

FORCE AND SIGNIFICATION IN THE IMMUNE SYSTEM: FOOTPRINTING IMMUNE SYNAPSIS

by

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A dissertation

Presented to the faculty of the Louis V. Gerstner, Jr.

Graduate School of Medical Sciences,

Memorial Sloan Kettering Cancer Center

in partial fulfillment of the requirements for the Degree of

Doctor of Philosophy

New York, NY

March 2024

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ABSTRACT

Immune cells regularly sense, process, and respond to mechanical information about other cells and their surrounding environment. Biophysical measurements have revealed that cytotoxic T lymphocytes (CTLs) exert efferent mechanical forces that distort their antigen-presenting targets through the immunological synapse. Importantly, these forces potentiate the biochemical activity of secreted Perforin-1, forging a link between a CTL's function (cytolysis) and mechanical activity (target membrane strain). This previous body of work, alongside studies in B cells and dendritic cells, **suggests that specific immune functions require particular patterns of mechanical output.**

To address this in the context of immune-mediated cytolysis, we developed a high-resolution microscopy and image analysis pipeline to measure CTL mechanical output on deformable polyacrylamide beads. This allowed us to record both the strength and patterning of individual CTL-bead interfaces. We found that *in vitro* cultured mouse CTLs compress 300-Pascal beads into synaptic craters *via* the actin cytoskeleton. Synapses thus appeared as convex rims circumscribing undulatory, concave basins with microscale reliefs and indentations. This stereotyped architecture forms within minutes of initial contact and is maintained indefinitely on the hydrogels. By contrast, bone-marrow-derived macrophages (BMDMs) form phagocytic contacts that are impressively protrusive but globally *tensile* rather than compressive. These findings demonstrated that distinct configurations of cell-cell contact form under distinct immune contexts.

As the cytotoxic payload of CTLs is exocytically released (degranulation) at the inner concave domain of the synapse, we reasoned that mechanical undulations formed by protrusive F-actin characterize an optimal mechanical environment for target cytolysis. A minimal model of target surface distension was constructed, containing terms for target stiffness, membrane tension, and membrane bending rigidity. Analysis of the simulated topographies revealed that clustering F-actin into progressively larger protrusive foci led

to a better balance between target membrane strain and degranulation capacity on the CTL surface, compared to diffuse, unclustered F-actin. Remarkably, this "goldilocks" behavior saturated beyond $1\text{-}\mu\text{m}$ —which matched indentation sizes in the real data. Thus, by generating undulatory synapses on target surfaces, CTLs straddle a Pareto frontier that maximizes both their biochemical and biomechanical outputs. We reasoned that the cytotoxic capacity of T cells relates to specific mechanical patterns, so we developed a novel method to parameterize single-cell topographies without positional averaging. In an approach analogous to Fourier analysis, we represented each synapse topography as a linear combination of Zernike polynomials, which allowed us to sort and arrange single-cell patterns by similarity using principal component analysis (PCA). Single-cell topographical patterns were highly heterogeneous, but defining discrete regions in PCA space by q-quantiling was sufficient to delineate pattern differences throughout these large datasets. Across all cell-type comparisons, the major axis of topographical pattern variation (PC1) specifically excluded patterns with C_∞ symmetry. We used this information to define a surface roughness metric (Spectral Complexity) using the Zernike expansions that reflects the (1) intensity, (2) radial asymmetry, and (3) wavelike distributions of local topographical features.

We applied this strategy to (1) contrast wild-type CTLs from cytotoxically impaired mutants, (2) track CTL mechanical output throughout a viral infection *in vivo*, and (3) contrast cytolytic CD8+ from non-cytolytic CD4+ effector T cells (T_{eff}). Remarkably, compressive strength, protrusive activity, and Spectral Complexity all independently distinguished cytolytic potential in these cases: (1) wild-type, WAVE2-knockout (KO), and PTEN-KO CTLs displayed greater mechanical output than Talin-KO or WASp-KO CTLs; (2) CD8+ T_{eff} displayed greater mechanical output than CD4+ T_{eff} cells; and (3) mechanical outputs rose and fell with the course of infections (Day 0 < Day 40 < Day 8).

Together, the phenotypes uncovered throughout this investigation strongly imply that the synaptic architecture described herein is optimized for effective T-cell-mediated cytolysis.

Finally, we measured how the patterning of synaptic F-actin depends on cell-intrinsic and cell-extrinsic factors. Applying the Zernike transform to sort cytoskeletal patterns revealed that upon encountering smaller, stiffer targets, CTLs form annular F-actin patterns at the synapse, resembling those formed by PTEN-KO CTLs. We posit that this mechanosensitive response is part of the greater regulatory behavior of CTL synapses.

This body of work demonstrates that specific patterns of mechanical output characterize particular immune cell functions, which we term *biomechanical signatures* or discrete *mechanotypes of immunity*. The cytolytic mechanotype is a compressive contact with undulating topography, while the phagocytic mechanotype is a tensile contact with protrusive character. Still other immune mechanotypes might exist, and further work is needed to uncover them. What emerges clearly is that interfacial mechanics expands our understanding of immune cell function, and that immune mechanotypes might present new targetable opportunities for immune modulation and therapy.

BIOGRAPHICAL SKETCH

Miguel was born in Mandaluyong City, Metro Manila, Philippines to Walberto Gerardo S. de Jesus and Melinda M. de Jesus on November 12, 1991. Too young to remember the Dissolution of the USSR in 1991 or the Asian Financial Crisis in 1997, Miguel lived a relatively idyllic childhood until undergoing a multiyear period of personal upheaval following his father's passing in 2004. Through Melinda's heroic efforts, the family of two regained a semblance of normalcy, and Miguel eventually entered the University of the Philippines where he would study Biochemistry, meet his future wife Camille V. Trinidad, and experience research work for the first time at the National Institutes of Health in Manila. This experience inspired a subsequent three-year career at the Center for Regenerative Medicine in Makati Medical Center, where he would manufacture dendritic cell vaccines and participate in the care of late-stage cancer patients. These experiences inspired and frustrated Miguel in equal measure, eventually prompting his application to Memorial Sloan Kettering Cancer Center to pursue graduate school. He moved to New York City in 2016, where he matriculated at the Louis V. Gerstner, Jr., Graduate School of Biomedical Sciences. In the laboratory of Morgan Huse, PhD at the Department of Immunology, Miguel combines chemical biology and high-resolution microscopy to uncover a vastly understudied mechanical principle of T-cell-mediated immunity. This document is the fruit of Miguel's nearly eight-year Chapter in the Huse Laboratory.

For Mom, Camille, and Pops

ACKNOWLEDGEMENTS

In eight years of graduate school, one accumulates a great many people to thank. First among these is my adviser Morgan Huse. This entire journey began with the question you asked me on that Monday afternoon when I was required to finally declare a thesis lab: "How would you like to try measuring T-cell forces in 3-D?" I appreciate all the advice and encouragement, as well as the boundless supply of enthusiasm and energy over the years (especially on Monday mornings...)—but far more important than these is the extremely ambitious, out-of-the-box, creative approach to science that I learned directly from the source. I will forever look back on this work of immunological hipsterism with a great sense of pride.

To the Huse lab, past and present, my deepest thanks for the meaningful companionship, scientific advice, and joy over the years. To the past lab members, thank you all for accepting and embracing me into the fold, and for really filling my memory bank with many golden years of karaoke, happy hours, and New York nights together. To Minggang Zhang and Ben Whitlock, thanks for being ever-reliable and sunny lab seniors; I tell people these days that I am the happiest eighth-year I've ever met, but then I realized I'm really standing on the shoulders of you two giants. To Fella Tamzalit, thanks for being Bay 3's spiritual leader, which has imparted onto me and Diana your unique blend of professionalism, dark humor with a kind heart, and self-advocacy, and which has truly made *sarcasm Bay 3's condition of truth* (to borrow from Roland Barthes). To Maria Tello-Lafoz, thanks for not only inspiring my science but for showing me the benefits of chaotic productivity/productive chaos, a spirit I inherited from you. I will always miss our bilingual/bicultural discussions in lab, and I'm so very glad to have been a part of the Lola Experience. To Ty El Rayes, thanks for your endlessly welcoming and warm lab energy. I appreciate each trip to your side of Bay 1, with its colorful discussions on the human condition and society, as well as all the memes we continue to exchange. To

Jackie Brown, thanks for all your support and encouragement during the difficult times in those early years, and for introducing the most beautiful Mister Pickles to me. To Brian Santich, thanks for your instrumental role in my decision not only to choose GSK, but also to consider the Huse lab for my PhD! To Carlos Jeronimo, thanks for your great sense of humor and calming presence in the lab—the Huse lab meme experience has always been great fun with you around. To Alex Ramadhin, thanks so much for all your administrative help and support over the years. To Lizzy Zale, thanks for your endlessly chipper spirit and for the full buy-in to the Huse lab philosophical experience; it was always fun talking about civilization and its discontents (to borrow from Freud). To Elisa Sanchez, thank you for your eternally sunny, positive, hopeful, and wholesome presence in my lab life; I’m very glad to have spent more time with you and Kevin in the later years of our PhDs, and to have met your lovely new family member! To Mitchell Wang, thanks for sharing in my endless spiraling, during those unforgettably philosophical nights where we cooperatively resisted Deleuzean defenestration with the power of Heidegger, Derrida, French cinema, and the never-ending search for aesthesis in New York City. (Also, thanks for all your constructs, which made their way into this work.) And finally, to my longest-tenured Baymate and Huselab littermate Diana, thanks for all the years of Bay 3 memes, our legendary Huse lab karaoke list, keeping me nice and caramelized with the constant roasting, our shared Asianhood, and of course, for introducing me to the greatness of Alien Taylor.

To the current lab members who came after me, thank you all for continuing to cultivate our happy and inspirational workplace vibe. To Ben Winer, thanks for the great scientific and social spirit you uphold in the lab, for various contributions to my project, and for really livening up the Madonna segment of our lab karaoke. To Yassmin Elbanna, thanks for adding so much color to these latter years of my PhD with your many truly bonkers stories and the lovely discussions on poetry and literature. We ought to have an

English major in every generation of the Huse lab from here on. To Aliya Holland, your chaos and humor are endlessly entertaining, and I feel a great kinship with you on the basis of our constant memeing. (Friendships built on the internet will never die.) To Alex Yakimov, many a lunch conversation is great fun with you around, and thanks also for the many book recs. To Esther Lee, thanks for being a great roton and for helping out with those last key experiments in my final stretch! To Nat Lovinger and Farah Mustapha, our Bay 3 spirit of "dark humor with a kind heart" is really well-maintained with you two. Thanks Farah for keeping the lab hangout spirit alive now that I am getting old and lazy, and thanks Nat for walking me through the many twists and turns of Gen Z slang. To Ann Wong, I will look back on our many wonderful afternoons chilling to Yellow Days, the beautiful dumpling nights, the discussions on art and philosophy, and your two masters with their combined total of seven legs. I see a lot of myself in you, and I am very proud to have been part of your journey in the Huse lab. And of course, thanks to the Huse lab Golden Boy Alex Settle for really developing this project with me. Without your great many intellectual contributions and hard work, this would not have been reached even a tenth of its hipsterness and uniqueness, which are, in my opinion, among its most enduring qualities. Very proud of what we've done here. And thanks for being a good senior for the next generation!

I also extend a great many thanks to my Thesis Committee: Sasha Rudensky, Philipp Niethammer, and Marilyn Resh. Your guidance over the years has been extremely important not only in the development of this project, but also in my own scientific development. I have always enjoyed our meetings and consider myself privileged to have been mentored by the three of you. Thanks also to Joe Sun, my PhD Candidacy exam chair, Justin Perry, my PhD Thesis Defense exam chair, and Janis Burkhardt, my external examiner for the dissertation—your valuable time and participation in this process means a lot to me.

Many thanks to all of my collaborators over the years, whom I am privileged to have encountered and worked with during this journey—Julie Theriot for the key insights about Zernike polynomials; the Theriot lab, for hosting me at Stanford and for always taking care of me during ASCB; to Daan Vorselen, for letting us in on this amazing experimental system and for all your patience on-boarding me (starting from *caxis[]* in MATLAB...); Endi Santosa, Joe Sun, and Andrea Schietinger for help with the LCMV experiments; Vitaly Boyko, Yevgeniy Romin, Eric Chan, and Eric Rosiek of the microscope core; Anthony Santella, Chris Brittin, and Zhirong Bao for tons of imaging support; Broseph Nick Kuhn of the Brentjens lab for virus; the master-membrane-modelers Steve Abel and Tom Gaetjens; Tian-Ming Fu for help on the azimuthal degeneracy problem; and Daigo Maeda of SPG Technology Co., for helping us set up and optimize the hydrogel synthesis process. Aside from all these great people, many fruitful discussions have helped me think about my project (and life) over the years—especially Clarissa Campbell and Joris Van der Veecken of the Rudensky lab, Mig Ordonez from the Ateneo De Manila University, and Arnold Lau from the Pew Research Center.

I am the product of many great formators who helped develop my mind and character in equal measure: thank you to all my Xavier Grade School and High School teachers, my teachers from the summer semester I spent at Ateneo, my many excellent professors from the University of the Philippines Manila (especially Drs. Maria Carrillo and Voltaire Organo), my first research mentors Drs. Raul Destura and Em Sevilleja, and Dr. Francisco Chung. I hope that my success in this endeavor, which I wholeheartedly share with all of you, shows the world how Filipinos, underrepresented in the sciences as we are, can nevertheless achieve great things given the right opportunities. I also thank Pavak Shah for setting off my love for microscopy, my many great mentors and teachers at the Marine Biological Laboratory's Advanced Quantitative Light Microscopy course (especially Chris Bjornsson from Zeiss and Louise Bertrand from Leica), my rotation mentors in

MSKCC who took a chance on me—Lydia Finley, Craig Thompson, Clarissa Campbell, and Sasha Rudensky—as well as my friends and mentors at Calico Life Sciences, from whom I learned so much even in my final year of grad school—Kat Labbe, Kayley Hake, Austin Lefebvre, Julia Dean, Alfred Millett-Sikking, Elliot Mount, Andy York, and Carmela Sidrauski.

The Louis V. Gerstner, Jr., Graduate School of Biomedical Sciences took a big chance on me in 2016, and I am forever grateful for the opportunity to be a part of this amazing graduate school. I hope this work makes you all proud. Thanks to our Founding Dean Ken Marians, former Associate Dean Linda Burnley, current Dean Michael Overholtzer, current Associate Dean Tom Magaldi, and all of the extremely helpful support staff over the years, especially Maria Torres, Ivan Gerena, Julie Masen, and David McDonagh. Through this graduate school, I have met many truly wonderful people over the years, including Dan Macalinao, Steve Albanese, Michelle Riegman, Emily Kansler, Rui Gao, Paige Arnold, and Gemma Regan-Mochrie who came before me, as well as Mariano Aufiero, Florisela Herrejon, Katelyn Mullen, Nan Xu, Brianna Naizir, and Zaza Gelashvili who came after. Of course, I have really enjoyed the time spent with my own cohort of 2016: Stella Paffenholtz, Amy Shyu, Zhongmin Wang, *shuaige yisheng* Yu-Jung Chen, Maria Sirenko, Ryan Smolkin, Renhe Luo, Mike Galiano, & Tyler Hitchman. A wonderful time was shared with you all.

Many friends around the Tri-I and MSK community also deserve my thanks for the companionship and good times: Beverley Royal, Godofredo Ebor, Lorenzo Requillas, Isiah Brockington, Nadeshka, Veronique, Jose Estrella, Howell Rose, Austin Thompson, Howard, Terrenze, and Ulises Pichardo; all my friends at the Scholar's pool table and the MSK bar; my many friends around the 16th floor over the years, including members of the Schietinger, Li, Tulpule, Hohl, Diehl, and Pamer labs over the years. I made some of my very best friends here throughout the years: Clarissa Campbell and Joris Van der

Veeken, who took me along a whirligig tour of science, jazz, and Japanese street food in New York in the early phase of my graduate school life; Michelle Riegman, Gemma Regan-Mochrie, and Rui Gao, with whom I have endlessly enjoyed drinks and cats and good times around the city; the Fungalmentals Kathleen Mills, Mariano Aufiero, and Loulou Leyre, who really gave me a wonderful second wind in a year most of my older friends left NYC—our many beach and upstate trips over these past four years have crystallized into my very happiest core memories. To Louise and Mitchell, thanks for sharing in many of the funniest war stories of my final grad school years. And finally, to my dedicated Sunday weebgroup Mariano Aufiero, Mike Galiano, Tyler Hitchman (I'm using the acceptable name of the group here), the fun and games with you three have been among the most important constants of an otherwise extremely stressful journey. Cheers to the next decades, and yes, I'll get to arranging the Asia visit one day. One day...

I thank my dear friends 9000 miles away in the Philippines, with whom I still remain in touch and enjoy home vicariously. I thank my high school and college friend groups, my friends from the Makati Medical Center, and those who jumped into the American Science Adventure with me: Dem Cordova, Aaron Balana, Camille Trinidad, Mao Reyes, and Geoffrey Li. Thanks also to all of my Pinoy friends in New York City and California for making life far from the motherland that much more fun—Chris Dee, Ryan Yu, Robby Sylianteng, Ozzie Purugganan, Cybelle Tabilas, Aileen Chang, Reese Sy, Gavin Kho, Clarence Ang, Emerson Ang, and Arnold Lau. And a very important source of joy in NYC has been my Capoeira Guatambu family—*muito obrigado a todos pelo seu apoio*. Thanks as well to my family members around the East Coast for the many good laughs, meals, and drinks shared together over the years: Elizabeth Martinez, Ray Fletcher, Tina Martinez, Maxine and Zandy Lizaso, Santi and Xavi Ablaza.

I end with thanking the five most important people in my life: my thanks and love to you all. Mig Ordonez, thanks for the constant laughs at deeply insomniac times in the

early morning, for helping me think about more balls than I ever want to for the rest of time, and for coming in second place in total number of miles traveled to visit me in New York City. May more aesthesis and good vibrations follow us into the next chapters of life. Timmy de Jesus, the person with whom I am most similar in human history—in pretentiousness, in personality, in humor, and in ambition—our parallel journeys over the past decade have always been a source of comfort and companionship. However deeply I spiral into the void, I always belong somewhere because of you. Camille, my better half in science and in life, my soon-to-be-wife, you are not just my home away from home: you *are* my home, period. Wherever I go in this life, as long we have each other, what is at first a scary unknown melts away into an exciting adventure, just like this whole "grad school thing." I can't wait to finally build a life together like we always dreamed. Thank you for taking care of me at my weakest, most vulnerable moments in this journey, and thank you for always choosing me. To Pops, who instilled in me the value of my own mind, who learned Chinese in his own idiosyncratic way (without understanding anything) so he could help me with schoolwork, who raised me on Pat Metheny and Dave Brubeck, who gave me a wonderfully happy childhood until his last day, who taught me not to take myself too seriously—this dissertation is for you.

Finally, to Mom. None of this happens without your resilience and love. I love you, and thank you for everything! Among all of the accomplishments in life, the one I am more proud of than anything is being your son.

