. **(iii):** The interaction of LFA-1 with ICAM-1 and β -actin allows for signal mechanotransduction along the β subunit. Tension (arrows) along the β 2-subunit has been measured at around the pN scale. Actin flow directs the facial orientation and location of LFA-1 at the immunological synapse. Stabilization of the integrin in its high affinity conformation via force generation requires adhesion to both the cytoskeleton and ICAM-1. The stiffness of the opposing surface will alter the overall capacity of force generation and associated outside-in signaling via LFA-1. Induction of Rho family GTPases via force exertion regulates cell proliferation and survival. This figure is adapted from [107].

i. Conformation Change



ii. Intracellular Effectors



iii. Mechanosensor

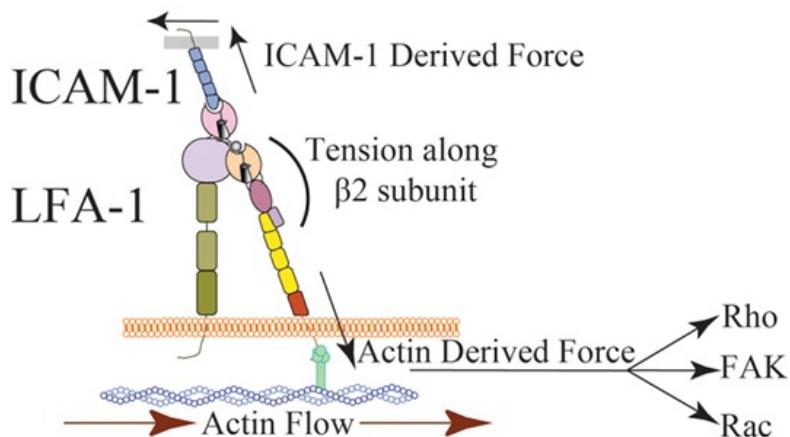


Figure 3.3 (Continued)

LFA-1 was a particular integrin of interest to study given its importance in CTL cytotoxicity and immune synapse formation, detailed previously in section 1.4 and more extensively in section 3.1.4. LFA-1 function is essential for the activation and proliferation of naïve T cells [109, 110]. Additionally, given that Rab27a was found to be a critical regulator of intracellular LFA-1 translocation to the cell surface [110] and that the lytic granules are transported to the immune synapse via a Rab27a-dependent mechanism [111], it seemed reasonable to investigate LFA-1 as a possible mechanotransducing molecule that could locally signal permissible regions for degranulation.

3.1.4 The actin cytoskeleton directs integrin localization

The actin cytoskeleton is intimately connected to integrin function, as well as integrin localization. (see section 3.1.3) [77]. The actin nucleation promoting factor WASP was found to constrain the high-affinity form of LFA-1 to local interstices in the actin network (see section 1.4) [77]. The activity of WASP promotes the formation of hundreds of LFA-1 nanoclusters in a radial belt (not unlike the canonical pSMAC description) for lytic granule docking. Defects in T cells lacking WASP (as measured from Wiskott-Aldrich syndrome patient-derived *CD8⁺* T cells) were associated with reduced levels of high-affinity LFA-1, possibly because of inability to scaffold with talin and mechanically open the bent LFA-1 heterodimer [77]. WASP-knockout T cells also displayed unstable synapse formation, and a delay in the lethal hit, phenotypically suggesting a defect in mechanical activity and lytic granule homing [77]. Given the importance of WASP in cytotoxicity (see Chapter 2) [77] and the implication of a degranulation defect that was possibly mechanical in nature [112], we were curious to investigate if

the LFA-1 integrin's mechanical activity homed lytic granules to the synapse for fusion.

3.2 Results

3.2.1 LFA-1 is required for synaptic force exertion, degranulation, and cytotoxicity

Lytic granules, the specialized secretory lysosomes that store perforin and granzyme, are known to accumulate at the IS and fuse selectively with the synaptic membrane [25, 92]. This behavior has long been attributed to the centrosome, which serves as a focal point for intracellular granules and polarizes toward the target cell during IS formation [25, 113]. Studies from our group and others, however, indicate that the centrosome is dispensable for synaptic secretion, implying the existence of other targeting mechanisms [100, 114, 76]. Cytotoxic lymphocytes exert nanonewton scale forces across the IS that have been implicated in both the activation of mechanosensitive cell surface receptors and the potentiation of perforin function [36, 115, 116, 106, 33, 117, 39]. We have found that lytic granule exocytosis (degranulation) tends to occur in regions of active force exertion within the IS [36], raising the possibility that local mechanosensing might play an instructive role in guiding perforin and granzyme release. To explore this hypothesis, we studied the degranulation requirements of primary murine CD8+ cytotoxic T lymphocytes (CTLs), which are activated by T cell receptor (TCR)-mediated recognition of antigenic peptide-major histocompatibility complex (pMHC) on the target cell. The CTLs we used for our experiments ex-

pressed the OT-1 TCR, which is specific for the ovalbumin257-264 peptide (OVA) bound to the class I MHC protein H₂-K^b. pMHC recognition by the TCR triggers IS formation and the “inside-out” activation of cell surface integrins [24, 118], including LFA-1 (Lymphocyte function-associated antigen-1), which establishes strong adhesion by binding to its cognate ligands Intercellular adhesion molecule-1 (ICAM-1) and ICAM-2 on the opposing membrane. Using micropillar-based traction force microscopy [34], we found that LFA-1 engagement substantially increased synaptic force exertion (Figure 3.4), implying that this integrin is a key player in IS mechanics and therefore a candidate for mediating coupling between mechanical and secretory output.

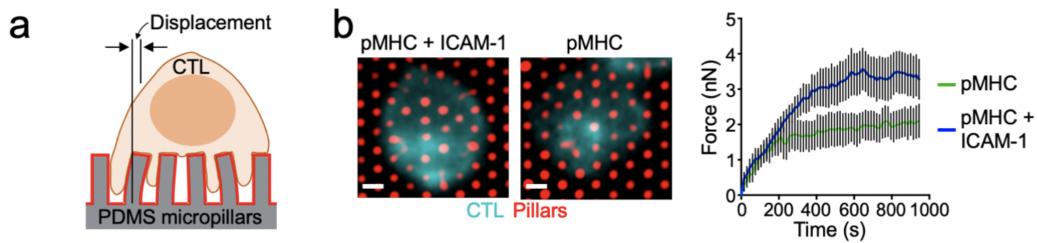


Figure 3.4: LFA-1 is required for synaptic force exertion.

(A): OT-1 CTLs labeled with fluorescent anti-CD45 Fab were stimulated on PDMS micropillar arrays coated with pMHC ± ICAM-1. Traction forces were derived from pillar displacement. **(B):** Left, representative images of CTLs interacting with the arrays. Scale bars = 2 μ m. Right, mean force exertion against the array graphed against time. N is \geq to 8 for each sample.

To evaluate the importance of LFA-1 for degranulation and cytotoxicity, we treated cocultures containing OT-1 CTLs and OVA-loaded RMA-s target cells with a neutralizing antibody that specifically disrupts LFA-1-ICAM-1/2 binding (Figure 3.5). Blocking LFA-1 in this manner dramatically inhibited antigen-induced degranulation, which we measured by surface exposure of the lysosomal marker Lamp1 (Fig. 1d and Fig. S1a) and by the depletion of intracellular granzyme B (Fig. S1b).

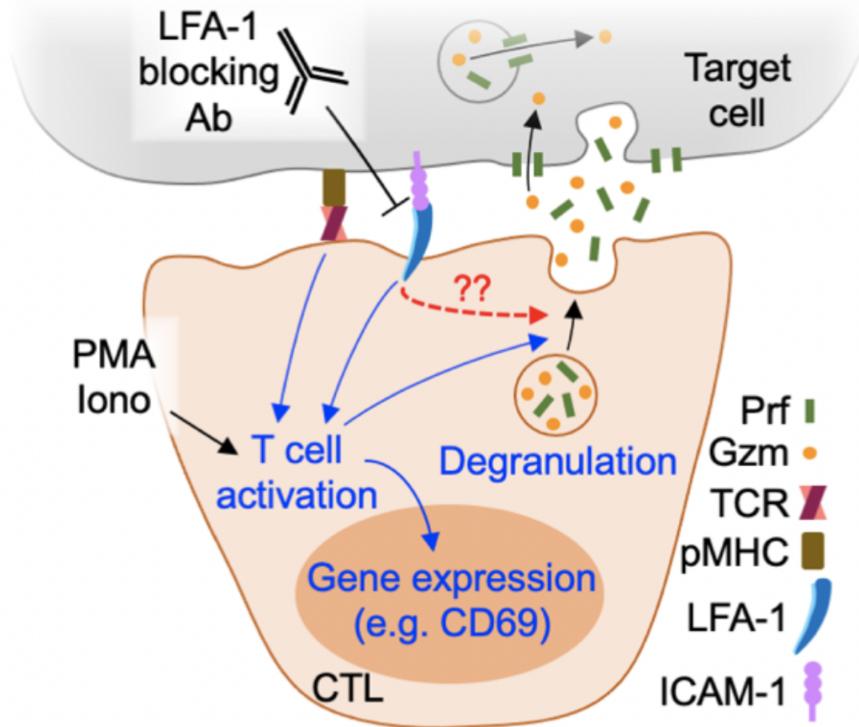


Figure 3.5: Model of antibody blocking of LFA-1 and small molecule-mediated T cell activation.

OVA-loaded RMA-s target cells were mixed with OT-1 CTLs in the presence of LFA1 blocking antibody (α LFA-1) or isotype control. PMA/Iono was applied to some samples in order to drive TCR independent CTL activation.

Anti-LFA-1 neutralizing antibodies also strongly suppressed cytotoxicity, as quantified by lysis of target cells (Fig. 1e). LFA-1 engagement was similarly important for degranulation responses elicited by stimulatory beads coated with pMHC and/or ICAM-1 (Fig. S1c-e).

Collectively, these results were consistent with a specific role for LFA-1 in degranulation, but they did not rule out the possibility that LFA-1 engagement might promote this response secondarily by augmenting T cell activation (Fig. 1c). Indeed, LFA-1 is known to function as a “costimulatory” receptor by lowering the

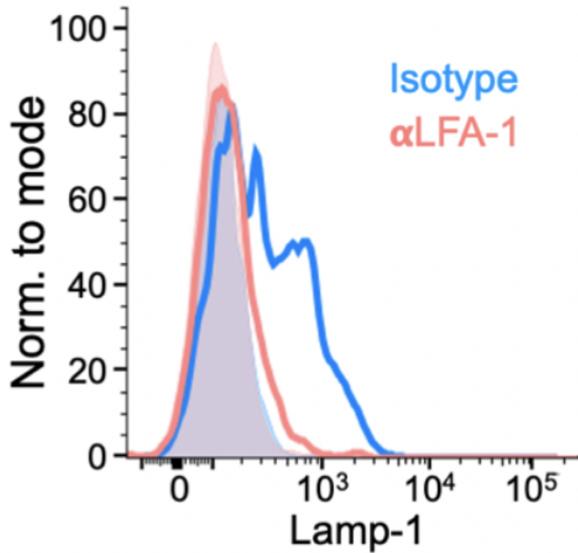


Figure 3.6: Measuring degranulation via flow cytometry.

A representative FACS histogram of CTL Lamp1 staining, measured 90 min after cell mixing.

antigen threshold required for signaling and TCR-induced gene expression [119, 120]. In our hands, LFA-1 blockade altered some, but not all, of these responses. We observed no effect on antigen-induced proliferation (Fig. S2a), assessed by dilution of CellTrace Violet dye. Signaling through the MAP kinase (MAPK) and PI-3 kinase (PI3K) pathways, which we measured by phosphorylation of Erk1/2 and Akt, respectively, was also normal (Fig. S2b). Conversely, antibody treatment dampened cytosolic calcium (Ca^{+}) influx (Fig. S2c) and inhibited the upregulation of CD69, an immediate early response gene (Fig. 1f and S1f).

To decouple these LFA-1 dependent effects on T cell activation from a distinct and specific role in degranulation, we used a combination of phorbol myristate acetate (PMA) and the Ca^{2+} ionophore A23187 (Iono) to induce T cell activation in the absence of TCR engagement (Fig. 1c, S1c). CTLs treated with PMA/Iono alone exhibited robust CD69 expression (Fig. 1f, S1f), indicative of strong activa-

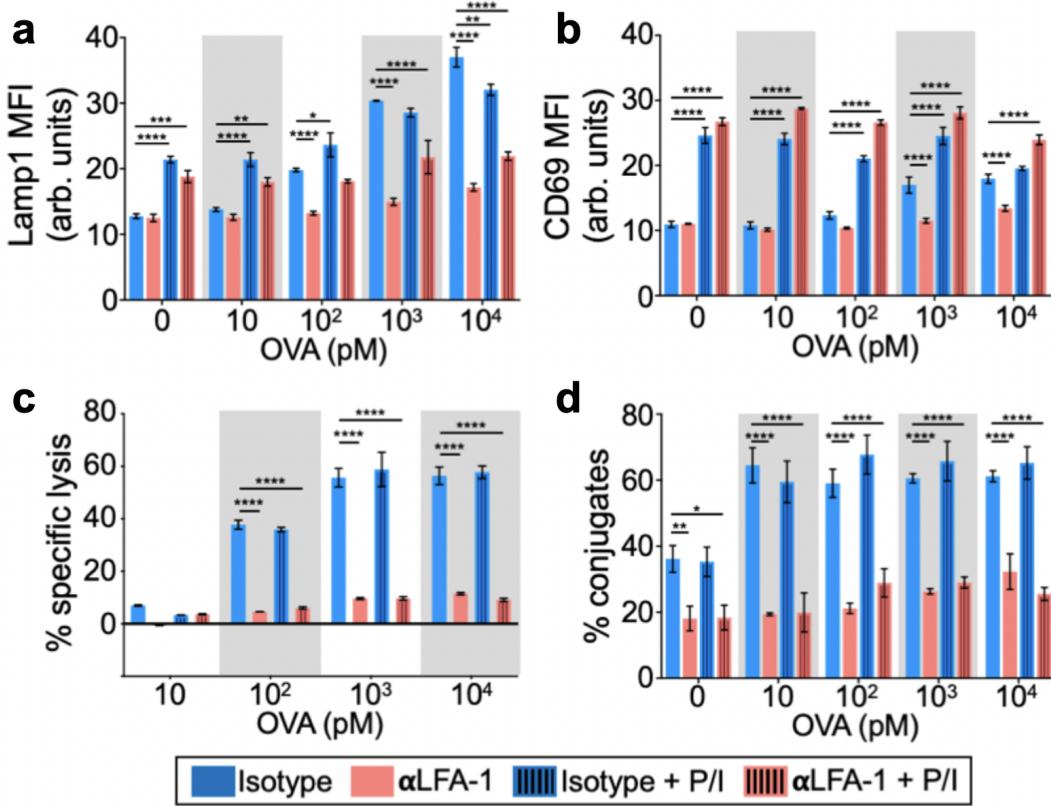


Figure 3.7: LFA-1 is required for synaptic force exertion, degranulation, and cytotoxicity.

(A): Lamp1 exposure (degranulation), measured 90 min after CTL-target cell mixing. (B): Target cell killing, measured 4 h after CTL-target cell mixing. (C): CD69 expression, measured 90 min after CTL-target cell mixing. (D): Conjugate formation, measured 90 min after CTL-target cell mixing. Data in (A-D) were derived from technical triplicates. All error bars denote SEM. *, **, ***, and **** denote $P \leq 0.05$, $P \leq 0.01$, $P \leq 0.001$, and $P \leq 0.0001$, calculated by 2way ANOVA. All data are representative of at least two independent experiments.

tion. Their degranulation responses were quite modest, however (Fig. 1d, S1e), pointing to the importance of target contact/proximity in stimulating cytotoxic secretion. Indeed, robust Lamp1 exposure was only observed in CTLs that were concomitantly exposed to antigenic pMHC and ICAM, either on target cells or on beads. Critically, this ligand-induced component of degranulation was completely inhibited by LFA-1 blockade (Fig. 1d, S1e). Taken together, these results indicate that LFA-1 engagement promotes cytotoxic secretion independently of T cell activation and at a level downstream of early signaling events. Notably,

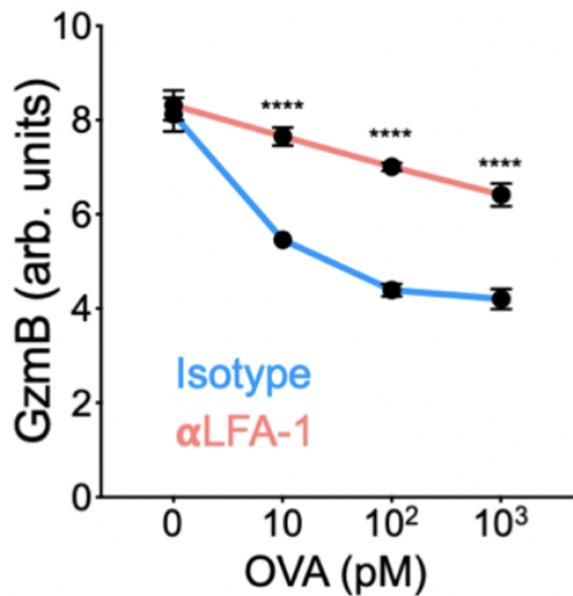


Figure 3.8: LFA block prevents release of granzyme B.

Staining for intracellular granzyme B (GzmB) in CTLs, performed 90 min after cell mixing.

LFA-1 was also required for the formation of strong CTL-target cell conjugates, both in the presence and in the absence of PMA/Iono (Fig. 1g). Hence, the capacity of LFA-1 to stimulate degranulation was phenotypically linked to its ability to mediate strong adhesion.

3.2.2 Degranulation occurs in IS domains containing ligand-bound TCR and ligand-bound LFA-1

That both the TCR and LFA-1 were required for robust degranulation (Fig. 1 and S1) raised the possibility that both receptor types must be engaged within the same cell-cell interface to elicit cytotoxic responses. This requirement would presumably enhance the specificity of killing by ensuring that toxic factors are

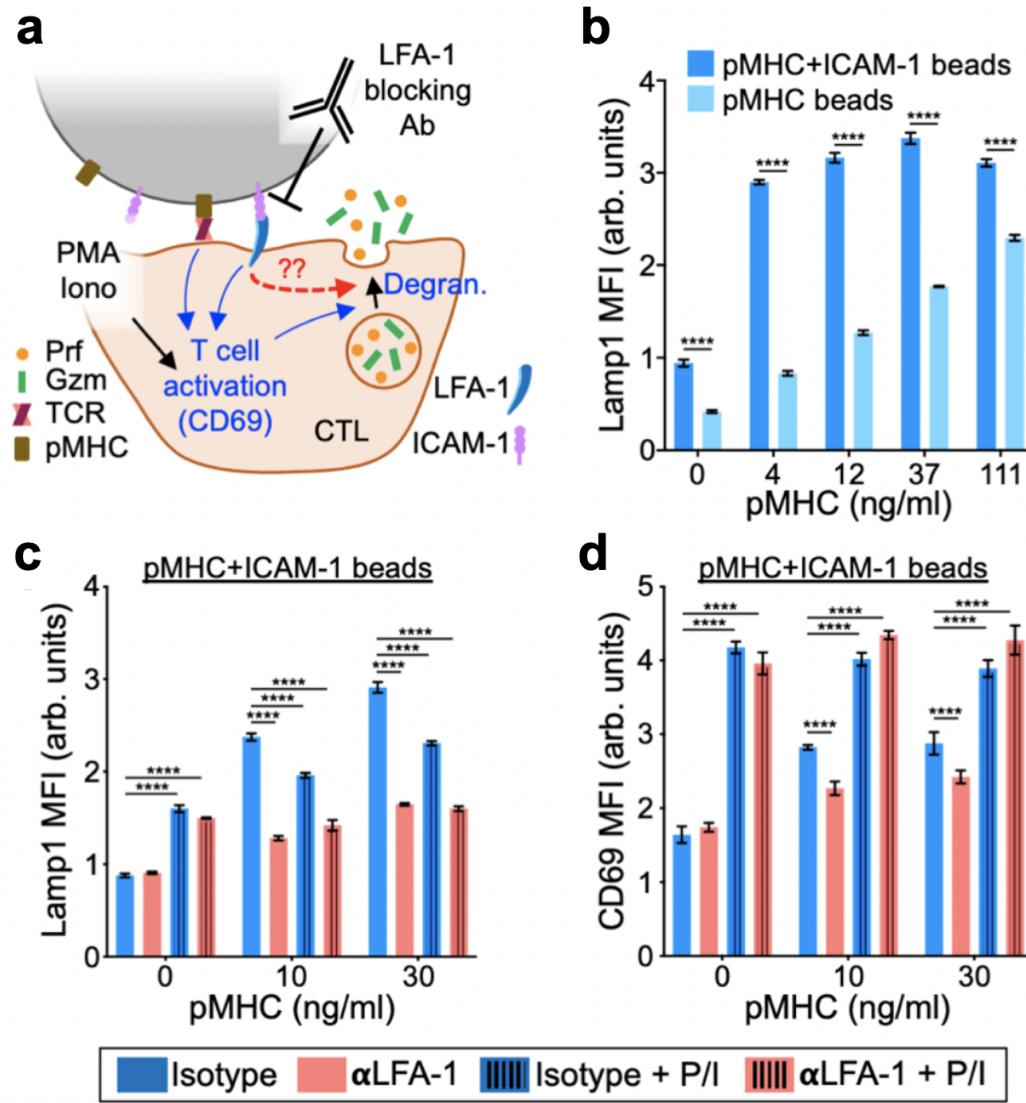


Figure 3.9: LFA-1 blockade disrupts CTL degranulation.

(A): A diagram schematizing CTL activation by stimulatory beads coated with pMHC (H-2K^b-OVA) ± ICAM-1. (B): OT-1 CTLs were mixed with beads coated with increasing amounts of pMHC in the presence or absence of ICAM-1. Degranulation was measured 90 min after CTL stimulation. (C-D) Beads coated with pMHC and ICAM-1 were incubated with OT-1 CTLs in the presence or absence of PMA/Iono and treated with either α LFA-1 or isotype control. Graphs show degranulation (C) and CD69 expression (D) measured 90 min after CTL stimulation. All error bars denote SEM. *, **, ***, and **** denote $P \leq 0.05$, $P \leq 0.01$, $P \leq 0.001$, and $P \leq 0.0001$, calculated by 2way ANOVA. All data are representative of at least two independent experiments.

