MECHANOTRANSDUCTION OF THE CYTOTOXIC T LYMPHOCYTE EFFECTOR RESPONSE

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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 Palo Alto, California in 1993, and grew up in the suburbs of Philadelphia, Pennsylvania. After high school, 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 in college, 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.

for Mama, Baba, and Didi

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0.1 Table of Abbreviations

Table 1: Table of Abbreviations

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
CD3ζ	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
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
ERK	Mitogen-activated protein kinase
F-actin	Filamentous actin
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
IFNγ	Interferon gamma
IκB	Inhibitor of kappa B
IL-2	Interleukin-2
ILP	Invadosome-like protrusion
IP_3	inositol 1,4,5-trisphosphate
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

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. For mammalian immune systems, this network is broadly broken into two major arms: the innate immune system and the adaptive immune system. 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. 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 specifically bind to antigens and identify their bound partners as foreign 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, 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 of most relevance 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-Tα 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-alpha chain against the now mature TCR-beta 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 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. Those T cells that are able to bind to an MHC complex presenting self-antigen receive a survival signal, while those with TCRs that cannot MHCs do not receive this survival signal. Therefore, the body positively filters/selects for T cells with TCRs that can recognize MHCs, leaving those that cannot recognize MHCs to die off.

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 signaling and the CD8+ T cell immune synapse

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 CD3ζ homodimer) bearing ITAM (immunoreceptor tyrosine-based activation motif) also bind to the TCR. The ITAMs on the CD3ζ get phosphorylated

1.4 T cell cytoskeleton

aaa

1.5 T cell degranulation and target cell death

Upon successful formation of the IS, T cells will coordinate their lytic granules

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 a 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 CTL immune synapse has been shown to be a physically dynamic structure, capable of exerting force against a resisting target or surface and

1.7 Integrins

aaa

1.8 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 directed 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 imaging experiments. 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 in: Tamzalit, F., Wang, M. S., Jin, W., Tello-Lafoz, M., Boyko, V., Heddleston, J. M., Black, C. T., Kam, L. C., and Huse, M. (2019). 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 [1]. 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 [2] (3). 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 (4). 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 (5).

IS formation is accompanied by dramatic reorganization of both microtubules and filamentous actin (F-actin) (6). 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 (3). 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 (7–10). 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 (3, 11, 12). 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 (10, 12–15). These dynamics enable T cells to impart mechanical force across the IS (16, 17). In CTLs, the capacity to exert synaptic force is notably correlated with cytotoxic potential (18). 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.

2.2 Results

2.2.1 CTLs form actin-rich protrusions on stimulatory micropillars (Fig 1, S1, S2, S3)

Synaptic force exertion can be measured by imaging T cells on arrays of flexible polydimethylsiloxane (PDMS) micropillars bearing immobilized TCR ligands and adhesion proteins (Fig. 1A) (17, 18). 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 (18). 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.

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 ad- hesion molecule 1), a ligand for the αLβ2 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) (Fig. 1A). 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 (Fig. 1B). 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 (Fig. 1B and movie S1). 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 (fig. S1). 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 (Fig. 1C). 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 (Fig. 1B and movie S1). However, once the radial size of the IS stabilized, after 60 s, 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 (3, 11, 19, 20). 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 5 μm from the cell front, roughly at the level of the pillar tops (Fig. 1D). This behavior implied a close association between granules and the centrosome, as previously reported (11, 20, 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) (fig. S2). 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 (Fig. 1D).

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 (Fig. 1E), 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, further supporting the idea that these structures transmit forces that promote cytotoxicity.

2.2.2 Granule fusion occurs at the base of synaptic protrusions (Fig 2)

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 (22). 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 (Fig. 2A). 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

tops (Fig. 2, B and C, and movie S2), the same vertical zone occupied by lytic granules and the centrosome. This position was well behind the leading edge of CTL protrusions, which extended 5 μm into the interpillar space.