TABLE OF CONTENTS

Abstract	i
Biographical Sketch	iv
Dedication	v
Acknowledgements	vi
Table of Contents	xiii
List of Tables	xvi
List of Figures	xvii
List of Abbreviations	xix
Preface	xxii
 1 INTRODUCTION: FORCE AND SIGNIFICATION IN THE IMMUNE SYSTEM	 6
1.1 The mechanical life of cells	6
1.1.1 The mechanical structures of cells	7
1.1.2 General trends in cellular rheology	16
1.1.3 Mechanosensing, mechanotransduction, mechanoreciprocity . .	21
1.2 Overview of the immune system	27
1.2.1 Organization of the immune response	27
1.2.2 T-cell activation, synapse formation, and target cytolysis . . .	34
1.3 Force and signification in T-cell biology	45
1.3.1 Mechanical input: Contributions to T-cell activation.	50
1.3.2 Mechanical output: Contributions to T-cell effector function. .	54
1.4 Thesis aims	58
 2 PHENOMENOLOGY OF T-CELL MECHANICAL ACTIVITY	 60
2.1 Background	60
2.1.1 Microparticle traction force microscopy: the advantages and limitations of a new force probe	60
2.2 Results	63
2.2.1 Hydrogel microparticles mimic T-cell activation by APCs . . .	63
2.2.2 The CTL synapse is a compressive crater with dynamic undulations	66
2.2.3 Actin remodeling fuels CTL mechanical activity	69
2.2.4 Bumpy craters are also formed on <i>bona fide</i> target cells	71
2.3 Discussion	72
2.4 Materials and methods	74
2.4.1 Cell culture, isolation, and retroviral transduction	74
2.4.2 Hydrogel synthesis, derivatization, and peptide coating	75
2.4.3 Ca^{2+} flux imaging	77
2.4.4 CD69 expression by flow cytometry	78
2.4.5 High-resolution imaging	78
2.4.6 Image processing and analysis	79

3	RATIONALIZING UNDULATION: INSIGHTS ON DEGRANULATION FROM A MINIMAL <i>IN SILICO</i> MODEL OF SYNAPSE DISTORTION	84
3.1	Background	84
3.1.1	T-cell degranulation through the cortical actin mesh	84
3.2	Results	87
3.2.1	CTL degranulation occurs at the undulatory crater floor of the synapse	87
3.2.2	Synapse simulations generated by an energy minimization approach resembles real synapse topographies	89
3.2.3	Degranulation and target membrane strain establish a fundamental trade-off for CTL activity	90
3.3	Discussion	94
3.4	Methods	96
3.4.1	Cell culture and retroviral transduction	96
3.4.2	Image processing and analysis	96
3.4.3	Design of the minimal model	97
4	LINKING SINGLE-CELL PATTERN ANALYSIS WITH CYTOLYTIC POTENTIAL <i>IN VITRO</i> (I) AND <i>IN VIVO</i> (II)	101
4.1	Background I	101
4.1.1	Expanding the concept of mechanical output: borrowing functions from ophthalmology and telescropy to capture <i>topographical patterning</i> by CTLs	101
4.1.2	Actin regulators and T-cell cytotoxicity	103
4.2	Results I	105
4.2.1	WASp, WAVE2, and Talin contribute distinctly to CTL mechanical output	105
4.2.2	A modified Zernike transform successfully sorts single-cell topographies by similarity	106
4.2.3	Spectral pattern complexity distinguishes killing-competent from perturbed CTLs	111
4.3	Discussion I	112
4.4	Materials and methods I	116
4.4.1	Cell culture and imaging	116
4.4.2	Image processing and analysis	117
4.5	Background II	121
4.5.1	The course of T-cell activation and differentiation during an infection	121
4.6	Results II	124
4.6.1	LCMV infection establishes cohorts of naive, effector, memory, and exhausted T cells <i>in vivo</i>	124
4.6.2	<i>Ex vivo</i> measurements of compression strength and spectral complexity follow the course of infection	125
4.7	Discussion II	129

4.8	Materials and methods II	131
4.8.1	Murine infection model	131
4.8.2	Cell isolation and T-cell phenotyping	131
5	THE LIMITS OF ONTOGENY: DEFINING SYNAPTOCYTES AND PHAGOCYTES ON THE BASIS OF FORM	133
5.1	Background	133
5.2	Results	134
5.2.1	Cytolytic synapses are stronger and more complex than non- cytolytic synapses	134
5.2.2	Primary macrophages form protrusive, undulatory contacts that are globally tensile	138
5.3	Discussion	141
5.3.1	A plurality of immune mechanotypes	141
5.3.2	Cell size as a determinant of contact mechanics: a brief, neu- trophilic detour	143
5.3.3	Preliminary exploration of cytoskeletal taxonomies within the immune system	144
5.4	Materials and methods	145
5.4.1	Cell culture, hydrogel derivatization	145
5.4.2	Radial profiling	147
6	MODULATING THE CYTOLYTIC F-ACTIN ARCHITECTURE	149
6.1	Background	149
6.1.1	Cell-intrinsic and cell-extrinsic influences on synaptic actin architecture	149
6.2	Results	150
6.2.1	PTEN-KO CTLs display enhanced cytolytic and mechanical activities	150
6.2.2	Applying the Zernike transform to Phalloidin maps reveals an emergent cytoskeletal pattern	153
6.3	Discussion	156
6.4	Materials and methods	158
6.4.1	Cell culture	158
6.4.2	Killing and degranulation by flow cytometry	158
6.4.3	Actin centralization measurement	159
7	OUTLOOK	160
7.1	The concept of an immune mechanotype	160
7.2	Future directions	167
7.3	Concluding remarks	168
	Bibliography	169

LIST OF TABLES

1.1	Summary of recent studies measuring T-cell force exertion.	56
5.1	Gene set comparison according to inter-sample Pearson's R.	146

LIST OF FIGURES

1.1	Mechanisms of actin polymerization.	9
1.2	Activation and engagement of the integrin-talin-vinculin molecular clutch.	14
1.3	Molecular, functional, and geographical strata of the molecular clutch.	23
1.4	Stages of an immune response.	28
1.5	Broad overview of adaptive immune responses.	31
1.6	Signaling network downstream of the TCR.	35
1.7	Functional and geographical stratification of the immune synapse according to actomyosin network architecture on a freely mobile bilayer.	39
1.8	Summary of critical cytoskeletal regulators in lymphocytes.	42
1.9	Overview of the CTL synapse.	44
2.1	Hydrogel bead synthesis and conjugation scheme.	61
2.2	Ca ²⁺ flux imaging of CD8+ CTLs.	64
2.3	CD69 detection by flow cytometry.	65
2.4	A representative synapse formed with the spherical hydrogel bead.	67
2.5	Measurement of hydrogel compression.	68
2.6	Active micropatterning by the T cell.	69
2.7	Topographical feature enumeration at the synapse.	70
2.8	Involvement of actin in CTL mechanical activity.	71
2.9	Measurement of compressions on <i>bona fide</i> APCs.	73
3.1	CTL degranulation through the cortical actin mesh.	85
3.2	CTL degranulation is gated by engagement of the integrin-talin clutch.	86
3.3	Mapping target topographies associated with degranulation.	88
3.4	Model parameters for simulating synapse topographies by an activated CTL.	89
3.5	Synapse simulations resemble real topographical data.	91
3.6	Definition of degranulation-permissible ("degranulatable") area, an index of chemical activity by the CTL.	92
3.7	Mechano-chemical tradeoffs in CTL activity.	93
3.8	Protrusion sizes of real CTL-hydrogel conjugates.	94
4.1	Zernike polynomials, arranged according to the Noll index convention.	102
4.2	Wavefront fitting <i>via</i> a linear combination of the Zernike polynomials.	103
4.3	Mechanical defects of CTLs after genetic perturbation of force regulators.	106
4.4	Representing individual synapse topographies as rotationally degenerate Zernike spectra.	108
4.5	PCA of Zernike spectra (Z-PCA plot) facilitates pattern sorting.	109
4.6	Z-PCA plot, with cell-type-specific galleries highlighted.	109
4.7	PC1 encodes information on radial asymmetry.	110
4.8	Definition of spectral complexity.	112
4.9	Spectral complexity as an index of CTL mechanical output.	113
4.10	Independence of spectral complexity and compression strength.	114

4.11	Zernike spectra and reconstructions of two synapse topographies. . . .	118
4.12	Differentiation landscape of CD8+ T cells.	122
4.13	Comparison of motility between T_{eff} and T_{exh} in a tissue slice culture. . . .	123
4.14	Experimental design for mechanical profiling of different T-cell differentiation states.	125
4.15	Purification and immunophenotyping of <i>in vivo</i> differentiated CD8+ T cells.	126
4.16	Comparison of T-cell compression strength and topographical features across the four differentiation states.	127
4.17	Comparison of T-cell topographical patterning across the four differentiation states.	128
4.18	Independence of strength and pattern complexity in this dataset.	129
5.1	Comparison between T-cell synapses and macrophage phagocytic contacts. . . .	134
5.2	Comparison of CD4+ and CD8+ mechanical outputs during synapse formation.	135
5.3	Difference map of topography as a function of radial location for CD4+ and CD8+ T_{eff} cells.	136
5.4	Comparative patterning studies of CD4+ and CD8+ T_{eff} cells.	137
5.5	Metrics of strength and local topographical feature generation of BMDMs and CD8+ CTLs.	139
5.6	Spectral pattern analysis of BMDMs and CTLs.	140
5.7	Similarity analysis of the ImmGen transcriptomes dataset.	144
6.1	PI3K and PTEN balance in driving synaptic F-actin polymerization. . . .	150
6.2	Cytotoxicity and degranulation of sgPTEN CTLs.	151
6.3	Comparison of compression strength and topographical feature generation by sgPTEN CTLs.	152
6.4	Comparison of topographical patterning by sgPTEN CTLs.	153
6.5	Sorting actin intensity patterns by Z-PCA.	154
6.6	An emergent, annular pattern of actin is generated by PTEN-KO cells. . . .	155
6.7	Actin centralization measured as a function of cell-intrinsic and cell-extrinsic features.	156

LIST OF ABBREVIATIONS

AP1	Activator protein 1
ADF	Actin-depolymerizing factor
AIBN	Azobisisobutyronitrile
AID	Activation-induced deaminase
AFM	Atomic force microscopy
APC	Antigen-presenting cell
APS	Ammonium persulfate
Arp2/3	Actin related protein-2/-3 complex
β PIX	PAK exchange factor β
BCR	B-cell receptor
BMDM	Bone-marrow-derived macrophage
C5a	Complement 5a fragment
Ca^{2+}	Calcium ion
CAR	Chimeric antigen receptor
CCL	Chemokine C-C motif ligand
CCR	C-C chemokine receptor
cGAS	cyclic GMP-AMP synthase (cGAS)
CRAC	Calcium release activated channel
CTL	Cytotoxic T lymphocyte
CTLA4	Cytotoxic T-lymphocyte associated protein 4
CXCL	Chemokine (C-X-C motif) ligand
DAAM	Deformable acrylamide-acrylic acid microparticle
DAG	Diacylglycerol
DC	Dendritic cell
DMSO	Dimethylsulfoxide
DPC	Distal pole complex
ECM	Extracellular matrix
EDC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
eGFP	Enhanced green fluorescent protein
ER	Endoplasmic reticulum
ERM	Ezrin, radixin, moesin
F-actin	Filamentous actin
FACS	Fluorescence-activated cell sorting
FAK	Focal adhesion kinase
FBS	Fetal bovine serum
FcR	Fc (crystallizable fragment) receptor
FDR	False discovery rate
fMLP	N-formylmethionine-leucyl-phenylalanine
FMNL1	Formin-like 1 protein
G-actin	Globular actin (monomeric)
GC	Germinal center

GEF	Guanine exchange factor
GPCR	G-protein-coupled receptor
ICAM1	Intercellular adhesion molecule 1
IF	Intermediate filaments
Ig	Immunoglobulin
IL	Interleukin
ILC	Innate lymphoid cell
IFN- γ	Interferon- γ
IP _n	Inositol trisphosphate (n = 1, 2, 3...)
IRF	Interferon-regulatory factor
iSIM	Instant structured illumination microscopy
ITAM	Immunoreceptor Tyrosine-based activation motif
FH1/2	Formin homology 1/2 domain
KD	Knockdown
KO	Knockout
kPa	Kilopascal
LAMP1 (CD107a)	Lysosomal-associated membrane protein 1
LatA	Latrunculin A
LCMV	Lymphocytic choriomeningitis virus
LFA-1	Lymphocyte function-associated antigen 1
LIM	Lin-11/Isl-1/Mec-3 (protein domains)
LPS	Lipopolysaccharide
LRR	Leucine-rich repeat
MAPK	Mitogen-activated protein kinase
MCA	Membrane-cortex attachment
MCT	Microcentrifuge tube
MEF	Mouse embryonic fibroblast
MES	4-morpholinoethanesulfonic acid
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MOPS	3-morpholinopropane-1-sulfonic acid
MP-TFM	Microparticle traction force microscopy
MTC	Magnetic twisting cytometry
MTOC	Microtubule-organizing center
N ₂	Nitrogen gas
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NF κ B	Nuclear factor kappa-light-chain-enhancer of activated B cells
NFAT	Nuclear factor of activated T cells
NHS	N-hydroxysuccinimide
NLR	NOD-like receptors
NK cell	Natural killer cell
NM2	Non-muscle myosin II
NPF	Nucleation-promoting factor

OVA	Chicken ovalbumin
Pa	Pascal
PAK	p21 (Rac1) activated kinase 1
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate-buffered saline
PCA	Principal component analysis
PD	Probability density
PD1, PDL1	Programmed cell death-1 & ligand
PFA	Para-formaldehyde
PhoE	Phoenix E cell line
PI3K	Phosphatidylinositol-3-kinase
PIP ₂ (n)	Phosphatidylinositol-4,5-bisphosphate (e.g., n: 4,5)
PIP ₃ (n)	Phosphatidylinositol-3,4,5-trisphosphate (e.g., n: 3,4,5)
PKB, Akt	Protein kinase B
PKC	Protein kinase C
PLC	Phospholipase C
PM	Plasma membrane
pMHC	Peptide-MHC
Prf1-/-	Perforin-knockout
psi	Pounds per square inch
PTFE	Polytetrafluoroethylene, Teflon
RAG	Recombination-activating gene
RIG-I	Retinoic acid-inducible gene I
ROCK	Rho-associated coiled-coil kinase
ROI	Region of interest
RTK	Receptor Tyrosine kinase
Scar	Suppressor of cyclic AMP receptor
SD	Standard deviation
SEM	Standard error of the mean
Shp2	Src homology region 2 domain-containing phosphatase-2
SIM	Structured illumination microscopy
SLB	Supported lipid bilayer
SMAC	Supramolecular activation cluster
SN _A	Nucleophilic acyl substitution reaction
SPG-SNM	Shirasu porous glass silica nanopore membrane
STIM	Stromal interaction molecule
STING	Stimulator of interferon genes
t-SNE	t-distributed Stochastic Neighbor Embedding
TCR	T-cell receptor
T _{eff}	Effector T cell
TEMED	Tetramethylethylenediamine
TFM	Traction force microscopy
TFS	Thermo Fisher Scientific
TGF- β	Transforming growth factor β

T_HC	helper T cell
TLR	Toll-like receptor
TMD	Transmembrane domain
T_n	Naive T cell
$TNF-\alpha$	Tumor necrosis factor α
T_{reg}	Regulatory T cell
UMAP	Uniform Manifold Approximation and Projection for Dimension Reduction
WASp	Wiskott-Aldrich syndrome protein
WAVE	WASp-family verprolin-homologous
WHD	WAVE homology domain
WIP	WASp-interacting protein
VDJ	Variable, Diversity, and Joining (gene segments)
VCAM1	Vascular cell adhesion molecule 1
VLA-4	Very late antigen 4
VLR	Variable Lymphocyte Receptor (gene segments)
YAP/TAZ	Yes-associated protein/transcriptional coactivator with PDZ-binding motif
Zap70	Zeta-activated protein 70 kDa

PREFACE

The possibility of communication

"Speaking frightens me because, by never saying enough, I also say too much."

—Jacques Derrida, *Force and Signification* (1963) [1]

If a scientific article is some kind of story, then is a PhD dissertation a kind of novel? Over the course of this study, my colleagues, mentors, collaborators, and I have developed novel analyses to make sense of some very interesting first-in-kind observations. One has, in principle, an infinite number of options by which to transform, present, and re-present data towards a study's ultimate *telos*. The major ethical question we readily confront, given this landscape of possibility, is how to make these choices and which choices we ultimately make. This is an issue of significance: are the data actually telling us something—for lack of a better word—real? And if so, is this sliver of reality—for lack of a better word—interesting? But there is another issue beyond significance that is best confronted from the onset, one of *signification*: what contexts shape our concepts? Which "right words" best express our interpretations of the results? Which metaphors?

Certain phrases and neologisms used repeatedly in this dissertation might fit about as comfortably as square pegs in the round holes of an immunological lexicon: "architecture", "topography", "patterning", the many uses of "complexity", etc. Of course, at first glance, the job of any good scientist in this position would be to stipulate, define, demarcate, and explicate each term of argument with as much exactitude as possible, minimizing linguistic freeplay; the reason "science works", after all, is because the rules of its language game are clear and rigid. And as a matter of course, we make conscious attempts to refrain from using words opaquely, equivocally, and loosely. However, there really is

no *a priori* benchmark for rigidity or fluidity except by the author's own good faith and sense. Furthermore (and more importantly), the very stability of the concept has been convincingly challenged (at least to me) in the recent century, not so much from a line of positivistic argumentation, but from the careful analysis of language. I believe the biomedical scientist must not ignore this development out of fear or inconvenience, but rather engage with it explicitly if we are to claim that our work has truly been conducted in the present moment.

Ferdinand De Saussure posthumously ushered in the age of structuralism in 1916, when published lecture notes from his *Course in General Linguistics* brought us past a simplistic, pictorial model of language to one in which each term (signifier) accrues meaning by the position it holds within a differential and arbitrary network of terms and meanings, i.e., within the language system as a whole. Jacques Derrida's great insight in 1967 was to push the Saussurean signifier toward its logical endpoint, which he found was toward its deconstruction: if a term has no necessary natural referent, then meaning emerges "like a trace" from the interplay of what is expressed (the word itself) and what is not expressed but still involved (other related terms it brings to mind, other words it sounds like, etc.) [2, 3]. So much for words, but this framework is extended towards meaning/s in general, towards ontology in general. We are confronted with conceptual structures (originally the human sciences, but why not also immunology or chemistry?) characterized *by symbolic freeplay* itself, structures whose very ground has to be imposed from outside of their own purview, lest they risk being replaced and destabilized [4]. On occasion, we see within the physical sciences discrete moments of such destabilization; Thomas Kuhn has called them paradigm shifts or revolutions [5]. To me, the overall movement is towards fuzziness, which I do not think is to be feared or avoided, but perhaps embraced as part of a scientific method. Gene usage patterns, surface marker expression patterns, and behavioral-phenotypic patterns that define any given immune

cell are growing, not shrinking, with time. Network-level and systems-level views of biological entities are increasing in number and complexity, not decreasing, with time.

Thankfully, fluidity and fuzziness do not necessarily contradict critical thought or rigor. (Equivocation does, but not the former two.) The scientist today still plays at tightrope-walking between Wittgenstein and Derrida. In so doing, however, we must make pragmatic choices to enable communication at all: we must deliberately restrict semiotic freeplay as we encounter the exciting new phenomena that make this occupation worthwhile. Reader and author alike must sign a temporary pact with modernity, so that we may commit to a belief in the same concepts, objects, and movements. But whenever a new idea crystallizes, we must also be open to a new turn of phrase, a new metaphor, a new image in the mind...

Immunity is a differential field

The immune system readily lends itself to questions about alterity, becoming, and relationality. After all, the immune system, chiefly among other biological systems, absolutely cannot be conceived without the Other. Nearly every subject of immunological thought, on all scales and within its vast scope, involves the study of some sort of "inter"—interface, interaction, interdigitation, interrelated network. Whether viewed from the top-down as the set of evolutionary solutions to specific problems (e.g., eliminating pathogenic threats, maintaining homeostasis, or defining selfhood) [6], or whether tackled from the bottom-up as the sets of biomolecules, cells, tissue architectures, and organ systems employed in each attempt at a solution [7], immunology may comprise boundaries and perhaps not much else. Boundaries of self & non-self, host & pathogen, lymphocyte & antigen-presenting cell, pathogen & commensal, autoimmunity & tolerance, immune

pathology & infection. This work focuses quite narrowly on one particular set of mesoscale boundaries, intercellular contacts. However, the insights derived from a study of leukocytes must not remain there for very long, because as the immunologist zooms out, it becomes evident that interfaces and interactions likewise constitute the greater intersubjective field that characterizes human life.

Cells of the immune system exist within microenvironmental milieus. Thinking spatially, we can describe immune microenvironments in terms of cells, proteins, genes, metabolites, secreted small molecules. In other words, as a mass or constellation of things. On the other hand, thinking temporally, we can also model immunity as process: cell-cell interactions, signaling pathways, circuits, hysteresis, memory. In other words, as an orchestra or a movement of the constellations. However, in my investigations of the time-bound cellular patterns of interfacial morphology, even when simplifying and abstracting their full richness and complexity, I feel at times forced to put into words the ineffable. In regards to this insecurity, a source of comfort is the view of post-structuralist giant Gilles Deleuze (1925-1995), who believed that "[t]hinking is a form of experimentation, where the aim is to determine concepts of the events which determine our fate." [8]

This seven-or-so-year intellectual project has been one in the business of pattern recognition, studying (immune-system-wide) patterns that comprise (individuated cellular) patterns within (leukocyte population-level) patterns. It's patterns all the way down, really, and we have to stop somewhere along the rabbit hole in order to say anything at all. In light of any potential scientific infelicities herein, all I can declare is that I have, in good faith, tried my very best to capture these appearances of truth, analyze them with great care, and in the end articulate what I hope to be interesting insights on how Nature's micromechanical activity folds itself into aspects of human personhood (e.g., health and

disease); or how, from within a possible set of physicochemical interfaces—or better yet, a *differential field*—tendencies we might call T-cell synopsis or myeloid cell phagocytosis crystallize in the determination of our own fates, beset as we are by challenges external (e.g., bacteria or viruses) and immanent to ourselves (e.g., autoimmune pathologies or cancer).

Herein lies a dissertation about the immune system. But I hope that this is not merely some document drafted in order to graduate, but a book with something to say about the world.

CHAPTER 1

INTRODUCTION: FORCE AND SIGNIFICATION IN THE IMMUNE SYSTEM

1.1 The mechanical life of cells

Life on all scales is rooted as deeply in physics and mechanics as in chemistry [9, 10, 11, 12, 13, 14]. The body plan of each organism has developed to account for its idiosyncratic experiences with gravity, fluid and air flow, stretching and compression, and all other physical encounters required for life. As far back as 1638, Galileo made the foundational mechanobiological observation that between animals of different sizes and weights, their long bones did not scale in a simple, linear fashion [15]. Additional studies on bones in the 1800s ossified the field as a bridging science between biology and physics, at a time when debates abounded on whether organismal development was driven by an underlying *telos* or by mechanical causality [16]. The internal architecture of bone trabeculae was likened to the trusses of a street lamp by Ward [17]; later, Culmann & von Meyer showed that trabeculae in the spongy bone arranged themselves precisely along stress lines (compression and tension) [18]. Not long after, Wolff formulated the principle of bone remodeling: the macroscale dimensions of a particular bone, as well as its internal trabecular architecture, dynamically remodel in response to the characteristic stresses experienced by that bone [19]. As summarized in D'arcy Thompson's *On Growth and Form* (1917) [20]: "The soles of our boots wear thin, but the soles of our feet grow thick, the more we walk upon them[.]"

Studies on *cellular rheology*—the material properties and responses of cells—and *mechanotransduction*—the biochemical processing of biomechanical signals—developed significantly and in parallel throughout the 20th century [10, 11, 12]. Beginning in the 1920s, flowing internal granules or microinjected particles were used as fiducial

markers to infer cytoplasmic viscosity by microscopy. It was appreciated by the 1940s that this internal viscosity changes with the cell cycle, various chemical treatments, temperature, and even external electrical fields. In 1954, the pioneering sliding filament theory was proposed to explain how actomyosin contractility powers muscle contraction [21, 22], providing a key conceptual link between biochemical reactions and physical force generation. Beyond the iconic sarcomeric architecture, other actomyosin network geometries were then shown to underpin cellular shape changes, mechanotransduction, efferent force exertions, and cellular motility [23, 24]. In the 1980s–1990s, technological advancements such as atomic force microscopy (AFM), traction force microscopy (TFM), magnetic tweezers, and optical traps rapidly fueled developments in our understanding of both cellular rheology and mechanotransduction: today, we have many sophisticated models that describe the complex material behaviors of cells, as well as a great body of literature on the mechanical activities of cellular adhesion receptors, the "molecular clutch", membrane lipids, various organelles, and many specific mechanically oriented biomolecules. These advances have now made their way past adherent, fibroblast-like cells, and into the more complex and amoeboid immune cells, making possible the rapidly developing field of mechanobiology.