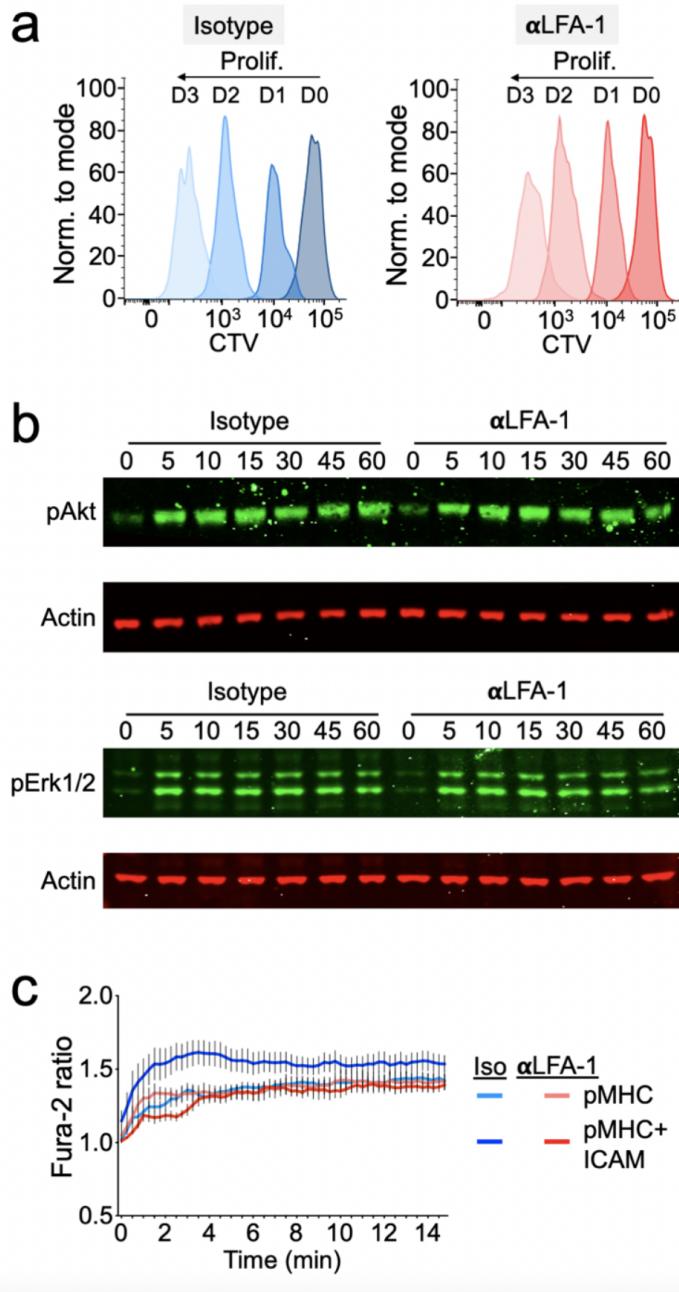


Figure 3.10: LFA-1 blockade alters only some indices of T cell activation.

(A): OT-1 CTLs were labeled with CellTrace Violet (CTV) and incubated with OVA-loaded splenocytes in the presence of α LFA-1 or isotype control. CTV dilution was monitored over 3 days (A) by flow cytometry. (B): OT-1 CTLs were mixed with pMHC ($H2-K^b$ -OVA) and ICAM-1 coated beads and, at the indicated timepoints, pAkt (top) and pErk1/2 (bottom) were assessed by immunoblot, with actin serving as a loading control. (C) OT-1 CTLs were loaded with Fura-2-AM and imaged on glass surfaces coated with the indicated proteins in the presence of α LFA-1 or isotype control. Graph shows the mean Fura-2 ratio of all CTLs in the imaging field, averaged over 6 positions. Error bars denote SEM. All data are representative of at least two independent experiments.

released only against bona fide target cells (expressing cognate pMHC) that are tightly associated with the CTL (via integrin adhesion). To test this idea, we stimulated CTLs using beads coated with both pMHC and ICAM-1 (cis) or using a mixture of beads coated separately with only pMHC or ICAM-1 (trans) (Fig. 2a). Care was taken to make sure that the total amount of accessible pMHC and ICAM-1 was identical in each experimental group and that CTLs could engage multiple beads simultaneously (Fig. S3a).

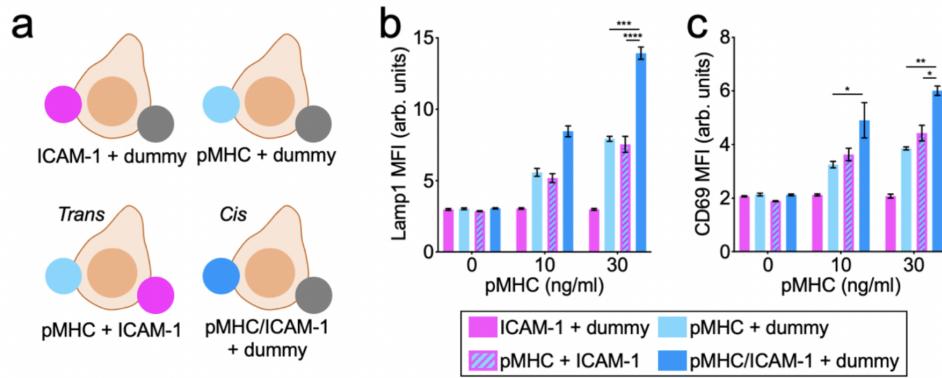


Figure 3.11: Degranulation occurs in IS domains containing both ligand-bound TCR and ligand-bound LFA-1.

(A-C): OT-1 CTLs were activated by equal mixtures of stimulatory beads bearing the indicated proteins. (A): A schematic of the experiment. Dummy denotes beads coated with nonstimulatory pMHC ($H2-K^b$ -KAVY) alone. Trans and cis copresentation of $H2-K^b$ -OVA and ICAM-1 are indicated. (B-C): Graphs showing CTL degranulation (B) and CD69 expression (C): measured 90 min after CTL stimulation. Data were derived from technical triplicates.

Strikingly, immobilized ICAM-1 boosted TCR-induced degranulation only when presented in the cis configuration (Fig. 2b). Indeed, CTLs stimulated with the trans mixture of pMHC- and ICAM-1- coated beads responded indistinguishably from CTLs stimulated with pMHC beads alone. We observed the same pattern of results using CD69 upregulation as the downstream readout (Fig. 2c). Hence, ligand-bound LFA-1 must share the same interface as the ligand-bound TCR in order to boost cytotoxicity and T cell activation. Building

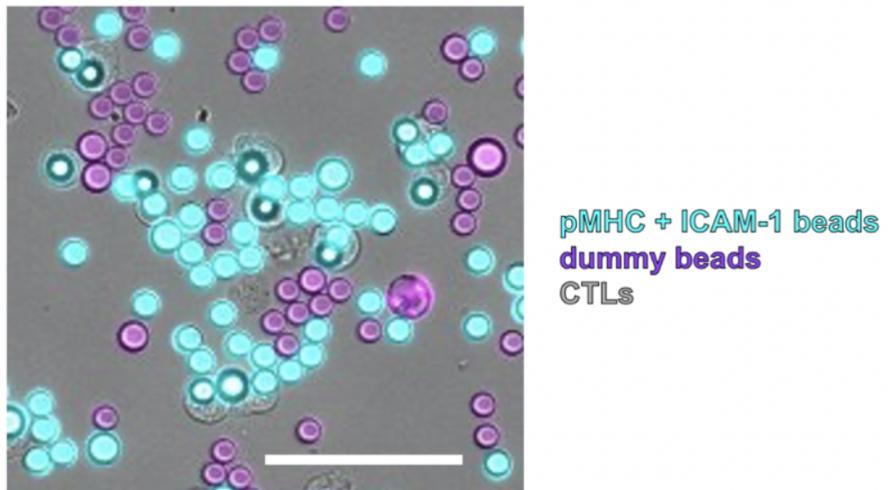


Figure 3.12: CTL stimulation with spatially segregated ligands on beads.

Representative image of OT-1 CTLs, pMHC-coated beads, and ICAM-1 coated beads mixed at the proportions used for Figure 2a-c. Scale bar = 50 μm .

upon this idea, we next examined whether local coengagement of LFA-1 and the TCR could control the position of degranulation events within the IS. Using protein microstamping¹⁹, we prepared glass coverslips containing 2 μm spots of fluorescent streptavidin spaced in a 10 $\mu\text{m} \times 10 \mu\text{m}$ square grid. These surfaces were then incubated with mixtures of unbiotinylated proteins to coat/block the empty glass between streptavidin spots, and then with biotinylated proteins to load the spots themselves. By varying the composition of coating and loading mixtures, we were able to generate a panel of distinct micropatterned substrates: 1) ICAM-1 spots within a uniform background of pMHC (ICAM-spot), 2) pMHC spots on an ICAM-1 background (Antigen-spot), and 3) spots containing both ICAM-1 and pMHC on a nonstimulatory (BSA coated) background (Dual-spot) (Fig. 2d). We also generated control surfaces containing empty streptavidin spots in a background of admixed pMHC and ICAM-1. CTLs plated on Dualspot surfaces evinced Ca^{2+} flux only during periods of spot contact (Fig. S3b and Movies

S1-2), confirming that biotinylated stimulatory ligands could be constrained in space by the patterned streptavidin.

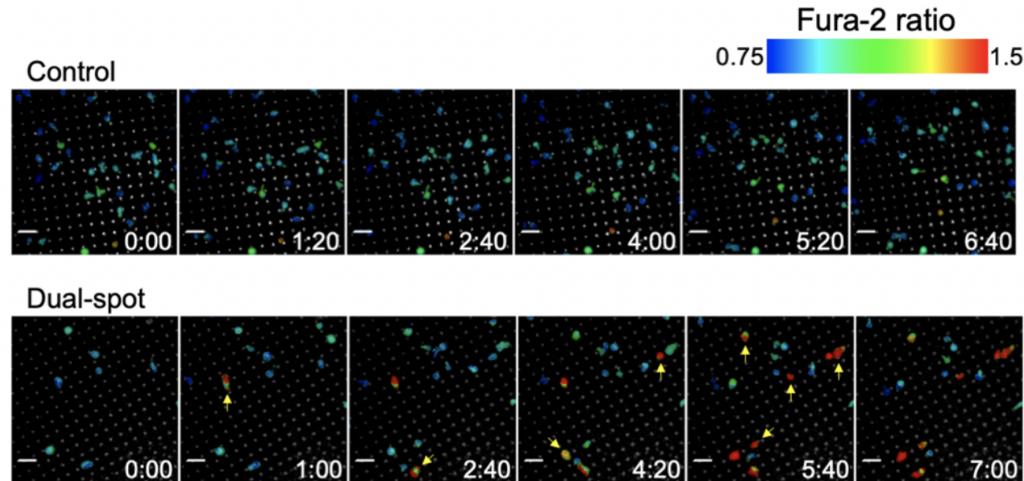


Figure 3.13: CTL stimulation with spatially segregated ligands via micropatterning.

Representative time-lapse montages of Fura-2-AM-loaded OT-1 CTLs imaged on Dual-spot and control surfaces. Yellow arrows denote the onset of Ca^{2+} flux in individual cells. Time in M:SS is shown in the bottom right corner of each image. Scale bars = 10 μm . All data are representative of at least two independent experiments.

To monitor degranulation position in this system, we employed a reporter construct in which the pH-sensitive fluorescent protein pHluorin is linked to the lytic granule resident protein Lamp1 [32]. Because pHluorin is quenched in the low pH granule environment, it becomes visible only upon fusion, generating a transient burst of fluorescence that reveals the position of the degranulation site within the IS. When CTLs expressing pHluorin-Lamp1 were imaged on ICAM-spot, Antigen-spot, or Dual-spot surfaces, degranulation events tended to cluster around the fluorescent spots containing ICAM-1 and/or pMHC (Fig. 2e-f and Movie S3). In all three cases, the mean distance between degranulation events and the spots closest to them was substantially lower than one would expect by chance (dotted line in Fig. 2f) and significantly less than the corresponding

distances measured between empty SA spots and degranulations on control surfaces. The enrichment of degranulation in zones where pMHC and ICAM-1 were either copresented (Dual-spot) or closely apposed (ICAM-spot and Antigen-spot) further supports a critical role for the coengagement of the TCR and LFA-1 in guiding cytotoxic secretion and suggests that permissive secretory domains of receptor coengagement can be substantially smaller than the IS itself [121].

Although all three micropatterned substrates elicited targeted degranulation, responses to the Dual-spot and Antigen-spot configurations were significantly more focused than what we observed on ICAM-spot surfaces (Fig. 2f). To explore the basis for this difference, we imaged CTLs expressing a fluorescent form of the LFA-1 α -chain (CD11a-YFP) on surfaces containing focal LFA-1 or TCR ligands. Because the CD11a-YFP CTLs were derived from a polyclonal animal rather than an OT-1 transgenic, we used an anti-CD3 antibody instead of antigenic pMHC to engage the TCR. On ICAM-spot surfaces (anti-CD3 in the background), LFA-1 tended to localize to the micropatterned ICAM-1 (Fig. 2g), consistent with ligand recognition. The Anti-CD3-spot configuration, however, induced even stronger focal accumulation of LFA-1 (Fig. 2h), which was surprising considering that ICAM-1 was coated in the background of these surfaces. Interestingly, LFA-1 recruitment on Anti-CD3-spot substrates was most apparent not over the anti-CD3 spot itself but in the surrounding 1-2 μm neighborhood. This could potentially reflect inside-out integrin activation and clustering induced by local TCR signaling [?]. The unexpectedly robust accumulation of LFA-1 to areas of focal TCR stimulation potentially explains why Antigen-spot surfaces elicited more focused degranulation than their ICAM-spot counterparts. Within the IS, both the TCR and LFA-1 are subjected to F-actin dependent pulling forces, which are thought to drive the formation of catch bonds between each receptor

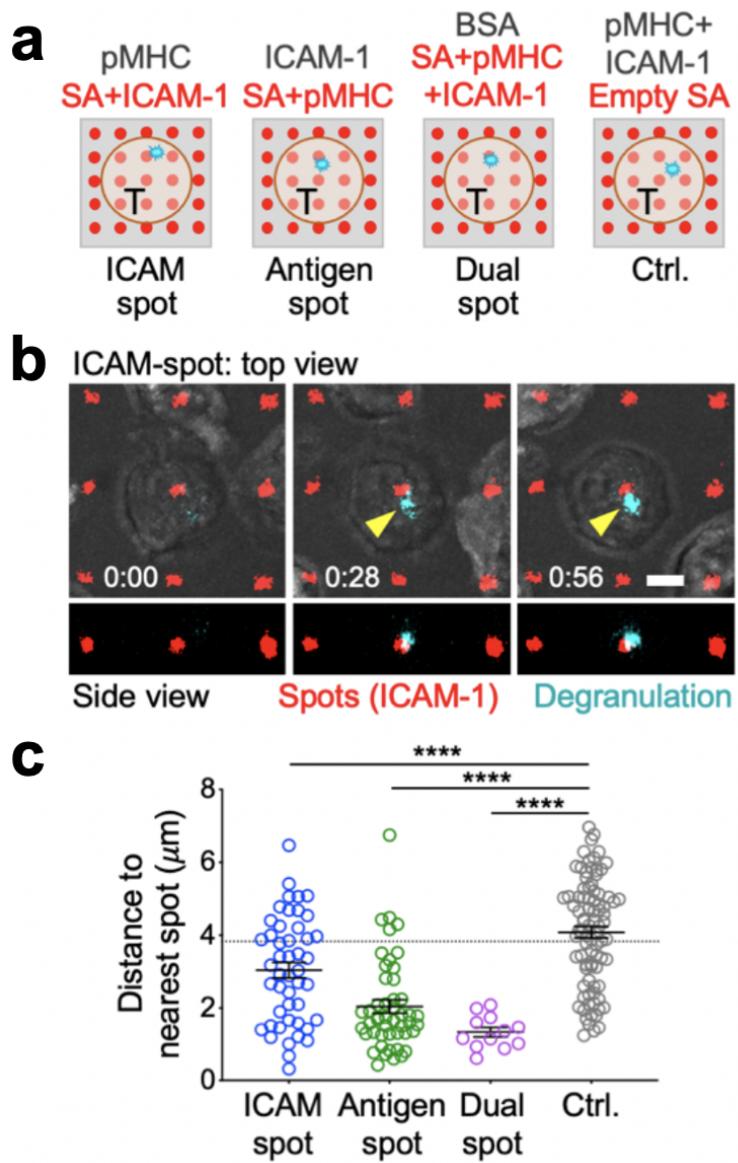


Figure 3.14: Degranulation occurs in IS domains containing both ligand-bound TCR and ligand-bound LFA-1.

(A-B): OT-1 CTLs expressing pHluorin-Lamp1 were imaged on micropatterned surfaces coated with stimulatory pMHC ($H2-K^b$ -OVA) and ICAM-1 in 4 different configurations (A). SA = streptavidin. (B) Time-lapse montage showing a representative degranulation event (indicated by the yellow arrowhead) on an ICAM-spot surface. In the top views, fluorescent signals have been superimposed on the brightfield image. Time in M:SS is shown at the bottom left corner of the top view images. Scale bars = $4 \mu\text{m}$. (C) Distance between each degranulation event and the closest fluorescent SA spot in the IS. Dotted line indicates the mean distance expected from randomly placed degranulation. N = 12 for Dual-spot and ≥ 47 for the other conditions.

and its respective ligand, promote conformational changes, and induce signal transduction [116, 106, ?, 104].

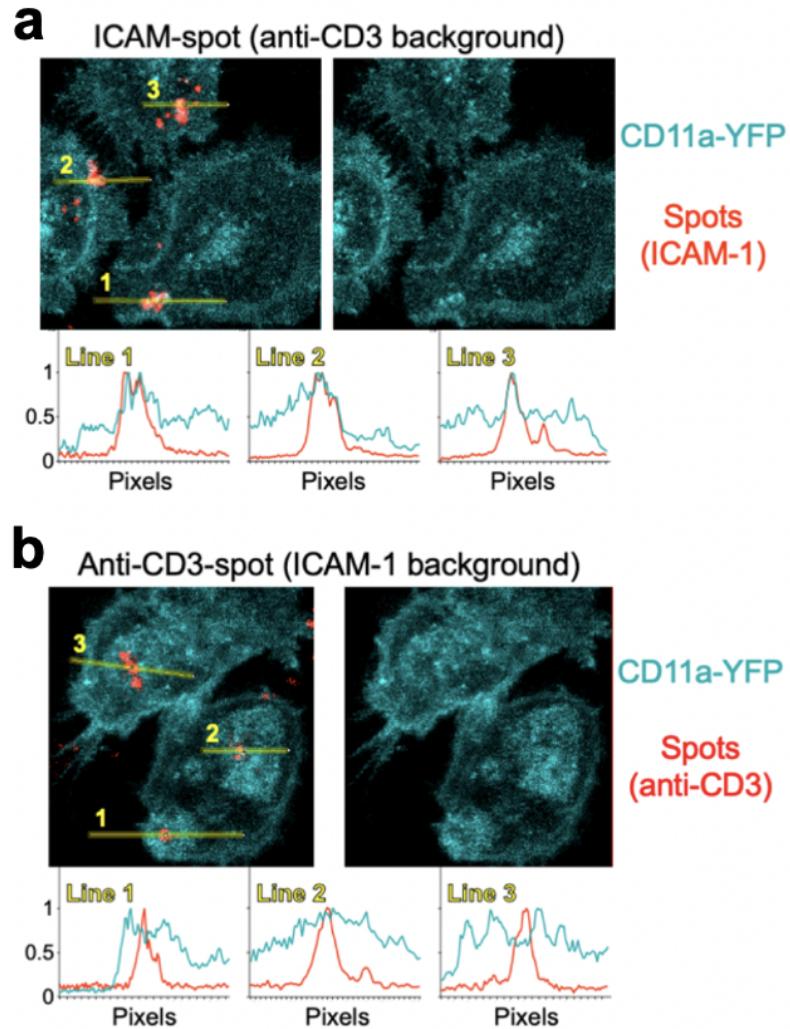


Figure 3.15: Local TCR signaling induces adjacent integrin activation.

(A-B): CD11a-YFP CTLs were imaged on ICAM-spot (A) and Anti-CD3-spot (B) surfaces. Representative images are shown in the top of each panel, with linescans through the fluorescent SA spots below. The lines used to generate the linescans are shown in the left image of each panel. All error bars denote SEM. *, **, ***, and **** denote $P \leq 0.05$, $P \leq 0.01$, $P \leq 0.001$, and $P \leq 0.0001$, calculated by 1way (f) and 2way (b-c) ANOVA. All data are representative of at least two independent experiments. Mitchell Wang and Morgan Huse performed these experiments.

3.2.3 LFA-1 pulling forces define degranulation domains

Given the importance of these forces for the function of each receptor, we reasoned that they might also play a role in guiding cytotoxic secretion. To investigate this hypothesis, we employed Förster resonance energy transfer (FRET)-based molecular tension probes (MTPs) [123] specific for the TCR and LFA-1. Each MTP comprised a stimulatory ligand (pMHC or ICAM1) attached to a DNA hairpin containing a fluorophore at one end (Atto647N or Cy3B, respectively) and a quencher (BHQ-2) at the other (Fig. 3a). When folded at resting state, MTPs do not fluoresce due to the close proximity between quencher and fluorophore. Applied forces capable of unwinding the hairpin (in this case, 4.7 pN) pull the quencher and fluorophore apart, dramatically increasing fluorescence. Consistent with prior reports [123, 124], surfaces coated with pMHC-MTPs and ICAM-1-MTPs induced IS formation by OT1 CTLs and the exertion of dynamic forces through both LFA-1 and the TCR (Fig. 3b and Movie S4), which we visualized by time-lapse imaging.

Figure 3.16: LFA-1 pulling forces define degranulation domains.

(A): Measuring correlations between degranulation (Degran.) and receptor specific pulling forces with MTPs. F = fluorophore, Q = quencher, G = pHluorin. **(B-E):** OT-1 CTLs expressing pHluorin-Lamp1 were imaged by TIRF microscopy on glass surfaces coated with pMHC ($H2-K^b$ -OVA) and ICAM-1 MTPs. **(B):** Representative image of pMHC-MTP and ICAM-1-MTP signals, overlaid onto the corresponding IRM image. Scale bar = $5 \mu\text{m}$. **(C):** Time-lapse montage showing a representative degranulation event (indicated by a white arrowhead) together with pMHC-MTP and ICAM-1-MTP signals. Time in M:SS is shown at the top left corner of each image. Scale bars = $2 \mu\text{m}$. **(D):** Left, image of a representative degranulation event, overlaid onto the corresponding IRM image. Regions defining the degranulation subdomain (R1) and the entire IS (R2) are indicated. Right, differences in mean fluorescence intensity between R1 and R2 at the moment of degranulation are shown for the indicated MTPs. N = 52 for each sample. Error bars denote SEM. P value calculated by unpaired Student's t test. **(E):** Left, image of a representative degranulation event, overlaid onto the corresponding IRM image. Linescans sampling the degranulating (L1) and inactive (L2) domains are indicated. Right, normalized ICAM-1-MTP fluorescence along L1 and L2 at the moment of degranulation (see Methods). The pHluorin-Lamp1 (Degran.) signal along L1 is shown for reference. Error bars denote SEM. N = 122 cells, pooled from two independent experiments. All other data are representative of at least two independent experiments. Mitchell Wang, Yuesong Hu, Xihe Xie, and Morgan Huse performed these experiments.