Granule fusion was not observed in zones of sustained F-actin depletion. Instead, it tended to occur in regions containing synaptic protrusions (Fig. 2B and movie S2). To quantify this effect, we determined the Lifeact-mRuby2 intensity over time in the 1-μm-diameter synaptic domain around each fusion site. F-actin accumulation within this domain actually increased modestly during granule fusion (Fig. 2D), 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 (Fig. 2E). 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 CK666 blocks protrusion formation (Fig 3, S4)

Having characterized the structure and dynamics of synaptic protrusions, we turned our attention to their molecular basis and biological function. We were particularly interested in the Arp2/3 complex, which nucleates actin polymerization from the sides of existing actin filaments (Fig. 3A) (23). To assess the importance of Arp2/3, we used CK666, a small-molecule inhibitor of the complex (24). Treatment with CK666 markedly attenuated protrusive activity on micropillar arrays (Fig. 3B and movies S3 and S4). We quantified these data by calculating the enrichment of F-actin (visualized using Lifeact-GFP) in the

region beneath the pillar tops (fig. S4A). This analysis revealed a dose-dependent reduction in synaptic protrusions (Fig. 3C). A small amount of protrusive activity was still observed even at high CK666 concentrations, possibly representing residual TCR-induced actin polymerization by formins (25, 26).

2.2.4 CK666 inhibits force exertion and cytotoxicity (Fig 4)

The capacity of CK666 to block protrusion formation provided a strategy for determining the importance of these structures for synaptic force exertion and cytotoxicity. To measure cellular forces, we imaged CTLs on stimulatory PDMS arrays con- taining narrow, deformable micropillars (Fig. 1A). Treatment with CK666 reduced force exertion by 75% (Fig. 4, A and B, and movies S5 and S6), indicating that Arp2/3-dependent protrusive activity is required for IS mechanics. Next, we assessed the role of Arp2/3 in cytotoxic function by incubating OT1 CTLs with OVA-loaded RMA-s target cells in the presence of CK666. Target cell lysis was inhibited by CK666 in a dose-dependent manner (Fig. 4C), implying a critical role for Arp2/3. To better define the basis for this defect, we examined several molecular and cellular events involved in T cell activation and cytotoxicity. CK666 treatment did not alter TCR-induced phosphorylation of extracellular signal–regulated kinase 1/2 and Akt and had little effect on the degradation of IκB (fig. S4B). This suggested that signaling through the MAPK (mitogen-activated protein kinase), PI3K, and NF-?B (nuclear factor–κB) pathways was intact. By contrast, we observed a modest inhibition of TCR-induced calcium (Ca^{2+}) flux in CK666-treated cells (fig. S4, C and D). Elevated intracellular Ca^{2+} is a prerequisite for lytic granule release (19, 27). Consistent with this idea, we found that CK666 significantly inhibited granule

fusion, which we measured by staining for surface exposure of Lamp1 (Fig. 4D). We also observed a marked inhibition of CTL–target cell conjugate formation, as quantified by flow cytometry (Fig. 4E).

Together, these data demonstrated that the Arp2/3 complex is required for the formation of synaptic protrusions and also for other TCR-dependent responses associated with cytotoxicity: synaptic force exertion, granule release, and adhesion to the target cell. These results were not inconsistent with our hypothesis that synaptic protrusions enhance killing via cytolytic mechanopotentiation. They could not, however, exclude the possibility that the killing defect induced by CK666 was caused entirely by reduced conjugate formation and/or cytolytic secretion, either of which may have occurred independently of the protrusion defect. Nor could we rule out that the cytotoxicity phenotype resulted from effects of CK666 on the target cells. Hence, to define the role of synaptic protrusions unambiguously, it was necessary to establish more specific molecular perturbations.

2.2.5 WASP and WAVE2 control distinct subsets of protrusions (Fig 5, S5)

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 (Fig. 3A). 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 (8, 28–30). WASP, for its part, func-

tions 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 (31–34). In humans, loss-of-function WASP mutations cause WAS, a primary immunodeficiency associated with increased incidence of auto immunity and cancer (35, 36).

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; Fig. 5A and movie S7). In subsequent time points, transient bursts of WAVE2-GFP appeared in isolated peripheral domains (Fig. 5A, magenta arrowheads), often occurring concomitantly with lateral movement of the IS toward the same side (movie S7). By contrast, WASP-GFP accumulated in annular structures that encircled individual pillars in central and intermediate synaptic domains (Fig. 5A and movie S8). WASP-GFP exhibited a significantly higher mean centralization factor than WAVE2-GFP at all time points (Fig. 5B and fig. S2), 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 (fig S5A and Materials and Methods). 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 (Fig. 5C and movie S11). 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 (Fig. 5D). Hence, WASP deficiency leads to a specific loss of central protrusions, whereas WAVE2 deficiency eliminates peripheral structures. 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.