1.1.1 The mechanical structures of cells

Reflecting the ubiquity of mechanical forces in cellular life, many distinct biochemical structures have evolved to sense, process, generate, and respond to force. Together, the higher-order assembly and concerted organization of these mechanical structures determine the material properties and direct the physical activities of a cell.

The cytoskeleton.

Foremost among the mechanical structures of a cell are the cytoskeleton [24] and the integrins [25, 26, 27, 28]. The cytoskeleton comprises: (1) filament-forming proteins (e.g., actin, tubulin, intermediate filaments [29], and septins [30]), (2) their adaptors and cross-linkers, (3) the regulators of their polymerization and depolymerization cycles, and (4) the motor proteins that act upon them [31]. Cytoskeletal architecture and dynamics define most of the mesoscale material properties and mechanical activities of cells [32, 33], including changes in shape, cell body polarization, motility, and physical force generation. Microtubules classically serve as tracks for vesicular trafficking and chromosomal sorting, but also bear compressive loads [34] and maintain a cell's ability to resist fragmentation under actin-mediated tensile stress [35]. Intermediate filaments are symmetrical fibers that resist shear and tensile stresses at steady state [24], but also serve as shock absorbers that buffer cells from the impact of large mechanical deformations [29]. Septins contribute to cell shape maintenance and mechanical coherence [36].

Actin is one of the most abundant and ancient proteins [32], forming the major cytoskeletal system for executing phenomena as diverse as vesicular trafficking, locomotion, shape changes, membrane bending, endocytosis, outward force application, and cytokinesis. Actin forms polarized helical filaments that elongate readily on the (+) or morphologically "barbed" end *via* actin-ATP assembly, and depolymerize on the (-) or "pointed" end *via* actin-ADP disassembly or by the action of severing proteins (e.g., Actin Depolymerizing Factor [ADF]/Cofilin, Severin). The complex pool of actin-regulatory proteins builds and disassembles filamentous actin (F-actin) in different orientations, leading to a large set of potential network geometries and utilities (Figure 1.1) [37].

Actin nucleates, elongates into filaments, and is capped (plugged) or severed. The rate of filament formation and elongation are, first and foremost, influenced by monomeric,

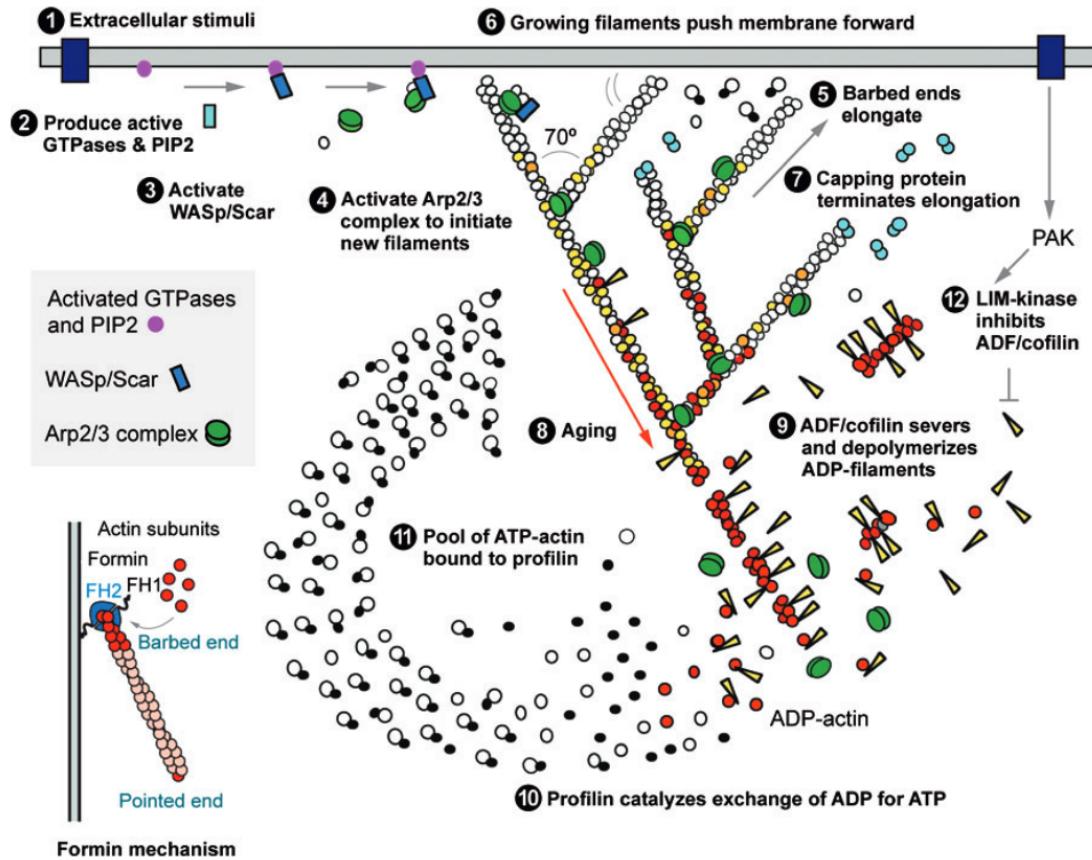


Figure 1.1: Mechanisms of actin polymerization.

Major events involved in actin polymerization within cells are summarized. Briefly, extracellular signals activate Rho family GTPases and mobilize phosphoinositides along the membrane, stimulating different types of actin polymerization. In T cells, Cdc42-GTP, Nck, and PIP₂ activate WASp; Rac1-GTP and PIP₃ activate WAVE2; while RhoA-GTP and PIP₂ stimulate the formins Dia and formin-like 1 protein (FMNL1) [38]. WASp and WAVE2 polymerize actin in branches, nucleating daughter filaments ~70° off the mother filament, while formins processively polymerize long, linear actin filaments from the membrane. Both these actin polymerization mechanisms compete for actin monomers; hence, actin network disassembly and dissolution into monomeric G-actin by severing proteins such as cofilin are necessary to maintain overall dynamics. Figure reproduced from [37] with permission from the copyright holder.

globular actin (G-actin) availability and sequestration (e.g., by Thymosin β 4 or Profilin), as well as by adenosine phosphorylation—with Thymosin and ADF/Cofilin inhibiting nucleotide exchange and Profilin promoting it. Actin filament nuclei (usually trimers) are then extended in two ways: in a linear fashion by the formins (e.g., FHOD1, mDia1) or in branched networks by the Actin-Related Protein complex (Arp2/3).

Formins processively elongate actin in long, linear bundles important for building the cytokinetic contractile ring, finger-like filopodial structures, and actin cables for structural integrity [37]. Formins share a common structure consisting of a Formin Homology 1 (FH1) domain that sponges profilin–G-actin complexes for efficient hand-off to and access by the C-terminal, catalytic FH2 domain. A regulatory N-terminal domain binds and inhibits the FH2 within an autoinhibition loop; this interaction can be displaced by active Rho family GTPases (e.g., Rho, CDC42), freeing and activating the FH1 and FH2 domains. The FH2 domain is a donut-shaped structure that executes the extension of the barbed end, rotating as it assembles fresh actin subunits onto the growing filament. Active FH2 competes with inhibitory capping proteins, effectively forming a "processive cap" that can elongate filaments at speeds of up to 100 actin units/second. Formin autoinhibition is relieved by RhoA-GTP and PIP₂ in T lymphocytes [38].

Actin branches are formed on the scaffold of the seven-membered Arp2/3 complex [37, 39]. Arp2/3 comprises actin-related proteins Arp2-3, which form a pseudo-dimer of actin that needs only one monomer to begin continuous elongation, and five accessory members ARPC1-5, which all bind a rigid interface along the mother filament. The pointed end of the shorter actin filament is held $\sim 70^\circ$ off to the side of the longer mother filament by activated Arp2/3. Nucleation of these Arp2/3 branches is stimulated by a family of nucleation-promoting factors (NPFs), which in mammals comprises WASp (Wiskott-Aldrich Syndrome protein), N-WASp, three isoforms of Scar/WAVE

(Suppressor of cyclic AMP receptor/WASp-family Verprolin-homologous), HS1, and Cortactin. These branched-actin NPFs share a general structure, with an N-terminal battery of regulatory and scaffolding regions followed by a C-terminal VCA (Verprolin homology, Connecting, and Acidic) region that simultaneously binds actin (V & C) and Arp2/3 (C & A). The N-terminal regions of both WASps contain: (1) a WH1 domain for binding WASp-interacting protein (WIP), influencing WASp activation, stabilization, and localization; (2) an electrostatically basic region that binds multiply phosphorylated phosphatidylinositols $\text{PIP}_2(4,5)$ and $\text{PIP}_3(3,4,5)$; (3) a GTPase-binding region (or CRIB domain) which responds to WASp activators from the Rho family of GTPases (e.g., CDC42); and a Proline-rich domain adjacent to the VCA region that binds Grb, Nck, and other SH3-containing proteins (e.g., immunosignaling proteins). On the other hand, the three WAVEs form pentameric complexes via their N-terminal WAVE homology domain (WHD), co-assembling with ABI, NAP1, SRA1, and HSPC300. Nap1 binds Nck while Sra1 binds Rac-GTP, the major upstream activator of WAVE. Between the WHD and VCA also lies a basic region that binds $\text{PIP}_3(3,4,5)$, and, in WAVE2 specifically, a Proline-rich region that bridges WAVE to Rac-GTP *via* IRSp53. WASp- and WAVE-mediated actin branching forms myriad functional structures in the cell: endocytic actin patches, cellular lamellipodia for shape changes and locomotion, as well as podosomes and filopodia for external palpation. In T lymphocytes, WASp autoinhibition is relieved by Cdc42-GTP, Nck, and PIP_2 , while WAVE autoinhibition is relieved by Rac1-GTP and PIP_3 [38].

Antegrade actin polymerization provides arguably the most important force-generating activity in cells, allowing them to protrude and directionally expand themselves [32]. The forward-directed motion of a polymerizing actin filament (F-actin) at the lamellipodium can be opposed by myosin contractility (which pulls actin backward) at the lamellum and by mechanical loads (e.g., the internal bending resistance of the plasma membrane [PM] or an external rigid body)—these counteracting forces cause actin mass to constantly

flow backward, with a "treadmilling" appearance. Overall actin network architecture is determined by filament assembly and disassembly kinetics, as well as by a diverse family of actin-bundling and crosslinking proteins [40], each with a particular angular preference and cell-type-dependent expression pattern. In turn, actin network architecture contributes significantly to steady-state cell rigidity and more complex, time-bound behaviors such as hysteresis [24, 41]. Complex spatiotemporal regulation of actin polymerization is driven by PIP metabolism and by membrane-proximal activation of Rho family GTPases; the contractile activity of myosins is controlled, on the other hand, by regulatory light chain kinases such as Rho-associated coiled-coil kinase (ROCK), themselves also activated by Rho family GTPases [38].

Integrins and the molecular clutch.

Importantly, retrograde actin flow generates energy that can be transmitted outwardly by the cell through bivalent, transmembrane structures such as integrins. Integrins are heterodimeric transmembrane receptors that bind ligands on the extracellular matrix (ECM) or surface-anchored adhesion molecules on other cells. Anchorage to the ECM typically occurs *via* integrin-rich protein assemblages called focal adhesions; in immune cells, integrins also facilitate many consequential intercellular interactions such as rolling adhesion, diapedesis, and immune synapse formation [25].

The activity of integrins depends on their conformational states at a given moment [28], which exist in an equilibrium landscape between a bent-closed state (low affinity, common) and an extended-closed state (intermediate affinity, rare). The bent-closed state precludes ligand binding and signaling: the exofacial "headpiece" of the integrin (containing the α -chain I/A, β -propeller, and thigh domains, as well as the β -chain I/A and hybrid domains) are bent downward towards the integrin's "legs" (α -chain calf domains,

β -chain EGF-like repeat domains) [26]. Furthermore, the cytosolic tails of both α - and β -chains are fastened together at their transmembrane domains (TMDs). In the rare extended-closed state, the headpiece is swung outward and capable of binding ligands, albeit suboptimally. When the integrin headpiece binds an immobilized ligand such as ICAM1 (intercellular adhesion molecule 1) or VCAM1 (vascular cell adhesion molecule 1) on an apposing cell, and when chemotactic signals act to unclasp the integrin tails, a third, extended-open state with high affinity and durable lifetime is stabilized [28].

Mechanical counterforces both from outside and from within are important for establishing the fully opened state of an integrin. A bound ECM ligand or cell adhesion molecule directly provides the integrin headpiece with a counterforce that maintains its "swung out" conformation; such a *catch bond* exhibits maximum lifetime under a small amount of tension (< 10 pN) [26, 28]. The generation of internal counterforces happens at the same time, and involves bridging proteins that couple the integrin tails to actin filaments, fully unclasp them from the inside [42]. First, binding of ligands to their G-protein coupled receptors (GPCRs) (e.g., chemokine receptors) leads to phospholipase C (PLC) activation and cleavage of bis-phosphoinositides (e.g., PIP_2 [4,5]) into triphosphorylated inositols (IP_3) and diacylglycerol (DAG). IP_3 leads to Ca^{2+} store release and cytosolic Ca^{2+} influx, which together with DAG activates guanine exchange factors (GEFs, e.g., CalDAG-GEF) that, in turn, activate and bring Rap1, a Ras-family GTPase, and its key adaptor RIAM to the plasma membrane. The Rap1-RIAM complex unfurls the mechanosensitive molecule Talin from its autoinhibited conformation and brings it into contact with the integrin β -chain tails ("inside-out signaling") [43]. Binding of the talin head domains and the structurally related Kindlin proteins to the integrin β -tails partially unclasps them; at the same time, unfurled talin displays cryptic actin binding sites that mechanically couple the physical force of actin treadmilling to the full opening of integrin tails, stabilizing them in the extended-open state and rotationally

realigning them along the direction of actin flow [43, 44]. This maximizes the capacity for tight and durable integrin-ligand binding and catch bond formation [26], allowing the cell to communicate bidirectionally: transducing biochemical signals ("outside-in" signaling), while transmitting forces externally towards the adhesion substrate.

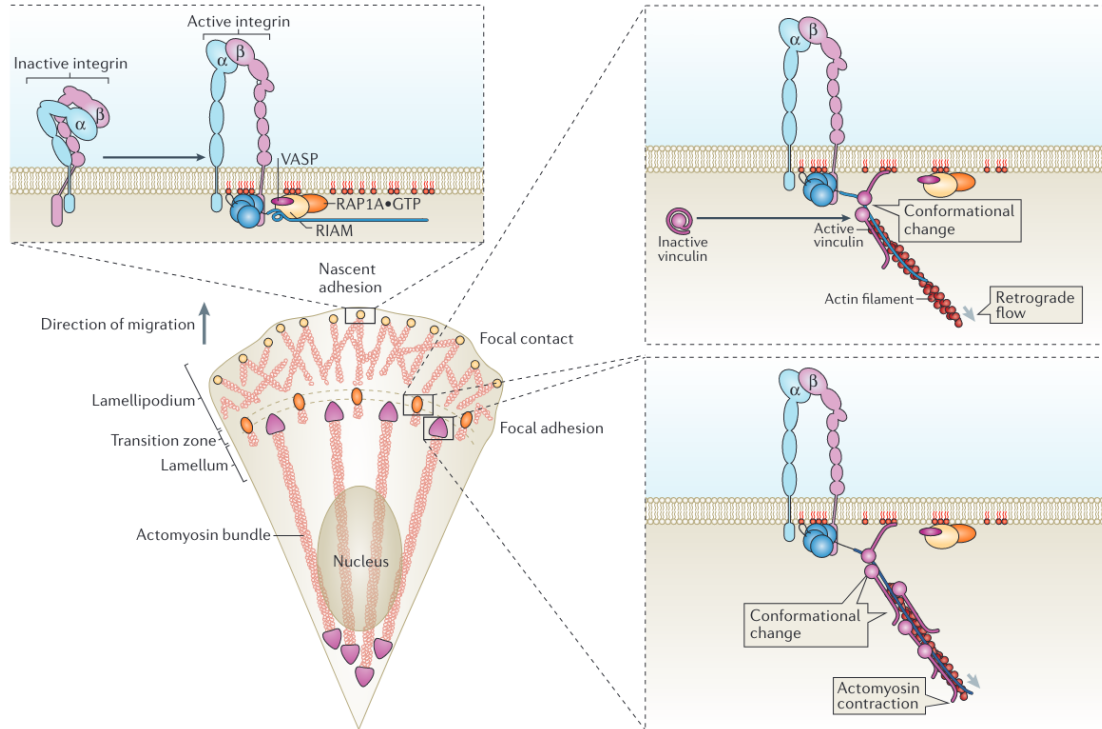


Figure 1.2: Activation and engagement of the integrin-talin-vinculin molecular clutch.

Downstream of receptor activation, Ras family GTPase Rap1 is activated and recruited to the membrane alongside its adaptor molecule RIAM. Here, talin (dark blue balls and rod) is unfolded and stabilized *via* interactions with the integrin β -tails and phosphoinositides. At the lamelliopodial cell front, nascent adhesions are initialized; as time passes and migration progresses forward, more contacts become reinforced by talin-vinculin interactions, which supplant RIAM binding and anchor the clutches to polymerizing actin. This frictional coupling increases the frequency of the opened integrins, which bind their ligands with maximal affinity and lifetime. At the lamellum, where myosin contractility further pulls on actin, retrograde flow is slowed down, but integrin reinforcement is enhanced, facilitating the assembly of many other focal adhesion-associated proteins. Figure reproduced from [43] permission from the copyright holder—Figure 2 Ba-Bc from the original publication, with figure panel labels removed.

Thus, integrins sense and transmit mechanical information by *frictionally coupling with retrograde actin flow*, in what is dubbed the "molecular clutch" mechanism (Figure 1.2) [12, 25]. Accordingly, integrin ligation and actomyosin activity are both crucial

determinants of leukocyte motility [45], contact-based activation [46], and force exertion [47, 48]. Leukocytes are particularly adaptive in their utilization of the molecular clutch: they can toggle between adhesion-dependent mesenchymal movement ("crawling") and protrusion-dependent amoeboid movement ("flowing" or "walking"), depending on the availability and distribution of integrin ligands in the microenvironment [45, 49, 50, 51]. In leukocytes, these interactions are brief and dynamic; in more stationary mesenchymal cells, activated integrins engaged with the molecular clutch assemble large-scale focal adhesions [25], which mediate strong attachment, dynamic force exertion on the substrate, and adhesion-dependent motility.

The plasma membrane and nucleus.

The lipid bilayer enclosing every animal cell is studded with proteins, many of which are mechanosensitive [52, 53]: stretch-activated ion channels [54, 55], G-proteins [52], curvature sensing proteins [56], integrins [57, 58], and still many others. The plasma membrane does not merely act alone as an isolated a glycan-rich, protein-rich, lipid-rich bilayer, but in concert with an underlying meshwork of membrane-proximal actin and associated proteins together called the *cortical cytoskeleton* [59]. In a process called the membrane-cortical attachment (MCA), proteins such as Ezrin can bridge cortical actin filaments with membrane lipids or transmembrane proteins in a manner that can be dynamically regulated by exofacial receptor engagement and endofacial leaflet composition (e.g., phosphoinositides and DAG, similarly to talin). The same way multivalent proteins anchor the molecular clutch, proteins also anchor the membrane and cortex in a manner that (1) regulates the rate of lateral membrane flow (tensional equilibration) [60, 61, 62], and (2) assembles diffusion barriers to regulate lateral transmembrane protein flow (picketing) [63, 64, 65]. Far from being a thin and mechanically inert layer, the plasma membrane is a

hub of signaling, mechanosensing, adhesion control, and mechanoregulation, presenting resistance against polymerizing (non-cortical) actin and thereby modulating cellular protrusion morphodynamics and substructure [66, 67, 68].

Further from the plasma membrane, the nucleus is the stiffest organelle in the cell, encapsulated by a double bilayer and associated cytoskeletal structures (the nucleoskeleton). Nuclear mechanical integrity is largely defined by the Lamin family of intermediate filaments, and the nucleus also maintains functional connections to actin, tubulin, and other intermediate filaments [69]. Being relatively stiff and bulky compared to most other cellular structures, the nucleus ultimately determines the environmental pore sizes and gaps through which cells can effectively migrate, which is of outstanding importance for ambulatory leukocytes [34, 70]. Cells in peripheral tissues can also detect osmotic imbalances, such as when local tissue disruption occurs, *via* nuclear swelling—stretched nuclear membranes accommodate the interdigitation of hydrophobic protein domains, leading to selective enzyme assembly and the subsequent production of leukocyte chemoattractants [71].

All these mechanical structures of a cell are far from static; they constantly remodel themselves and interact with one another in space and time, endowing cells with complex time-dependent and state-dependent material characteristics, and making the development of a unifying rheological model of the animal cell a difficult but exciting goal [13, 31, 72, 73, 74].