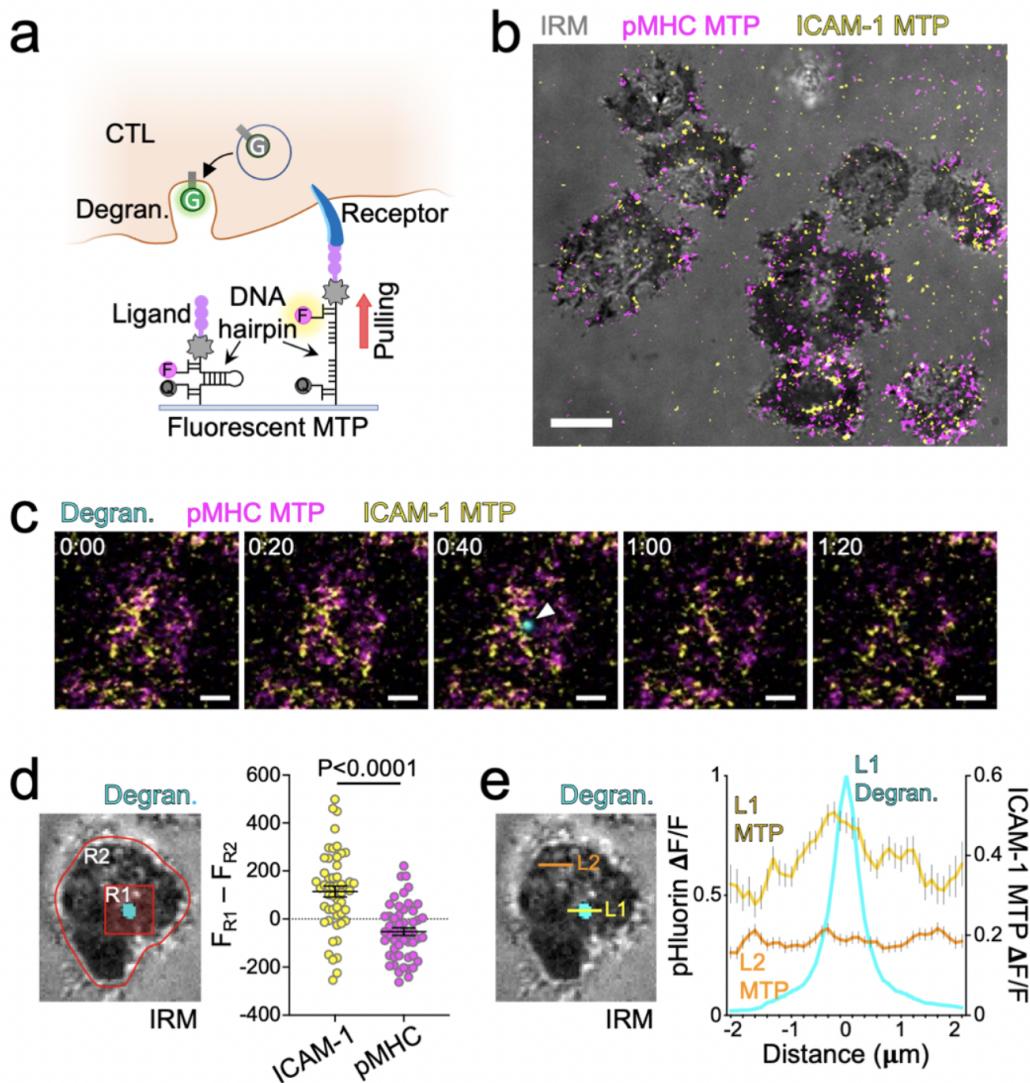


Figure 3.16 (Continued)

To measure the association between degranulation and receptor-specific forces, we used CTLs expressing pHluorin-Lamp1 to record exocytic events elicited by stimulatory MTPs (Fig. 3c). The mean MTP fluorescence in the immediate vicinity of each event ($2 \mu\text{m}$ box) was then compared with the mean fluorescence of the entire IS. This approach revealed a marked enrichment of ICAM-1-MTP signal in the degranulation zone (Fig. 3d), indicative of a spa-

tial correlation between cytotoxic secretion and force exertion through LFA-1. pMHC-MTP pulling was not associated with degranulation in this way (Fig. 3d), arguing against a role for the TCR as a critical force bearing receptor in this context. To further characterize the pattern of LFA-1 mechanics, we examined ICAM-1-MTP fluorescence along linescans bisecting the degranulation peak. Mean LFA-1 forces reached a local maximum in the $1\text{ }\mu\text{m}$ diameter region surrounding each event (Fig. 3e), consistent with the idea that mechanically active LFA-1 defines permissive zones for cytotoxic secretion. A degranulation zone of this size would accommodate the approach of a typical lytic granule ($0.5\text{-}1\text{ }\mu\text{m}$ in diameter) [125].

3.2.4 Talin is required for LFA-1 mediated force exertion, degranulation, and cytotoxicity

Integrins are coupled to the F-actin cytoskeleton via talin, a mechanosensitive scaffolding protein that is critical for the formation and signaling of integrin adhesions [126] (Fig. 4a).

To evaluate the importance of integrin-cytoskeletal linkage for synaptic force exertion, we used CRISPR/Cas9 to deplete talin from OT1 CTLs (Fig. S4a) and then compared the physical output of these cells to that of controls expressing a nontargeting guide RNA. Talin depletion strongly suppressed ICAM-1 pulling on MTP surfaces (Fig. 4b-c) indicative of a profound defect in LFA-1 dependent force exertion. By contrast, pMHC-MTP forces were essentially unchanged (Fig. 4bc and Movie S6), indicating that the mechanical effects of talin were restricted to LFA-1 in this system.

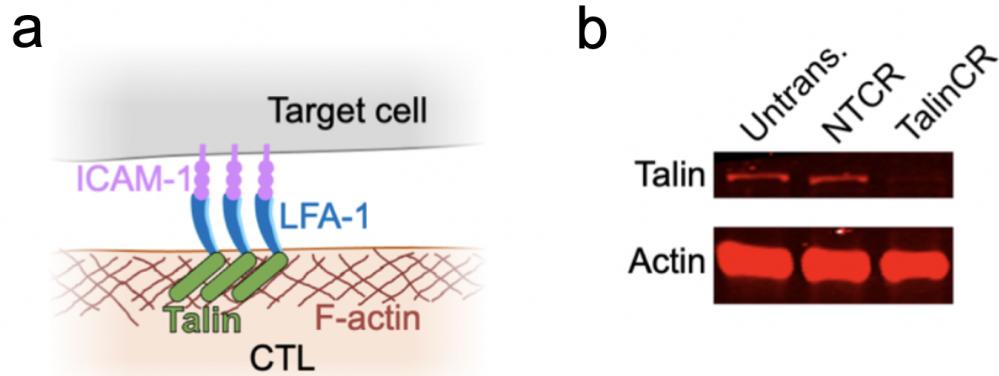


Figure 3.17: Talin is required for LFA-1 mediated force exertion, degranulation, and cytotoxicity.

(A): Talin couples ligand-bound integrins to the F-actin cytoskeleton. **(B):** Immunoblot analysis of talin expression in OT-1 Cas9 CTLs transduced with the indicated gRNAs. Untrans. = untransduced. Actin served as a loading control.

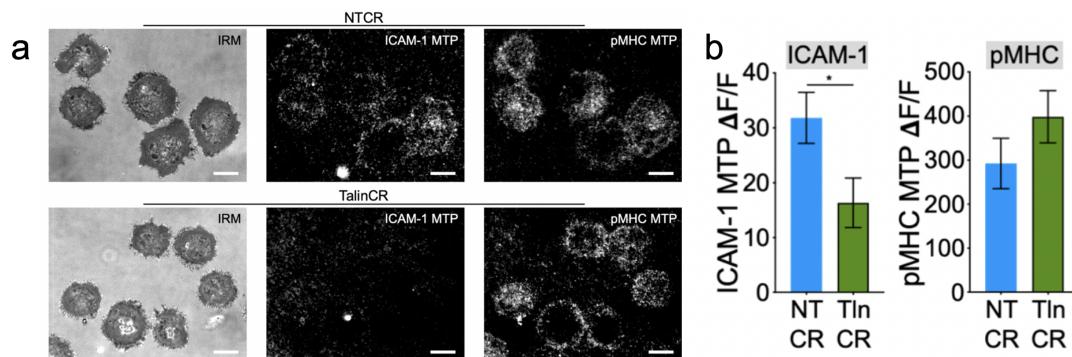


Figure 3.18: Talin is required for LFA-1 mediated force exertion.

(A): Representative images showing ligand specific pulling in synapses. Scale bars = 5 μm. **(c)** Mean ICAM-1-MTP (left) and pMHC ($H2-K^b$ -OVA)-MTP (right) signals, assessed 30 min after the addition of CTLs to the MTP surface. $N \geq 14$ cells for each sample. Error bars denote SEM (*). $P \leq 0.05$, calculated by unpaired Student's t test. Mitchell Wang, Yuesong Hu, and Morgan Huse performed these experiments.

The selectivity of the talin loss-of-function phenotype allowed us to interrogate the specific role of integrin mechanotransduction in cytotoxicity assays. In cocultures with OVA-loaded RMA-s cells, CTLs lacking talin exhibited sharply reduced degranulation and target cell lysis (Fig. 4de), implying a central role for integrin adhesions in both processes. These loss-of-function phenotypes were not rescued by the application of PMA/Iono (Fig. 4d-e), indicating that they were not caused by impaired T cell activation.

Consistent with this interpretation, depletion of talin did not affect TCR-induced MAPK and PI3K signaling (Fig. S4b), and it only modestly suppressed CD69 responses (Fig. 4f). The disproportionately large effect of talin deficiency on cytotoxic secretion was particularly obvious in two-dimensional plots of Lamp1 and CD69 (Fig. S4c), which confirmed that, for a given level of activation, CTLs lacking talin consistently degranulated more weakly than nontargeting controls.

Talin depletion also suppressed CTL-target cell conjugate formation (Fig. 4g), similar to the effects of LFA-1 blockade (Fig. 1g). Collectively, these results support a critical role for talin in LFA-1 dependent IS mechanics and strongly suggest that it is force exertion through LFA-1, rather than the TCR, that imposes spatiotemporal control over CTL degranulation.

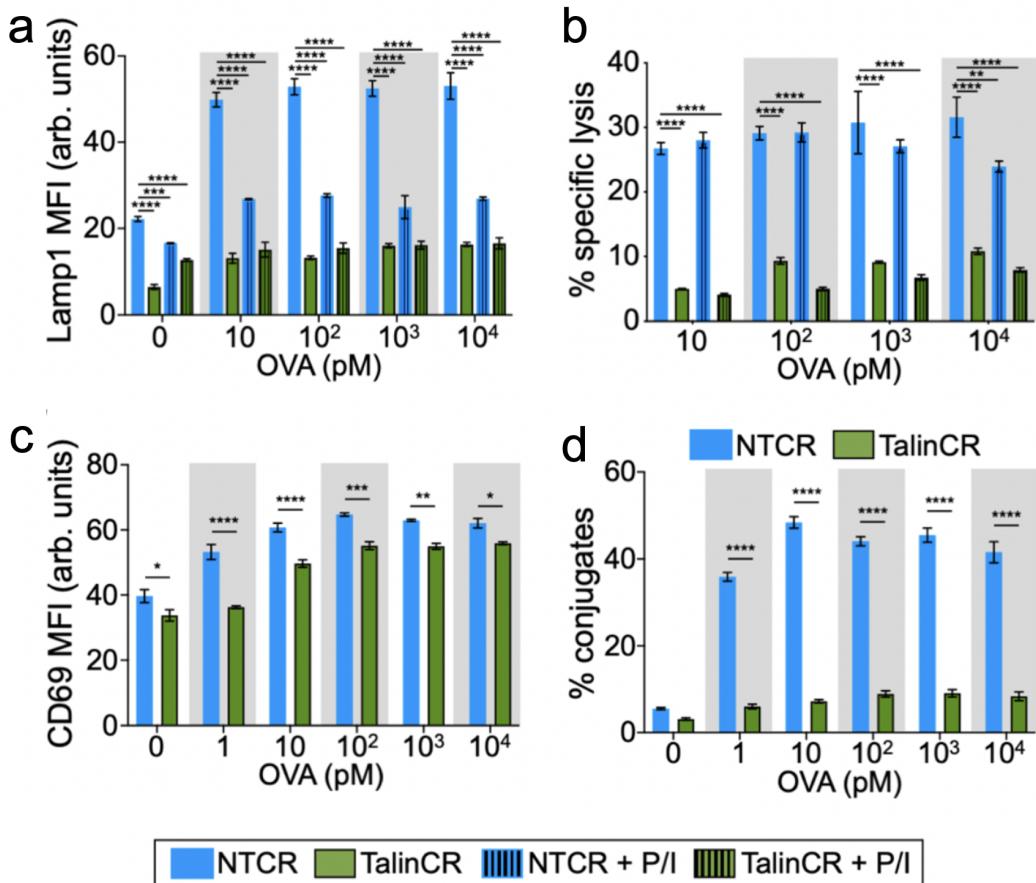


Figure 3.19: Talin is required for LFA-1 mediated degranulation, and cytotoxicity.

(A-D): OVA-loaded RMA-s target cells were mixed with OT-1 Cas9 CTLs expressing the indicated gRNAs. PMA/Iono was applied to some samples in order to drive TCR independent CTL activation. **(A):** Lamp1 exposure (degranulation), measured 90 min after CTL-target cell mixing. **(B):** Target cell killing, measured 4 h after CTL-target cell mixing. **(C):** CD69 expression, measured 90 min after CTL-target cell mixing. **(D):** Conjugate formation, measured 90 min after CTL-target cell mixing. Data in d-g were derived from technical triplicates. All error bars denote SEM. *, **, ***, and **** denote $P \leq 0.05$, $P \leq 0.01$, $P \leq 0.001$, and $P \leq 0.0001$, calculated by 2way ANOVA (A-D). All data are representative of at least two independent experiments.

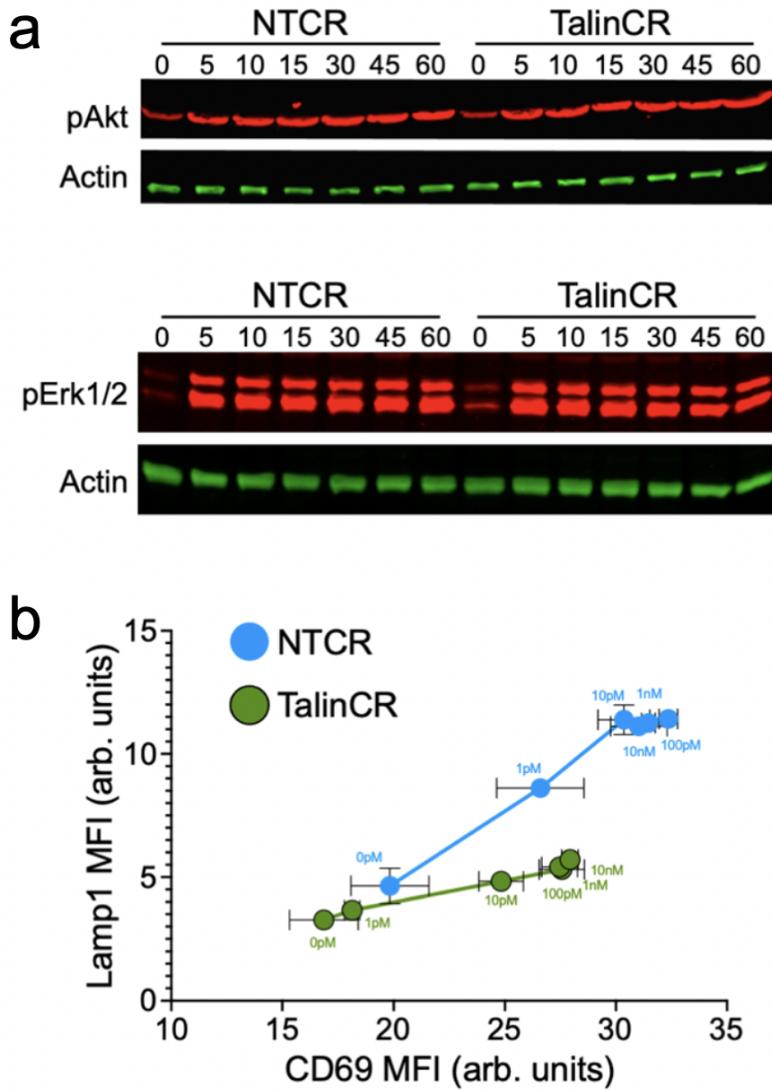


Figure 3.20: Talin depletion inhibits degranulation but not early TCR signaling.

(A): OT-1 Cas9 CTLs transduced with the indicated gRNAs were mixed with pMHC ($H2-K^b$ -OVA) and ICAM-1 coated beads and, at the indicated timepoints, pAkt (top) and pErk1/2 (bottom) were assessed by immunoblot. Actin served as a loading control. (B): 2-dimensional plot correlating degranulation (Lamp1) and T cell activation (CD69), taken from the same data set used to generate Fig. 4d and 4f. Error bars denote SEM. All data are representative of at least two independent experiments.

3.2.5 Talin, but not LFA-1, is required for CTL-mediated killing of B16F10 cells

Not all cells express LFA-1 ligands, raising the question of whether integrin mechanotransduction controls CTL-mediated killing across a broad spectrum of targets. The capacity of talin deficiency to interrogate integrin function independently of LFA-1 allowed us to address this issue. B16F10 melanoma cells do not express ICAM-1, implying that they cannot engage LFA-1 across the IS (Fig. 5a-b). Nevertheless, they are reasonable targets for OT-1 CTLs, eliciting robust degranulation and cytotoxicity responses in the presence of OVA.

LFA-1 blockade failed to inhibit either of these responses (Fig. 5c), consistent with the idea that LFA-1 is not involved in the recognition and killing of B16F10 cells. By contrast, talin depletion abrogated both target cell lysis and degranulation (Fig. 5d), strongly suggesting that integrins other than LFA-1 contribute to the killing of ICAM deficient targets. We conclude that integrin-mediated control of cytotoxic secretion is likely to be a general feature of the IS.

3.3 Discussion

Taken together, our data suggest a model in which degranulation occurs at permissive secretory subdomains within the IS that are defined by mechanically active integrins (see Figure 1.4). TCR signaling plays critical role in this process by inducing close contact formation and also by triggering Ca^{2+} influx, which is required for granule fusion [56, 127] (28). In the absence of integrin dependent force exertion, however, TCR signaling alone is insufficient for robust cytotoxic

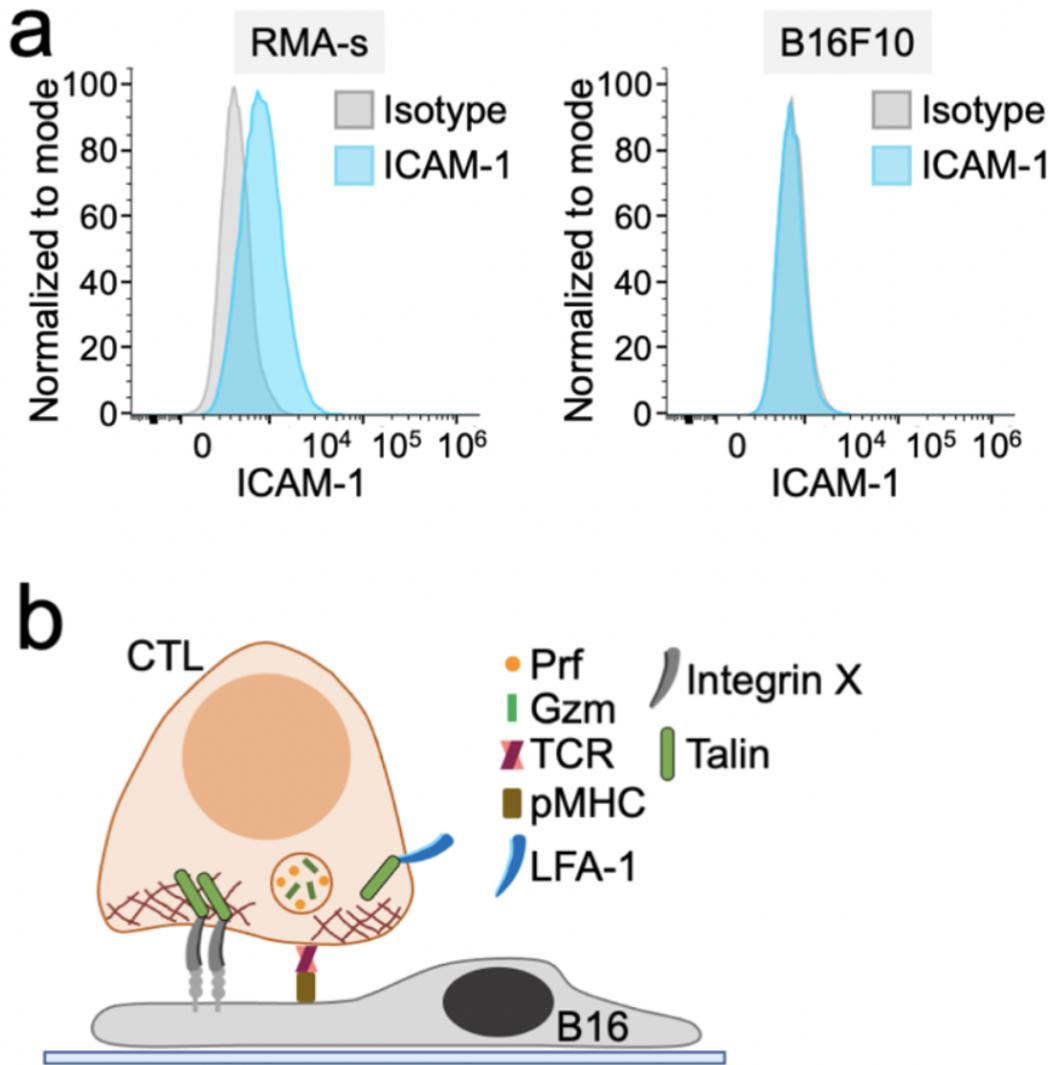


Figure 3.21: B16F10 cells do not express ICAM-1.

(A): Representative histograms showing ICAM-1 expression in RMA-s (left) and B16F10 (right) cells. (B): Model of CTL-mediated killing of B16F10 cells that requires both the TCR and an unidentified integrin X.