2.2.6 WASP and WAVE2 depletion induce distinct functional phenotypes (Fig 6)

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 (Figs. 1A and 6A and movies S12 to S14). 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 (Fig. 6B). By contrast, depletion of WAVE2 delayed the onset of force exertion but did not affect its overall magnitude (Fig. 6, A and B). 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 (fig. S5B). NT-CR and WAVE2-CR CTLs induced pillar deflections in both the central IS (¡3 μm from the COG) and the periphery (¿3 μm from the COG). By contrast, in WASP-CR CTLs, there was a marked absence of centrally localized events (Fig. 6C). 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 (Fig. 6D and fig. S5C). This defect was most pronounced (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 (Fig. 6E), 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 somewhat 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 (Fig. 6, D and E, and fig. S5D). WAVE2-CR CTLs, but not their WASP-CR counterparts, exhibited significantly reduced conjugate formation (Fig. 6F), 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 (fig. S5E). 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-κB pathways (fig. S5, F and G). 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 (33, 37). 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.

2.2.7 WASP controls target cell deformation at the IS (Fig 7, S6)

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 (13, 31). 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 (Fig. 7A). 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 (Fig. 7B, 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 (Fig. 7B and movie S15). 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 (fig. S6A), 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 (fig. S6B), consistent with previous work (13). Last, imaging of CTLs expressing Lamp1-GFP revealed that lytic granules accumulated close to areas of deformation (fig. S6C), implying that physical manipulation of the target cell contributes to perforin- and granzyme-mediated killing.

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 (Fig. 7B and movie S16). 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 (fig. S6D). Analysis of this "target IS volume" parameter confirmed that WASP depletion significantly reduced the target cell displacement response (Fig. 7, B and C). 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 (Fig. 7, B and C, and movie S17). Collectively, these results mirror the force exertion analysis of WASP-CR and WAVE2-CR CTLs (Fig. 6, A to C), and they suggest that WASP-dependent synaptic protrusions play a particularly important role in the physical deformation of target cells.

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 (Fig. 8). 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 (19, 22, 38–40). However, the converse relationship may be worth considering, namely, that lytic granule docking and fusion might influence local F-actin architecture and IS mechanics.

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 (11, 12, 22, 40). 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 (42–44). Granule delivery via microtubules is another possibility (45, 46). 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 (47). 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 (13, 14, 20, 34, 48, 49). 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 (34). Subsequently, ILPs were also found in antigen-induced synapses formed between T cells and endothelial cells, dendritic cells, or B cells (13). ILPs are podosomal structures that are enriched in LFA1 and require WASP and Arp2/3 for their formation (31, 34). 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 (31, 33, 50-52). WASP is expressed by all lymphoid and myeloid lineages (35, 36), 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 (36). 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 (53, 54), T cell priming by dendritic cells (55, 56), and the induction of B cell class switching by follicular T helper cells (57). 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 (28–30). 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 (58, 59). 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 synap-

tic 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 (17). 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 μg/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 μg/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 (60). 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-μM 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 (60) 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 (fig. S3C), 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 (Fig. 3C), 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 (fig. S4A). Force exertion against 0.7 μm-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 (Fig. 6C) were generated by calculating the distances between strongly deflected pillars (¿0.6 μm deflection) and the IS COG at each time point

(fig. S5B). COG coordinates for the IS were generated from masks derived from the Alexa Fluor 488 (CTL) channel. To calculate the centralization factor (Figs. 1D and 5, B and D), 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) (fig. S2). Granule polarization (Fig. 1D) 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 (Fig. 7, B and C), 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 (fig. S6D).

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 (61).