1.1.2 General trends in cellular rheology

Active microrheological approaches involve tracking a cell’s time-dependent material response to known, externally applied mechanical forces (stresses). On the other

hand, passive methods involve optically tracking intracellular fiducial markers such as membrane-bound organelles or granules. In spite of the underlying biochemical complexity and the potential sources of experimental variation across microrheological studies [13, 31, 74, 75], at least three general trends have emerged in the literature:

1. Under the influence of small stresses, cells present as soft and viscoelastic materials.

Materials can be deformed in a number of ways: by compression, tension, indentation, shear, etc. [13, 31, 72, 73, 76] An elastic material stores the energy applied to deform it and manifests a reactionary restoring force that returns it to its original shape upon cessation of the stress. A viscous material exhibits the opposite behavior, not storing the applied energy but dissipating it as heat, changing the material's shape permanently. Thus, viscous materials are said to flow, behaving as macroscale fluids that conform to the lowest-energy shape made available by their containers. Viscoelastic materials exhibit both of these behaviors, storing and dissipating energy to different extents while undergoing structural changes.

When a cell is subjected to an externally applied oscillating stress $\sigma(\omega)$ with frequency ω , its resulting deformation or *strain* $\gamma(\omega)$ can be measured and related to the stress *via* a linear modulus $G(\omega)$:

$$\sigma(\omega) = G(\omega) \times \gamma(\omega) \quad (1.1)$$

Because $G(\omega)$ oscillates, a productive quantitative treatment is to model it as a complex-valued function spanning a unit circle [77, 78]. The same way that any complex number $x + iy$ can be written in a polar form with an "in-phase" cosine component projecting onto the x-axis and an orthogonal, "out-of-phase" sine component projecting onto y,

$$re^{i\theta} = x + iy = r\cos(\theta) + ir\sin(\theta) \quad (1.2)$$

we can express the complex modulus $G(\omega)$ as:

$$G(\omega) = G'(\omega) + iG''(\omega) \quad (1.3)$$

where the real component $G'(\omega)$ is an in-phase stress-strain response and the imaginary component $G''(\omega)$ is an out-of-phase response. $G'(\omega)$ reflects the degree of elastic, solid-like energy storage into the material— $G'(\omega)$ is thus called the *elastic or storage modulus*. $G''(\omega)$, on the other hand, characterizes the *viscous or loss modulus*, reflecting the degree of viscous behavior and energy dissipation by the material. By definition, their vector relation also defines a *phase lag* or *loss angle* δ between $G'(\omega)$ and $G''(\omega)$:

$$\delta = \tan^{-1}\left(\frac{G''(\omega)}{G'(\omega)}\right) \quad (1.4)$$

Thus, a perfectly elastic material is dominated by the in-phase component $G'(\omega)$, and it follows that $G''(\omega) \sim \sin(\omega) = 0$, and $\delta = 0$. On the other hand, purely viscous fluids are dominated by the out-of-phase component $G''(\omega)$, whereby $G'(\omega) \sim \cos(\omega) = 0$, and $\delta = \pi/2$. For values that fall in between either extreme, both elastic and viscous behaviors contribute to an overall *viscoelastic* material response, which is influenced by the timescale of the applied stress oscillations.

Small-scale deformations of cells at lower frequencies follow a remarkably consistent behavior across cell types, cytoskeletal perturbations, and even measurement techniques: the complex modulus of a cell $G(\omega)$ scales with weak exponential values ($\beta < 1$) when plotted against oscillation frequency ω . Cells have therefore been described as following a *universal power law* [14, 72, 74, 79, 80]. This power-scaling term β can be measured in a creep experiment, wherein the material (cell) is stressed with a constant force F :

$$\frac{\gamma(t)}{F} = j_0 \left(\frac{t}{\tau_0}\right)^\beta \quad (1.5)$$

Here, j_0 is the material *compliance* (inverse-stiffness) and t/τ_0 is a scale-free/normalized time of force application [74]. If a sinusoidal force is applied to the cell, which defines the complex modulus, the relation above transforms to:

$$G(\omega) = j_0^{-1} (i\omega\tau_0)^\beta \Gamma(1 - \beta) \quad (1.6)$$

In this form, when $\beta = 0$, the equations simplify into Hooke's law and define elastic solids; when $\beta = 1$, the equations collapse into Newton's law of viscosity and define viscous fluids. When $0 < \beta < 1$, as in cells, the equations define non-equilibrium soft glassy materials, which include pastes, slurries, and various complex colloids. In this conceptual paradigm, material properties emerge from disordered, metastable ensembles of nanoscale interactions [74]. Indeed, whereas early microrheological studies modeled cells as linearly viscoelastic materials, more recent work suggests that they are more complex and better modeled as soft glassy materials [31, 74] (e.g., the *glassy worm-like chain model* and its modifications).

2. Cells are internally prestressed (tensed) due to the contractile activity of actomyosin networks. The internal build-up of tension in a cell is balanced to an extent by microtubules, which can bear compressive loads [34], and by adhesions that tug on the external environment, manifesting as traction forces [81, 82]. The extent of contractile prestress influences cell stiffness and their mechanical responses to external forces [83, 74, 81] (e.g., the extent of fluidization in response to strong deformations). Cellular prestress also leads to tensional homeostasis across cellular collectives such as epithelial sheets, where mechanical extrusion of cells harboring oncogenic genetic lesions [84, 85] or intracellular pathogens [86] may represent a pre-hematological tissue defense mechanism.

3. Cells are molecular networks operating far from thermodynamic equilibrium.

Gralka & Kroy liken the molecular construction of the cytoskeleton to an electrical circuit made up of "excessive amounts of burned fuses" [31] . These short fuses (e.g., dynamic linkages between actin monomers or tubulin monomers) form higher-order filaments that continually undergo cycles of breakage and re-assembly, thus leading to their remarkable adaptability to large-scale deformations [87, 88, 89, 90] and ever-changing mechanical environments [35, 91, 41, 92, 93, 94]. Importantly, this type of network architecture facilitates complex, active mechanical behavior. For example, tuning myosin activity (actomyosin contractility) allows cells to either stiffen [95, 96] or fluidize [97] under stress, depending on context and demand [73, 97, 98]: active motors can dynamically reshape the network, contributing to global fluidity and remodeling, while inactive motors act more like static cross-linkers, contributing to global elasticity and stiffness [31, 73]. This bioenergetically costly, non-equilibrium cytoskeletal steady-state allows cells to exhibit not only the viscoelastic power-law behavior within small deformation regimes, but also large-scale plastic and inelastic responses (that also follow power-law behavior) under the influence of strong external forces.

Together, these rheological properties of cells define them as complex biomaterials with a remarkable ability to interact with mechanical environments, whether they are acting autonomously as single immune cells, or within higher-order assemblies such as epithelial sheets.

1.1.3 Mechanosensing, mechanotransduction, mechanoreciprocity

Palpation and pathfinding: cells sense and respond to the mechanical features of their environment.

The literature on rigidity sensing by cells is rich, due in no small part to advances in polymer chemistry that have made the routine creation of hydrogel substrates spanning a wide range of Young's modulus values experimentally tractable [99]. Provided an ample supply of adhesion ligands, cells will typically spread out thinly and/or stiffen on a stiff substrate, but remain somewhat rounded and more compliant on a soft one (termed *mechanoreciprocity*) [100]; they will divide faster, move faster, and metabolize faster on a stiff one [99]; cytoskeletal structures such as focal adhesions and actin stress fibers are formed and stabilized more readily on a stiffer substrate [13, 99]. These "more is more" trends with regards to substrate rigidity are observed in many cell types, such as fibroblasts, epithelial cells, cancer cells, and muscle cells; on the other hand, certain cell types such as neurons are either unaffected by stiffness or exhibit enhanced activity on soft substrates [101, 99]. Many immune cells, being amoeboid and not fibroblast-like in their actomyosin network organization, display many of these mechanosensitive behaviors [102] without forming canonical focal adhesion structures. Thus, different cell types appear adapted to distinct mechanical environments, necessitating a mechanism for dynamic sensing and adjustment. The following section is in no way meant to be an exhaustive catalog of mechanobiology, but is rather intended to establish the perspective that cells are fully integrated mechanochemical entities, living in spaces that are as much physical as they are chemical.

Importantly, a cell's concept of "stiffness" is not what is directly measured by AFM or optical traps by the macroscale experimenter; on the molecular scale, rigid substrates

present counterforces to various ligand-receptor bonds, placing them under significantly more tension than do soft substrates [103]. The aforementioned *molecular clutch* embodied by the integrin-talin-actin linkage provides cells with a means to measure substrate stiffness, and to transduce these measurements into appropriate transcriptional and metabolic programs: as talin unfolds and couples the force of actin treadmilling with integrin activation and realignment, hundreds of other proteins assemble onto this nascent focal adhesion to form a large supramolecular complex. This focal adhesion complex actively signals, continuously exerts forces on the engaged substrate, and reinforces or disassembles itself accordingly [104, 105, 25].

Interestingly, focal adhesions can be functionally divided into geographical strata (Figure 1.3): a plasma-membrane-proximal *integrin signaling layer*, an intermediary *force transduction layer*, and a cytoplasmic *actin regulatory layer* [104, 25]. In the integrin signaling layer, alongside the endofacial integrin tails are Focal Adhesion Kinase (FAK) and some of its phosphorylation targets such as paxillin, an adaptor for many other adhesion proteins (e.g., p21/Rac1-Activated Kinase [PAK] and PAK exchange factor β [β PIX] [106]). Importantly, activated FAK also phosphorylates receptor Tyrosine kinases (RTKs) and phosphatidylinositol-3-kinase (PI3K), leading to mitogen-activated protein kinase (MAPK) pathway activation [13]. The force transduction layer is where talin unfolding and co-assembly with Vinculin occurs, reinforcing the entire linkage *via* mutual interactions with acidic phospholipids, talin, and actin filaments [43]. The actin regulatory layer contains actin-associated proteins, actin bundling proteins, cross-linkers, and motors, which together establish and modulate the force of actin flow, as well as form larger-scale actin bundles (i.e., stress fibers) that can bear tensional loads across the entire cell body [12]. Focal adhesion complexes across a cell are mechanically active, repeatedly "tugging" on the bound substrate as if to sample its rigidity—they reinforce themselves where tractions are strong enough to maintain the entire *agencement*, and

disassemble where tractions become too low. The dynamic life cycle and functional independence of individual focal adhesions [105] allows a cell to effectively generate a map of the substrate at any given moment by sampling distinct coordinates along its cell body, maintaining only those subjected to a certain regime of stiffness. On a substrate with mixed rigidities, this palpation and mechanical sampling facilitates net movement toward some optimal, typically stiffer region—a phenomenon dubbed mechanotaxis or *durotaxis* [105, 99].

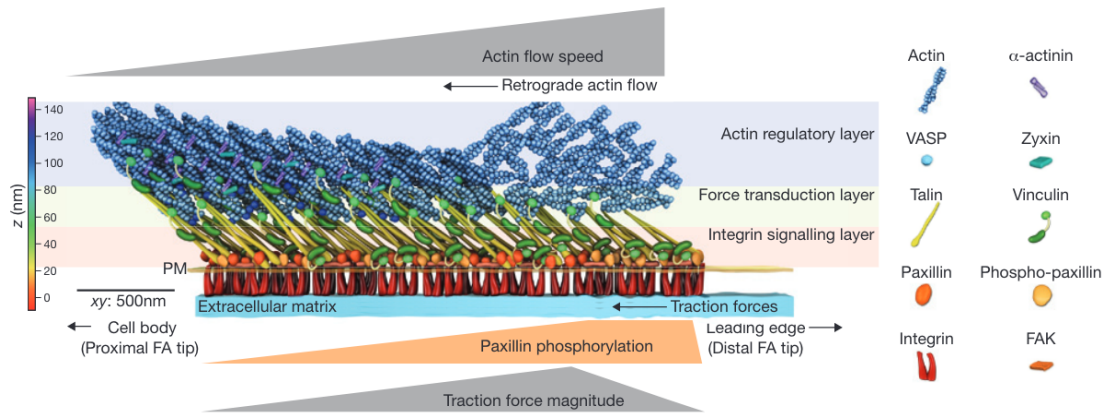


Figure 1.3: Molecular, functional, and geographical strata of the molecular clutch.

Integrin-based adhesions are organized into strata both along the direction of actin flow (x-axis) and normal to the surface (y-axis). The velocity of retrograde actin flow, efferent traction forces, and signaling output from adhesion-associated kinases segregate along the direction of actin flow. Compositionally, adhesions are also stratified normal to the surface, into a surface-proximal integrin signaling layer, an intermediate transduction layer with the talin-vinculin clutch, and an actin regulatory layer. Figure reproduced from [25] with permission from the copyright holder.

What determines the substrate stiffness favored by a given cell? One recently developed model suggests that a balance between motor activity and clutch reinforcement is a major determinant of cellular tractions and durotaxis [107]. Molecular diversity in the focal adhesion strata, targets of the mechanosensitive kinases [99], and actin regulatory networks would no doubt impact these parameters and the resultant Goldilocks zone of a given cell [105]. A cell’s developmental and functional state also makes an impact, as shown by pioneering studies using *in vitro* cultured mesenchymal stem cells (MSC), the

differentiation of which into different lineages could be influenced by substrate stiffness, cell density & compaction, tissue shape, and actomyosin activity [108, 109]. Signaling pathway activation can also impact cell mechanosensing, as shown by the effects of lipopolysaccharide (LPS) treatment on microglia durotaxis [110]. The effects of LPS, and indeed the other toll-like receptor (TLR) ligands on leukocyte actomyosin dynamics, clutch reinforcement, or focal adhesion composition have not been systematically catalogued, but are likely operative in the determination of their mechanical behaviors [92]. In support of this connection between lineage and mechanosensation, the lamellipodial growth cones of neurite processes generate particularly weak tractions [111], possibly representing an adaptation that sensitizes them to the softer stiffness ranges of the brain [101].

A number of mechanosensitive supramolecular complexes functionally related to, but compositionally distinct from, focal adhesions exist. Adherens junctions are one such analogous structure [112, 113]: in place of integrins, cadherin receptors serve to bind two cells along their adjoining faces. Onto their endofacial tail domains, an assemblage of p120-catenin, β -catenin, and α -catenin are formed. In place of talin, α -catenin exhibits the mechanosensitive unfolding that frictionally couples the receptor tails to actin flow. Whereas integrin-rich focal adhesions cluster due to the multimeric, filamentous ECM proteins, cadherin-based junctions are primarily seeded by the oligomerization of cadherin ectodomains, which leads to diffusion trapping and nascent assembly of the catenin core [113]. Just like in focal adhesions, vinculin reinforces the cadherin- α -catenin clutch, leading also to junction stabilization and compositional maturation under tensional loading. Yet other mechanosensitive molecules such as p130Cas or Kindlin exist, and play roles in mechanosensitive cytoskeletal reorganization [114, 43, 13, 26, 115, 28]. Aside from integrins, the hyaluronic acid receptor CD44 has also been proposed to frictionally couple the ECM to actin flow in the manner of a clutch receptor [116, 117] *via* the ezrin, radixin, moesin (ERM) family of proteins of the MCA.

Integrating mechanical cues transcriptionally *via* the Hippo pathway.

The question remains: how do cells interpret and process their mechanical state genetically? The Hippo pathway, originally discovered as a regulator of organ size in *Drosophila*, integrates signals engendered at mechanosensitive assemblages (e.g., focal adhesions, adherens junctions) and leads to changes in transcription [118, 119]. The hippo pathway is turned "off" by default: its core effector kinases Mst1/2 and Lats1/2 (mammalian *hippo* & *warts* homologs, respectively) maintain the transcriptional effectors YAP/TAZ (*yorkie* homolog) phosphorylated, sequestering them from the nucleus. Upon pathway activation, Mst1/2 and Lats1/2 are inhibited and YAP/TAZ can enter the nucleus, where they bind a panoply of DNA-binding proteins to promote cell survival and proliferation—including the TEAD family of transcription enhancer factors, Smad1, RUNX, PPAR γ , etc. [118] YAP/TAZ are excluded from the nucleus by phosphorylation-dependent (Ser127) and phosphorylation-independent mechanisms, as well as by phosphorylation-dependent (Ser381) ubiquitinylation and degradation. In general, pathway activation is stimulated by actin polymerization and increased tension along adherens junctions & focal adhesions [119]. Actin polymerization inhibits Lats through a number of mechanisms, prominent among which seems to be the competitive binding of F-actin for angiomotins, which keep YAP/TAZ sequestered in the cytoplasm. In parallel, nuclear F-actin assembly also prevents a YAP/TAZ inhibitor from binding it—Arid1A of the Swi/SNF chromatin remodeling complex. In adherens junctions, under conditions of high tension (low cell density), the mechanically activated catenin junctions sequester Lats *via* Lin-11/Isl-1/Mec-3 (LIM)-domain-containing proteins, as well as Merlin, which can both activate Lats and serve as a YAP/TAZ nuclear export factor. When cells are dense, tension across adherens junctions is low, and Merlin and Lats are free to inhibit YAP/TAZ. Finally, in focal adhesions, multiple kinases such as FAK and Src positively regulate the pathway: by directly activating YAP/TAZ through

phosphorylation (Tyr341/Tyr357/Tyr394); inhibiting its inhibitors (e.g., MOB1, Merlin, and Lats) through a number of direct & indirect mechanisms; and by a multi-step pathway involving reduction of PIP₂(4,5) and phosphatidic acid on the membrane, which activates Lats-activating protein RAP2 [119]. Focal adhesions cannot maintain stable signaling output when engaged with compliant substrates or when ECM ligands are sparse, leading to YAP/TAZ inhibition and reduction in hippo pathway output.

The hippo pathway is central for cell survival and maintenance under mechanical cues. By integrating the state of focal adhesions, the hippo pathway maintains cell viability and proliferation on a sufficiently stiff and ECM-rich substrate; otherwise, cells die in a process called *anoikis* [13, 119, 99]. Another consequence of the pathway is familiar to any scientist who has left a tissue culture to overgrow: in cellular collectives such as epithelial sheets, the hippo pathway integrates information from adherens junctions to impose restraint on the cell cycle upon reaching a certain density, a process called contact inhibition. Among the transcriptional outputs of the pathway are also genes coding for the production of ECM ligands, by which cells can dynamically and actively remodel their environment when the conditions are right [13, 99]. The hippo pathway also appears to influence the regulation of topological mechanosensing in epithelial sheets [84], as well as the decision between stem cell maintenance and differentiation [120, 119].

While substrate rigidity is the most well studied mechanical determinant of cell behavior and fate, cells also respond to the local topography—that is, to the microscale and nanoscale *curvatures* [121, 122, 123, 124]—of their solid environments, as well as to fluid flow [125, 126, 127, 128, 129]. In what follows, I provide a summary and overview of the immune system, specifically on the roles of cytotoxic T cells, and then end with remarks on what is known about leukocyte rheology and T-cell mechanical output.

1.2 Overview of the immune system

1.2.1 Organization of the immune response

As demonstrated by the coronavirus pandemic of December 2019, the very same organ system we have evolved to recognize and clear pathological agents, when operating far from optimal conditions suited to our flourishing, is capable of causing long-lasting and damaging pathologies [130]. Whether one's perspective of the path taken is of the organism as a holistic, self-preserving unity or as an emergent product of internal Darwinian struggle among its parts [16], as far as one can tell, the practical *telos* of the immune system is: "*Kill the pathogen, and [not] harm the host.*" (Harris Goldstein, in a lecture delivered to the Nelson R. Mandela School of Medicine, South Africa, 2010) The study of immunology spans a number of interconnected scales—diverse biomolecules with distinct modes of action, diverse cell types harnessing distinct biomolecules and pathways, diverse tissue contexts and architectures harboring distinct cell types, and diverse immune profiles within a population exhibiting distinct, individuated tendencies and susceptibilities [131]. In spite of this kaleidoscopic complexity, a general multi-layered logic characterizes the overall organization of the mammalian immune system (Figure 1.4) [131].

The body plan and innate immunity. The body plan is the first line of defense, encasing the internal organs and exposing to the external environment only anatomical barriers such as the skin and mucosa, which are loaded with pre-formed anti-bacterial peptides, sentinel cells, and commensal microbes. If the skin is breached or some mucosal compartment overwhelmed with a slew of incoming pathogens, an innate immune response against the infection begins to orchestrate [132]. First, tissue-resident sentinel

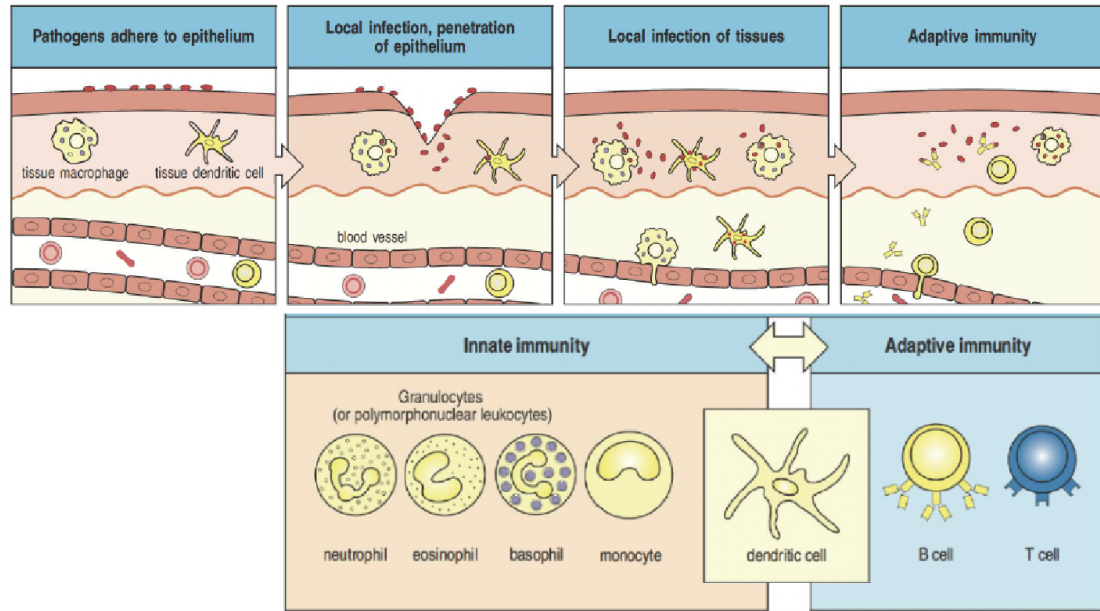


Figure 1.4: Stages of an immune response.