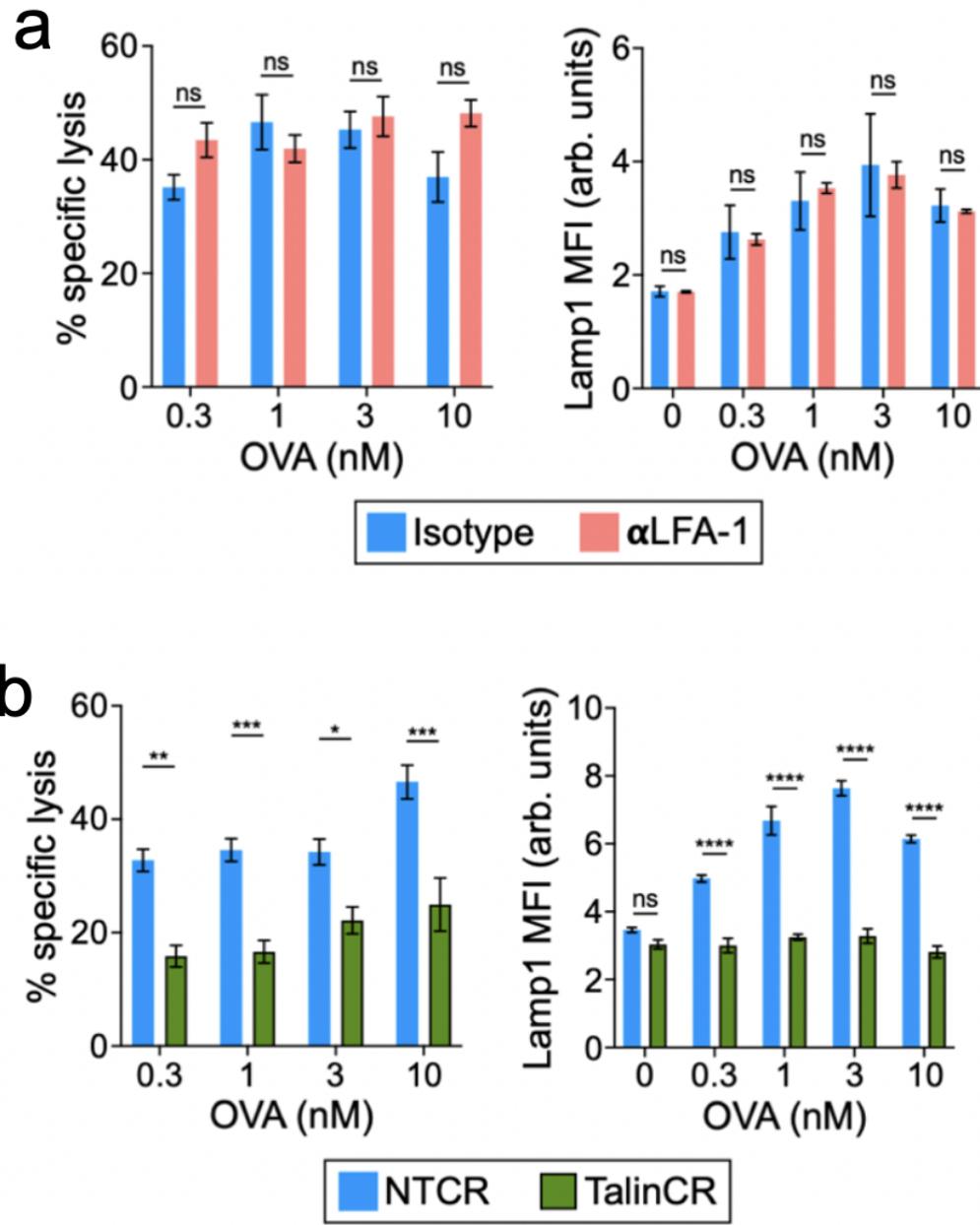


Figure 3.22: Talin, but not LFA-1, is required for CTL-mediated killing of B16F10 cells.

(A): OVA-loaded B16F10 target cells were mixed with OT-1 CTLs in the presence of LFA1 blocking antibody (α LFA-1) or isotype control. Left, specific lysis, measured after 4 h. Right, Lamp1 exposure, measured after 90 min. (d) OVA-loaded B16F10 target cells were mixed with OT-1 Cas9 CTLs transduced with the indicated gRNAs. Left, specific lysis, measured after 4 h. Right, Lamp1 exposure, measured after 90 min. Data in c-d were derived from technical triplicates. All error bars denote SEM. *, **, and *** denote $P \leq 0.05$, $P \leq 0.01$, and $P \leq 0.001$, calculated by 2way ANOVA. All data are representative of at least two independent experiments. Elisa Sanchez performed these experiments.

secretion. Indeed, our results imply that one of the major ways that the TCR promotes killing is by locally activating LFA-1 within the IS. This integrin-based model for degranulation both explains our data and is consistent with prior work documenting lytic granule accumulation and perforin release in synaptic subdomains defined by the engagement of adhesive and activating receptors [77, 121]. In the absence of LFA-1 ligands, we speculate that other integrins may assume its licensing role. Indeed, the fact that B16F10 cell killing requires talin, but not LFA-1, strongly implies the existence of alternative integrin activators. CTLs express both VLA-4 ($\alpha 4\beta 1$) and CD103 ($\alpha E\beta 7$), which recognize protein ligands (VCAM-1/2 and E-Cadherin, respectively) found on subsets of potential target cells. CD103 is a particularly interesting candidate, as it has been shown to promote granule polarization and release toward E-Cadherin expressing tumor cells [128]. Previous studies indicate that synaptic forces enhance the pore forming activity of perforin by straining the target membrane [36, 33]. The integrin dependent targeting model described above would be expected to facilitate this process by directing perforin to mechanically active subdomains within the IS. Integrin-mediated mechanotransduction also provides an elegant mechanism for identifying regions of synaptic membrane that are tightly engaged with the target cell, where extensive mechanical coupling between directly opposing membranes would enable the CTL to “feel” the presence of the target by pushing or pulling against it. Guiding degranulation to these regions of close apposition would ensure that only the target is exposed to perforin and granzyme, thereby limiting damage to innocent bystander cells. Hence, using mechanically active integrins to license cytotoxic secretion likely promotes both the potency and the specificity of killing responses.

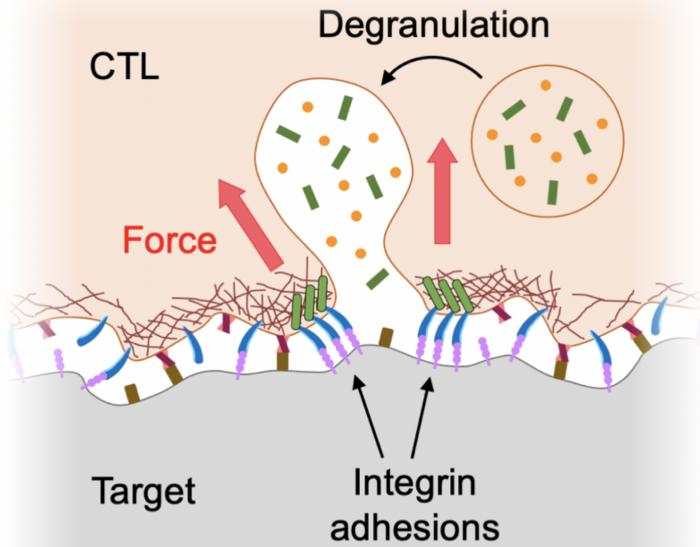


Figure 3.23: Mechanical licensing of degranulation by integrin adhesions.

Lytic granules fuse within synaptic subdomains defined by ligand-bound integrins under tension.

The centrosome and its associated microtubules are key determinants of lytic granule localization [25, 113]. Target recognition induces the trafficking of lytic granules along microtubules toward the centrosome, which concomitantly reorients to a position just beneath the center of the IS, thereby positioning granules close to the synaptic membrane. Although this mechanism is thought to promote polarized secretion, studies from multiple labs indicate that it is dispensable for the process [100, 114, 76]. Indeed, we have found that CTLs lacking a functional centrosome or even the entire microtubule cytoskeleton retain the capacity to release perforin and granzyme directionally into the IS [100]. The integrin licensing model described here explains these prior observations by providing an alternative targeting mechanism. That being said, our results do not exclude an important role for the centrosome and microtubules in enhancing the speed

and efficiency of cytotoxic secretion. Indeed, we have shown that microtubule depletion, while failing to disrupt the directionality of degranulation, nevertheless profoundly reduces the magnitude of the secretory response. Accordingly, we favor a model in which centrosome polarization delivers granules into the IS neighborhood, at which point their site of fusion is dictated by integrin licensing. The coupling of mechanical input to secretory output is unlikely to be unique to the cytotoxic IS. Indeed, one can imagine analogous mechanisms regulating other mechanically active processes, like phagocytosis and cell-cell fusion, that involve secretion and/or polarized membrane remodeling. We anticipate that biophysical analysis of systems like these will further illuminate the scope and functional relevance of mechano-secretory crosstalk in communicative cell-cell interactions.

3.4 Materials and Methods

3.4.1 Constructs

The retroviral expression construct for pHluorin-Lamp1 has been described²⁰. A CRISPR gRNA construct targeting talin was prepared according to a published protocol [129] using the following targeting sequence: 5'-GCTTGGCTTGTGAGGCCAGT-3'. A non-targeting control construct was also prepared using the sequence 5'-GCGAGGTATTGGCTCCGCG-3'. After PCR amplification, DNA fragments encoding these guide sequences were subcloned into the pMRIG vector using the BamHI and MfeI restriction sites.

3.4.2 Proteins

Class I MHC proteins ($H2-K^b$ and $H2-D^b$) were overexpressed in E. Coli, purified as inclusion bodies, and refolded by rapid dilution in the presence of β 2-microglobulin and either OVA (for $H2-K^b$) or KAVYDFATL (KAVY, for $H2-D^b$). Monomeric MHC proteins were biotinylated using the BirA enzyme and purified by size exclusion chromatography. The extracellular domain of mouse ICAM-1 (a.a. 28-485, polyhistidine-tagged) was expressed by baculoviral infection of Hi-5 cells and purified by Ni^{2+} chromatography. After BirA-mediated biotinylation, the ICAM-1 was further purified by size exclusion.

3.4.3 Cells

The animal protocols used for this study were approved by the Institutional Animal Care and Use Committee of Memorial Sloan Kettering Cancer Center. Primary CTL blasts were prepared by pulsing splenocytes from an OT1 $\alpha\beta$ TCR transgenic mice with 100 nM OVA in RPMI medium containing 10% (vol/vol) FCS. Cells were supplemented with 30 IU/mL IL-2 after 24 h and were split as needed in RPMI medium containing 10% (vol/vol) FCS and IL-2. RMA-s cells were maintained in RPMI containing 10% (vol/vol) FCS. B16F10 cells were maintained in DMEM medium containing 10% (vol/vol) FCS.

3.4.4 Traction force microscopy

Arrays of PDMS (Sylgard 184; Dow Corning) micropillars (0.7 μ m in diameter, 6 μ m in height, spaced hexagonally with a 2- μ m center-to-center distance) were

cast onto glass coverslips using the inverse PDMS mold method [130]. After an ethanol wash and stepwise exchange into phosphate buffered saline (PBS), pillars were stained with fluorescently labeled streptavidin (20 μ g/mL Alexa Fluor 647, Thermo Fisher Scientific) for 2 hours at room temperature. Following additional PBS washes, the arrays were incubated with biotinylated H2-K^b-OVA and ICAM-1 (10 μ g/mL each) overnight at 4°C. The pillars were then washed into RPMI containing 5% (v/v) FCS and lacking phenol red for imaging. T cells stained with Alexa Fluor 488-labeled anti-CD45.2 Fab (clone 104-2) were then added to the arrays and imaged using an inverted fluorescence microscope (Olympus IX-81) fitted with a 100× objective lens and a mercury lamp for excitation. Images in the 488-nm (CTLs) and 647-nm (pillars) channels were collected every 15 s using MetaMorph software.

3.4.5 Antibody blockade and pharmacolog. activation/inhibition

To assess the importance of LFA-1, CTLs were pre-incubated with LFA-1 blocking antibody (20 μ g/mL, Clone M17/4, BioXCell) or an IgG2ak isotype control antibody (20 μ g/mL, Clone RTK2758, BioLegend) at 37°C for 5 minutes before the addition of target cells/stimulatory beads. The final concentration of antibodies during the assay was 10 μ g/mL. To induce T cell activation independently of the TCR, CTLs were pre-incubated with phorbol myristate acetate (PMA, 20 ng/mL, Sigma Aldrich) and the Ca^{2+} ionophore A23187 (2 μ M, Tocris Bioscience) at 37°C for 5 minutes before the addition of target cells/stimulatory beads, yielding final concentrations of 10 ng/mL PMA and 1 μ M A23187.

3.4.6 Retroviral transduction

Phoenix E cells were transfected with expression vectors and packaging plasmids using the calcium phosphate method. Ecotropic viral supernatants were collected after 48 h at 37°C and added to 1.5×10^6 OT-1 blasts 24 h after primary peptide stimulation. Mixtures were centrifuged at 1400g in the presence of polybrene (4 μ g/mL) at 35°C, after which the cells were split 1:3 in RPMI medium containing 10% (vol/vol) FCS and 30 IU/mL IL-2 and allowed to grow for an additional 4-6 days.

3.4.7 Functional assays

To measure cytotoxicity, RMA-s target cells were labeled with CellTrace Violet (CTV), loaded with increasing concentrations of OVA, and mixed 3:1 with PKH26-stained OT-1 CTLs in a 96-well V-bottomed plate. Specific lysis of CTV+ target cells was determined by flow cytometry after 4 h at 37°C [91]. To quantify degranulation, OT-1 CTLs were mixed with RMA-s target cells as described above and incubated at 37°C for 90 min in the presence of eFluor 660-labeled anti-Lamp1 (clone eBio1D4B; eBioscience). Cells were then stained with FITC-labeled anti-CD69 (clone) and subjected to flow cytometric analysis to quantify Lamp1 and CD69 staining. To measure conjugate formation, labeled OT-1 CTLs and RMA-s targets were mixed 1:1, lightly centrifuged (100g) to encourage cell contact, and incubated 20 min at 37°C. Cells were then resuspended in the presence of 2% paraformaldehyde, washed in fluorescence-activated cell sorting buffer (PBS + 4% FCS), and analyzed by flow cytometry. Conjugate formation was quantified as $(\text{PKH26+CTV+}) / (\text{PKH26+})$. For B16F10 killing assays, B16F10 targets were

cultured overnight on fibronectin and then pulsed with varying concentrations of OVA for 2 hours. OT1 CTLs were added at an 8:1 E:T ratio and incubated for 3-4 hours at 37°C in RPMI medium supplemented with IL-2 (30 IU/mL). Target cell death was quantified with an LDH (lactate dehydrogenase) cytotoxicity assay kit (Clontech) using the manufacturer's recommended protocol. To measure intracellular granzyme B depletion, OT1 CTLs were mixed 1:3 with OVA-loaded RMA-s cells and incubated for 4-6 hours at 37°C. Intracellular granzyme B levels were then measured by flow cytometry after fixation, permeabilization, and staining with Alexa 647 labeled anti-granzyme B (clone GRB11, Biolegend). All functional assays were performed in triplicate. To quantify ICAM-1 expression, RMA-s or B16F10 cells were stained with a fluorescently labeled anti-ICAM-1 antibody CD54 (Clone YN1/1.7.4, BioLegend) or isotype control antibody (Clone RTK4530, Biolegend).

3.4.8 CTL activation with stimulatory beads

Streptavidin-conjugated polystyrene beads (Spherotech) were coated with 1 μ g/mL biotinylated ICAM-1 and/or various concentrations of biotinylated H₂-K^b-OVA. Nonstimulatory pMHC (H₂-D^b) was used if necessary to adjust the total biotinylated protein concentration of each mixture 2 μ g/mL. After overnight incubation at 4°C, excess unbound protein was washed out and the beads were transferred into RPMI medium containing 10% (vol/vol) FCS (+/- phenol red) for use in experiments. For immunoblot analysis of signaling, beads were mixed with OT-1 CTLs at a 1:1 ratio. For functional studies (e.g. degranulation), the CTL to bead ratio was 1:3. For 2 bead stimulation (Fig. 2a-c), Nile Red and Purple streptavidin beads were coated as described above with H-2Kb-OVA and

ICAM-1 in either cis or trans configurations. H-2Db was used to fill empty spaces in the trans configuration and also to generate “dummy” coated beads. In each experimental condition, CTLs were mixed with two kinds of beads at a 1:3:3 ratio. Degranulation and CD69 upregulation were quantified by flow cytometry as described above.

3.4.9 Micropatterning experiments

PDMS stamps for imprinting 2 μm in diameter spots in 10 μm center-to-center square arrays were prepared using microfabricated silicon masters as previously described¹⁹. Stamps were washed in ethanol and water, then coated with fluorescently-labeled streptavidin (10 $\mu\text{g}/\text{mL}$ Alexa Fluor 647, Thermo Fisher Scientific) for 1 hour at room temperature. After PBS washing to remove excess proteins, the stamps were pressed onto 35 mm glass coverslips (# 1.5) to transfer the streptavidin. Coverslips were then incubated with the following unbiotinylated proteins to coat the spaces between streptavidin dots: (1) ICAM-spot – 10 $\mu\text{g}/\text{mL}$ H2-K^b-OVA, (2) Antigen-spot – 10 $\mu\text{g}/\text{mL}$ ICAM-1, (3) Dual-spot – 5% BSA, (4) ICAM-spot with anti-CD3 background – 10 $\mu\text{g}/\text{mL}$ anti-CD3 antibody (Clone 145-2C11, eBioScience), (5) Anti-CD3-spot – 10 $\mu\text{g}/\text{mL}$ ICAM-1, and (6) control – 10 $\mu\text{g}/\text{mL}$ unlabeled streptavidin. After 1 h at room temperature, coverslips were rinsed with PBS 3 times to wash away uncoated protein and blocked with 5% BSA at room temperature for 1 hour before another round of PBS washes. Coverslips were then incubated with the following biotinylated proteins to load the streptavidin: (1) ICAM-spot – 2 $\mu\text{g}/\text{mL}$ ICAM-1, (2) Antigen-spot – 2 $\mu\text{g}/\text{mL}$ H2-K^b-OVA, (3) Dual-spot – 2 $\mu\text{g}/\text{mL}$ H2-K^b-OVA and 2 $\mu\text{g}/\text{mL}$ ICAM-1, (4) ICAM-spot with anti-CD3 background – 2 $\mu\text{g}/\text{mL}$ ICAM-1, (5) Anti-

CD3-spot – 2 µg/mL anti-CD3 antibody (Clone 145-2C11, eBioScience), and (6) control – 2 µg/mL H2-K^b-OVA and 2 µg/mL ICAM-1. After 1 h at room temperature, the surfaces were washed into RPMI containing 5% (v/v) fetal calf serum (FCS) and lacking phenol red for imaging. Cells were then added and imaging performed using either a Leica SP5-inverted confocal laser scanning microscope fitted with 488 nm, 563 nm, and 647 nm lasers, or a Leica SP8-inverted confocal laser scanning microscope fitted with a white light laser. In general, samples were imaged every 15 s for 30 min.

3.4.10 Ca²⁺ imaging

CTLs were loaded with 5 µg/mL Fura2-AM (ThermoFisher Scientific), washed, and then imaged on stimulatory glass surfaces coated with H2-K^b-OVA and ICAM-1 as previously described [8]. 340 nm and 380 nm excitation images were acquired every 30 seconds for 30 min using a 20x objective lens (Olympus).

3.4.11 DNA hairpins

Name Sequence (5' to 3')

A21B - /5AmMC6/- CGC ATC TGT GCG GTA TTT CAC TTT - /3Bio/

Quencher strand - /5DBCON/- TTT GCT GGG CTA CGT GGC GCT CTT - /3BHQ_2/

Hairpin strand - GTG AAA TAC CGC ACA GAT GCG TTT GTA TAA ATG
TTT TTT TCA TTT ATA CTT TAA GAG CGC CAC GTA GCC CAG C

A mixture of oligo A21B (10 nmol) and excess Cy3B-NHS ester or Atto647N-NHS ester (50 μ g) in 0.1 M sodium bicarbonate solution was allowed to react at room temperature overnight. The derivatized oligo was then purified by gel filtration and reversed phase HPLC.

3.4.12 MTP surface preparation and imaging

Glass coverslips (# 1.5H, Ibidi) were sonicated in MilliQ H₂O and ethanol, rinsed in H₂O, and then immersed in piranha solution (3:1 sulfuric acid:H₂O₂) for 30 min to remove organic residues and activate hydroxyl groups on the glass. Subsequently, the cleaned substrates were rinsed with more H₂O and ethanol and then transferred to a 200 mL beaker containing 3% APTES in ethanol for 1 h, washed with ethanol and baked at 100 °C for 30 min. After cooling, slides were mounted to 6-channel microfluidic cells (Sticky-Slide VI 0.4, ibidi). To each channel, 50 μ L of 10 mg/mL of NHS-PEG4-azide in 0.1 M NaHCO₃ (pH = 9) was added and incubated for 1 h. The channels were then washed with H₂O, blocked with 0.1% BSA in PBS for 30 min, and washed with PBS. 50 μ L of PBS solution was retained inside the channel after washing to prevent drying. Subsequently, the hairpin tension probes were assembled in 1M NaCl by mixing the Atto647N labeled A21B strand (220 nM), quencher strand (220 nM) and hairpin strand (200 nM) in the ratio of 1.1: 1.1:1. The mixture was heat annealed by incubating at 95°C for 5 min, followed by cool down to 25°C over 30 min. 50 μ L of the assembled probe was added to the channels (total volume = 100

μL) and incubated overnight at room temperature. The following day, unbound DNA probes were removed by PBS wash. Then, 10 $\mu\text{g}/\text{mL}$ of streptavidin was incubated in the channels for 45 min at room temperature. The surfaces were cleaned with PBS and incubated with 5 $\mu\text{g}/\text{mL}$ of biotinylated pMHC ligand for 45 min at room temperature. After PBS washing, a second DNA tension probe (Cy3B labeled) was assembled and attached as described above, followed by loading with streptavidin and 5 $\mu\text{g}/\text{mL}$ of biotinylated ICAM-1. Monomeric ICAM-1 was used for pHluorin-Lamp1 experiments (Fig. 3) and dimeric Fc-ICAM1 for talin-KO experiments (Fig. 4). After washing off the unbound ICAM-1 protein, surfaces were rinsed in complete RPMI (no phenol red, supplemented with IL-2) in preparation for imaging with CTLs. MTP imaging was performed on a Nikon Eclipse Ti microscope attached to an electron multiplying charge coupled device (EMCCD; Photometrics), an Intensilight epifluorescence source (Nikon), a CFI Apo 100x (NA 1.49) objective lens (Nikon), and a TIRF launcher with 488 nm, 561 nm, and 638 nm laser lines. In general, IRM, 488 nm, 561 nm, and 638 nm images were collected every 20 s for 30 min. TIRF illumination was used to image pHluorin-Lamp1 and epifluorescence to image the MTPs.