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 (8, 22). 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 (61). 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) (8) were subcloned into the miR30E vector (62) using the following primers: miRE-Xho-fw (5′-*TGAACTCGAGAAGGTATAT TGCTGTTGACAGTGAGCG*-3′) and miRE-EcoOligo-rev (5′-TCTCGAATTCT AGCCCCTTGAAGTCCGAGGCAGTAGGC -3′). gRNAs targeting WASP and WAVE2 were generated as previously described (63, 64) using the following oligos: NT gRNA: oligo-1 (5′- CACCGGGATACCTGGGCCGACTTTC -3′) and oligo-2 (5′- AAACGAAAGTCGGCCCAGGTATCCC -3′). WASP gRNA: oligo-1 (5′- CACCGCTGGACCATGGAACACTGCG -3′) and oligo-2 (5′- AAACCGCAGTGTTCCATGGTCCAGC -3′). WAVE2 gRNA: oligo-1 (5′-CACCGAGCAAGGGAGTTTACTCGGG-3′) and oligo-2 (AAACCCCGAGTAAACTCCCTTGCTC -3′). Each gRNA was cloned into the LentiGuide-Puro vector and then amplified by PCR using the following primers: LMP BamHI F2 (5′- TTTTTGGATCCTAGTAGGAGGCTTGGTAG -3′) and LMP EcoRI R2 (5′- TTTTTGAATTCTGTCTACTATTCTTTCCC -3′). The resulting fragments were digested with BamHI and EcoRI and ligated into miR30E previously digested with EcoRI and BglII.

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 µM, 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 × g in the presence of polybrene (4 μg/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 M14/7; 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 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 (62). 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 μg/ml streptavidin in PBS followed by increasing concentrations of biotinylated ICAM1 in the presence or absence of 1 μg/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 μg/ml by the addition of nonspecific biotinylated MHC (H2-Db). OT1 CTLs were fluorescently labeled with cell trace violet (CTV), resuspended in adhesion buffer (PBS with 0.5% BSA, 2 mM MgCl2, 1 mM CaCl2), 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 (62), and the bound cells quantified by fluorimetry.

2.4.14 Immunoblot

0.2-1 × 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 × 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-κB 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 IκB (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. Mechanically active integrins directed cytotoxic secretion at the immune synapse.

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 destroy1. Cytotoxic lymphocytes kill their targets by channeling a mixture of granzyme proteases and the pore forming protein perforin directly into the IS2, 3. 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 αLβ2 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 Results

3.1.1 LFA-1 is required for synaptic force exertion, degranulation, and cytotoxicity (Fig 1, S1, S2)

Lytic granules, the specialized secretory lysosomes that store perforin and granzyme, are known to accumulate at the IS and fuse selectively with the synaptic membrane2, 3. 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 formation2, 4. Studies from our group and others, however, indicate that the centrosome is dispensable for synaptic secretion, implying the existence of other targeting mechanisms5-7. 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 function8-14. We have found that lytic granule exocytosis (degranulation) tends to occur in regions of active force exertion within the IS8, 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 expressed the OT-1 TCR, which is spe-

cific 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 integrins1, 15, 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 microscopy16, we found that LFA-1 engagement substantially increased synaptic force exertion (Fig. 1a-b), implying that this integrin is a key player in IS mechanics and therefore a candidate for mediating coupling between mechanical and secretory output. 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 (Fig. 1c). 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). 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 antigen threshold required for signaling and TCR-induced gene expression17, 18. 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 activation. 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, 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.

3.1.2 Degranulation occurs in IS domains containing ligand-bound TCR and ligand-bound LFA-1 (Fig 2, S3)

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 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). 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 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 μm x 10 μm 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. 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 Lamp120. 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 ICAMspot, 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. 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 a-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 signaling15. 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 and its respective ligand, promote conformational changes, and induce signal transduction10, 11, 14, 21.

3.1.3 LFA-1 pulling forces define degranulation domains (Fig 3)

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)22 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 reports23, 24, 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. 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 μm 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 spatial 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 μm 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 (0.5-1 μm in diameter)25.

3.1.4 Talin is required for LFA-1 mediated force exertion, degranulation, and cytotoxicity (Fig 4, S4)

Integrins are coupled to the F-actin cytoskeleton via talin, a mechanosensitive scaffolding protein that is critical for the formation and signaling of integrin adhesions26 (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 and Movie S5), 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. 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.

3.1.5 Talin, but not LFA-1, is required for CTL-mediated killing of B16F10 cells (Fig 5, Fig S5)

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.