Shown on the top row are the stages of an infection through a breach in the skin. Below are the major leukocyte subsets involved in each of these phases. Figure modified from Janeway's Immunobiology [131].

cells such as macrophages, neutrophils, and dendritic cells (DCs) can directly engage with the target. These first-responder cells not only clear pathogens *via* phagocytosis, but also undergo transcriptional activation upon recognition of pathogen-associated molecular patterns (PAMPs) through a number of innate receptors: phagocytic receptors, toll-like receptors (TLRs), NOD-like receptors (NLRs), retinoic acid-inducible gene I (RIG-I)-like receptors, cyclic GMP-AMP synthase (cGAS)-stimulator of interferon genes (STING), etc. These PAMP receptors signal through different pathways which converge onto key transcription factors (e.g., nuclear factor kappa-light-chain-enhancer of activated B cells [NF κ B], interferon regulatory factors [IRF] 3 & 7), which produce effector molecules such as cytokines that simultaneously enhance the local immune response and activate systematic responses over long distances. During this *early induced innate response*, secreted cytokines (interleukins [ILs]) and chemokines from the activated innate cells perform the following functions: (1) activate the endothelium to enhance adhesiveness

(e.g., IL-1 β , tumor necrosis factor [TNF]- α), (2) increase vascular permeability (e.g., IL-1 β , TNF- α), (3) recruit a larger-scale immune response to the site of infection (e.g., Chemokine C-X-C motif ligand 8 [CXCL8]/IL-8), and (4) activate other immune cells in the vicinity (e.g., IL-6, IL-12, IL-18). At the same time, innate lymphoid cells (ILCs) seeded in various mucosal tissues can also become activated and help establish the cytokine milieu [133], while natural killer (NK) cells directly kill compromised host cells. In addition, epithelial cells of the tissue independently orchestrate a protective response by signaling to the immune system *via* alarmins (e.g., IL-33), and by extruding infected, damaged, and compromised members of the cellular sheet [134]. In the case of a barrier breach, disruptions to osmotic balance [135] and electrical field distributions [136] also stimulate an immediate epithelial wound closure response that contains the damage and pathogen load.

Adaptive immunity. The innate immune response is relatively quick and efficient, relying on pre-formed pattern recognition receptors that engage stereotyped molecular features of *pathogen classes*. However, another immune response, slower and more energetically expensive, engenders protection against specific molecular features of *individual pathogens* [137]. The linchpin of this *adaptive immunity* is the evolution of mechanisms for genetic diversification in the loci responsible for target recognition, which facilitate the formation of an immunoreceptor "repertoire" against a wide array of potential antigens. Innate pattern recognition receptors (e.g., TLR, NLR) are shared by vertebrates, invertebrates, and even plants; on the other hand, from the jawed fishes onwards, two recombination-activating genes (RAGs) from the transib transposon family were acquired that generate such a repertoire through genetic recombination of multi-segment Variable, Diversity, and Joining (VDJ) gene arrays. VDJ recombination in these animals leads to the development of specialized B-cell receptor (BCR) and T-cell receptor

(TCR) repertoires, which become expressed by the respective lymphocyte subsets after developmental pruning. Interestingly, in the cyclostomes (e.g., lampreys & hagfish), genetic recombination of Variable Lymphocyte Receptor (VLR) segments quite like in higher chordates has been observed through mechanisms distinct from RAG. This evolutionary convergence suggests the existence of a vertebrate ancestor with all three VLR, BCR, and TCR gene segments—in one vertebrate lineage, which acquired RAG activity, an immunoglobulin (Ig) superfamily gene templated BCRs and TCRs; in the other, a leucine-rich repeat (LRR) protein templated the VLRs [137]. Importantly, *in lieu* of a dedicated recombinase to generate a diverse immunoreceptor repertoire, plants have dramatically expanded PAMP receptor families and an adaptive response based on systemic RNA interference [138]—either way, a target-specific adaptive immunity layered on top of broadly neutralizing innate immunity potentially protects organisms not only from threats encountered throughout our evolutionary history, but also to new ones that we have never seen before.

A few days after the initiation of the induced innate response, the adaptive immune response is stimulated (Figure 1.5). Tissue-resident DCs, which at steady-state patrol the barrier tissues, are among the first to encounter and phagocytose target pathogens during the initial invasion and colonization. They are potently activated by this initial encounter, which induces a dramatic phenotype switch *via* TLR4-MyD88 signaling: front-loaded actin, presumably used by immature DCs for macropinocytic antigen sampling and environmental exploration, polarizes toward the back of the cell in mature DCs, fueling fast and directionally persistent movement [139, 140, 90]. Mature DCs at first follow a CCL21 chemokine gradient through lymphatic vessels into the draining lymph nodes, and then a CCL19 gradient within the lymph nodes towards the paracortical T-cell zones, where they encounter 500–5000 naive T (T_n) cells an hour [141]. Mature DCs digest the phagocytosed pathogens and load fragments of antigenic peptides onto their Major

Histocompatibility Complex (MHC) molecules MHC-I or MHC-II, for presentation to and selection of naive CD8+ or CD4+ T cells, respectively.

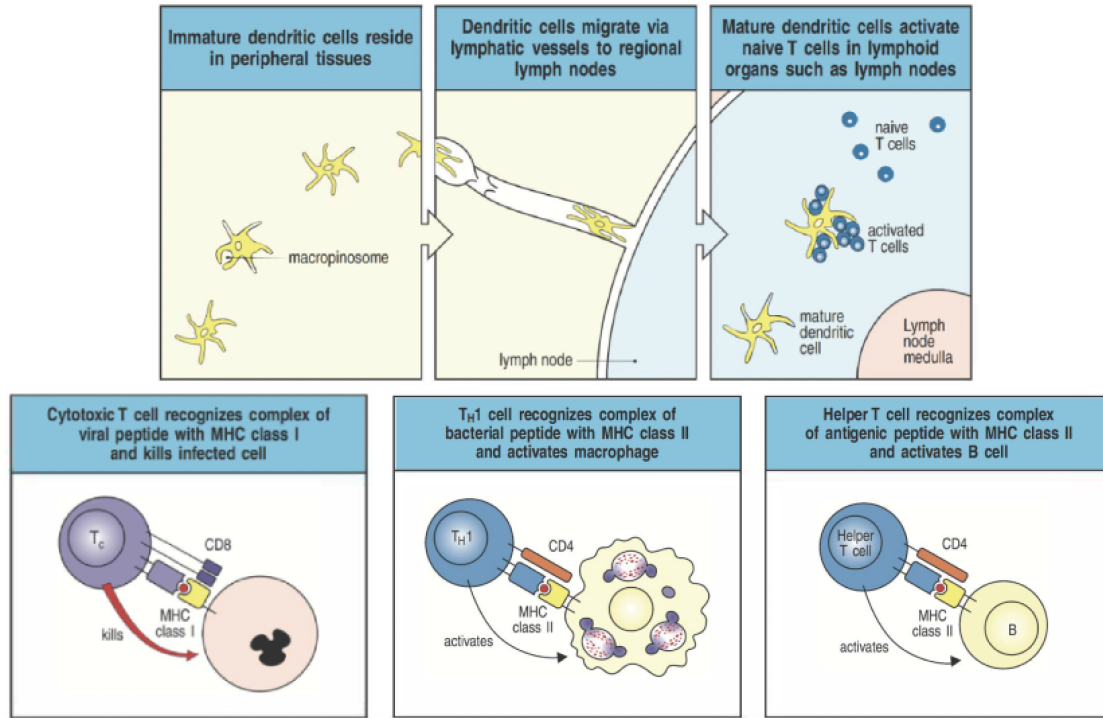


Figure 1.5: Broad overview of adaptive immune responses.

Shown on the top row are the events involved in T-cell priming by dendritic cells during an infection. Below are some lymphocyte effector subsets involved in the adaptive immune response; not shown are antibody producing plasma cells that develop from the activated B cells, regulatory T cells, and memory T cells. Figure modified from Janeway's Immunobiology [131].

T_n cells are *primed* by DCs—that is, activated—based on sufficient shape complementarity of their cell-surface TCRs with the peptide-MHC (pMHC) molecules on antigen-presenting cells (APCs), in this case mature DCs. Upon finding its cognate antigen, a T cell halts, polarizes, and forms a dynamic intercellular contact with the APC called the *immunological synapse*—the analogy to neuronal synapses based upon intercellular receptor-ligand clustering, exocytic secretion into the intercellular space, and the presence of neuronal protein Thy1 at the contact [142, 143]. At the synapse, a signaling network is triggered beginning from the TCR that leads to full transcriptional activation, cellular differentiation & maturation, and clonal proliferation [144, 145].

In this way, from an array of cells expressing the vast repertoire of potential TCRs, only those cells that can specifically recognize fragments of antigen derived from the peripheral source of inflammation are built up as an army in the draining lymph nodes. Primed CD4+ T cells develop into helper T cells (T_H Cs), which support and enhance the functions of other immune cells through the secretion of context-specific cytokines. T_H C developmental polarization is tuned by cytokines in their environment: IL-12 leads to T_H1 polarization and secretion of IFN- γ & TNF- α , which boost the activities of innate cells in the inflamed site, while IL-6 and IL-21 lead to follicular helper T cell (T_F H) polarization, by which B-cell activation at the lymph nodes is achieved. High amounts of IL-2 alongside transforming growth factor β (TGF- β) lead to induction of regulatory T cells (T_{reg}), which can sequester IL-2 from pro-inflammatory effector T cells, or secrete IL-10, a potentially anti-inflammatory cytokine that dampens the activities of many immune cell types. On the other hand, primed CD8+ T cells develop into cytotoxic/cytolytic T lymphocytes (CTL). These mature effector cells home to sites of inflammation and directly kill host cells that present a matching antigen. In this way, they restrict the spread of infection from cell to cell, as well as clear tumors expressing mutation-associated neoantigens.

In contrast to T-cell recognition, BCRs directly probe an antigen in its native conformation (i.e., unprocessed/undigested). B cells reside in cell-dense follicles of the draining lymph nodes and spleen, where they can encounter native antigen in at least two forms: (1) soluble, low-molecular-weight antigens can flow directly into the B-cell zones through the afferent lymphatic drainage, while (2) large particles or immune complexes can be presented to B cells by subcapsular sinus macrophages and follicular dendritic cells [146]. Antigen-activated B cells initially enter the T-cell zone, where they can encounter and be primed by T_F H cells. Upon ligation of CD40 on the B-cell surface by T-cell-expressed CD154 (CD40 ligand), the former migrate into the primary follicles and

undergo massive proliferation at follicular germinal centers (GCs). Proliferative B cells in the densest "dark zones" undergo somatic hypermutation, whereby activation induced deaminase (AID) introduces deoxyCytidine-to-deoxyUracil mutations in the Ig variable regions of their BCRs. The cells that successfully hypermutate their BCRs then migrate out into the "light zone", where their new receptors undergo affinity-based selection against limiting amounts of antigen presented by follicular dendritic cells. At the same time, they are coaxed by T_H Cs and/or local cytokine milieus to undergo *class switching*, in which the "stalk" (constant region) of their initial IgM BCR is supplanted by more specialized IgG, IgA, or IgE isotypes. Those that emerge from this stringent, pseudo-Darwinian selection finally differentiate into antibody-secreting plasma cells. Secreted antibodies have many functions depending upon the specific isotype to which their producer cells class-switched; however, they all act by directly binding and neutralizing pathogens at the sites of infection, as well as by bridging the bound antigens to pre-formed Fc receptors (FcRs) expressed by innate immune cells.

In most cases, these two cell-mediated adaptive immune strategies support the innate immune response enough to resolve the infection. As the pathogen load diminishes, signaling cascades that led to the inflamed tissue remodeling cease, anti-inflammatory and tissue-regenerative cells (e.g., T_{regs}) downregulate the inflammatory milieu, most responder T cells in the vicinity die, and the tissue returns to homeostasis. However, a small subset of effector T and B cells differentiate into long-lived "memory" subtypes (T_{mem} & B_{mem}), which can much more quickly regenerate a specific clonal response to the same pathogen in case of a subsequent reinfection. This way, a fully orchestrated immune response also generates protective *immune memory*, in the form of (1) a humoral/antibody response and cell-mediated (2) cytotoxic & (3) helper responses.

1.2.2 T-cell activation, synapse formation, and target cytolysis

T cells are generally activated through direct intercellular contact, i.e., through immune synapse formation with APCs. Here, I briefly summarize the biochemical and cytoskeletal events at the immune synapse leading to T_h activation (Figure 1.6) [144, 147, 148]; with a few key differences, similar events occur at CTL immune synapses that mediate target cell destruction.

Summary of TCR signaling.

The TCR is a multimeric transmembrane complex (CD3) with numerous immunoreceptor Tyrosine-based activation motifs (ITAMs) on its endofacial "tails". An initial conformational change in the complex upon antigen binding exposes these tails, which the Src kinase Lck, recruited to the TCR complex *via* co-receptors CD4 or CD8, then phosphorylates. Phospho-Tyrosines on CD3 are recognized by SH2 domains expressed by multiple proteins in the TCR signaling network; among the first to recognize phosphorylated TCR is Zeta-activated protein 70 kDa (Zap70), which docks onto the complex and in turn phosphorylates Lat, a scaffolding protein. Slp76 associates with phospho-Lat and is likewise phosphorylated by Zap70, forming the pLat-pSlp76 scaffold onto which many other proteins assemble in parallel to propagate downstream signals. Together, these proteins form what is sometimes dubbed the TCR signalsome, which are visible as mobile clusters at the synaptic membrane when stained or tagged with a fluorescent protein fused to one of its constituents.

One of the proteins that assembles onto pLat-pSlp76 is PLC- γ , which, as in GPCR signaling, cleaves PIP₂ into its headgroup IP₃ and lipid tail DAG. Each of these messengers then plays downstream roles that lead to T-cell activation [144]. DAG recruits a number

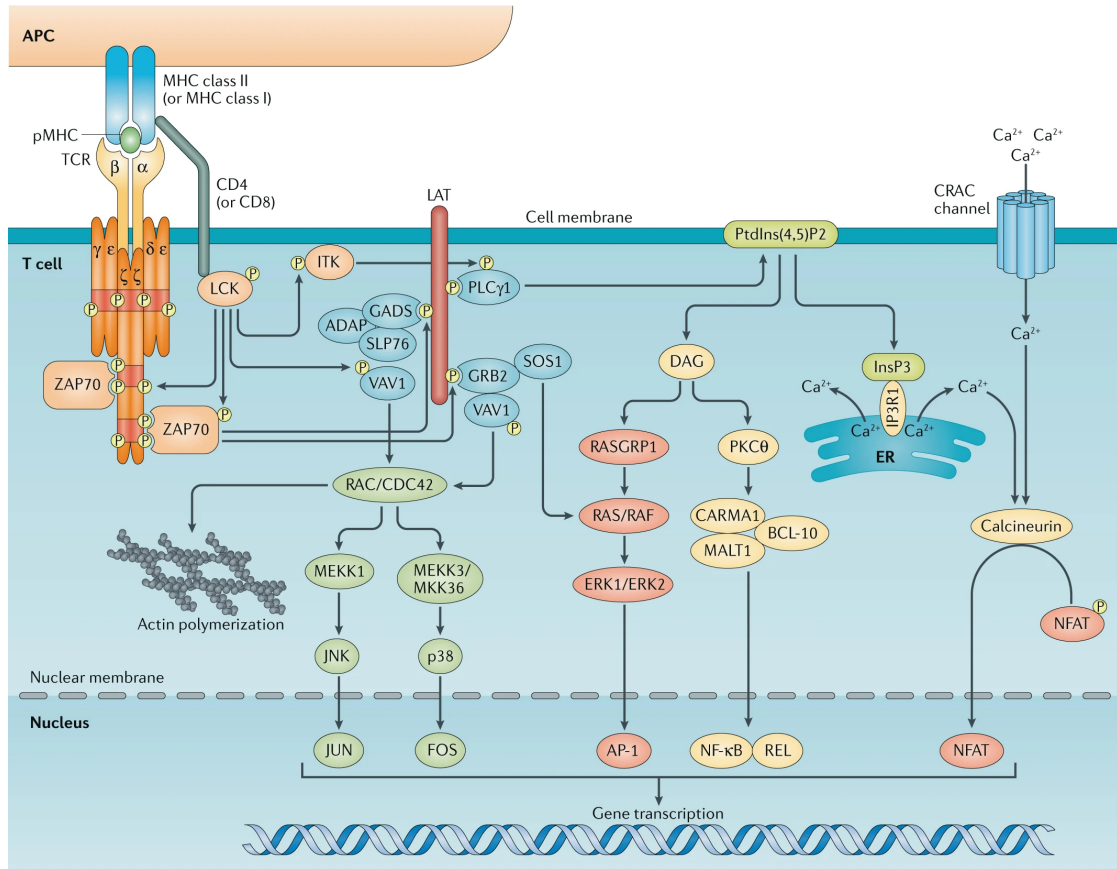


Figure 1.6: Signaling network downstream of the TCR.

Early TCR signaling mobilizes a cascade of phosphorylation events that together lead to assembly of a (multiply phosphorylated) Lat-Slp76 scaffold on the TCR-proximal membrane. From here, multiple parallel mechanisms are engaged that lead to transcriptional changes through at least four distinct transcription factors. Simultaneously, actin polymerization is engaged, leading to the cessation of motility, formation of mature adhesions, and key architectural changes that drive the dynamics of the immune synapse. Figure reproduced from [147] with permission from the copyright holder.

of important proteins to the membrane: Protein kinase C (PKC) θ docks onto DAG, and acts through CARMA1-BCL10-MALT1 and the IKK complex to degrade the NF κ B inhibitor I κ B, facilitating NF κ B-mediated transcriptional activation. PKC- θ also leads to integrin and talin activation *via* RapGEF2, which activates Rap. Other PKCs - η and - ϵ act in concert with - θ to draw the microtubule-organizing center (MTOC) toward the synapse *via* reciprocal induction of dynein accumulation and clearance of non-muscle myosin II (NM2) at the synapse [149]. At the same time, Ras and Ras GEFs also dock onto

DAG, leading to Jun-Fos (activator protein 1 [AP1])-mediated transcriptional activation through MEKK1-MKK3/6-p38 & MEKK1-JNKK-JNK cascades. Ras also activates PI3K, leading to generation of PIP₃ at the synapse.

While all this is happening, IP₃ leads to Ca²⁺ store release from the endoplasmic reticulum (ER), which leads to oligomerization of ER-bound Stromal Interaction Molecule (STIM) and co-assembly with membrane-bound Orai1. STIM-Orai1 assembly at PM-ER junctions facilitates massive Ca²⁺ influx from the extracellular space, leading to transcriptional activation *via* calmodulin- & calcineurin-mediated dephosphorylation-activation of Nuclear Factor of Activated T cells (NFAT). NFAT-inhibitory GSK3 activity is also antagonized by PI3K through protein kinase B (PKB, Akt) activation, which then targets GSK3 for phosphorylation-inhibition.

Grb2 and Sos also assemble onto Lat-Slp76. Sos, another Ras GEF, activates Ras independently from the DAG-inducible Ras GEF RasGRP. Actin cytoskeletal regulators Vav and Nck also assemble onto the Lat-Slp76 scaffolding complex. Nck binds the actin NPF WASp through WIP [150], while Vav serves as a GEF for Rac1 and Cdc42, which then activate actin NPFs WAVE2 and WASp, respectively. PI3K also helps in templating actin throughout the contact, as PIP₃ recruits a complex of DOCK2 & ELMO1, which together serve as a Rac1 GEF [151]. Together, these mechanisms link the actin cytoskeleton to the TCR signalsome, which then becomes subject to tangential flow along the membrane for translocation and, possibly, force-mediated activation.

This TCR signaling architecture, in which multiple parallel cascades are fired and sustained by networks of phosphorylation/dephosphorylation and protein-protein interactions, requires prolonged encounter with cognate antigens relative to non-cognate ones in order to proceed. This *kinetic mechanism of proofreading* ensures that only those TCR-pMHC bonds with sufficient affinity (bond lifetime) in a population can receive the

full gamut of nuclear signals for transcriptional activation [152]. Other *co-stimulatory* signals synergize with the TCR to enhance the signaling output of the synapse. Mature DCs express B7 ligands (B7.1 [CD80], B7.2 [CD86]) and ICOSL that bind CD28 and ICOS on the T-cell side, respectively, leading in both cases to PI3K activation. CD28 supports actin polymerization not only through PI3K activation, but also *via* interactions with Filamin A, an actin bundling protein, and Vav through Grb2 and Slp76 [148]. Co-inhibitory signals, on the other hand, depress the signaling output of the synapse, leading to a failure of T-cell activation. Cytotoxic T-Lymphocyte Associated protein 4 (CTLA4) is an activation-induced CD28 family member that has higher affinity for B7 ligands than CD28, which it outcompetes [153]. In addition, CTLA4 can facilitate the trans-endocytic sequestration of B7 ligands from the surface of APCs, potentially affecting the activation of other cells *in trans* as well [154]. CTLA4 can also impact T-cell priming *in trans* by inducing PI3K activity on the DC side, leading to a reduction of autophagy necessary for efficient antigen processing and presentation. Programmed cell Death-1 (PD-1) is another important co-inhibitory receptor on T cells that can be toggled by expression of PD-L1 or PD-L2 by the APC. Ligated PD-1 recruits the inhibitory Tyrosine phosphatase Src homology region 2 domain-containing phosphatase-2 (Shp2) to the synapse, whereupon it deactivates co-stimulatory signaling through CD28 [155]. Interestingly, PD-1 engagement also downregulates the actin-based mechanical activity necessary for synapse maintenance in a non-canonical manner independent from its signaling motifs [156].