3.4.13 Imaging analysis

Imaging data were analyzed using SlideBook (3i), Imaris (Bitplane), Excel (Microsoft), Prism (GraphPad), and Python in Jupyter Notebook [131]. Ca^{2+} signaling was quantified by determining the mean Fura2 ratio for all cells in the imaging field using a mask thresholded on the 340 nm excitation signal. To quantify force exertion in traction force microscopy experiments, custom MATLAB scripts were used to extract pillar displacements from the imaging

data, which were then converted into force vectors. To measure the distance between degranulation events and the closest streptavidin spot on micropatterned surfaces, pHluorin-Lamp1 and streptavidin Alexa Fluor 647 signals were converted into Imaris spot constructs using Imaris scripts. The distances (μm) between each pHluorin-Lamp1 signal of interest and the closest streptavidin spot within the synaptic boundary of the CTL were then determined using the Imaris ‘Shortest Distance’ function. The expected distance between randomly placed degranulation events and ligand spots was determined in silico. First, the unit cell of the micropattern was modeled as a $5\ \mu\text{m} \times 5\ \mu\text{m}$ square with a quarter-circle representing the stamped protein at one corner. Then, the unit square was divided into 1×10^6 points (evenly sampling 10-nm spaces in both x and y), and the Euclidean distance of each point to the stamped protein corner was calculated. The mean distance of this distribution is $3.8266\ \mu\text{m}$. MTP data were analyzed by comparing the mean fluorescence intensity of each MTP within the $2\ \mu\text{m} \times 2\ \mu\text{m}$ box centered on a pHluorin-Lamp1 signal of interest with the mean fluorescence intensity of the MTP within the entire IS, defined by threshold masking of IRM images (Fig. 3d). Linescan analysis of ICAM-1-MTP fluorescence at degranulation sites (Fig. 3e) was performed by generating a series of $2\ \mu\text{m}$ linescans bisecting degranulation events of interest. The linescan intensities were aligned around the degranulation, averaged over each pixel, and normalized per linescan. An analogous set of control linescans, collected from parts of the IS lacking degranulation events, were processed in parallel using the same scripts.

3.4.14 Proliferation Assay

Day 7 OT-1 CTLs were stained with CellTrace Violet at room temperature for 20 min, washed in serum containing medium, and then incubated with irradiated OVA-loaded C57BL/6 splenocytes (0.5×10^6 CTL with 4.0×10^6 splenocytes) the presence of $10 \mu\text{g}/\text{mL}$ anti-LFA-1 or an isotype control antibody. Subsequent dilution of CellTrace Violet was assessed by flow cytometry.

3.4.15 Immunoblot

$0.2\text{-}1 \times 10^6$ CTLs were lysed using cold cell lysis buffer containing 50 mM TrisHCl, 0.15 M NaCl, 1 mM EDTA, 1% NP-40 and 0.25% sodium deoxycholate. Suppression of talin 1 was confirmed using an anti-talin 1 antibody (clone 8D4, Abcam). Actin served as a loading control (clone AC-15, Sigma). For signaling assays, serum and IL-2 starved OT1 CTLs were incubated with streptavidin polystyrene beads (Spherotech) coated with H2-K^b-OVA and ICAM-1 at a 1:1 ratio for various times at 37°C and immediately lysed in 2x cold lysis buffer containing phosphatase inhibitors (1 mM NaF and 0.1 mM Na3VO4) and protease inhibitors (cOmplete mini cocktail, EDTAfree, Roche). Activation of PI3K and MAP kinase signaling was assessed by immunoblot for pAkt (Phospho-Akt (Ser473) Ab; Cell Signaling Technology) and pErk1/2 (Phospho-Thr202/ Tyr204; clone D13.14.4E; Cell Signaling Technology).

CHAPTER 4

DISCUSSION

4.1 Conclusion

For my doctoral work, I performed research investigating the mechanoregulation of cytotoxic T lymphocyte killing. Together, we found that CTLs form synaptic actin protrusions against target cells [33]. This act sensitizes the target cell to perforin lysis and granzyme entry, heightening the overall cytotoxic effector response. This potentiation is molecularly controlled by the actin nucleation promoting factors WASP and WAVE2, which are concentrically organized (from the center to the periphery, respectively). WASP promotes central actin protrusions associated with lytic granule fusion and content release, while WAVE2 is linked with peripheral actin protrusions and robust conjugate formation. Phenotypically, the increased application of force against targets is associated with an overall better effector response. This biomechanical force-effector relationship again appears in our model of degranulation, in which T cells use mechanical signals via integrins to home their lytic granules towards synaptic 'hotspots' in a novel manner that is distinct from their canonical signaling capacity through focal adhesions. These synaptic sites of granule fusion are immunologically verified to be foreign and accordingly sensitized to perforin. Although our data supports this model and is in line with published work in the field, there are key outstanding questions to be answered. A discussion of some of these questions and how the results of my thesis work might inform future studies follows.

4.1.1 Integrin signaling in focal adhesions

We established a necessary mechanical requirement for integrin-mediated degranulation, but much work remains to be done in characterizing how other molecules in focal adhesion machinery contribute to degranulation. Vinculin is a cytoskeletal molecule that consists of a head region that binds to talin or α -actinin, and a tail region that binds to f-actin, lipids, and paxillin [132]. Vinculin is regulated by phosphoinositide expression and reinforces the connection between talin and the f-actin cytoskeleton, stabilizing the mechanical action on integrins and talin [133, 132]. As the disruption of vinculin activity is known to affect the capacity of T cells to form proper focal adhesions and adhere [132], it would be a good place to begin investigation of focal adhesion machinery to degranulation using genetic means of perturbation and measuring force exertion and cytotoxic capacity using similar methods and techniques as described in Chapter 3 (e.g. CRISPR, DNA MTP force experiments, TCR signaling, etc.). Of course, any of the other components of focal adhesions are also fair game for study.

In Chapter 3, we hypothesized that the outside-in mechanical activity of LFA-1 drove lytic granule fusion at the synapse. However, one outstanding question is the respective contributions of inside-out vs. outside-in signaling of integrins to degranulation homing. As we used methods that would abolish both inside-out and outside-in signaling, experiments that only perturb the outside-in signaling would be necessary. One possible method could involve the rescue of inside-out signaling after talin knock out via CRISPR by overexpressing the talin-head domain only which has been carefully characterized in the literature to rescue inside-out signaling [134, 135, 136]. The talin head domain will bind to the cytosolic tail of the β -integrin domain and trigger inside-out signaling (see

refig:lfa for further detail) but be unable to bind the actin cytoskeleton, which is mediated by the tail domain. We predict that T cells that have been depleted for talin but express the talin-head domain will have active conformation LFA-1 on the surface and be able to adhere and form synapses, but be unable to home and fuse lytic granules at the synapse. Other alternative approaches could involve the use of the mangnese ion (Mn^{2+}) , which is known to stabilize the MIDAS domain of LFA-1 [137, 138, 139], to stablize the LFA-1/ICAM-1 function and yield more mechanical force, possibly enhancing degranulation.

4.1.2 Optimizing degranulation to escape T cell suicide

The observation that T cells tend to granulate at pillar tops (see Figure 2.9) aligns with existing knowledge in the field that lytic granules require actin hypo-dense/cleared regions for membrane fusion [11]. However, the transient burst of f-actin associated with the moment of degranulation (Figure 2.9) is ripe to consider. It could be associated with a burst of local mechanopotentiating synaptic force, generating actin-flow mediated traction force to potentiate integrin granule homing (Figure 3.17). It could also be involved in actively polymerizing f-actin polymers, creating actin-rich micro-protrusions/structure at a resolution scale below detection via confocal microscopy. FRET-based actin polymerization probes could be used to interrogate if T cells were creating microdomains of actin polymerization at sites of degranulation, as Lifeact-GFP only detects mature f-actin. Other strategies involve photobleaching f-actin and watching its recovery (fluorescence recovery after photobleaching, or FRAP) [140] relative to where cells degranulate, or using a fluorescent WASP, WAVE2, or Arp2/3

probe to monitor the relative location of actin nucleation promoting factors to lytic granule secretion [141, 33].

Additional cytotoxic benefits may also be realized by degranulating at the base of actin-rich protrusions. Given the the concave orientation of the protrusion 'pit' relative to the corresponding target cell surface, we hypothesize that the perforin molecule preferentially binds to convex lipid membrane curvatures, due to its the asymmetric, "key-like" sterics [26]. We could directly test this hypothesis by adapting gramicidin-based techniques from studies on fluorescence-based small unilamellar vesicles (SUVs) [142, 143]. Lipids will be constituted in buffer containing the fluorophore 8-Aminonaphthalene-1,3,6-Trisulfonic Acid (ANTS) and one of its quenchers p-Xylene-Bis-Pyridinium Bromide (DPX) to reform into large multilamellar vesicles (LMVs). Preference for convexity will be tested by treating the fluorescent SUVs with perforin, triggering oligomerization with calcium in the reaction buffer, and then measuring the amount of liberated fluorescence as a marker for pore formation. We expect the group in which perforin engages a convex lipid membrane to liberate more fluorophore than the group in which perforin engages a concave lipid membrane. Preference for concavity will be tested by constituting perforin into SUVs and triggering oligomerization and fluorophore leakage using A23187, a small molecule calcium ionophore (this ionophore can similarly be added to the convexity sample for full control). Should it bear out that perforin has a preference for convex lipid membranes, it might potentially explain how T cells avoid being killed by their own secreted perforin, which remains an outstanding question in the field.

4.1.3 SNARE complex crosstalk to integrins

SNARE proteins are a large family of proteins that are responsible for mediating vesicle fusion and are directly responsible for the fusion of lytic granules [92]. SNARE proteins are localized to the vesicle membrane or target membranes and are called v- and t-SNARES, respectively [144]. Synaptobrevin2 is the v-SNARE and syntaxin11 is the t-SNARE that mediates lytic granule fusion in cytotoxic lymphocytes [145, 146, 147]. The Habc domain of the Q-SNARE domain folds back and allows the interaction between the v-SNARE and the t-SNARE, forming a *trans*-SNARE complex [144]. The SNARE activating function is regulated by Munc18 [148, 144, 149]. Finally, the R-SNARE binds to the trimeric complex, forming a mature SNARE complex that works as a molecular zipper to bring the vesicle and plasma membranes closer together for fusion [150].

It is likely that the finely tuned LFA-1 mediated mechanical homing of degranulation is communicated to the t-/v-SNARE complex, as SNARE complexes themselves are mechanosensitive [151]. One group observed that neuronal SNARE complexes placed under tension (around 10 pN) can maintain a partially-assembled form in which the v- and t-SNARES are in a 'frayed' state [152]. The observed mechanoregulation of SNARE complexes suggests that their mechanical activity can lead to increased biological activity (higher formation of the fusion stalk and vesicle fusion), but has yet to be directly tested in immune/T cells. There are reports of functional associations between the SNARE complex and integrin-mediated recycling at the membrane [153, 154, 155], but whether or not any signaling molecules liase in-/directly between the two signalosomes remains unclear and would be a fecund area for future study.

To investigate possible integrin mechano-crosstalk to the SNARE complex, we could investigate the Munc-family group of proteins, known regulators of t-/v-SNARE fusion [156, 144]. Two possible candidates to study are Munc13-1 and Munc18-1 proteins, which are in direct control of the assembly of SNARE complex components [157] and have been studied extensively in neuronal synapses. In neurons, Munc18-1 activity is directly regulated by diacylglycerol and PKC θ , bearing two PKC θ phosphorylation sites that, when phosphorylated, induces fusion of vesicles bearing neurotransmitters [158, 155, 159]. Munc13-1 bears a C1 diacylglycerol binding domain [160] and markedly accelerates the kinetics of this interaction and vesicle fusion [156, 160]. Whether or not outside-in signaling from integrins affects SNARE fusion could be tested by knocking out talin in T cells (using the gRNA we characterized in Chapter 3) profiling the phosphorylation of the Munc18-1 protein against T cells transduced with a non-targeting control gRNA. Additionally, one could track the localization of integrins and Munc13-1 at the synapse (using fluorescent probes) in response to a photoactivated TCR [161], which is known to induce diacylglycerol localization [8]. If there is crosstalk, photoactivation of the TCR should induce integrin localization. One could even test the mechanical strength's influence over Munc13-1 (and by proxy, SNARE localization) by generating photoactivatable agonists on peptides of different TCR signal strengths (i.e. different primary sequences), which are known to induce different mechanical strengths [162, 163, 164, 165]. These experiments would be illuminating to see if mechano-regulation of integrins directly affects SNARE complex formation and function for lytic granule fusion.

4.1.4 TCR signaling strength and integrin-mediated degranulation

The mechanical activity of integrins is regulated by TCR signaling strength, as force experiments in our lab and others on force-measuring surfaces without stimulatory peptide have indicated [164, 166, 167, 168]. Different peptides presented in MHC are capable of stimulating different TCR signaling strengths as well [169]. These observations lead us to ask how TCR signaling strengths affect integrin clustering and if there is a minimal number of integrin clusters needed for degranulation. Experimental techniques designed to ask the same question of minimal number of TCRs needed for T cell activation could be adapted to answer this question, such as restricting the clustering of integrins to test minimal requirements for degranulation [170, 171, 172].

Another outstanding question is how physiologically relevant the OT-1/OVA-SIINFEKL peptide system is, given its high TCR signaling strength [169]. For this question, one could repeat the experiments outlined in Chapter 2 and Chapter 3 using a model peptide system more similar to what T cells encounter *in vivo*. However, given that mechanical defects in human cells also produce lowered immune responses (such as in Wiskott-Aldrich syndrome or HIV [77]), we expect the results to be similar.

4.1.5 Fas-ligand mediated cytotoxicity

Although the T cell primarily kills through the secretion of perforin and granzyme [173], T cells have other ways in killing target cells as well. A well-

known alternative pathway is the Fas ligand (FasL) pathway. Binding of FasL against the Fas receptor (FasR, or CD95) leads to the formation of a trimeric "death-inducing signaling complex"(DISC) FasL-FasR complex [174]. Upon aggregation of DISCs and corresponding FasL-FasR downstream signaling, caspases 2, 3, 8, and 9 are released leading to DNA degradation, membrane blebbing, and apoptosis [175, 174]. Fascinatingly, Fas ligand has been observed to localize to cytotoxic granules containing perforin and granzyme [176], with some groups even reporting a distinct granule subsets that contain Fas but no perforin or granzymes [92, 177, 178].

From the perspective of this study, a natural question to consider is whether or not subpopulations of granules are segregated mechanically and for what purpose they might be. Probably the first pair of questions to investigate is whether or not Fas ligand or Fas receptor signaling is mechanically regulated, which could be studied using the techniques used in this study. One study has observed co-immunoprecipitation of FasR with ezrin, a known linker molecule to the actin cytoskeleton, suggesting that FasR could be mechanically regulated [175]. To directly test this hypothesis, one could imagine an experiment involving placing Fas-receptor positive target cells expressing fluorescent probes for the Fas-receptor mediated apoptosis pathway (e.g. caspases) onto traction force microscopy-compatible micropillars (thinner). These pillars could be coated with adhesive proteins and Fas ligand, which could trigger FasL-mediated apoptosis. Colocalization studies of caspases against areas of target cell force exertion could be informative.

4.2 Future directions

This study attempted to comprehensively cover the mechanobiology of cytotoxicity (Chapter 2) and lytic granule secretion (Chapter 3), which are important but still ultimately only one region of the T cell field of study. What follows is an informed discussion of the future of mechanobiology as it pertains to *other* T cell fields of study, and how the author speculates how the field of mechanobiology might change in the years to come.

4.2.1 Population-scale CTL force dynamics

Going forward, it would be interesting to investigate dynamics of synaptic force exertion in a single CTL against a target cell. In particular, one wonders if the CTL at first pass deploys a standard range of force exertion and protrusion-type or cellular structure (in a "probing phase"), and then must dynamically change its range of force exertion or arsenal of structures for force exertion according to local biostructural architecture (i.e. what is the lifetime force action of a standard CTL encounter?). Certainly these events are correspondingly influenced by the signaling molecules present at the synapse, each of which live their own biophysical lives by mere virtue of their presence. Answering such questions would require direct interrogation of a live CTL-target cell synapse. Previous technologies for measuring membrane tension include atomic force microscopy or an optical trap - but both remain insufficiently precise to monitor real-time *in vitro* or *in vivo* synapse dynamics. Certain fluorescence-based technologies have been recently been developed in order to measure synapse dynamics that ignore restrictive planar geometries [22]. Combined with fluorescent probes, the use of

these microparticles could elucidate "phases" of the T cell physical response as it (putatively) probes, adheres, degranulates, kills, and releases, the target cell.

Thinking outside the context of single-T cell force exertions, it would be fascinating to investigate the host CTL-ome at large, if certain "battalions" or "legions" of CTLs exist, organized according to their physical strengths and effector capabilities. Certainly a reasonable first-pass method of organization is expression levels of certain effector genes and molecules (e.g. *Prf*, *Grzb*, *Ifng*), but these measures obviously omit the mechanoregulation of these molecules and thus miss their effects in totality. One would require experimental techniques capable of not only measuring single-cell T cell force exertion *en masse*, but also the possible swarm behavior of responding T cells in which an initial "less capable" group of T cells is replaced by stronger effector cells (or weaker, in a "clean up" phase). Certain molecular perturbation platforms that are designed to measure CRISPR-based perturbations at the protein level [179] come closer to characterizing all of the effects that a molecular perturbation truly encompasses. Other platforms for monitoring infiltrating immune cells into locally perturbed regions of the tumor microenvironment also exist [180]. One could imagine choosing a tumor system for this platform that has been spatially perturbed heterogeneously for known cytoskeletal/cell stiffness molecules and investigate how T cells of well-characterized strengths segregate (and presumably kill). Characterizing T cell swarm or group behavior would be a massive undertaking that would take many years to perform.

Many of the experiments conducted in order to complete this doctoral thesis were performed using *in vitro* systems. These systems were designed to interrogate force activity by holding the force threshold constant (e.g. micropillars,

DNA membrane tension probes), while observing the cell's force activity in time. While these arrangements are useful for ease of investigation, holding constant a variable as broad as the force threshold creates major scientific blind spots even in real-time studies (much less bulk studies via co-culture assays). This is true particularly in studies focused on the effector cell, in which the arrangement ignores half of the cellular conversation: the target cell [181]. The target cell is capable of dynamically responding to the effector cell's efforts to kill (presumably in a cooperative or antagonistic manner), which is entirely neglected in these experimental systems.

4.2.2 The mechanobiology of exhausted T cells

To date, there has been no direct study of mechanobiology of exhausted T cells. Nevertheless, given what we know about the immunological behavior of exhausted T cells and how mechanobiological principles influence these behaviors in other T cell contexts (such as naïve, proliferating, or activated T cells), we can comfortably speculate on how we might think mechanical principles might influence exhausted T cell (dys)function.

The label 'exhausted T cells' can be quite wide, but general agreement can be found in a definition that includes a molecular signature of increased inhibitory receptor expression, such as B lymphocyte induced maturation protein 1 (Blimp-1), basic leucine zipper transcription factor, ATF-like (BATF), nuclear factor of activated T cells (NFAT), T-box expressed in T cells (T-bet), eomesodermin (Eomes), and PD-1 (programmed cell death protein 1) [182, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192]. In particular, exhausted T cells are strongly associated

with a $PD - 1^{hi}$ phenotype [184]. Persistent antigen stimulation in the context of chronic infection triggers epigenetic alternations to the *Pdcd1* locus, resulting in this higher surface expression of the PD-1 inhibitory receptor. The PD-1/PD-L1 signaling axis is known to attenuate TCR activation [193], proliferation [194], and T cell migration [195]. Although direct evidence is needed, it seems reasonable to suggest that exhausted T cells might display diminished physical capacity, further dampening their effector capabilities. It is likely, as TCR mechanical signaling is a known requirement for T cell activation [115].

Exhausted T cells also present a diminished metabolic state. Accordingly, it is likely that their effector capacity is severely weakened, considering that cytoskeletal remodeling is a highly energy intensive activity [196]. Reports on the energy dynamics of actin-polymerization inhibited cells indicate that ATP consumption is up to 50% slower [196, 197]. Depletion of glucose/ATP from the medium also immobilizes the motor protein myosin [198], which is known to be involved in potentiating lytic granule fusion [36].

4.2.3 Mechanically enhancing the success of engineered T cells

There has been some study of the CAR immune synapse, although it remains in the preliminary stages and the relationship between the unique organization of the CAR IS and CAR efficiency have yet to be fully explored. Most strikingly, the CAR IS does not present an annular bull's eye structure, which is a characteristic feature of TCR IS. The organization of the actin ring in CAR IS is poor, and actin may not be completely diminished at the center of CAR IS [199], which may disrupt smooth granule secretion. LFA-1 is disorganized and MHC-bound CAR

microclusters are randomly distributed at the CAR IS [200]. While TCR IS forms a bull's eye's structure in 5–10 min, the CAR IS induces significant CAR-proximal signaling much faster (<2 min), suggesting that CARs may not need to form these stable structures.

Another important part of IS biology is the delivery of cytotoxic granules, including perforin and granzymes, to the IS mediated by MTOC [201]. The rapid proximal signaling of CAR IS also induces MTOC migration to the IS and accelerates the delivery of granules [200]. Although the mechanisms of CAR IS have gradually been revealed, it is still unclear whether the differences in CAR IS structure correlate with the efficacy of CAR T cells [202, 203].

Much work remains to be done to achieve the full potential of CARs as a sure and accessible therapy. Despite significant public and private sector effort in designing and developing novel cancer immunotherapies, these efforts are almost ubiquitously underappreciate the biomechanical aspects of T cell killing, which play a large role in their cytotoxicity. In addition to their effector function, T cells participate in mechanical communication throughout their entire lifecycle, surveying organs and tissues for foreign cells and experiencing both harsh and forgiving physical environments such as the shear force of lymphatic transport or the broad range tissue stiffnesses that exist within the body. These biomechanical properties are significantly altered in disease states. T cells take significant cues from their mechanical environment in their activity, altering their development, differentiation, migration, activation, and effector functions, all of which can tip the balance of successful vs. unsuccessful immune system clearance of pathogens or cancers. Biomechanical communication has been extensively studied at a fundamental research, but the knowledge yielded has

yet to reach a significant level of incorporation in immunotherapy design for both knowledge-based and practical reasons. Genetically modifying the cancer cell is near impossible, and the *ex vivo* modification of T cells remains exceedingly expensive and inaccessible.

Nonetheless, growing knowledge of T cell mechanobiology has enabled the creation of mechanically-based engineering strategies to modulate T cell immunity, a nascent field termed “mechanical immunoengineering”. Mechanical immunoengineering takes advantage of biomechanical cues such as stiffness and external forces in order to modulate T cell proliferation and effector functions for therapeutic applications. Of course, this is in complement to well-established modes of modulating biochemical cues such as antigen recognition (chimeric antigen receptors) and co-stimulatory signals (checkpoint blockade) that curate the anti-tumor T cell immune response and enhance therapeutic efficacy. For example, stiffer antigen-presenting matrices have been shown to enhance T cell proliferation independently of the intensity of biochemical stimulatory signals [204] and can be adapted and used *ex vivo* in order to improve patient outcomes. Fabrication of planar surfaces presenting T cell ligands has demonstrated that focal clustering of TCR can be inhibited by presenting antigens in a hollow ring configuration, which in turn reduces IFN γ and proliferation of T cells [205]. In contrast, TCR clustering signaling can be promoted by introducing regions of ligand for multi-valent receptor binding on the activating surface, which boosts the effector function of T cells [206, 205]. These studies suggest that spatial organization of the TCR clusters control the T cell response, which should strongly inform the design of these immunoengineering strategies [207].