3.2 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 (Fig. S5). 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 fusion27-29. In the absence of integrin dependent force exertion, however, TCR signaling alone is insufficient for robust cytotoxic 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 receptors30, 31. 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 ($\alpha \mathrm{E}\beta 7$), 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 cells32. Previous studies indicate that synaptic forces enhance the pore forming activity of perforin by straining the target membrane8, 12. 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.

The centrosome and its associated microtubules are key determinants of lytic granule localization2, 4. 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 process5-7. 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 IS5. 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.

3.3 Materials and Methods

3.3.1 Constructs

3.3.2 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 β2-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.

3.3.3 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.

3.3.4 Traction force microscopy

Arrays of PDMS (Sylgard 184; Dow Corning) micropillars (0.7 μm in diameter, 6 μm in height, spaced hexagonally with a 2-μm center-to-center distance) were cast onto glass coverslips using the inverse PDMS mold method34. After an ethanol wash and stepwise exchange into phosphate buffered saline (PBS), pillars were stained with fluorescently labeled streptavidin (20 μg/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 μg/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.

3.3.5 Antibody blockade and pharmacological activation/inhibition

To assess the importance of LFA-1, CTLs were pre-incubated with LFA-1 blocking antibody (20 μg/mL, Clone M17/4, BioXCell) or an IgG2ak isotype control antibody (20 μg/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 μg/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 μM, 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 μM A23187.

3.3.6 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 x 10^6 OT-1 blasts 24 h after primary peptide stimulation. Mixtures were centrifuged at 1400g in the presence of polybrene (4 μg/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.

3.3.7 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 Vbottomed plate. Specific lysis of CTV+ target cells was determined by flow cytometry after 4 h at 37°C35. 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 (PKH26+CTV+)/(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).

3.3.8 CTL activation with stimulatory beads

Streptavidin-conjugated polystyrene beads (Spherotech) were coated with 1 μg/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 μg/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.

3.3.9 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 μg/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 μg/mL H2-K^b-OVA, (2) Antigen-spot – 10 μg/mL ICAM-1, (3) Dual-spot – 5% BSA, (4) ICAM-spot with anti-CD3 background – 10 μg/mL anti-CD3 antibody (Clone 145-2C11, eBioScience), (5) Anti-CD3-spot – 10 μg/mL ICAM-1, and (6) control – 10 μg/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 μg/mL ICAM-1, (2) Antigen-spot – 2 μg/mL H2-K^b-OVA, (3) Dual-spot – 2 μg/mL H2-K^b-OVA and 2 μg/mL ICAM-1, (4) ICAM-spot with anti-CD3 background – 2 μg/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.

Table 3.1: DNA hairpins

A21B	5AmMC6 - CGC ATC TGT GCG GTA TTT CAC TTT - 3Bio
Quencher strand	5DBCON - TTT GCT GGG CTA CGT GGC GCT CTT - 3BHQ2
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

3.3.10 Calcium 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 described36. 340 nm and 380 nm excitation images were acquired every 30 seconds for 30 min using a 20x objective lens (Olympus).

3.3.11 DNA hairpins

Name Sequence (5′ to 3′)

A mixture of oligo A21B (10 nmol) and excess Cy3B-NHS ester or Atto647N-NHS ester (50 μg) 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.

3.3.12 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 μL 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 μL 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 μL 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 μg/mL of streptavidin was incubated in the channels for 45 min at room temperature. The surfaces were cleaned with PBS and incubated with 5 μg/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 μg/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.

3.3.13 Imaging analysis

Imaging data were analyzed using SlideBook (3i), Imaris (Bitplane), Excel (Microsoft), Prism (GraphPad), and Python in Jupyter Notebook37. 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 μm x 5 μm square with a quarter-circle representing the stamped

protein at one corner. Then, the unit square was divided into 1 x 106 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 μm. MTP data were analyzed by comparing the mean fluorescence intensity of each MTP within the 2 μm x 2 μm 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 μm 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.

3.3.14 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 x 10^6 CTL with 4.0 x 10^6 splenocytes) the presence of 10 μg/mL anti-LFA-1 or an isotype control antibody. Subsequent dilution of CellTrace Violet was assessed by flow cytometry.

3.3.15 Immunoblot

0.2-1 x 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

CONCLUSION

4-1. FUTURE DIRECTIONS To be added

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