Architecture of the T-cell immune synapse.

"Wherever anything lives, there is, open somewhere, a register in which time is being inscribed." —Henri Bergson, *L'Évolution Créatrice*, 1907 [157]

The T-cell synapse is a dynamic interface, with radial and tangential movements of biomolecules being driven by actomyosin network dynamics (Figure 1.7) [158, 38, 159]. Pioneering imaging studies of the T-cell synapse on supported lipid bilayers (SLBs) *in vitro* showed that molecular segregation along the radial axis occurs from the inception of the contact, leading to the self-organization of concentric domains called the central, peripheral, and distal supramolecular activation clusters (SMACs) [160, 161]. The cSMAC is the destination of the TCR/pMHC, CD28/B7, Fyn, Lck, and PKC- θ [162, 160, 163, 164, 165, 166, 167]; the pSMAC is the site of LFA-1/ICAM1, talin, PIP₃, and actin enrichment [167, 151, 168]; and at the dSMAC, the TCR-inhibitory CD45 phosphatase is excluded [167, 169]. At the opposite end of the cell, a distal pole complex (DPC) is also organized, where ezrin, moesin, CD43, RhoGDI, PKC- ζ , Dlg, and Scrib localize [170, 158]. It must be noted that this bull's eye organization readily forms on freely mobile bilayers, while other more diffuse architectures tend to form on surfaces coated with immobile stimulatory molecules. The mobility of surface molecules on actual APCs lies somewhere between these two extremes [171, 64], and by consequence, so does the type of biomolecular organization ultimately formed.

High-speed, high-resolution imaging of T-cell synapses led to the discovery of at least four distinct types of actin structures at the immune synapse. Interestingly, these synaptic structures not only neatly map onto the concentric SMAC domains formed on fluid bilayers (with one exception), but are also required for the maintenance of their morphology and dynamics, implying that actin network activity throughout the T-cell synapse establishes the overall functional & molecular architecture of the contact [148, 38]. A helpful framework is to consider the immune synapse as a radially symmetric analogue of migrating T cells [158, 172]. For instance, the dSMAC closely resembles the Arp2/3-driven lamellipodia of motile cells, in which rapid actin flow of $\sim 0.1 \mu\text{m/s}$ is observed. The actin network here is dense due to a high degree of branching, fueled by

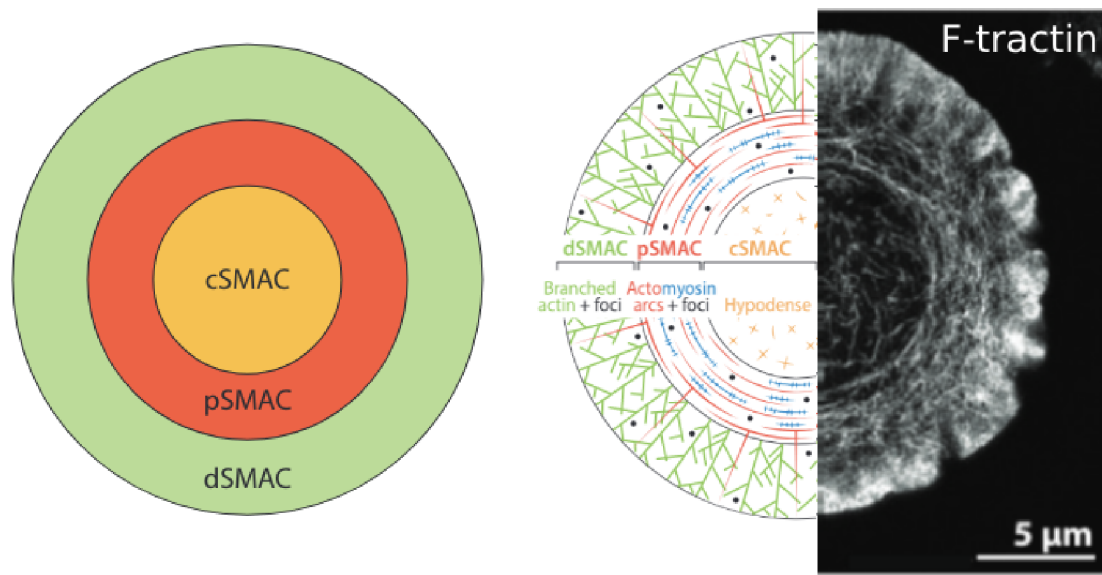


Figure 1.7: Functional and geographical stratification of the immune synapse according to actomyosin network architecture on a freely mobile bilayer.

On the left, the concentric domain segregation of the T-cell synapse into central, peripheral, and distal SMACs is diagrammed. On the right, these domains are related to at least four different actinaceous structures, which each adopt distinct architectures in different strata along the radial axis of the synapse. The distal SMAC resembles lamellipodia, the peripheral SMAC lamella, and the central SMAC a relatively actin-free region through which exocytic release of intracellular endosomes can be achieved. A more elusive set of actin focal structures (black dots) is peppered throughout the dSMAC and pSMAC. Figure modified from [38] with permission from the copyright holder—Figures 1a-b, 1e, and 2a were merged for ease of visualization.

WAVE2 through Rac1-GTP, and appears as a thick outer ring. TCR signaling is often initiated at this zone, and is microscopically visible as focal *microclusters* that travel inward. Through frictional coupling with actin flow, TCR microclusters move inward at about the same speed. Interestingly, TCR signaling output is highest per microcluster at the dSMAC (as shown by pLck, pZap70, and pLAT staining), and decreases as they move inward toward the cSMAC [173, 174, 175]. As mentioned earlier, their signaling is protected from CD45 activity by its steric exclusion from the inward flow beyond the dSMAC, whereas TCRs readily flow through to the lamellar pSMAC.

At the pSMAC, an abrupt change in actin morphology occurs: *in lieu* of a dense band of branched actin, actin networks adopt the form of circular arcs/cords of linear actin fibers

that move inward at about a third of the lamellipodial speed ($\sim 0.035 \mu\text{m/s}$) [176, 177]. Driven by RhoA and formin activity, these linear bundles are generated proximal to the cell edge and are pulled into the pSMAC, where they are bent and dragged inward to form arcs. At the pSMAC, they not only travel centripetally but are telescopically contracted through NM2A activity (as in cytokinetic contractile rings). NM2A in the lamellum/pSMAC self-organizes into bundled, bipolar filaments after phosphorylation by ROCK and induce the global contraction of the arc networks in this zone. Notably, this region is also enriched in integrins that form a tight adhesive gasket with the target cell; it is possible that the enhancement of contractile tension by myosin filaments optimizes the mechanosensitive clutch engagement required for integrin activation at the pSMAC.

Importantly, adhesion molecules such as ICAM1, VCAM1, or CD2 in this region can tune the efficiency of T-cell priming by modulating the signaling output of engaged TCRs [178, 179, 46]. ICAM1 on the APC binds and activates the $\alpha\text{L}\beta 2$ integrin (also called lymphocyte function-associated antigen 1 [LFA-1]) on T cells, facilitating outside-in signaling, mechanotransduction, and establishment of the intercellular adhesions. TLR signaling on the DC side (e.g., through LPS treatment) not only induces the phenotype switch of macropinocytic immature DCs to highly motile mature DCs, but also a lateral constraint of ICAM1 along the DC membrane. This occurs *via* moesin-ICAM1 interactions [171] and provides the necessary counterforces to fully activate T-cell LFA-1, leading to maximal priming. Indeed, adhesion forces between T cells and DCs correlate with both T-cell intracellular Ca^{2+} and IL-2 secretion, as measured by AFM of two-cell conjugates [180]. Interestingly, the frictional coupling of integrin and actin flow leads to a proportional relationship between phospho-Tyrosine levels and actin speed: integrin ligation slows down actin flow at the pSMAC, and this is associated with lower, not higher, levels of pZap70 & pPLC- $\gamma 1$ [46]. It is possible, therefore, that optimal T-cell activation necessitates a dampening of individual TCR microcluster signaling output by

integrin ligation at the pSMAC; on the other hand, because they move three-fold slower, the *integrated signaling output over time* per microcluster may actually be higher. Since phospho-Tyrosine staining requires fixation, based on current evidence, it is difficult to choose between these possibilities. Furthermore, integrins do not all act in the same way upon ligation: against the backdrop of TCR stimulation *via* α CD3 ϵ , LFA-1 engagement by target ICAM1 leads to more dramatic slowing of actin flow than does ligation of VLA-4 (integrin α 4 β 1) by VCAM1. Accordingly, in the context of migration (no antigen), LFA-1/ICAM1 led to strongly polarized migration *against shear flow*, while VLA-4/VCAM1 led to migration *along the direction of flow*—with much attenuated levels of pAkt, pERK, pCasL, pPyk2, and pcCbl relative to LFA-1/ICAM1 [51]. It is important to note that TCR signalsome mobility appears to be a function of substrate membrane fluidity, and is selectively observed on supported lipid bilayers or target cells.

Beyond the pSMAC, a large-scale disassembly of actomyosin networks occurs at the cSMAC. Originally described as "cleared" of actin, this central zone is now rather appreciated to contain a relatively thin but extant meshwork of membrane-proximal actin [181, 182]. CD28 disengages from TCRs at the cSMAC concurrently with the termination of TCR signaling [183, 175], potentially related to receptor dephosphorylation, ubiquitination, and internalization [174, 184]. In effector T cells, granule exocytosis must occur through this actin mesh, and indeed, synaptic actin "loading" has been shown to negatively correlate with phases of degranulation [185, 186]. It is currently believed that the cSMAC of CTLs is segregated further into sub-domains for degranulation and signal extermination [187, 188] .

Another set of actin-based structures, WASp-dependent actin foci, are found peppered throughout the contact. These structures are associated with sites of active TCR signaling [189], although some evidence in this work suggests that WASp also contributes to target

cell deformation through protrusive activity [190], thereby modulating the efficacy of CD8+ CTL-mediated cytotoxicity [191]. Furthermore, WASp regulates synapse stability and symmetry breaking (i.e., synapse termination into "kinapses") [192, 172].

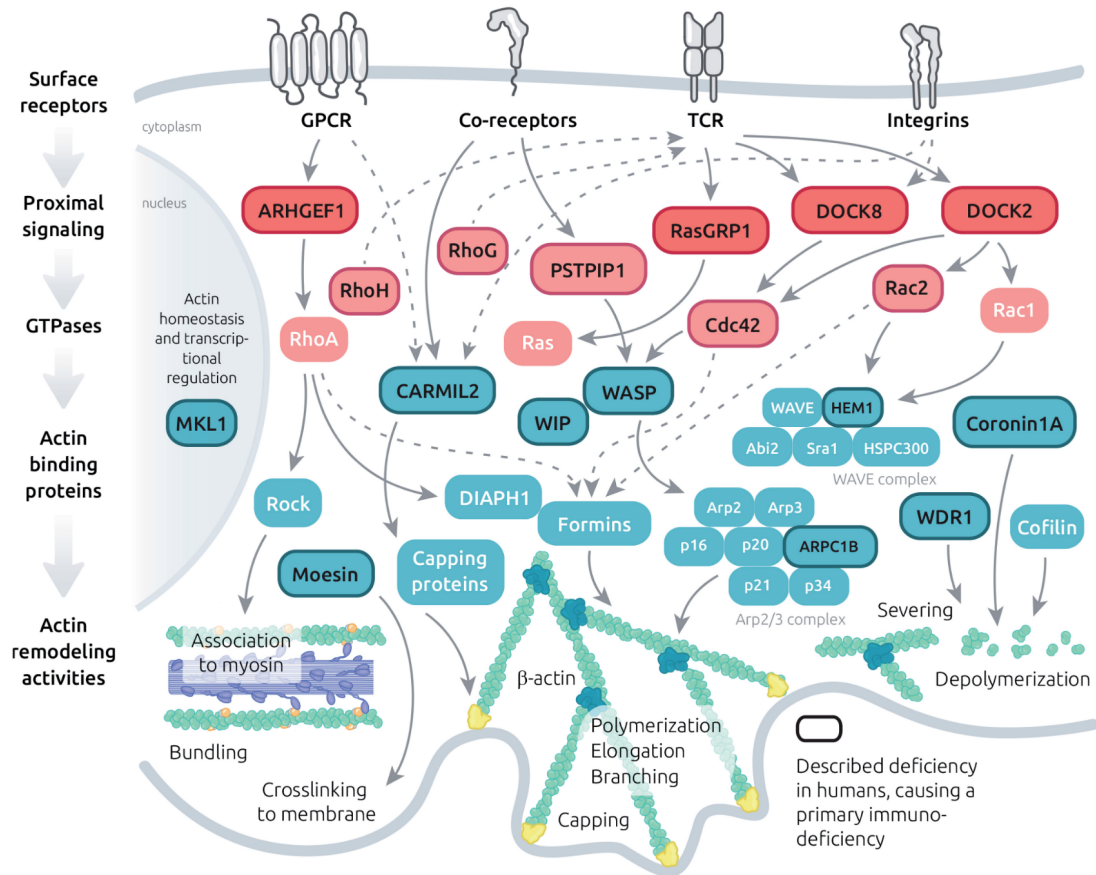


Figure 1.8: Summary of critical cytoskeletal regulators in lymphocytes.

Proteins involved in lymphocyte actin network regulation are critical for immune function; hypomorphic or nullomorph mutations in many of these genes lead to known primary immunodeficiencies (bold outlines). Figure reproduced from Figure 3 of [159] in accordance with the Creative Commons Attribution License CCBY.

Proper regulation of the actin network is paramount for all mechanical functions of T cells (Figure 1.8): migration, contact formation, synapse maintenance, TCR signaling, and contact cessation required for multiple rounds of cell-cell contact [159]. Indeed, deficiencies in many genes and proteins involved in actomyosin network regulation lead to T- and B-cell dysfunction, and ultimately to immunodeficiency.

Effector synapses.

Upon activation by APCs (mature DCs) in the secondary lymphoid organs, T_n cells are coaxed to produce IL-2 and other molecules associated with their destined effector T cell (T_{eff}) functions, and to proliferate. They then leave the lymph node, adhering under shear flow to the activated endothelium in the vicinity of the infection, subsequently migrating through the endothelial cells, and ultimately finding their antigen-presenting target cells following cytokine gradients emanating from the sources of inflammation. $CD4^+$ T_H Cs find "professional" APCs bearing cognate MHC-II such as macrophages, and secrete effector cytokines associated with their functional polarization. In the case of T_H1 Cs, IFN- γ secretion towards macrophages enhances their ability to degrade phagocytosed targets resistant to digestion. On the other hand, $CD8^+$ CTLs, the major subject of this dissertation work, produce cytolytic molecules such as Perforin-1 and granzymes that destroy antigen-presenting host cells (i.e., those bearing viral antigens or mutational neoantigens).

$CD8^+$ CTLs and $CD3^-$ natural killer (NK) cells kill target cells in similar ways, although the "antigen" that they recognize is very different: CTLs recognize presented pathogen-derived peptides in the context of MHC-I presentation (co-stimulation is not necessary at this point); on the other hand, NK cells are naturally inhibited by surface MHC-I presentation on most host cells, and instead detect compromised presentation by virally infected cells ("altered self" presenting no inhibitory signal). Upon recognizing a suitable target cell, CTLs and NK cells will form an immune synapse within ~5 minutes and polarize both the actin cytoskeleton and the MTOC toward the target cell [194, 193]. MTOC polarization is accompanied by the docking of specialized lysosomes called *lytic granules*, which contain the cytolytic perforin-1 and granzymes in large, pre-formed clusters [195]. After docking of granules, a subset of them exocytically fuses

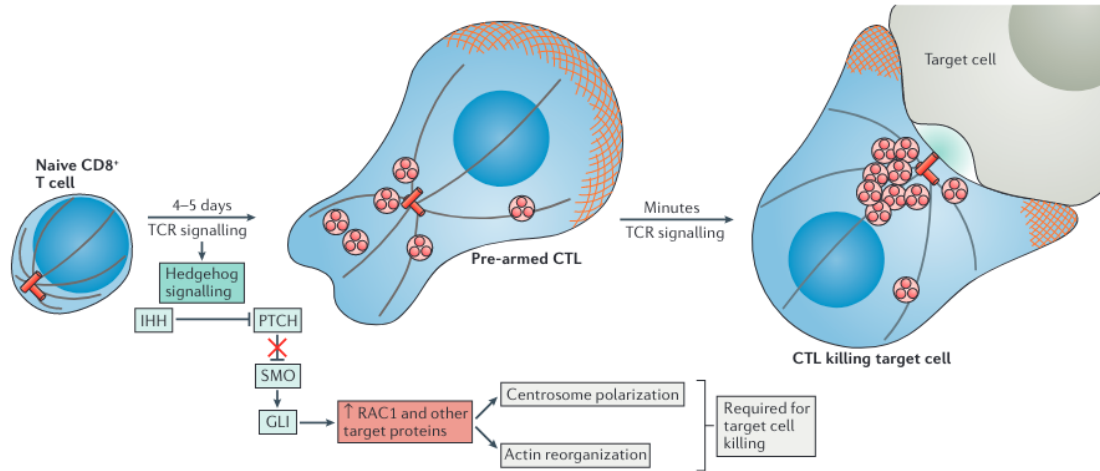


Figure 1.9: Overview of the CTL synapse.

CD8⁺ T_n cells become larger and produce lytic granules upon priming and activation in the lymph node. The effector CTLs that develop from these T_n cells are able to release pre-formed lytic granules quickly (within minutes) and directionally, by forming immune synapses with target cells presenting cognate antigen. Figure modified from [193] with permission from the copyright holder.

with the plasma membrane to release their contents into the extracellular space (Figure 1.9). Perforin-1 selectively forms oligomeric channels on the APC membranes, which were originally thought to mediate direct influx of granzymes into the target cytoplasm. Rather, a membrane repair response is initiated in target cells that facilitates synaptic membrane endocytosis into large vesicles containing both perforin-1 and granzymes from the intercellular space. The granzymes slowly leak out of these "gigantosomes" and cleave myriad substrates to effect target cell apoptosis: ICAD, Bid, Lamins, PARP1, Tubulin, Histones, Caspases 3 and 7, etc. [196] Because the pSMAC forms a tight "gasket" binding the conjugate, target specificity is ensured and bystander killing is minimized.

In the next section, in preparation for discussing my thesis aims, I summarize T-cell mechanobiology after a brief detour through studies on the material properties of leukocytes.

1.3 Force and signification in T-cell biology

The biomechanical idiosyncrasies of leukocytes. For several reasons, the immune system may represent a unique context for mechanobiology [197]. Aside from primary and secondary lymphoid tissues, most "immune tissue" comprises autonomously acting single cells. Leukocytes adopt mechanically active lifestyles that involve constant circulation, migration, and penetration into the periphery [198]. The effect of shear flow on immune cell adhesion to vasculature *en route* to inflamed tissues has long been studied [199], but many other events in immune homeostasis and inflammation warrant similar attention. For example, some immune cells take up residence within the peripheral tissues—which vary widely in mechanical properties [198, 100, 200]—patrolling them for pathogens or regulating homeostatic cell turnover therein [201]. Furthermore, during the course of an immune response, most immune cells will necessarily interact with many different biomaterials within the target tissues: epithelial cells and stroma, other immune cells, non-cellular structural elements (the extracellular matrix), and in some cases, the pathogens themselves (decorated with various host-derived effector molecules). These environments are potentially rich sources of reciprocal mechanical interactions between immune and non-immune tissue. "Immune mechanobiology", then, must consider the immune system's vast threefold undertaking: it must (1) uphold host tissue homeostasis, (2) reject pathogens from these tissues upon demand, and (3) orchestrate appropriate responses in many possible microenvironments and tissue contexts.

The last point in particular presents an idiosyncratic mechanobiological challenge to leukocytes, who must adapt to such varied materials as fluids (bloodstream and lymph), softer tissues (skin and adipose), and stiffer tissues (muscle and bone). One potential solution would be for cells to evolve a mechanically agnostic disposition, activating uniformly on all stimulatory surfaces regardless of material properties. Yet, evidence

argues that immune cells are instead mechanically adaptive, processing mechanical information across different regimes. In this section, I summarize what is known about leukocyte material properties, the biophysical events during synapse formation, and recent work on the specific mechanobiology of T-cells.

Leukocyte rheology.

Impacts of the cytoskeleton and substrate contact. A landmark study used magnetic twisting cytometry (MTC) to demonstrate that human neutrophils and mouse J774A.1 macrophages exhibit universal power law rheology, similar to adherent cells [79]. The mechanical properties of leukocytes are not isotropically distributed, however. In another study, neutrophil granules were subjected to an optical trap, to passively measure material properties across their cell-body segments: the lamellipodial leading edge, cell body, and uropod [202]. The leading edge in these cells was consistently less stiff and more fluid than the cell body or uropod. Both studies also noted significant cell softening and fluidization with cytochalasin D treatment, which blocks the polymerizing (barbed) ends of actin filaments. A later study utilized an optical trap-based cell stretcher (optical stretcher) and a microfluidic device with small constrictions in series (microchannel) to study the effects of myosin activity on HL-60 human leukemia cells, *in vitro* differentiated neutrophils, differentiated monocytes, and adherent cell lines [97]. Here, naturally and enzymatically suspended cells alike stiffened and became less fluid upon myosin inhibition, implying that suspended cells utilize myosin to soften and maintain fluidity. This stood in contrast to the effects of actomyosin contractility in adherent cells. Importantly, while cytochalasin D softened and fluidized the cells in this study, jasplakinolide (which stabilizes F-actin filaments) stiffened them.

Microtubules were perturbed in studies that used a microchannel with single constrictions to image the deformation of non-adherent K562 leukemia cells [203, 204]. Reminiscent of the trends with actin, cells stiffened and became less fluid when treated with paclitaxel, which prevents microtubule disassembly; on the other hand, they softened and fluidized upon treatment with nocodazole, which induces net tubulin disassembly. Perturbations of the nucleus also drove rheological changes in the expected directions: lamin A overexpression, as well as nuclear condensation by treatment with divalent cations, stiffened the cells; the opposite was found with the nuclear decondensing agent aza-Cytidine.