A recent report details the development of a CAR T cell that upon activation, secretes an enzyme that cleaves the administered pro-drug for localized drug administration [208]. This platform could be adapted in other immune cells in order to make the local immune environment more stiff, thereby mechanically potentiating T cell activation and effector function.

4.2.4 On nutrient mechano-sensing

Nutrient sensing is a necessary activity for cell survival and is a meticulously regulated process. mTOR (the mammalian target of rapamycin) is a master regulator of nutrient sensing and receives significant research interest [209]. It is a kinase that upon activation promotes anabolism and macromolecule synthesis. mTOR can be found on lysosomes [210] and localizes to focal adhesions [210]. Focal adhesions are major nutrient-sensing sites/hubs [211] because of their proximity to endo-/exosomal membrane fusion-related traffic. In this sense, it is significantly advantageous for mTOR to position itself at the membrane in order to sense and metabolize amino acids [212] and other nutrients.

Since FAs are mechanically active, are sites of mTOR localization, and that mTOR is in close proximity to talin in focal adhesions (<10nm) [213], an exciting possibility to consider is that mTOR is mechanically driven to home towards focal adhesions. The questions raised in subsection ?? reappear - what mechanosensitive molecules are homing mTOR to focal adhesions? What molecules dynamically regulate the level of mTOR activity with corresponding mechanical force? These are all exciting potential lines of investigation. The purpose of such an arrangement within T cells might be to ensure contact with a foreign and dying

cell, so that its nutrients might be scavenged or used for mTOR signaling, but this is only speculative.

4.2.5 Nuclear mechanotransduction

The T cell nucleus is mechanosensitive as well, which is not surprising given its substantial size in T cells [214], taking up almost 90% of the cell volume [215]. T cell forces are mechanotransduced through the lamina, a network of lamin and lamin-binding proteins that are anchored to the actin cytoskeleton [216, 217, 218]. When T cells migrate, squeezing and stretching can deform not only the plasma membrane, but also the nucleus, which triggers mechano-sensitive receptors located on the nuclear membrane [218, 219]. The mechanical tension applied to these receptors modify the heterochromatin state of cellular DNA, and transcription activity of the T cell [220]. This mechanical tension can also trigger the proinflammatory eicosanoid pathway [217], which is typically activated in wound healing contexts.

Eicosanoids are proinflammatory signaling molecules that regulate a number of T lymphocyte functions [221]. There is significant therapeutic focus in targeting these pathways, and future research may benefit from incorporating mechanical dimensions of T cell activation into their therapeutic strategies.

4.3 *La fin*

A number of outstanding questions surrounding T cell mechanobiology remain open for investigation. These questions remain unanswered today for

either knowledge or technically-based reasons, but they will surely be answered in due time.

BIBLIOGRAPHY

- [1] Martin F. Flajnik and Masanori Kasahara. Origin and evolution of the adaptive immune system: Genetic events and selective pressures. *Nature Reviews Genetics*, 11(1):47–59, 2010.
- [2] P. W. Dempsey, S. A. Vaidya, and G. Cheng. The Art of War: Innate and adaptive immune responses. *Cellular and Molecular Life Sciences*, 60(12):2604–2621, 2003.
- [3] Martin Heidegger. Sein und Zeit. pages 114–129, 1927.
- [4] Uzma Alam. Immunity: The Immune Response to Infectious and Inflammatory Disease. *The Yale Journal of Biology and Medicine*, 80(3):137–137, 2007.
- [5] Ronald N. Germain. t-cell development and the CD4-CD8 lineage decision. *Nature Reviews Immunology*, 2(5):309–322, 2002.
- [6] Ed Palmer. Negative selection - Clearing out the bad apples from the T-cell repertoire. *Nature Reviews Immunology*, 3(5):383–391, 2003.
- [7] Rebecca J. Brownlie and Rose Zamoyska. T cell receptor signalling networks: branched, diversified and bounded. *Nature Reviews Immunology* 2013 13:4, 13(4):257–269, mar 2013.
- [8] E J Quann, E Merino, T Furuta, and M Huse. Localized diacylglycerol drives the polarization of the microtubule-organizing center in T cells. *Nat Immunol*, 10(6):627–635, 2009.
- [9] Jane C. Stinchcombe, Endre Majorovits, Giovanna Bossi, Stephen Fuller, and Gillian M. Griffiths. Centrosome polarization delivers secretory granules to the immunological synapse. *Nature*, 443(7110):462–465, 2006.
- [10] Andrea Maul-Pavicic, Samuel C.C. Chiang, Anne Rensing-Ehl, Birthe Jessen, Cyril Fauriat, Stephanie M. Wood, Sebastian Sjöqvist, Markus Hufnagel, Ilka Schulze, Thilo Bass, Wolfgang W. Schamel, Sebastian Fuchs, Hanspeter Pircher, Christie Ann McCarl, Katsuhiko Mikoshiba, Klaus Schwarz, Stefan Feske, Yenan T. Bryceson, and Stephan Ehl. ORAI1-mediated calcium influx is required for human cytotoxic lymphocyte degranulation and target cell lysis. *Proceedings of the National Academy of Sciences of the United States of America*, 108(8):3324–3329, 2011.

- [11] Alex T. Ritter, Yukako Asano, Jane C. Stinchcombe, N. M G Dieckmann, Bi Chang Chen, C. Gawden-Bone, Schuyler van Engelenburg, Wesley Legant, Liang Gao, Michael W. Davidson, Eric Betzig, Jennifer Lippincott-Schwartz, and Gillian M. Griffiths. Actin Depletion Initiates Events Leading to Granule Secretion at the Immunological Synapse. *Immunity*, 42(5):864–876, 2015.
- [12] Yan Yu, Alexander A. Smolgovets, and Jay T. Groves. Modulation of t cell signaling by the actin cytoskeleton. *Journal of Cell Science*, 126(5):1049–1058, 2013.
- [13] Audrey Le Floc'h, Yoshihiko Tanaka, Niels S Bantilan, Guillaume Voisinne, Grégoire Altan-Bonnet, Yoshinori Fukui, and Morgan Huse. Annular PIP3 accumulation controls actin architecture and modulates cytotoxicity at the immunological synapse. *The Journal of experimental medicine*, 210(12):2721–37, 2013.
- [14] Emmanuel Derivery and Alexis Gautreau. Generation of branched actin networks: Assembly and regulation of the N-WASP and WAVE molecular machines. *BioEssays*, 32(2):119–131, 2010.
- [15] Karon Abe, Kent L. Rossman, Betty Liu, Kimberly D. Ritola, Derek Chiang, Sharon L. Campbell, Keith Burridge, and Channing J. Der. Vav2 is an activator of Cdc42, Rac1, and RhoA. *Journal of Biological Chemistry*, 275(14):10141–10149, 2000.
- [16] Wendell A. Lim Kenneth E. Prehoda, Jessica A. Scott, R. Dyche Mullins. Integration of Multiple Signals Through Cooperative Regulation of the N-WASP-Arp2/3 Complex. *Integration of Multiple Signals Through Cooperative Regulation of the N-WASP-Arp213 Complex*, 45(October), 2000.
- [17] Louise Weston, Amanda S. Coutts, and Nicholas B. La Thangué. Actin nucleators in the nucleus: An emerging theme. *Journal of Cell Science*, 125(15):3519–3527, 2012.
- [18] Colin R.F. Monks, Benjamin A. Freiberg, Hannah Kupfer, Noah Sciaky, and Abraham Kupfer. Three-dimensional segregation of supramolecular activation clusters in T cells. *Nature*, 395(6697):82–86, 1998.
- [19] Su-Yi Tseng, Janelle C. Waite, Mengling Liu, Santosh Vardhana, and Michael L. Dustin. T Cell-Dendritic Cell Immunological Synapses Contain TCR-dependent CD28-CD80 Clusters That Recruit Protein Kinase C θ . *The Journal of Immunology*, 181(7):4852–4863, 2008.

- [20] Kenneth G. Johnson, Shannon K. Bromley, Michael L. Dustin, and Matthew L. Thomas. A supramolecular basis for CD45 tyrosine phosphatase regulation in sustained T cell activation. *Proceedings of the National Academy of Sciences of the United States of America*, 97(18):10138–10143, 2000.
- [21] Hye Ran Kim, Ye Vin Mun, Kyung Sik Lee, Yoo Jin Park, Jeong Su Park, Jin Hwa Park, Bu Nam Jeon, Chang Hyun Kim, Youngsoo Jun, Young Min Hyun, Minsoo Kim, Sang Myeong Lee, Chul Seung Park, Sin Hyeog Im, and Chang Duk Jun. T cell microvilli constitute immunological synaptosomes that carry messages to antigen-presenting cells. *Nature Communications*, 9(1), 2018.
- [22] Daan Vorselen, Yifan Wang, Miguel M. de Jesus, Pavak K. Shah, Matthew J. Footer, Morgan Huse, Wei Cai, and Julie A. Theriot. Microparticle traction force microscopy reveals subcellular force exertion patterns in immune cell-target interactions. *Nature Communications*, 11(1), 2020.
- [23] Weiyang Jin, Fella Tamzalit, Parthiv Kant Chaudhuri, Charles T. Black, Morgan Huse, and Lance C. Kam. T cell activation and immune synapse organization respond to the microscale mechanics of structured surfaces. *Proceedings of the National Academy of Sciences of the United States of America*, 116(40):19835–19840, 2019.
- [24] Michael L. Dustin and Eric O. Long. Cytotoxic immunological synapses. *Immunological Reviews*, 235(1):24–34, 2010.
- [25] Jane C. Stinchcombe and Gillian M. Griffiths. Secretory Mechanisms in Cell-Mediated Cytotoxicity. <http://dx.doi.org/10.1146/annurev.cellbio.23.090506.123521>, 23:495–517, oct 2007.
- [26] Ruby H P Law, Natalya Lukyanova, Ilia Voskoboinik, Tom T Caradoc-Davies, Katherine Baran, Michelle a Dunstone, Michael E D’Angelo, Elena V Orlova, Fasséli Coulibaly, Sandra Verschoor, Kylie a Browne, Annette Ciccone, Michael J Kuiper, Phillip I Bird, Joseph a Trapani, Helen R Saibil, and James C Whisstock. The structural basis for membrane binding and pore formation by lymphocyte perforin. *Nature*, 468(7322):447–51, 2010.
- [27] Adam N R Cartwright, Jeremy Griggs, and Daniel M Davis. The immune synapse clears and excludes molecules above a size threshold. *Nature Communications*, 5:5479, 2014.

- [28] Dennis Keefe, Lianfa Shi, Stefan Feske, Ramiro Massol, Francisco Navarro, Tomas Kirchhausen, and Judy Lieberman. Perforin triggers a plasma membrane-repair response that facilitates CTL induction of apoptosis. *Immunity*, 23(3):249–262, 2005.
- [29] Yovan Sanchez-Ruiz, Salvatore Valitutti, and Loic Dupre. Stepwise Maturation of Lytic Granules during Differentiation and Activation of Human CD8+ T Lymphocytes. *PLOS ONE*, 6(11):e27057, nov 2011.
- [30] Jerome Thiery and Judy Lieberman. Perforin: A Key Pore-Forming Protein for Immune Control of Viruses and Cancer. *Sub-Cellular Biochemistry*, 80:197–220, may 2014.
- [31] Dennis Keefe, Lianfa Shi, Stefan Feske, Ramiro Massol, Francisco Navarro, Tomas Kirchhausen, and Judy Lieberman. Perforin triggers a plasma membrane-repair response that facilitates CTL induction of apoptosis. *Immunity*, 23(3):249–262, 2005.
- [32] Gregory D. Rak, Emily M. Mace, Pinaki P. Banerjee, Tatyana Svitkina, and Jordan S. Orange. Natural Killer cell lytic granule secretion occurs through a pervasive actin network at the immune synapse. *PLoS Biology*, 9(9), 2011.
- [33] Fella Tamzalit, Mitchell S Wang, Weiyang Jin, Vitaly Boyko, John M Heddleston, Charles T Black, Lance C Kam, and Morgan Huse. Interfacial actin protrusions mechanically potentiate killing by cytotoxic T cells. *Science Immunology*, 5445(March):443309, 2018.
- [34] Keenan T Bashour, Alexander Gondarenko, Haoqian Chen, Keyue Shen, Xin Liu, Morgan Huse, and James C Hone. CD28 and CD3 have complementary roles in T-cell traction forces. 111(6):2241–2246, 2014.
- [35] Roddy S O Connor, Xueli Hao, Keyue Shen, Tatiana Akimova, Wayne W Hancock, C Lance, and Michael C Milone. Substrate Rigidity Regulates Human T Cell Activation and Proliferation. 2012.
- [36] Roshni Basu, Benjamin M. Whitlock, Julien Husson, Audrey Le Floc'h, Weiyang Jin, Alon Oyler-Yaniv, Farokh Dotiwala, Gregory Giannone, Claire Hivroz, Nicolas Biais, Judy Lieberman, Lance C. Kam, and Morgan Huse. Cytotoxic T Cells Use Mechanical Force to Potentiate Target Cell Killing. *Cell*, 165(1):100–110, 2016.

- [37] Maria Tello-Lafoz, Miguel M. de Jesus, and Morgan Huse. Harder , better , faster , stronger : biochemistry and biophysics in the immunosurveillance concert. 2022.
- [38] Dikran Aivazian and Lawrence J. Stern. Phosphorylation of T cell receptor ζ is regulated by a lipid dependent folding transition. *Nature Structural Biology*, 7(11):1023–1026, 2000.
- [39] Baoyu Liu, Wei Chen, Brian D. Evavold, and Cheng Zhu. Accumulation of dynamic catch bonds between TCR and agonist peptide-MHC triggers T cell signaling. *Cell*, 157(2):357–368, 2014.
- [40] Mojdeh Shakiba, Paul Zumbo, Gabriel Espinosa-carrasco, Laura Menocal, Friederike Dündar, Sandra E Carson, Nicholas D Socci, Benjamin Whitlock, Fella Tamzalit, Morgan Huse, Matthew D Hellmann, and Daniel K Wells. TCR signal strength defines distinct mechanisms of T cell dysfunction and cancer evasion. 219(2), 2021.
- [41] Mirko Moroni, M Rocio Servin-vences, Raluca Fleischer, and Gary R Lewin. Voltage gating of mechanosensitive PIEZO channels. (2018):1–15, 2018.
- [42] Adam J Iliff and X Z Shawn Xu. A Mechanosensitive GPCR that Detects the Bloody Force. *Cell*, 173(3):542–544, 2018.
- [43] Zhiqi Sun, Shengzhen S Guo, and Reinhard Fässler. Integrin-mediated mechanotransduction. 215(4), 2016.
- [44] Facundo D. Batista and Michael L. Dustin. Cell: Cell interactions in the immune system. *Immunological Reviews*, 251(1):7–12, jan 2013.
- [45] Luis Martínez-Lostao, Alberto Anel, and Julián Pardo. How Do Cytotoxic Lymphocytes Kill Cancer Cells? *Clinical Cancer Research*, 21(22):5047–5056, 2015.
- [46] Xin Liu and Morgan Huse. Immunological synapse formation: Cell polarity during T cell–APC interaction. *Cell Polarity 1: Biological Role and Basic Mechanisms*, 50(3):247–275, 2015.
- [47] Stephen C. Bunnell, Veena Kapoor, Ronald P. Trible, Weigu Zhang, and Lawrence E. Samelson. Dynamic actin polymerization drives T cell receptor-induced spreading: A role for the signal transduction adaptor LAT. *Immunity*, 14(3):315–329, 2001.

- [48] Yoshihisa Kaizuka, Adam D. Douglass, Rajat Varma, Michael L. Dustin, and Ronald D. Vale. Mechanisms for segregating T cell receptor and adhesion molecules during immunological synapse formation in Jurkat T cells. *Proceedings of the National Academy of Sciences of the United States of America*, 104(51):20296–20301, 2007.
- [49] Jason Yi, Xufeng S. Wu, Travis Crites, and John A. Hammer. Actin retrograde flow and actomyosin II arc contraction drive receptor cluster dynamics at the immunological synapse in Jurkat T cells. *Molecular Biology of the Cell*, 23(5):834–852, 2012.
- [50] P. T. Sage, L. M. Varghese, R. Martinelli, T. E. Sciuto, M. Kamei, A. M. Dvorak, T. A. Springer, A. H. Sharpe, and C. V. Carman. Antigen Recognition Is Facilitated by Invadosome-like Protrusions Formed by Memory/Effecter T Cells. *The Journal of Immunology*, 188(8):3686–3699, 2012.
- [51] En Cai, Kyle Marchuk, Peter Beemiller, Casey Beppler, Matthew G. Rubashkin, Valerie M. Weaver, Audrey Gérard, Tsung Li Liu, Bi Chang Chen, Eric Betzig, Frederic Bartumeus, and Matthew F. Krummel. Visualizing dynamic microvillar search and stabilization during ligand detection by T cells. *Science*, 356(6338), 2017.
- [52] Ken Nguyen, Nicholas R. Sylvain, and Stephen C. Bunnell. T Cell Costimulation via the Integrin VLA-4 Inhibits the Actin-Dependent Centralization of Signaling Microclusters Containing the Adaptor SLP-76. *Immunity*, 28(6):810–821, 2008.
- [53] Julien Husson, Karine Chemin, Armelle Bohineust, Claire Hivroz, and Nelly Henry. Force generation upon T cell receptor engagement. *PLoS ONE*, 6(5), 2011.
- [54] Johannes Pettmann, Ana Mafalda Santos, Omer Dushek, and Simon J. Davis. Membrane ultrastructure and T cell activation. *Frontiers in Immunology*, 9(SEP):1–9, 2018.
- [55] Ewa Sitarska and Alba Diz-Muñoz. Pay attention to membrane tension: Mechanobiology of the cell surface. *Current Opinion in Cell Biology*, 66:11–18, 2020.
- [56] Allison M. Beal, Nadia Anikeeva, Rajat Varma, Thomas O. Cameron, Gaia Vasiliver-Shamis, Philip J. Norris, Michael L. Dustin, and Yuri Sykulev. Kinetics of Early T Cell Receptor Signaling Regulate the Pathway of Lytic Granule Delivery to the Secretory Domain. *Immunity*, 31(4):632–642, 2009.

- [57] Jane C. Stinchcombe, Giovanna Bossi, Sarah Booth, and Gillian M. Griffiths. The immunological synapse of CTL contains a secretory domain and membrane bridges. *Immunity*, 15(5):751–761, 2001.
- [58] Daniel Friedman, Poppy Simmonds, Alexander Hale, Leoma Bere, and W Nigel. Natural Killer cell immune synapse formation and cytotoxicity are controlled by tension of the target interface. 44(March), 2020.
- [59] Jeffrey C. Nolz, Timothy S. Gomez, Peimin Zhu, Shuixing Li, Ricardo B. Medeiros, Yoji Shimizu, Janis K. Burkhardt, Bruce D. Freedman, and Daniel D. Billadeau. The WAVE2 complex regulates actin cytoskeletal reorganization and CRAC-mediated calcium entry during T cell activation. *Current Biology*, 16(1):24–34, 2006.
- [60] Patricia A. Zipfel, Stephen C. Bunnell, D. Scott Witherow, Jing Jin Gu, Elizabeth M. Chislock, Colleen Ring, and Ann Marie Pendergast. Role for the Abi/Wave protein complex in T cell receptor-mediated proliferation and cytoskeletal remodeling. *Current Biology*, 16(1):35–46, 2006.
- [61] Jeffrey C. Nolz, Lucas P. Nacusi, Colin M. Segovis, Ricardo B. Medeiros, Jason S. Mitchell, Yoji Shimizu, and Daniel D. Billadeau. The WAVE2 complex regulates T cell receptor signaling to integrins via Abl- and CrkL-C3G-mediated activation of Rap1. *Journal of Cell Biology*, 182(6):1231–1244, 2008.
- [62] Sudha Kumari, David Depoil, Roberta Martinelli, Edward Judokusumo, Guillaume Carmona, Frank B Gertler, Lance C Kam, Christopher V Carman, Janis K Burkhardt, Darrell J Irvine, and Michael L Dustin. Actin foci facilitate activation of the phospholipase C- γ in primary T lymphocytes via the WASP pathway. pages 1–31, 2015.
- [63] Tasha N. Sims, Timothy J. Soos, Harry S. Xenias, Benjamin Dubin-Thaler, Jake M. Hofman, Janelle C. Waite, Thomas O. Cameron, V. Kaye Thomas, Rajat Varma, Chris H. Wiggins, Michael P. Sheetz, Dan R. Littman, and Michael L. Dustin. Opposing Effects of PKC θ and WASp on Symmetry Breaking and Relocation of the Immunological Synapse. *Cell*, 129(4):773–785, 2007.
- [64] Ronan Calvez, Fanny Lafouresse, Julie de Meester, Anne Galy, Salvatore Valitutti, and Loïc Dupré. The wiskott-aldrich syndrome protein permits assembly of a focused immunological synapse enabling sustained T-cell receptor signaling. *Haematologica*, 96(10):1415–1423, 2011.

- [65] Christopher V Carman, Peter T Sage, Tracey E Sciuto, Miguel A De Fuente, S Geha, Hans D Ochs, Harold F Dvorak, Ann M Dvorak, and Timothy A Springer. Transcellular Diapedesis Is Initiated by Invasive Podosomes. *26*(6):784–797, 2007.
- [66] Elizabeth Rivers and Adrian J. Thrasher. Wiskott-Aldrich syndrome protein: Emerging mechanisms in immunity. *European Journal of Immunology*, 47(11):1857–1866, 2017.
- [67] Michel J. Massaad, Narayanaswamy Ramesh, and Raif S. Geha. Wiskott-Aldrich syndrome: A comprehensive review. *Annals of the New York Academy of Sciences*, 1285(1):26–43, 2013.
- [68] Julie De Meester, Ronan Calvez, Salvatore Valitutti, and Loïc Dupré. The Wiskott-Aldrich syndrome protein regulates CTL cytotoxicity and is required for efficient killing of B cell lymphoma targets. *Journal of Leukocyte Biology*, 88(5):1031–1040, 2010.
- [69] Alex T. Ritter, Senta M. Kapnick, Srisharan Murugesan, Pamela L. Schwartzberg, Gillian M. Griffiths, and Jennifer Lippincott-Schwartz. Cortical actin recovery at the immunological synapse leads to termination of lytic granule secretion in cytotoxic T lymphocytes. *Proceedings of the National Academy of Sciences of the United States of America*, 114(32):E6585–E6594, 2017.
- [70] Bin Qu, Varsha Pattu, Christian Junker, Eva C. Schwarz, Shruthi S. Bhat, Carsten Kummerow, Misty Marshall, Ulf Matti, Frank Neumann, Michael Pfreundschuh, Ute Becherer, Heiko Rieger, Jens Rettig, and Markus Hoth. Docking of Lytic Granules at the Immunological Synapse in Human CTL Requires Vti1b-Dependent Pairing with CD3 Endosomes. *The Journal of Immunology*, 186(12):6894–6904, 2011.
- [71] Alice C.N. Brown, Stephane Oddos, Ian M. Dobbie, Juha Matti Alakoskela, Richard M. Parton, Philipp Eissmann, Mark A.A. Neil, Christopher Dunsby, Paul M.W. French, Ilan Davis, and Daniel M. Davis. Remodelling of cortical actin where lytic granules dock at Natural Killer cell immune synapses revealed by super-resolution microscopy. *PLoS Biology*, 9(9), 2011.
- [72] Anne Chauveau, Audrey Le Floc'h, Niels S Bantilan, Gary A Koretzky, and Morgan Huse. Diacylglycerol kinase- α controls T cell polarity by shaping diacylglycerol accumulation at the immune synapse. *Science signaling*, 7(340):ra82, 2014.