The differences upon myosin inhibition between suspended and adherent cells pointed to the potential role of substrate contact on leukocyte material properties. In an AFM-based study on rat neutrophils, [205], cells were measured on passivated glass, which precluded adhesion. On passivated regions, actin was mostly cortical and cells remained spherical; on bare zones (control), cells spread more, polarized their actin, became stiffer, and became less fluid. These studies implied that both cytoskeletal dynamics and substrate contact actively determine leukocyte material properties.

Impact of cell lineage. Different leukocytes each specialize in conducting a subset of immunoprotective functions. There is reason, therefore, to speculate that cell-type-specific and cell-state-specific mechanical distinctions might likewise exist. Ekpenyong and colleagues explicitly investigated how mechanical properties track with cell fate, contrasting HL-60 (progenitor) cells from *in vitro* derived monocytes, macrophages, and neutrophils [206]. According to optical stretching measurements, HL-60-derived macrophages at steady state and without substrate contact were the stiffest, followed by undifferentiated cells, monocytes, and neutrophils. Interestingly, after extended periods of stretching (> 15 minutes), macrophages became softer than the HL-60 cells.

In the same work, primary human CD34+ hematopoietic stem cells were found to be softer than differentiated and primary neutrophils. Another group used image-based deformability measurements of immune cells flowing through a microchannel to show that human red blood cells, neutrophils, B cells, and CD4+ T cells could all be distinguished mechanically, without any prior labeling [207]. Here, the lymphocytes were stiffer than the granulocytes, and RBCs were softer than the rest. In addition, an AFM-based study found that CD4+ Jurkat leukemia T cells and primary CD4+ T cells were much softer than antigen-presenting cells (APCs) at steady-state by about one-third [92], which could impact cell-cell communication at lymph nodes and inflamed sites. In line with this, another AFM-based study compared HL-60 cells, Jurkat cells, and primary human neutrophils immobilized within inert microwells (no adhesion) [208]: here, HL-60 cells were approximately 18-fold stiffer than primary neutrophils, while Jurkat cells were the softest. In a more recent study, optical tweezers were used to profile human macrophages differentiated using GM-CSF or M-CSF, and found that M-CSF-differentiated cells were stiffer, more solid-like/elastic, and better able to migrate persistently [209]. These differences appear to mirror the cytoskeletal differences found between immature and mature DCs [140]. In all, these findings demonstrate that different immune cell types and differentiation states are associated with distinct mechanical properties, even prior to biochemical stimulation.

Impact of biochemical stimulation. Within leukocyte subtypes, the possibility remains that distinct activation states are associated with mechanical properties suited to their distinct functions. The peptide N-formylmethionine-leucyl-phenylalanine (fMLP), an early bacterial translation product and PAMP recognized by various innate immune cells, has been shown to soften HL-60-derived macrophages, while stiffening neutrophils and monocytes derived from the same cell line [206]. A subsequent study noted stiffening of

primary human neutrophils in response to fMLP and granulocyte-macrophage colony stimulating factor (GM-CSF) [210]. On the other hand, primary human neutrophils became more deformable upon treatment with the chemotactic Complement 5a fragment (C5a) [211]. Like fMLP and GM-CSF, C5a induced neutrophil cytoskeletal polarization, suggesting that multiple pathways converge on cytoskeletal activation, leading to a variety of possible morphological and rheological changes.

Illustrating the same principle, *in vitro* differentiated human DCs and macrophages both stiffened as measured by AFM in response to IFN- γ , and softened upon dual treatment with TNF- α and prostaglandin E2 (PGE2) [92]. LPS treatment of DCs *in vitro*, as noted earlier, changes their global actin distribution and migratory patterns. One study showed that human monocyte-derived DCs treated with LPS adopted more solid/elastic characteristics, while differentiated macrophages did not change appreciably [140]. These macrophage findings are contrasted by work showing significant softening and fluidization upon LPS treatment, although the methods of macrophage differentiation were different [212].

These studies emphasize that stimulatory molecules affect the material character of immune cells differentially (even to the level of *in vitro* differentiation methods), and that the dose thresholds for inducing bulk mechanical responses depends on cell type. LPS-induced DC stiffening may improve their ability to prime naive T cells at the lymph nodes, a function that is not typically shared by activated macrophages [171]. LPS-induced stiffening also occurs in human neutrophils [213] and mouse microglia [110]. A dramatic level of activation-induced rigidification was observed in primary human CD4+ T cells: the average Young's modulus of these cells more than doubled within a minute of contacting stimulatory α -CD3/ α -CD28 beads, as measured by micropipette-based microindentation [48]. It is tempting to speculate that such bulk changes to stiffness are

sensed by a lymphocyte's binding partners during cell contact, through mechanosensitive adhesion pathways.

Perspectives and open questions. These studies all hint towards a complex mechanochemical network amongst immune cells. The findings summarized above do not simply suggest, for example, that all stimulation leads to stiffening. In this regard, systematic cataloguing of how different innate and adaptive signaling arms influence cell rheology is warranted (e.g., various TLRs, intracellular nucleic acid sensors, cytokines, activating and inhibitory lymphocyte receptors, etc.). Distinct mechanical changes due to pro- and anti-inflammatory stimuli would also be illuminating. As high-throughput technology continues to develop, mechanical profiling ought to join the routine battery of assays used for characterizing immune cells of interest.

In the following pages, I narrow down on the literature concerning T-cell mechanobiology, highlighting the ways by which mechanical forces contribute to T-cell activation (input) and function (output).

1.3.1 Mechanical input: Contributions to T-cell activation.

T cells live very mechanically active lifestyles, considering the numerous instances of long-distance migration and synapse formation. Whereas haptotactic cells (e.g., fibroblasts) move *via* controlled cycles of incremental protrusion and adhesion to the substratum, leukocytes, by virtue of their functions, must be exploratory, highly motile, not prone to sedimentation on the ECM, and readily able to exert traction forces when needed [198]. Accordingly, leukocytes utilize an amoeboid mode of migration whereby actin-driven exploration pushes the cell front, while myosin-driven contraction drags

the body through the available space, in what has been termed "flowing and squeezing" [50]. Furthermore, DCs and T cells dynamically adjust to the adhesiveness of their substrata as they move [49, 45]. The lamellipodial cell front of these cells seems to act as an exploratory module rather than the major source of traction for locomotion, in keeping with their cellular functions [50, 45, 214, 66]. A constant, "fractal" surface flow of TCRs driven by actin dynamics is thought to facilitate efficient exploration of the T-cell's surrounding space, so that they can encounter even very rare antigens on the target surface [215, 216].

Forces felt by a T cell's surface receptors. Little is known about the biomechanics of thymic development and lymph node residence, but recent *in vitro* work has uncovered intriguing mechanical aspects during TCR triggering, the antigenic search, and formation of the immunological synapse. While most cell-surface receptors generally "slip" under tension, weakening and unloading after reaching a threshold amount of force, a subset of them exhibit "catch bond" behavior, in which an optimal amount of tension enhances bond lifetime and apparent strength [217]. Some immunostimulatory receptors such as the Fc γ R on NK cells [218] and the T-cell receptor (TCR) [219, 220, 221] have also demonstrated catch bond character, potentially representing an integrated physicochemical immune strategy for "self versus non-self" discrimination. Force may trigger initial TCR signaling by exposing key ITAMs on the CD3 tails, which by default can dock onto the plasma membrane *via* a combination of intermolecular forces [222]; force then also stabilizes key intramolecular interactions that control catch-bond behavior [223, 220]. However, results in cell-free, single-molecule systems are varied (for example, see [223] and [224]). This implies that observables resembling catch bond behavior and cyclic mechanical reinforcement [225] could also emerge from mechanoreceptors being studded on a functional plasma membrane, where they are subject to (1) longitudinal

[65, 63, 64, 62] and transverse membrane flows [226, 216], (2) cooperativity with other mechanoreceptors [227], and to (3) architectural reinforcement by the focal adhesion machinery [25].

Using optical tweezers, "conversion" of a non-agonist antibody into a stimulatory one was achieved by applying shear (tangential), but not axial, forces on T cells [228]. In support of this, activation of T cells on an elongated, non-activating ligand (pMHC attached *via* a long linker) was dramatically enhanced under the influence of shear flow [229]. Work soon followed that demonstrated maximum bond strength at ~ 10 piconewtons (pN) of applied force between T cells and antigen-coated biophysical force probes (BFPs) [219, 223]. In these experiments, only cognate TCR-pMHC interactions resulted in bond lifetime enhancement (catch bond behavior); altered peptide ligands all weakened with applied forces (slip bond behavior). A pioneering DNA-based tension sensor has also been used to measure nanoscale forces across the T cell-substrate contact area [230]. On sensors presenting pMHC and ICAM1, early "tugging" forces on the order of 20 pN were measured, and signaling molecules Lck and Zap70 co-localized within these force exertion zones. Opposite, nanoscale "poking" forces by migrating T cells was found using quantum dots on lipid bilayers [216]. All of these studies suggest that force application, delivered by T cells through microvilli [231, 216] or invadosome-like protrusions (ILPs) [232, 233], allows them to discriminate between the affinities of ~ 15 -nm TCR-pMHC bonds against the backdrop of a 50–500-nm thick glycocalyx [233, 216, 48, 178]. This is further supported by observations of TCR enrichment at microvillar tips [226], and by the finding that engaged microvilli persist for much longer than unengaged ones [216].

Because retrograde actin flow is important for forming and transporting TCR micro-clusters, lateral friction across the synaptic plane, especially at the dSMAC and pSMAC, is thought to regulate signaling. *In vitro* experiments using chromium grids as physical

barriers to lateral flow [183, 234, 235], bilayers with restricted phospholipid mobility (formed using the Langmuir-Blodgett method) [236], and ICAM1 or VCAM1 as a source of frictional drag [179, 46, 237, 51], all support the importance of frictional coupling both for actin focusing and signaling persistence. An important source of lateral, frictional counterforce at the synapse is the mature DC cytoskeleton, which polarizes towards T cells during their interactions [238, 239, 240, 241]. A number of compelling studies have demonstrated the influence of lateral mobility of stimulatory molecules along the plasma membrane on the signaling output of immunoreceptors [171, 242, 64]. Using fluorescence recovery after photobleaching (FRAP) of ICAM-1 molecules on *in vitro* differentiated mouse DCs, lateral restraint and reduced mobility of the integrin ligands were detected in response to LPS treatment [171], which were postulated to optimize naive T-cell activation and priming through tension-modulated conformational maturation [242]. In parallel, diffusion of macrophage phagocytic Fc- γ R is also regulated, through "picket fences" formed by the hyaluronan receptor CD44 on the exofacial side bridged by ezrin to the cortical actin mesh on the endofacial side.

Impacts of macroscale stiffness on T-cell activation. A number of *in vitro* studies have established the mechanosensitivity of T-cell activation to substrate stiffness [243, 244, 245, 47] (summarized in [246]). The studies used different cell sources, subtypes, and activation states (e.g., mouse vs. human, CD4+ or CD8+, naive or blasts); they spanned different regimes of stiffness within the large range of 1–2000 kPa; they used different methods of stimulation (e.g., α CD3 alone, with α CD28, or with ICAM1); and they measured different indices of T-cell activation (e.g., IL-2/IFN- γ secretion or phospho-Tyrosine levels). In most assays, increased T-cell activation was observed with increased stiffness; furthermore, mechanosensitive T-cell spreading and signaling output were dependent on integrin ligation and myosin contractility [47, 247]. However, only

one of these earlier studies actually varied stiffness across a physiological range of 1–5 kPa, and this study used Jurkat T cells [245].

Are there protective benefits to T-cell mechanosensitivity? It could allow T cells to read an additional layer of functional distinctions among APCs, particularly those reliant on cytoskeletal activity [92, 140, 248]. More recently, the important phenomenological cataloging of mechanosensitive activation both by mouse CD4+ and CD8+ T cells under many different parameter sweeps has been conducted [248]. Remarkably, naive CD4+ T cells exhibited a greater degree of mechanosensitivity than naive CD8+ T cells across the physiologically relevant Young's modulus range of 2–25 kPa, using CD69, CD25 (the high-affinity IL-2 receptor α chain), and proliferation as indices of T-cell activation. These mechanosensitive behaviors may act in part through YAP-mediated control of NFAT activity [249], and reflect a greater need for co-stimulation by CD4+ T cells in general, or perhaps reflect a functional benefit to cytolytic immune cells adapting a mechanically agnostic disposition. In contradiction to the latter, *in vitro* differentiated CTLs do exhibit mechanosensitive degranulation [248] and cytokine secretion [250, 251]—and the latter has been shown to impact metastatic tumor cell immunosurveillance, which leads to the selective eradication of tumor cells that are stiffer [250].

1.3.2 Mechanical output: Contributions to T-cell effector function.

By the integration of actin into the TCR and co-stimulatory signaling pathways, T-cell activation leads to a cessation of motion and formation of the immune synapse on a cognate APC. Through the synapse, the activated T cell then also generates mechanical forces of its own, transmitted to their targets/partners. Different force probes measure different types of mechanical output—and no single system fully captures all components of T-cell

mechanical output—so care must be taken in the intuition of general phenomenological trends. Experimental systems can be grouped into those that measure: (1) bulk axial pushing & pulling forces along the axis of the cell-cell contact, (2) traction forces along the plane of the immune contact interface, and (3) nanoscale pushing & pulling forces protruding *into* the target surface (Table 1.1). AFM and micropipette assays measure the axial displacement of stimulatory beads by T cells; however, because these particles are rigid, no sense of the nanoscale forces "felt" by the target cell surface can be gleaned. On the other hand, flat compliant surfaces such as traction force hydrogels or micropillar arrays measure lateral displacement (spreading, squeezing) along the planar area of the immune synapse, but axial information is lost. This is because hydrogel slabs are thick and flat, precluding side-on imaging of the contact, while micropillars can be bent along the x,y-plane but are rigid along the z-axis. Some force probes are designed to specifically measure piconewton, nanometer-scale forces along the immune cell contact: the prototypical example is a DNA hairpin that unfolds when the attached ligand is tugged by a receptor on the cell. These probes measure the pulling or shearing of individual receptor-ligand bonds across a rigid surface; therefore, depending on the DNA hairpin design, they can report on "3-D" nanoscale axial forces along z (poking or tugging) or traction (shearing) forces along x & y.

Table 1.1: Summary of recent studies measuring T-cell force exertion.

Orientation	Method	Cell type	Stimulation	Key findings	Ref.
1-D axial (bulk pushing/pulling)	Atomic force microscopy	Mouse CD4+	α -CD3, pMHC	Stronger pulling forces from antibody than antigen; similar pushing forces	[252]
		Mouse CD8+	α -CD3	Actin & myosin required for both; CRAC channels required for pulling but not pushing	
	Micropipette assay	Mouse T-cell hybridoma	pMHC dimer-Fc fusion	Co-receptor engagement affects adhesion rate but not force exertion	[253]
		Human CD4+	α -CD3/ α -CD28	CD28 increases "punch length"	[48]
			α -CD3/ α -CD18	LFA-1 ligation decreases punch length and loading rate	[254]
2-D planar (spreading/squeezing traction forces)	Traction force hydrogel	Jurkat CD4+	α -CD3	Target stiffness increases loading rate	
			α -CD3	Actin and ROCK are required, but not dynein and tubulin; mixed results with myosin inhibition	[245]
	Micropillar array ("bed of needles")	Human CD4+	α -CD3/ α -CD28	Squeezing is strengthened by CD28 (whether immobilized or soluble)	[255]
		Mouse CD8+	α -CD3/ α -CD28	LFA-1 and formins increase traction	[47]
			pMHC/ICAM1	Arp2/3, WASp, WAVE are required	[190]
3-D axial (tugging, possibly shearing)	DNA-based tension probe	Mouse CD8+	α -CD3, pMHC (affinity series)	Antigen affinity scales with tugging forces	
			α -CD3/ICAM1	LFA-1 strengthens tugging forces	[230]
	Micropillar array		Unclear	Cdc42 and actin are required; myosin, Arp2/3, ROCK inhibit tugging	
3-D axial (poking/protrusions)	Electron microscopy		pMHC/ICAM1	Arp2/3 required for protrusions	[190]
	Light-sheet microscopy		Antigen-pulsed target cells	T cells protrude into target cells	[256, 233]
					WASP enhances target cell distension, protrusion

Using AFM, BFP, and micropipette systems, one-dimensional forces exerted by T cells have been measured. Activated T cells first undergo a pushing phase, followed by a stronger pulling phase [254, 180, 252, 48]. The phasing and magnitudes measured are difficult to compare across studies because of the different stimulatory conditions used. However, it appears that T cells pull with loading rates (applied force over time) on the order of 1–10 pN/s. Two-dimensional synaptic forces have also been measured along the plane of the synapse *via* TFM on hydrogel slabs or micropillar assays, in which pillars of known bending stiffness are bent by engaged cells. On α CD3 ϵ -coated TFM hydrogels (OKT3 clone), Jurkat T cells exerted $\sim$ 2-nN forces directed centripetally, with a peak traction stress of $\sim$ 50 Pa [245]. In addition, these forces scaled somewhat with hydrogel stiffness, suggesting possible mechanosensitivity of mechanical output as well. On OKT3-coated micropillars, synaptic forces on the nanonewton scale were exerted by naive CD4⁺ T cells [255]. Furthermore, CD28 costimulation was shown to modulate the strength of these forces in a pathway involving PI3K, phosphoinositide-dependent protein kinase 1 (PDK1), and actin, but not Akt. Interestingly, in this OKT3/B7 micropillar system, forces did not seem to scale with micropillar stiffness (height), arguing against the mechanosensitivity found in the OKT3-only TFM hydrogels. However, it could be argued that co-stimulation leads to a change in overall synaptic actin architecture, which could selectively manifest in components of force (e.g., axial, as in [48]) that would be missed in the TFM assay.

On beads coated with H2K^b-OVA and ICAM1, OT-I primary CD8⁺ T cells pulled with loading rates of $\sim$ 10 pN/s [191], while primary CD4⁺s pulled at $\sim$ 1.5 pN/s. On similarly coated micropillars, much stronger tractions of $\sim$ 4 pN/pillar were measured, compared to naive CD4⁺ T cell tugging on the order of $\sim$ 0.1 nN/pillar. In addition, by way of genetic perturbations that increase (PTEN knockdown) and decrease (DOCK2 knockdown) force output, a proportionality between forces and cytotoxicity was demonstrated [151, 191].

This work proposed that T-cell exertions act in part to generate zones of high membrane tension on target cells, which sensitizes them to lysis by exocytosed perforin molecules. In a phenomenon dubbed *mechanopotential*, T cells coordinate the cytotoxic program both biochemically (degranulation) and biomechanically (force exertion).

Although these one- and two-dimensional force measurements have proven to be instructive, limited evidence suggests that T cells may simultaneously exert axial and planar forces across the synapse—indicating a three-dimensional complexity that cannot be revealed by these methods. On stimulatory micropillar arrays, T-cell actin polymerization was observed into the space between rigid pillars [190], reflecting protrusive activity indirectly [190]; electron micrographs (EM) of T cells interacting with targets confirms that submicron-scale protrusions "poke" into targets, distorting even the relatively stiff nuclei [233, 256]. T cell-DC conjugates are also enriched in microvillar protrusions and "veils", which indicate a three-dimensionally complex contact [257].

1.4 Thesis aims

Upon target recognition (through cognate pMHC or anti-TCR antibodies), activated T cells not only process mechanical signals from the apposing surface, but also begin to exert forces of their own towards the stimulatory targets. They push and pull on their targets, spread and squeeze on them, and on the sub-micron scale, poke and tug on them as well. These forces are TCR-dependent and influenced differentially by co-stimulation from CD28-B7 and integrin ligation. Actin polymerization *via* formins and Arp2/3 is necessary for synaptic force evolution, but the requirement for myosin is subtler, possibly being more selective for the axial forces. Importantly, these different orchestrations of the T-cell cytoskeleton likely happen in concert, and none of the above methods fully

captures the mechanical activity of activated T cells in 3-D. A compliant system on the size order of individual cells would, in principle, be able to provide more information on cellular strength and mechanical force patterning than any of the previous techniques.

This dissertation work grew from an interest in capturing the 3-D mechanical force exertions by CTLs, based on the studies summarized above. In Chapter 2, I build upon a novel hydrogel-based system introduced in 2020 for macrophage phagocytosis [258] to describe the mechanical features of the cytotoxic immune synapse. In Chapter 3, I attempt to link this mechanical description to cytotoxicity, and rationalize the way in which the mechanopotential of perforin could be conceived as a mechanochemical optimization problem. In Chapter 4, reflecting on the limitations of describing mechanical activity solely in terms of strength, I develop a method for describing the topographical patterns of the synapse in frequency space, using the principles of Fourier signal analysis. From Chapters 4-5, I apply these complementary measurements to link cytotoxic function to mechanical activity in three different biological contexts. In Chapter 6, I explore the mechanosensitivity of synaptic mechanical activities.