- [73] Xin Liu, Tarun M. Kapoor, James K. Chen, and Morgan Huse. Diacylglycerol promotes centrosome polarization in T cells via reciprocal localization of dynein and myosin II. *Proceedings of the National Academy of Sciences of the United States of America*, 110(29):11976–11981, 2013.
- [74] Roshni Basu, Yuedan Chen, Emily J. Quann, and Morgan Huse. The variable hinge region of novel PKCs determines localization to distinct regions of the immunological synapse. *PLoS ONE*, 9(4):1–8, 2014.
- [75] Aurelie Wiedemann, David Depoil, Mustapha Faroudi, and Salvatore Valitutti. Cytotoxic T lymphocytes kill multiple targets simultaneously via spatiotemporal uncoupling of lytic and stimulatory synapses. *Proceedings of the National Academy of Sciences of the United States of America*, 103(29):10985–10990, 2006.
- [76] Boyd Butler and John A. Cooper. Distinct Roles for the Actin Nucleators Arp2/3 and hDia1 during NK-Mediated Cytotoxicity. *Current Biology*, 19(22):1886–1896, 2009.
- [77] Raïssa Houmadi, Delphine Guipouy, Javier Rey-Barroso, Zilton Vasconcelos, Julie Cornet, Manoel Manghi, Nicolas Destainville, Salvatore Valitutti, Sophie Allart, and Loïc Dupré. The Wiskott-Aldrich Syndrome Protein Contributes to the Assembly of the LFA-1 Nanocluster Belt at the Lytic Synapse. *Cell Reports*, 22(4):979–991, 2018.
- [78] Hironori Ueda, Mary K. Morphew, J. Richard McIntosh, and Mark M. Davis. CD4 + T-cell synapses involve multiple distinct stages. *Proceedings of the National Academy of Sciences of the United States of America*, 108(41):17099–17104, 2011.
- [79] Yunmin Jung, Inbal Riven, Sara W. Feigelson, Elena Kartvelishvily, Kazuo Tohya, Masayuki Miyasaka, Ronen Alon, and Gilad Haran. Three-dimensional localization of T-cell receptors in relation to microvilli using a combination of superresolution microscopies. *Proceedings of the National Academy of Sciences of the United States of America*, 113(40):E5916–E5924, 2016.
- [80] Judy L. Cannon and Janis K. Burkhardt. Differential Roles for Wiskott-Aldrich Syndrome Protein in Immune Synapse Formation and IL-2 Production. *The Journal of Immunology*, 173(3):1658–1662, 2004.
- [81] Loïc Dupré, Alessandro Aiuti, Sara Trifari, Silvana Martino, Paola Saracco, Claudio Bordignon, and Maria Grazia Roncarolo. Wiskott-Aldrich syn-

- drome protein regulates lipid raft dynamics during immunological synapse formation. *Immunity*, 17(2):157–166, 2002.
- [82] Jinyi Zhang, Amro Shehabeldin, Luis A.G. Da Cruz, Jeffrey Butler, Ally Khan Somani, Mary McGavin, Ivona Kozieradzki, Antonio O. Dos Santos, Andras Nagy, Sergio Grinstein, Josef M. Penninger, and Katherine A. Siminovitch. Antigen receptor-induced activation and cytoskeletal rearrangement are impaired in Wiskott-Aldrich syndrome protein-deficient lymphocytes. *Journal of Experimental Medicine*, 190(9):1329–1341, 1999.
- [83] Yann Leverrier, Roberto Lorenzi, Michael P. Blundell, Paul Brickell, Christine Kinnon, Anne J. Ridley, and Adrian J. Thrasher. Cutting Edge: The Wiskott-Aldrich Syndrome Protein Is Required for Efficient Phagocytosis of Apoptotic Cells. *The Journal of Immunology*, 166(8):4831–4834, 2001.
- [84] Roberto Lorenzi, Paul M. Brickell, David R. Katz, Christine Kinnon, and Adrian J. Thrasher. Wiskott-Aldrich syndrome protein is necessary for efficient IgG-mediated phagocytosis. *Blood*, 95(9):2943–2946, 2000.
- [85] D. Malinova, M. Fritzsche, C. R. Nowosad, H. Armer, P. M. G. Munro, M. P. Blundell, G. Charras, P. Tolar, G. Bouma, and A. J. Thrasher. WASp-dependent actin cytoskeleton stability at the dendritic cell immunological synapse is required for extensive, functional T cell contacts. *Journal of Leukocyte Biology*, 99(5), 2016.
- [86] Gerben Bouma, Ariadna Mendoza-Naranjo, Michael P. Blundell, Elena De Falco, Kathryn L. Parsley, Siobhan O. Burns, and Adrian J. Thrasher. Cytoskeletal remodeling mediated by WASp in dendritic cells is necessary for normal immune synapse formation and T-cell priming. *Blood*, 118(9):2492–2501, 2011.
- [87] Xuan Zhang, Rongxin Dai, Wenyan Li, Hongyi Zhao, Yongjie Zhang, Lina Zhou, Hongqiang Du, Guangjin Luo, Junfeng Wu, Linlin Niu, Yunfei An, Zhiyong Zhang, Yuan Ding, Wenxia Song, Chaohong Liu, and Xiaodong Zhao. Abnormalities of follicular helper T-cell number and function in Wiskott-Aldrich syndrome. *Blood*, 127(25):3180–3191, 2016.
- [88] Michael Dustin. Insights into function of the immunological synapse from studies with supported planar bilayers. *Current Topics in Microbiology and Immunology*, 340(1):1–24, 2010.

- [89] Lakshmi Balagopalan, Eilon Sherman, Valarie A. Barr, and Lawrence E. Samelson. Imaging techniques for assaying lymphocyte activation in action. *Nature Reviews Immunology*, 11(1):21–33, 2011.
- [90] Bi Chang Chen, Wesley R. Legant, Kai Wang, Lin Shao, Daniel E. Milkie, Michael W. Davidson, Chris Janetopoulos, Xufeng S. Wu, John A. Hammer, Zhe Liu, Brian P. English, Yuko Mimori-Kiyosue, Daniel P. Romero, Alex T. Ritter, Jennifer Lippincott-Schwartz, Lillian Fritz-Laylin, R. Dyche Mullins, Diana M. Mitchell, Joshua N. Bembeneck, Anne Cecile Reymann, Ralph Böhme, Stephan W. Grill, Jennifer T. Wang, Geraldine Seydoux, U. Serdar Tulu, Daniel P. Kiehart, and Eric Betzig. Lattice light-sheet microscopy: Imaging molecules to embryos at high spatiotemporal resolution. *Science*, 346(6208), 2014.
- [91] Marco A. Purbhoo, Darrell J. Irvine, Johannes B. Huppa, and Mark M. Davis. T cell killing does not require the formation of a stable mature immunological synapse. *Nature Immunology*, 5(5):524–530, 2004.
- [92] Hsin Fang Chang, Hawraa Bzeih, Praneeth Chitirala, Keerthana Ravichandran, Marwa Sleiman, Elmar Krause, Ulrike Hahn, Varsha Pattu, and Jens Rettig. Preparing the lethal hit: interplay between exo- and endocytic pathways in cytotoxic T lymphocytes. *Cellular and Molecular Life Sciences*, 74(3):399–408, 2017.
- [93] Mónica Bettencourt-Dias and David M. Glover. Centrosome biogenesis and function: Centrosomics brings new understanding. *Nature Reviews Molecular Cell Biology*, 8(6):451–463, 2007.
- [94] German A. Pihan. Centrosome dysfunction contributes to chromosome instability, chromoanagenesis, and genome reprogramming in cancer. *Frontiers in Oncology*, 3 NOV(November):1–26, 2013.
- [95] Judith Pineau, Léa Pinon, Olivier Mesdjian, Jacques Fattacioli, and Ana-maria Lennon Duménil. Microtubules restrict F-actin polymerization to the immune synapse via GEF-H1 to maintain polarity in lymphocytes. pages 1–23, 2022.
- [96] Alex T. Ritter, Karen L. Angus, and Gillian M. Griffiths. The role of the cytoskeleton at the immunological synapse. *Immunological Reviews*, 256(1):107–117, 2013.
- [97] Andy Tsun, Ihjaaz Qureshi, Jane C. Stinchcombe, Misty R. Jenkins, Maike De La Roche, Joanna Kleczkowska, Rose Zamoyska, and Gillian M. Grif-

- fiths. Centrosome docking at the immunological synapse is controlled by Lck signaling. *Journal of Cell Biology*, 192(4):663–674, 2011.
- [98] Maike De La Roche, Alex T. Ritter, Karen L. Angus, Colin Dinsmore, Charles H. Earnshaw, Jeremy F. Reiter, and Gillian M. Griffiths. Hedgehog signaling controls T cell killing at the immunological synapse. *Science*, 342(6163):1247–1250, 2013.
- [99] Misty R. Jenkins, Jane C. Stinchcombe, Byron B. Au-Yeung, Yukako Asano, Alex T. Ritter, Arthur Weiss, and Gillian M. Griffiths. Distinct structural and catalytic roles for Zap70 in formation of the immunological synapse in CTL. *eLife*, 2014(3):1–21, 2014.
- [100] Fella Tamzalit, Diana Tran, Weiyang Jin, Vitaly Boyko, Hisham Bazzi, Ariella Kepecs, Lance C. Kam, Kathryn V. Anderson, and Morgan Huse. Centrioles control the capacity, but not the specificity, of cytotoxic T cell killing. *Proceedings of the National Academy of Sciences of the United States of America*, 117(8):4310–4319, feb 2020.
- [101] Rapid and unidirectional perforin pore delivery at the cytotoxic immune synapse. *Journal of Immunology*, 191(5):2328–34, 2013.
- [102] Kithiganahalli N Balaji, Norbert Schaschke, Werner Machleidt, Marta Catal-famo, and Pierre A Henkart. Surface Cathepsin B Protects Cytotoxic Lymphocytes from Self-destruction after Degranulation. 196(4), 2002.
- [103] Jesse A Rudd-schmidt, Adrian W Hodel, Tahereh Noori, Jamie A Lopez, Hyun-jung Cho, Sandra Verschoor, Annette Ciccone, Joseph A Trapani, and Bart W Hoogenboom. Lipid order and charge protect killer T cells from accidental death. *Nature Communications*, (2019):1–13, 2019.
- [104] Wei Chen, Jizhong Lou, and Cheng Zhu. Forcing Switch from Short- to Intermediate- and Long-lived States of the A Domain Generates LFA-1 / ICAM-1 Catch Bonds. *Journal of Biological Chemistry*, 285(46):35967–35978, 2010.
- [105] Katarzyna I. Jankowska, Edward K. Williamson, Nathan H. Roy, Daniel Blumenthal, Vidhi Chandra, Tobias Baumgart, and Janis K. Burkhardt. Integrins modulate T cell receptor signaling by constraining actin flow at the immunological synapse. *Frontiers in Immunology*, 9(JAN):1–19, 2018.

- [106] William A. Comrie, Alexander Babich, and Janis K. Burkhardt. F-actin flow drives affinity maturation and spatial organization of LFA-1 at the immunological synapse. *Journal of Cell Biology*, 208(4):475–491, 2015.
- [107] Brandon L. Walling and Minsoo Kim. LFA-1 in T cell migration and differentiation. *Frontiers in Immunology*, 9(MAY), 2018.
- [108] Pontus Nordenfelt, Hunter L. Elliott, and Timothy A. Springer. Coordinated integrin activation by actin-dependent force during T-cell migration. *Nature Communications*, 7:1–15, 2016.
- [109] Yang Wang, Dan Li, Roza Nurieva, Justin Yang, Mehmet Sen, Roberto Carreño, Sijie Lu, Bradley W. McIntyre, Jeffrey J. Molldrem, Glen B. Legge, and Qing Ma. LFA-1 affinity regulation is necessary for the activation and proliferation of naive T cells. *Journal of Biological Chemistry*, 284(19):12645–12653, 2009.
- [110] Tara Capece, Brandon L. Walling, Kihong Lim, Kyun Do Kim, Seyeon Bae, Hung Li Chung, David J. Topham, and Minsoo Kim. A novel intracellular pool of LFA-1 is critical for asymmetric CD8+ T cell activation and differentiation. *Journal of Cell Biology*, 216(11):3817–3829, 2017.
- [111] Mathieu Kurowska, Nicolas Goudin, Nadine T Nehme, and Magali Court. Terminal transport of lytic granules to the immune synapse is mediated by the kinesin-1 / Slp3 / Rab27a complex. *119(17):4–6*, 2016.
- [112] Kristina Somersalo, Nadja Anikeeva, Tasha N. Sims, V. Kaye Thomas, Roland K. Strong, Thomas Spies, Tatiana Lebedeva, Yuri Sykulev, and Michael L. Dustin. Cytotoxic T lymphocytes form an antigen-independent ring junction. *Journal of Clinical Investigation*, 113(1):49–57, 2004.
- [113] Morgan Huse, Audrey Le Floc'h, and Xin Liu. From lipid second messengers to molecular motors: Microtubule-organizing center reorientation in T cells. *Immunological Reviews*, 256(1):95–106, 2013.
- [114] Florie Bertrand, Sabina Müller, Kyung Ho Roh, Camille Laurent, Loïc Dupré, and Salvatore Valitutti. An initial and rapid step of lytic granule secretion precedes microtubule organizing center polarization at the cytotoxic T lymphocyte/target cell synapse. *Proceedings of the National Academy of Sciences of the United States of America*, 110(15):6073–6078, 2013.
- [115] Kenneth H. Hu and Manish J. Butte. T cell activation requires force generation. *Journal of Cell Biology*, 213(5):535–542, 2016.

- [116] Mark S. Lee, Caleb R. Glassman, Neha R. Deshpande, Hemant B. Badgandi, Heather L. Parrish, Chayasith Uttamapinant, Philipp S. Stawski, Alice Y. Ting, and Michael S. Kuhns. A Mechanical Switch Couples T Cell Receptor Triggering to the Cytoplasmic Juxtamembrane Regions of CD3 $\zeta\zeta$. *Immunity*, 43(2):227–239, 2015.
- [117] King Lam Hui, Lakshmi Balagopalan, Lawrence E. Samelson, and Arpita Upadhyaya. Cytoskeletal forces during signaling activation in Jurkat T-cells. *Molecular Biology of the Cell*, 26(4):685–695, 2015.
- [118] Tatsuo Kinashi. Intracellular signalling controlling integrin activation in lymphocytes. *Nature Reviews Immunology*, 5(7):546–559, 2005.
- [119] Omar D. Perez, Dennis Mitchell, Gina C. Jager, Sharon South, Chris Murriel, Jacqueline McBride, Lee A. Herzenberg, Shigemi Kinoshita, and Garry P. Nolan. Leukocyte functional antigen 1 lowers T cell activation thresholds and signaling through cytohesin-1 and Jun-activating binding protein 1. *Nature Immunology*, 4(11):1083–1092, 2003.
- [120] Jun Ichiro Suzuki, Sho Yamasaki, Jennifer Wu, Gary A. Koretzky, and Takashi Saito. The actin cloud induced by LFA-1-mediated outside-in signals lowers the threshold for T-cell activation. *Blood*, 109(1):168–175, 2007.
- [121] Quentin Verron, Elin Forslund, Ludwig Brandt, Mattias Leino, Thomas W. Frisk, Per E. Olofsson, and Björn Önfelt. NK cells integrate signals over large areas when building immune synapses but require local stimuli for degranulation. *Science Signaling*, 14(684), 2021.
- [122] Yang Liu, Galior Kornelia, Victor Pui-Yan Ma, and Khalid Salaita. Molecular Tension Probes for Imaging Forces at the Cell Surface. *Physiology behavior*, 176(3):139–148, 2016.
- [123] Yang Liu, Lori Blanchfield, Victor Pui-Yan Ma, Rakieb Andargachew, Kornelia Galior, Zheng Liu, Brian Evavold, and Khalid Salaita. DNA-based nanoparticle tension sensors reveal that T-cell receptors transmit defined pN forces to their antigens for enhanced fidelity. *Proceedings of the National Academy of Sciences of the United States of America*, 113(20):5610–5615, 2016.
- [124] Yun Zhang, Chenghao Ge, Cheng Zhu, and Khalid Salaita. DNA-based digital tension probes reveal integrin forces during early cell adhesion. *Nature Communications*, 5:1–10, 2014.

- [125] J. K. Burkhardt, S. Hester, C. K. Lapham, and Y. Argon. The lytic granules of natural killer cells are dual-function organelles combining secretory and pre-lysosomal compartments. *Journal of Cell Biology*, 111(6 I):2327–2340, 1990.
- [126] Chungho Kim, Feng Ye, and Mark H. Ginsberg. Regulation of integrin activation. *Annual Review of Cell and Developmental Biology*, 27:321–345, 2011.
- [127] B Y Hajime Takayama and Michail V Sitkovsky. ANTIGEN RECEPTOR-REGULATED EXOCYTOSIS IN CYTOTOXIC T LYMPHOCYTES. 1987.
- [128] Audrey Le Floc'h, Abdelali Jalil, Isabelle Vergnon, Béatrice Le Maux Chansac, Vladimir Lazar, Georges Bismuth, Salem Chouaib, and Fathia Mami-Chouaib. α E β 7 integrin interaction with E-cadherin promotes anti-tumor CTL activity by triggering lytic granule polarization and exocytosis. *Journal of Experimental Medicine*, 204(3):559–570, 2007.
- [129] Bonnie Huang, Kristoffer Haurum Johansen, and Pamela L. Schwartzberg. Efficient CRISPR/Cas9-Mediated Mutagenesis in Primary Murine T Lymphocytes. *Current Protocols in Immunology*, 124(1):1–21, 2019.
- [130] Weiyang Jin, Charles T. Black, Lance C. Kam, and Morgan Huse. Probing Synaptic Biomechanics Using Micropillar Arrays. 1584:497–515, 2017.
- [131] Thomas Kluyver, Benjamin Ragan-Kelley, Fernando Pérez, Brian Granger, Matthias Bussonnier, Jonathan Frederic, Kyle Kelley, Jessica Hamrick, Jason Grout, Sylvain Corlay, Paul Ivanov, Damián Avila, Safia Abdalla, and Carol Willing. Jupyter Notebooks—a publishing format for reproducible computational workflows. *Positioning and Power in Academic Publishing: Players, Agents and Agendas - Proceedings of the 20th International Conference on Electronic Publishing, ELPUB 2016*, pages 87–90, 2016.
- [132] Wolfgang H. Goldmann and Donald E. Ingber. Intact vinculin protein is required for control of cell shape, cell mechanics, and rac-dependent lamellipodia formation. *Biochemical and Biophysical Research Communications*, 290(2):749–755, 2002.
- [133] Robert M. Ezzell, Wolfgang H. Goldmann, Ning Wang, Natesh Parasharama, and Donald E. Ingber. Vinculin promotes cell spreading by mechanically coupling integrins to the cytoskeleton. *Experimental Cell Research*, 231(1):14–26, 1997.

- [134] Corina Ciobanasu, Hong Wang, Véronique Henriot, Cécile Mathieu, Annabelle Fente, Sandrine Csillag, Clémence Vigouroux, Bruno Faivre, and Christophe Le Clainche. Integrin-bound talin head inhibits actin filament barbed-end elongation. *Journal of Biological Chemistry*, 293(7):2586–2596, 2018.
- [135] Paul R. Elliott, Benjamin T. Goult, Petra M. Kopp, Neil Bate, J. Günter Grossmann, Gordon C.K. Roberts, David R. Critchley, and Igor L. Barsukov. The Structure of the Talin Head Reveals a Novel Extended Conformation of the FERM Domain. *Structure*, 18(10):1289–1299, 2010.
- [136] Stephanie J. Ellis, Emily Lostchuck, Benjamin T. Goult, Mohamed Bouaouina, Michael J. Fairchild, Pablo López-Ceballos, David A. Calderwood, and Guy Tanentzapf. The Talin Head Domain Reinforces Integrin-Mediated Adhesion by Promoting Adhesion Complex Stability and Clustering. *PLoS Genetics*, 10(11), 2014.
- [137] Alexandra M.L. Meijne, Mariëtte H.E. Driessens, Geertje La Rivière, Diane Casey, Constance A. Feltkamp, and Ed Roos. LFA-1 integrin redistribution during T-cell hybridoma invasion of hepatocyte cultures and manganese-induced adhesion to ICAM-1. *Journal of Cell Science*, 107(9):2557–2566, 1994.
- [138] Mehmet Sen, Adem C. Koksal, Koichi Yuki, Jianchuan Wang, and Timothy A. Springer. Ligand- and cation-induced structural alterations of the leukocyte integrin LFA-1. *Journal of Biological Chemistry*, 293(17):6565–6577, 2018.
- [139] Neha Dixit, Itsukyo Yamayoshi, Ari Nazarian, and Scott I. Simon. Migrational Guidance of Neutrophils Is Mechanotransduced via High-Affinity LFA-1 and Calcium Flux. *The Journal of Immunology*, 187(1):472–481, 2011.
- [140] J. R. Simon, A. Gough, E. Urbanik, F. Wang, F. Lanni, B. R. Ware, and D. L. Taylor. Analysis of rhodamine and fluorescein-labeled F-actin diffusion in vitro by fluorescence photobleaching recovery. *Biophysical Journal*, 54(5):801–815, 1988.
- [141] Dorian Obino, Francesca Farina, Odile Malbec, Pablo J. Sáez, Mathieu Maurin, Jérémie Gaillard, Florent Dingli, Damarys Loew, Alexis Gautreau, Maria Isabel Yuseff, Laurent Blanchoin, Manuel Théry, and Ana Maria Lennon-Duménil. Actin nucleation at the centrosome controls lymphocyte polarity. *Nature Communications*, 7, 2016.

- [142] Helgi I Ingólfsson and Olaf S Andersen. Screening for small molecules' bilayer-modifying potential using a gramicidin-based fluorescence assay. *Assay and drug development technologies*, 8(4):427–36, 2010.
- [143] I.V. Polozov, G.M. Anantharamaiah, J.P. Segrest, and R.M. Epand. Osmotically Induced Membrane Tension Modulates Membrane Permeabilization by Class L Amphipathic Helical Peptides: Nucleation Model of Defect Formation. *Biophysical Journal*, 81(2):949–959, 2001.
- [144] Tae Young Yoon and Mary Munson. SNARE complex assembly and disassembly. *Current Biology*, 28(8):R397–R401, 2018.
- [145] Mahantappa Halimani, Varsha Patti, Misty R. Marshall, Hsin Fang Chang, Ulf Matti, Martin Jung, Ute Becherer, Elmar Krause, Markus Hoth, Eva C. Schwarz, and Jens Rettig. Syntaxin11 serves as a t-SNARE for the fusion of lytic granules in human cytotoxic T lymphocytes. *European Journal of Immunology*, 44(2):573–584, 2014.
- [146] Praneeth Chitirala, Keerthana Ravichandran, Donatella Galgano, Marwa Sleiman, Elmar Krause, Yenan T. Bryceson, and Jens Rettig. Cytotoxic granule exocytosis from human cytotoxic T lymphocytes is mediated by VAMP7. *Frontiers in Immunology*, 10(AUG):1–14, 2019.
- [147] Ulf Matti, Varsha Patti, Mahantappa Halimani, Claudia Schirra, Elmar Krause, Yuanyuan Liu, Lisa Weins, Hsin Fang Chang, Raul Guzman, Jenny Olausson, Marc Freichel, Frank Schmitz, Mathias Pasche, Ute Becherer, Dieter Bruns, and Jens Rettig. Synaptobrevin2 is the v-SNARE required for cytotoxic T-lymphocyte lytic granule fusion. *Nature Communications*, 4:1–8, 2013.
- [148] Waldo A. Spessott, Maria L. Sanmillan, Margaret E. McCormick, Vineet V. Kulkarni, and Claudio G. Giraudo. SM protein Munc18-2 facilitates transition of Syntaxin 11-mediated lipid mixing to complete fusion for T-lymphocyte cytotoxicity. *Proceedings of the National Academy of Sciences of the United States of America*, 114(11):E2176–E2185, 2017.
- [149] Richard W. Baker, Philip D. Jeffrey, Michael Zick, Ben P. Phillips, William T. Wickner, and Frederick M. Hughson. A direct role for the Sec1/Munc18-family protein Vps33 as a template for SNARE assembly. *Science*, 349(6252):1111–1114, 2015.

- [150] Massimo D. Agostino, Herre J. Risselada, Anna Lürick, Christian Ungermann, and Andreas Mayer. A tethering complex drives the terminal stage of SNARE-dependent membrane fusion. *Nature*, 551(7682):634–638, 2017.
- [151] Herre Jelger Risselada and Andreas Mayer. SNAREs , tethers and SM proteins : how to overcome the final barriers to membrane fusion ? (January):243–258, 2020.
- [152] Qiangjun Zhou, Peng Zhou, Austin L. Wang, Dick Wu, Minglei Zhao, Thomas C. Südhof, and Axel T. Brunger. The primed SNARE-complexin-synaptotagmin complex for neuronal exocytosis. *Nature*, 548(7668):420–425, 2017.
- [153] Krista A. Riggs, Nazarul Hasan, David Humphrey, Christy Raleigh, Chris Nevitt, Deborah Corbin, and Chuan Hu. Regulation of integrin endocytic recycling and chemotactic cell migration by syntaxin 6 and VAMP3 interaction. *Journal of Cell Science*, 125(16):3827–3839, 2012.
- [154] Mingfeng Zhuang, Mingming Song, Dadong Liu, Jiamin Huang, and Bingwei Sun. Exogenous carbon monoxide suppresses LPS-Induced platelet SNAREs complex assembly and α -granule exocytosis via integrin α IIb β 3-Mediated PKC θ /Munc18a pathway. *Experimental Cell Research*, 386(2):111735, 2020.
- [155] Bela Nagy, Kamala Bhavaraju, Todd Getz, Yamini S. Bynagari, Soochong Kim, and Satya P. Kunapuli. Impaired activation of platelets lacking protein kinase C- θ isoform. *Blood*, 113(11):2557–2567, 2009.
- [156] Cong Ma, Wei Li, Yibin Xu, and Josep Rizo. Munc13 mediates the transition from the closed syntaxin-Munc18 complex to the SNARE complex. *Nature Structural and Molecular Biology*, 18(5):542–549, 2011.
- [157] Jiajie Diao, Zengliu Su, Xiaobing Lu, Tae Young Yoon, Yeon Kyun Shin, and Taekjip Ha. Single-vesicle fusion assay reveals Munc18-1 binding to the SNARE core is sufficient for stimulating membrane fusion. *ACS Chemical Neuroscience*, 1(3):168–174, 2010.
- [158] Özgür Genç, Olexiy Kochubey, Ruud F Toonen, Matthijs Verhage, and Ralf Schneggenburger. Munc18-1 is a dynamically regulated PKC target during short-term enhancement of transmitter release. *eLife*, 3:1–19, 2014.
- [159] Natalya Korogod, Xuelin Lou, and Ralf Schneggenburger. Posttetanic potentiation critically depends on an enhanced Ca $^{2+}$ sensitivity of vesicle

- fusion mediated by presynaptic PKC. *Proceedings of the National Academy of Sciences of the United States of America*, 104(40):15923–15928, 2007.
- [160] Yong Song, Menachem Ailenberg, and Mel Silverman. Human munc13 is a diacylglycerol receptor that induces apoptosis and may contribute to renal cell injury in hyperglycemia. *Molecular Biology of the Cell*, 10(5):1609–1619, 1999.
- [161] Morgan Huse, Lawrence O. Klein, Andrew T. Girvin, Joycelyn M. Faraj, Qi Jing Li, Michael S. Kuhns, and Mark M. Davis. Spatial and Temporal Dynamics of T Cell Receptor Signaling with a Photoactivatable Agonist. *Immunity*, 27(1):76–88, 2007.
- [162] P. Anton Van der Merwe. The TCR triggering puzzle. *Immunity*, 14(6):665–668, 2001.
- [163] Zhengyu Ma and Terri H. Finkel. T cell receptor triggering by force. *Trends in Immunology*, 31(1):1–6, 2010.
- [164] Zhengyu Ma, Dennis E. Discher, and Terri H. Finkel. Mechanical force in T cell receptor signal initiation. *Frontiers in Immunology*, 3(JUL):2–4, 2012.
- [165] Antonio Lanzavecchia, Giandomenica Iezzi, and Antonella Viola. From TCR engagement to T cell activation: A kinetic view of T cell behavior. *Cell*, 96(1):1–4, 1999.
- [166] Yinnian Feng, Ellis L. Reinherz, and Matthew J. Lang. $\alpha\beta$ T Cell Receptor Mechanosensing Forces out Serial Engagement. *Trends in Immunology*, 39(8):596–609, 2018.
- [167] Wei Chen and Cheng Zhu. Mechanical regulation of T-cell functions. *Immunological Reviews*, 256(1):160–176, 2013.
- [168] Devin L. Harrison, Yun Fang, and Jun Huang. T-cell mechanobiology: Force sensation, potentiation, and translation. *Frontiers in Physics*, 7(APR):1–18, 2019.
- [169] Amy E. Moran, Keli L. Holzapfel, Yan Xing, Nicole R. Cunningham, Jonathan S. Maltzman, Jennifer Punt, and Kristin A. Hogquist. T cell receptor signal strength in Treg and iNKT cell development demonstrated by a novel fluorescent reporter mouse. *Journal of Experimental Medicine*, 208(6):1279–1289, 2011.

- [170] Akiko Hashimoto-Tane and Takashi Saito. Dynamic regulation of TCR-microclusters and the microsynapse for T cell activation. *Frontiers in Immunology*, 7(JUN):1–8, 2016.
- [171] Nathalie Labrecque, Liam Simon Whitfield, Reinhard Obst, Caroline Waltzinger, Christophe Benoist, and Diane Mathis. How much TCR does a T cell need? *Immunity*, 15(1):71–82, 2001.
- [172] Boryana N. Manz, Bryan L. Jackson, Rebecca S. Petit, Michael L. Dustin, and Jay Groves. T-cell triggering thresholds are modulated by the number of antigen within individual T-cell receptor clusters. *Proceedings of the National Academy of Sciences of the United States of America*, 108(22):9089–9094, 2011.
- [173] Masaki Yasukawa, Hideki Ohminami, Junko Arai, Yoshihito Kasahara, Yasushi Ishida, and Shigeru Fujita. Granule exocytosis, and not the Fas/Fas ligand system, is the main pathway of cytotoxicity mediated by alloantigen-specific CD4+ as well as CD8+ cytotoxic T lymphocytes in humans. *Blood*, 95(7):2352–2355, 2000.
- [174] Akiko Yamada, Rieko Arakaki, Masako Saito, Yasusei Kudo, and Naozumi Ishimaru. Dual role of Fas/FasL-mediated signal in peripheral immune tolerance. *Frontiers in Immunology*, 8(APR):1–10, 2017.
- [175] Stefania Parlato, Anna Maria Giammarioli, Mariantonio Logozzi, Francesco Lozupone, Paola Matarrese, Francesca Luciani, Mario Falchi, Walter Malorni, and Stefano Fais. CD95 (APO-1/Fas) linkage to the actin cytoskeleton through ezrin in human T lymphocytes: A novel regulatory mechanism of the CD95 apoptotic pathway. *EMBO Journal*, 19(19):5123–5134, 2000.
- [176] Yuko Kojima, Akemi Kawasaki-Koyanagi, Noriyoshi Sueyoshi, Atsushi Kanai, Hideo Yagita, and Ko Okumura. Localization of Fas ligand in cytoplasmic granules of CD8+ cytotoxic T lymphocytes and natural killer cells: Participation of Fas ligand in granule exocytosis model of cytotoxicity. *Biochemical and Biophysical Research Communications*, 296(2):328–336, 2002.
- [177] Hendrik Schmidt, Christoph Gelhaus, Melanie Nebendahl, Marcus Lettau, Ralph Lucius, Matthias Leippe, Dietrich Kabelitz, and Ottmar Janssen. Effector granules in human T lymphocytes: The luminal proteome of secretory lysosomes from human T cells. *Cell Communication and Signaling*, 9(1):4, 2011.

- [178] Hendrik Schmidt, Christoph Gelhaus, Melanie Nebendahl, Marcus Lettau, Ralph Lucius, Matthias Leippe, Dietrich Kabelitz, and Ottmar Janssen. Effector granules in human T lymphocytes: Proteomic evidence for two distinct species of cytotoxic effector vesicles. *Journal of Proteome Research*, 10(4):1603–1620, 2011.
- [179] Aleksandra Wroblewska, Maxime Dhainaut, Benjamin Ben-Zvi, Samuel A. Rose, Eun Sook Park, El Ad David Amir, Anela Bektesevic, Alessia Baccarini, Miriam Merad, Adeeb H. Rahman, and Brian D. Brown. Protein Barcodes Enable High-Dimensional Single-Cell CRISPR Screens. *Cell*, 175(4):1141–1155.e16, 2018.
- [180] Maxime Dhainaut. Perturb-map enables CRISPR genomics with spatial resolution and identifies regulators of tumor immune composition. 2021.
- [181] Maria Tello-lafoz, Katja Srpan, Elisa E Sanchez, and Joan Massague. Cytotoxic lymphocytes target characteristic biophysical vulnerabilities in cancer. pages 1–18, 2021.
- [182] E. John Wherry. T cell exhaustion. *Nature Immunology*, 12(6):492–499, 2011.
- [183] Michael Quigley, Florencia Pereyra, Björn Nilsson, Filippos Porichis, Catia Fonseca, Quentin Eichbaum, Boris Julg, Jonathan L Jesneck, Kathleen Brosnahan, Sabrina Imam, Kate Russell, Ildiko Toth, Alicja Piechocka-trocha, Douglas Dolfi, Jill Angelosanto, Alison Crawford, Haina Shin, Douglas S Kwon, Jennifer Zupkosky, Loise Francisco, Gordon J Freeman, E John Wherry, Daniel E Kaufmann, Bruce D Walker, Benjamin Ebert, and W Nicholas Haining. Transcriptional analysis of HIV-specific CD8 + T cells shows that PD-1 inhibits T cell function by upregulating BATF. *Nature Medicine*, 16(10):1147–1151, 2010.
- [184] Mary E. Keir, Manish J. Butte, Gordon J. Freeman, and Arlene H. Sharpe. PD-1 and its ligands in tolerance and immunity. *Annual Review of Immunology*, 26:677–704, 2008.
- [185] Travis A Doering, Alison Crawford, Jill M Angelosanto, Michael A Paley, Carly G Ziegler, and E John Wherry. Network Analysis Reveals Centrally Connected Genes and Pathways Involved in CD8 + T Cell Exhaustion versus Memory. *Immunity*, 37(6):1130–1144, 2012.
- [186] E John Wherry, Sang-jun Ha, Susan M Kaech, W Nicholas Haining, Surojit Sarkar, Vandana Kalia, Shruti Subramaniam, Joseph N Blattman, Daniel L

- Barber, and Rafi Ahmed. Molecular Signature of CD8 + T Cell Exhaustion during Chronic Viral Infection. 2007.
- [187] Chao Wang, Ann J Mcpherson, R Brad Jones, Kim S Kawamura, Gloria H Y Lin, Philipp A Lang, Thanuja Ambagala, Marc Pellegrini, Thomas Calzascia, Nasra Aidarus, Alisha R Elford, Feng Yun Yue, Elisabeth Kremmer, Colin M Kovacs, Erika Benko, Cecile Tremblay, Jean-pierre Routy, Nicole F Bernard, Mario A Ostrowski, Pamela S Ohashi, and Tania H Watts. Loss of the signaling adaptor TRAF1 causes CD8 + T cell dysregulation during human and murine chronic infection. 209(1), 2012.
- [188] Vanja Lazarevic, Laurie H. Glimcher, and Graham M. Lord. T-bet: A bridge between innate and adaptive immunity. *Nature Reviews Immunology*, 13(11):777–789, 2013.
- [189] Manu Rangachari, Chen Zhu, Kaori Sakuishi, Sheng Xiao, Jozsef Karman, Andrew Chen, Mathieu Angin, Andrew Wakeham, Edward A Greenfield, Raymond A Sobel, Hitoshi Okada, Peter J Mckinnon, Tak W Mak, Marylyn M Addo, Ana C Anderson, and Vijay K Kuchroo. Bat3 promotes T cell responses and autoimmunity by repressing Tim-3 – mediated cell death and exhaustion. *Nature Medicine*, 18(9):1394–1400, 2012.
- [190] Haina Shin, Shawn D Blackburn, Andrew M Intlekofer, Charlly Kao, Jill M Angelosanto, Steven L Reiner, and E John Wherry. A Role for the Transcriptional Repressor Blimp-1 in CD8 + T Cell Exhaustion during Chronic Viral Infection. *Immunity*, 31(2):309–320, 2009.
- [191] Michael A. Paley, Daniela C. Kroy, Pamela M. Odorizzi, Jonathan B. Johnnidis, Douglas V. Dolfi, Burton E. Barnett, Elizabeth K. Bikoff, Elizabeth J. Robertson, Georg M. Lauer, Steven L. Reiner, and Wherry E. John. Progenitor and Terminal Subsets of CD8+ T Cells Cooperate to Contain Chronic Viral Infection. 1220, 2012.
- [192] Marie Larsson, Esaki M Shankar, Karlhans F Che, Alireza Saeidi, Rada Ellegrd, and Muttiah Barathan. Molecular signatures of T-cell inhibition in HIV-1 infection. 7:1–14, 2013.
- [193] Reina Mizuno, Daisuke Sugiura, Kenji Shimizu, Takumi Maruhashi, and Mizuki Watada. PD-1 Primarily Targets TCR Signal in the Inhibition of Functional T Cell Activation. 10(March):1–14, 2019.

- [194] Andrea Schietinger and Philip D. Greenberg. Tolerance and exhaustion: Defining mechanisms of T cell dysfunction. *Trends in Immunology*, 35(2):51–60, 2014.
- [195] Bernd H Zinselmeyer, Sara Heydari, Catarina Sacristán, Debasis Nayak, Michael Cammer, Jasmin Herz, Xiaoxiao Cheng, Simon J Davis, Michael L Dustin, and Dorian B McGavern. PD-1 promotes immune exhaustion by inducing antiviral T cell motility paralysis. *Immunity*, 41(4):757–774, 2013.
- [196] Barbara W Bernstein and James R Bamburg. Actin-ATP Hydrolysis Is a Major Energy Drain for Neurons. *Journal of Cell Biology*, 158(1):1–6, 2002.
- [197] Wylie W. Ahmed, Étienne Fodor, and Timo Betz. Active cell mechanics: Measurement and theory. *Biochimica et Biophysica Acta - Molecular Cell Research*, 1853(11):3083–3094, 2015.
- [198] Li Xu and Anthony Bretscher. Report Rapid Glucose Depletion Immobilizes Active Myosin V on Stabilized Actin Cables. *Journal of Cell Biology*, 205(10):2471–2479, 2014.
- [199] Wei Xiong, Yuhui Chen, Xi Kang, Zhiying Chen, Peilin Zheng, Yi Hsin Hsu, Joon Hee Jang, Lidong Qin, Hao Liu, Gianpietro Dotti, and Dongfang Liu. Immunological Synapse Predicts Effectiveness of Chimeric Antigen Receptor Cells. *Molecular Therapy*, 26(4):963–975, 2018.
- [200] A. J. Davenport, R. S. Cross, K. A. Watson, Y. Liao, W. Shi, H. M. Prince, P. A. Beavis, J. A. Trapani, M. H. Kershaw, D. S. Ritchie, P. K. Darcy, P. J. Neeson, and M. R. Jenkins. Chimeric antigen receptor T cells form non-classical and potent immune synapses driving rapid cytotoxicity. *Proceedings of the National Academy of Sciences of the United States of America*, 116(22):11075, 2019.
- [201] Malini Mukherjee, Emily M. Mace, Alexandre F. Carisey, Nabil Ahmed, and Jordan S. Orange. Quantitative Imaging Approaches to Study the CAR Immunological Synapse. *Molecular Therapy*, 25(8):1757–1768, 2017.
- [202] Rui Li, Chao Ma, Haogang Cai, and Weiqiang Chen. The CAR T-Cell Mechanoimmunology at a Glance. *Advanced Science*, 7(24):1–17, 2020.
- [203] Keisuke Watanabe, Shunichiro Kuramitsu, Avery D Posey Jr, Carl H June, and Carl H June. Expanding the Therapeutic Window for CAR T Cell Therapy in Solid Tumors : The Knowns and Unknowns of CAR T Cell Biology. *Cancer Research*, 78(9):1–12, 2018.

- [204] Kewen Lei, Armand Kurum, and Li Tang. Mechanical Immunoengineering of T cells for Therapeutic Applications. 2020.
- [205] Barbara U. Schraml and Caetano Reis e Sousa. Defining dendritic cells. *Current Opinion in Immunology*, 32:13–20, 2015.
- [206] Roddy S. O'Connor, Xueli Hao, Keyue Shen, Keenan Bashour, Tatiana Akimova, Wayne W. Hancock, Lance C. Kam, and Michael C. Milone. Substrate Rigidity Regulates Human T Cell Activation and Proliferation. *The Journal of Immunology*, 189(3):1330–1339, 2012.
- [207] Morteza Aramesh, Diana Stoycheva, Lion Raaz, and Enrico Klotzsch. Engineering T-cell activation for immunotherapy by mechanical forces. *Current Opinion in Biomedical Engineering*, 10:134–141, 2019.
- [208] Thomas J. Gardner, J. Peter Lee, Christopher M. Bourne, Dinali Wijewarnasuriya, Nihar Kinarivala, Keifer G. Kurtz, Broderick C. Corless, Megan M. Dacek, Aaron Y. Chang, George Mo, Kha M. Nguyen, Renier J. Brentjens, Derek S. Tan, and David A. Scheinberg. Engineering CAR-T cells to activate small-molecule drugs in situ. *Nature Chemical Biology*, 2021.
- [209] Jin H. Park, Gina Lee, and John Blenis. Structural Insights into the Activation of mTORC1 on the Lysosomal Surface. *Trends in Biochemical Sciences*, 45(5):367–369, 2020.
- [210] Yoana Rabanal-Ruiz and Viktor I. Korolchuk. mTORC1 and nutrient homeostasis: The central role of the lysosome. *International Journal of Molecular Sciences*, 19(3), 2018.
- [211] Hellyeh Hamidi and Johanna Ivaska. Food for thought: How cell adhesion coordinates nutrient sensing. *Journal of Cell Biology*, 220(5):4–5, 2021.
- [212] Mitsugu Shimobayashi and Michael N. Hall. Multiple amino acid sensing inputs to mTORC1. *Cell Research*, 26(1):7–20, 2016.
- [213] Yoana Rabanal-Ruiz, Adam Byron, Alexander Wirth, Ralitsa Madsen, Lucia Sedlackova, Graeme Hewitt, Glyn Nelson, Julian Stingele, Jimi C. Wills, Tong Zhang, André Zeug, Reinhard Fässler, Bart Vanhaesebroeck, Oliver D.K. Maddocks, Evgeni Ponimaskin, Bernadette Carroll, and Viktor I. Korolchuk. mTORC1 activity is supported by spatial association with focal adhesions. *Journal of Cell Biology*, 220(5), 2021.

- [214] Alexandra Lynn McGregor, Chieh Ren Hsia, and Jan Lammerding. Squish and squeeze - the nucleus as a physical barrier during migration in confined environments. *Current Opinion in Cell Biology*, 40:32–40, 2016.
- [215] Dmitry I. Strokotov, Maxim A. Yurkin, Konstantin V. Gilev, Dirk R. van Bockstaele, Alfons G. Hoekstra, Nikolay B. Rubtsov, and Valeri P. Maltsev. Is there a difference between T- and B-lymphocyte morphology? *Journal of Biomedical Optics*, 14(6):064036, 2009.
- [216] Jérémie Rossy, Julia M. Laufer, and Daniel F. Legler. Role of mechanotransduction and tension in t cell function, 2018.
- [217] Balázs Enyedi and Philipp Niethammer. Nuclear membrane stretch and its role in mechanotransduction. *Nucleus*, 8(2):156–161, 2017.
- [218] Kris Noel Dahl, Alexandre J.S. Ribeiro, and Jan Lammerding. Nuclear shape, mechanics, and mechanotransduction. *Circulation Research*, 102(11):1307–1318, 2008.
- [219] Christophe Guilluy, Lukas D. Osborne, Laurianne Van Landeghem, Lisa Sharek, Richard Superfine, Rafael Garcia-Mata, and Keith Burridge. Isolated nuclei adapt to force and reveal a mechanotransduction pathway in the nucleus. *Nature Cell Biology*, 16(4):376–381, 2014.
- [220] Huy Quang Le, Sushmita Ghatak, Ching Yan Chloé Yeung, Frederik Tellkamp, Christian Günschmann, Christoph Dieterich, Assa Yeroslaviz, Bianca Habermann, Ana Pombo, Carien M. Niessen, and Sara A. Wickström. Mechanical regulation of transcription controls Polycomb-mediated gene silencing during lineage commitment. *Nature Cell Biology*, 18(8):864–875, 2016.
- [221] Anna Mari Lone and Kjetil Taskén. Proinflammatory and immunoregulatory roles of eicosanoids in T cells. *Frontiers in Immunology*, 4(JUN):1–15, 2013.