CHAPTER 2

PHENOMENOLOGY OF T-CELL MECHANICAL ACTIVITY

2.1 Background

2.1.1 Microparticle traction force microscopy: the advantages and limitations of a new force probe

In 2020, Dr. Daan Vorselen led a research project in the group of Dr. Julie Theriot at Stanford University to develop a method for the high-yield synthesis and derivatization of spherical microparticle hydrogels [258]. These Deformable Acrylamide-Acrylic acid Microparticles (DAAM particles, hereafter "beads", "hydrogel beads", or "particles") were produced by (1) preparing standard pre-polymerization mixtures of acrylamide and bis-acrylamide co-polymers (similar to compositions used in protein gel electrophoresis); (2) extruding this aqueous mixture into a stable oil emulsion through a Shirasu porous glass silica nanopore membrane (Al_2O_3 - SiO_2 coated in hydrophobic silicone, hereafter [SPG-SNM]), which causes them to bud off as spherical droplets; and then (3) polymerizing the droplets *in situ* using an oil-soluble radical initiator (e.g., azobisisobutyronitrile [AIBN] instead of ammonium persulfate [APS]) (Figure 2.1). Billions of beads can be generated from a 10-mL pre-polymerization mixture under optimal conditions. By adjusting the relative molar proportion of bis-acrylamide, the post-polymerization Young's modulus of particle preparations can be tuned: particles can range from 200 Pa to 18 kPa (not shown). By using SPG-SNMs of different pore diameter sizes, monodisperse bead populations ranging from 7–30 μm can be produced. Importantly, by doping sodium acrylate into the co-polymer mixture, the final polymerized product is a population of

polyacrylamide hydrogel microspheres with exposed acrylate groups (prop-2-enoate), which through acyl substitution reactions can be functionalized with immune-stimulatory peptides, fluorophores, and other aminated functionalities.

This hydrogel bead system offers numerous advantages over other force probe methods: the synthesis of these beads is extremely high-yield, tunable, relatively straightforward, and reproducible, once the optimal extrusion pressure conditions for a given SPG-SNM are empirically determined. Furthermore, the functionalization strategies are simple and flexible, allowing experimenters to generate beads that stimulate different immune cell subsets, fluoresce in different colors, and even change their properties when exposed to specific conditions [258, 259, 260, 261, 262, 263, 264]. Fluorophore functionalization can be adjusted to uniformly highlight the entire particle (by conjugating aminated fluorophores directly onto acrylates) or only the surface outline (through an intermediary fluorescent protein layer that selectively binds the bead exterior), offering further flexibility in terms of shape analysis strategies.

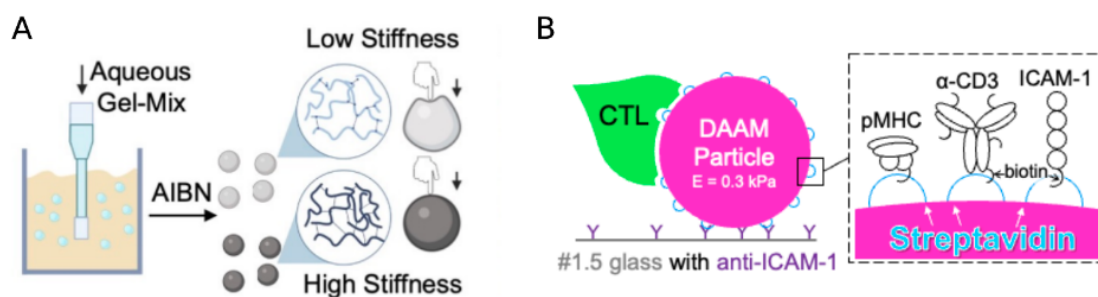


Figure 2.1: Hydrogel bead synthesis and conjugation scheme.

A: A pre-polymerization mixture of acrylamide and other co-polymers are extruded into an organic phase of mixed hexane isomers and detergent Span-80. Stiffness is determined by the degree of cross-linking, which depends on the ratio of mono- and bis-acrylamide in the mixture. B: The acrylic acid moieties on the hydrogel can be used in nucleophilic acyl substitution (S_NA) reactions for functionalization. In this work, streptavidin and aminated fluorophores are first "snapped" onto these acrylate groups, and then biotinylated peptides are co-incubated with the streptavidinylated beads. Figure reproduced from [262, 264] in accordance with the Creative Commons Attribution License CCBY—2.1A is taken from Figure 1A of [264], and 2.1B is taken from Figure 1A of [262].

While these chemical properties are shared by other activated hydrogel systems, the spherical form of these hydrogels allows the simultaneous observation of compression and tension, shear traction forces, and nanoscale protrusive forces. Indeed, the original paper describing this *microparticle traction force microscopy* (MP-TFM) method estimated the three-dimensional force profiles of macrophages during phagocytosis using an iterative energy minimization procedure [258]. In this work, I used surface topography (curvature maps) and volume displacement of the beads as indices of cellular force patterning and strength, respectively.

Two distinct disadvantages cannot be overcome by this hydrogel bead system: bead porosity and the lack of membrane fluidity. The beads are essentially cross-linked co-polymer slabs of acrylamide, where cross-linking density (related to the bis-acrylamide:mono-acrylamide ratio) determines their mechanical properties. Stiffer beads are less porous, while softer beads are significantly more porous, facilitating protein diffusion to different extents into the bead cores (not shown). These considerations must be taken into account when conducting studies across bead stiffnesses. Importantly, soft bead porosity impacts CTL viability to a certain extent, possibly due to compressive stresses [265, 266, 267]. The other notable disadvantage of this bead system is the lack of lateral membrane fluidity along the surface of the hydrogel. Unlike in synapses formed against oil droplet-based force probes [268, 269], T cells engaged with hydrogel beads cannot drive lateral movement of ligated receptor-ligand pairs along the plane of the contact, as they would against APCs. This prevents straightforward interpretation of spatiotemporal correlations between known biochemical signaling events (e.g., central translocation of TSG101 & TCR, peripheral exclusion of CD45, etc.) and T-cell mechanical force exertion. A hybrid system combining realistic mechanical resistance from a hydrogel with the membrane fluidity of the oil droplet or supported bilayer systems would bring

together the physiological elements of both. However, at present, such a system does not exist.

In our hands, some modifications to the original method in Vorselen et al., were necessary to synthesize the hydrogel beads at the desired yield and monodispersion. Importantly, the concentration of surfactant Span-80 had to be lowered from the reported 3 % (v/v) to 1 % (v/v); otherwise, extrusions were unstable and tended not to form proper water-in-oil emulsions. For most of this work, a single batch of 300-Pa was used consistently; for later experiments in which multiple bead batches are used, a range of bis-/mono-acrylamide ratios were used and the appropriate stiffnesses determined empirically by AFM.

In this section, I first validated that the functionalized beads act as biomimetic substrates for T-cell activation by measuring Calcium flux and CD69 expression on CTLs, as well as the dependence of synapse mechanics on the actin cytoskeleton. Next, I documented the general phenomenological presentation of immune-synaptic forces on these substrates and correlated these behaviors with deformations observed on real target cells.

2.2 Results

2.2.1 Hydrogel microparticles mimic T-cell activation by APCs

TCR-proximal signaling by Ca^{2+} flux. The ability of these biomimetic hydrogel beads to stimulate TCR activation in a native manner was measured using Ca^{2+} flux imaging, in which ovalbumin (OVA)-specific TCR transgenic CTLs (OT-I^{tr-tg}, hereafter OT-I) were

loaded with the ratiometric Ca^{2+} -responsive dye fura-2, then co-incubated with particles and followed for ~30 minutes (Figure 2.2).

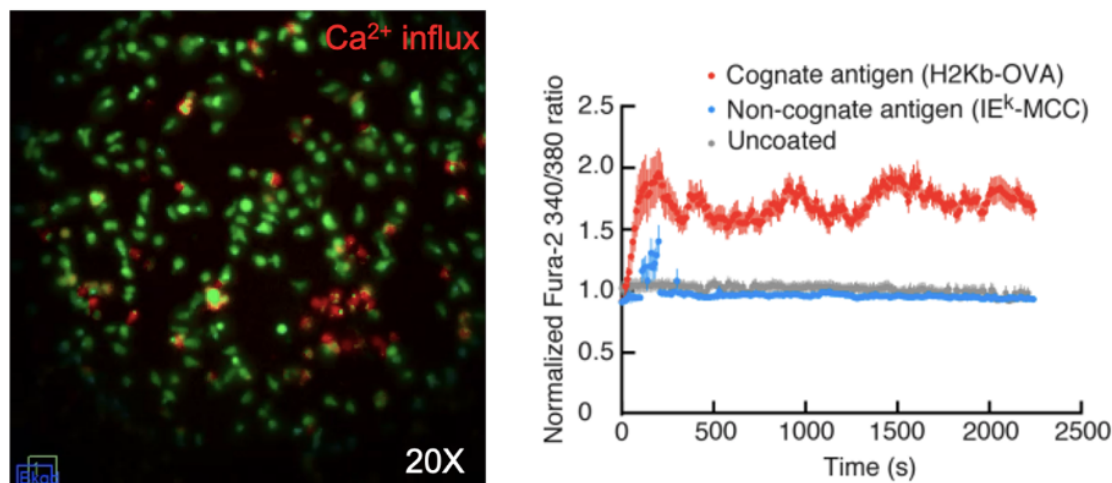


Figure 2.2: Ca^{2+} flux imaging of CD8+ CTLs.

Left: CTLs engaged with stimulatory beads, pseudocolored by the fura-2 ratiometric signal A_{340nm}/A_{380nm} . Right: Quantification of the population data over time, plotted as mean fluorescence intensity (MFI) $\pm$ standard error of the mean (SEM). Data is representative of at least $n=3$ independent experiments. Figure reproduced from Figure S12 of [258] in accordance with the Creative Commons Attribution License CCBY.

Antigen-specific TCR activation could be measured as a sharp rise in the ratiometric fura-2 signal upon encountering cognate antigen and ICAM1 on the beads (for the OT-I TCR, OVA 257-264 [SIINFEKL] in complex with H2K^b MHC-I molecules [hereafter, H2K^b -OVA]). As a control, beads containing mismatched MHC-I-restricted antigen (e.g., H2D^b -gp33) or an MHC-II-restricted antigen (e.g., moth cytochrome c on IE^k [IE^k -MCC]) can be used as a comparator. In experiments conducted on antigen-pulsed target cells, this initial sharp rise is followed by an oscillatory response owing to multiple cycles of antigen recognition on target cells, killing of the target cells, and deconjugation from the apoptotic corpses; however, on hydrogel beads, which do not undergo any death-associated changes sensed by CTLs [270], the sharp rise was followed by a slow decay. The cells formed stable conjugates only with beads bearing cognate antigen, and rarely, if ever, let go of the hydrogel beads over the monitoring period (data not shown).

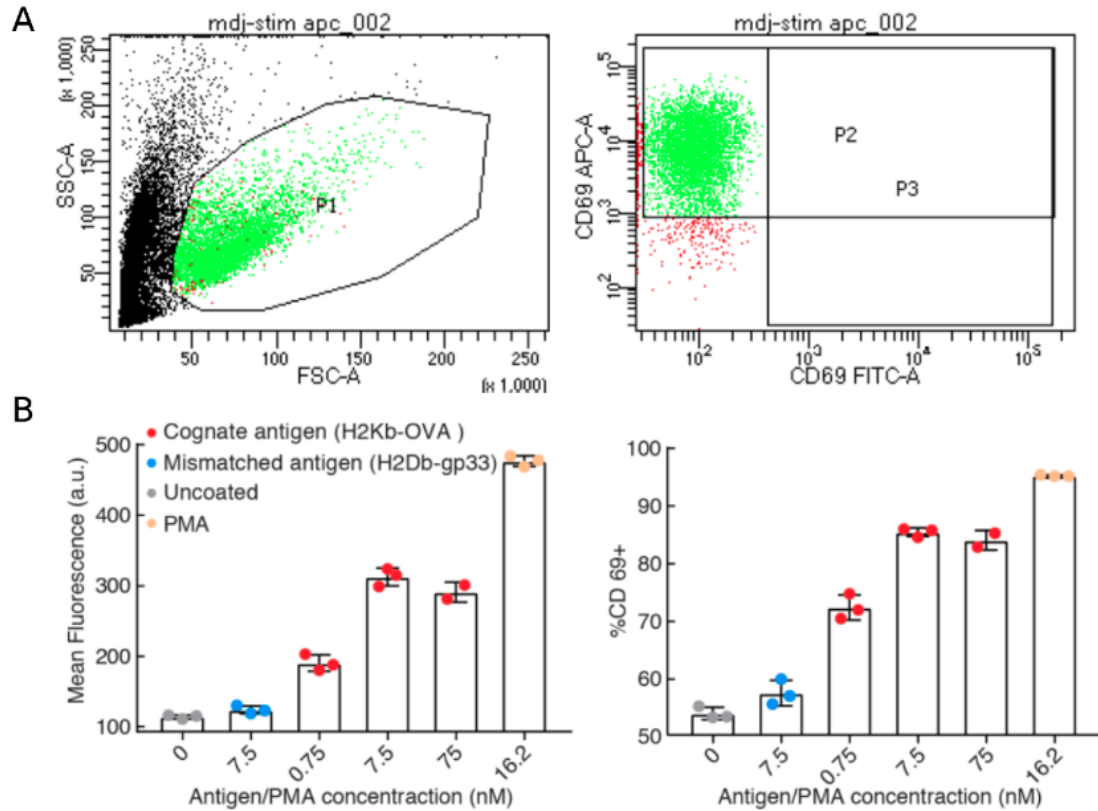


Figure 2.3: CD69 detection by flow cytometry.

A: Example gating strategy for CD69 surface expression. B: Quantification after 5 hours of co-incubation with pMHC/ICAM1-coated hydrogels. Plotted as mean $\pm$ sample standard deviation (SD). Data is representative of at least $n=2$ independent experiments. Panel B was modified from Figure S12 of [258] in accordance with the Creative Commons Attribution License CCBY.

Cell activation by surface CD69 expression. CD69 is an early activation marker in T cells that appears on the cell surface within hours of TCR stimulation [271]. The levels of CD69 expression of CTLs in a population can thus be assayed by flow cytometry (Figure 2.3). For this experiment, T-cells were incubated with 1-kPa beads bearing the indicated amounts of antigen (or phorbol 12-myristate 13-acetate as indicated), all with 1×10^{-16} mol/bead of ICAM-1 for five hours. CD69 was then assayed by flow cytometry, and it was determined that a coating concentration of approximately 1.5×10^{-18} mol/bead was sufficient to attain saturable T-cell activation based on % CD69+ or CD69 geometric MFI (gMFI), suggesting that our system is exquisitely sensitive. Using TCR-proximal and

TCR-distal indices of cell activation, the ability of these hydrogels to stimulate T cells in a native manner was demonstrated.

2.2.2 The CTL synapse is a compressive crater with dynamic undulations

To profile synapse mechanics at high resolution, beads with a nominal Young's modulus (E) of 300 Pa were functionalized with H2K^b-OVA and ICAM1. Hydrogels were deposited onto super-resolution-grade glass imaging chambers and captured *via* α ICAM1 antibodies. OT-I and P14 CTLs (antigen: H2D^b-gp33) both formed physically active synapses with derivatized DAAM particles, which we imaged by high-speed structured illumination microscopy (Visitech instant SIM) [272]. CTLs not only spread over the beads but also polymerized their actin *into* them, compressing the contact area into a synaptic "crater" $\sim 10\ \mu\text{m}$ in diameter (Figure 2.4).

To process the images, we first segmented the 3-D hydrogel stacks then applied the surface edge detection algorithm developed in the original MP-TFM paper, finally rendering the hydrogels as 3-D Delaunay triangulations [258]. Normally, points in 3-D Cartesian space (i.e., x-y-z) are "triangulated" into tetrahedrons; however, by expressing the points in radial/polar coordinates (i.e., $r\text{-}\theta\text{-}\phi$), the Delaunay triangulation can be simplified to a data structure containing the vertices (e.g., "point 1 has values of $x=3.8$, $y=27.1$, $z=500.8$ " [or polar equivalent]), as well as the connectivity matrix of the vertices (e.g., "triangle 1 comprises points 51, 3174, and 5002") [273]. Surface triangulation meshes can be used to calculate curvature values—where *mean curvature* is the degree of convexity or concavity of a point on the object surface (in units of inverse distance). Using this representation, topographical maps of the hydrogel bead surfaces could be

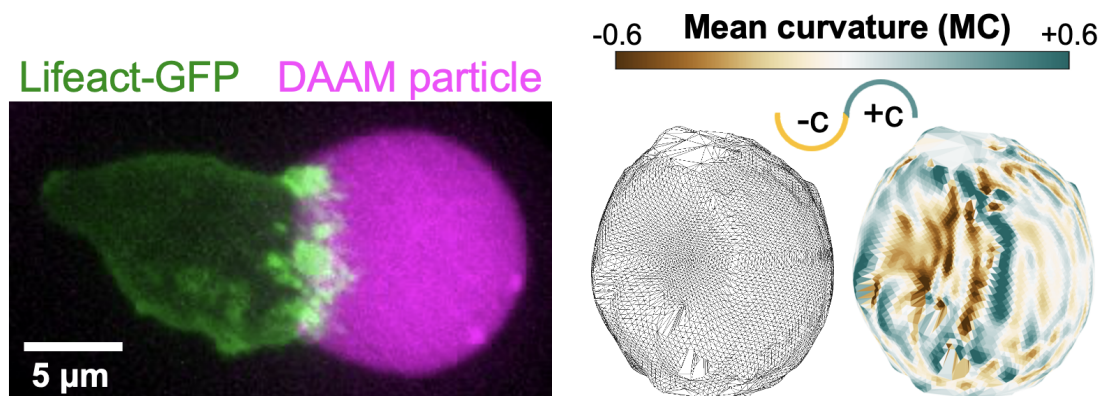


Figure 2.4: A representative synapse formed with the spherical hydrogel bead.

Left: A live image of an OT-I CTL expressing Lifeact fused to enhanced green fluorescent protein (Lifeact-eGFP) is shown in conjugation with an H2K^b-OVA/ICAM1-coated hydrogel ($E = 300$ Pa). Right: The hydrogel image stack is then rendered as a triangulation (black-and-white meshwork), which can be colored by surface curvature across the entire particle (turquoise-brown coloring). This manner of visualizing the surface topography emphasizes the compressive, micropatterned crater-like configuration of the CTL synapse. These panels are similar to data in [262], namely Figure 1B.

generated, where different areas of convexity or concavity all around the bead could be readily visualized.

The CTL synapses could topographically be divided into two zones: a peripheral rim dominated by positive mean curvature, and an inner crater floor characterized by concave depressions. To quantify the overall compressive strength of the synapse, we assumed that unengaged particles, or those being brushed by a CTL without synapse formation, are close to resembling perfect spheres along the x-y plane—thus, synapse strength would be reflected in the deviation of the particle’s volume from that of a size-matched sphere (Figure 2.5). This was valid except at the bottom of the beads, which flattened out on the adhesive substrate. This analysis confirmed our observation that CTLs induce substantial, quite dramatic particle compression. Live-imaging experiments demonstrated that the compression forces peak within 5 minutes of establishing contact and are maintained indefinitely. This compression may reflect the CTL’s axial mechanical activity in the micropipette bead assays [254, 48].

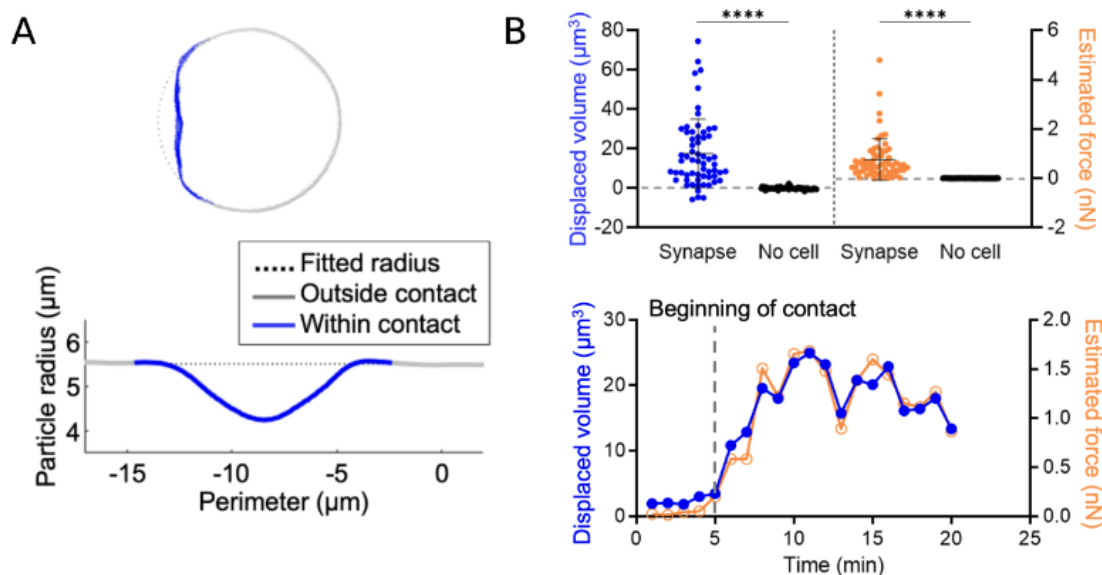


Figure 2.5: Measurement of hydrogel compression.

A: A cross-sectional rendering of the hydrogel outline, where blue points span the T-cell contact. At the bottom, the radii of all points along these cross-sections are plotted in an "unfolded" version of the synapse. B: On top, compressed volume and estimated compression forces of CTL-particle contacts compared to unengaged particles in the same image ($n = 61$ synapses, $n = 56$ unindented spheres). **** denotes $P \leq 0.0001$, calculated by unpaired Welch's t-test. Error bars indicate SD. At the bottom, a representative time-course is shown that demonstrates how CTL compressions peak within five minutes and are actively maintained. These panels are similar to data in [262], namely Figure 1D-F.

The crater floor is not smooth, but rather contains discrete topological indentations, reliefs (hills), and ridges. Though CTLs maintained a rather constant level of compressive activity for well over thirty minutes, the precise pattern of surface deformation, as indicated by the *en face* distribution of curvatures, was highly dynamic. Individual indentations and reliefs pulsed in and out of the crater bowl, and the topographical patterns turn over fully within 1 minute (Figure 2.6). For these representative observations, OT-I or P14 CTLs were imaged while interacting with antigen/ICAM-1-coated hydrogels using the Vt-iSIM microscope.

To measure the number and curvature of individual distortions within the synapse, we developed a watershed approach for identifying convex reliefs and concave protru-

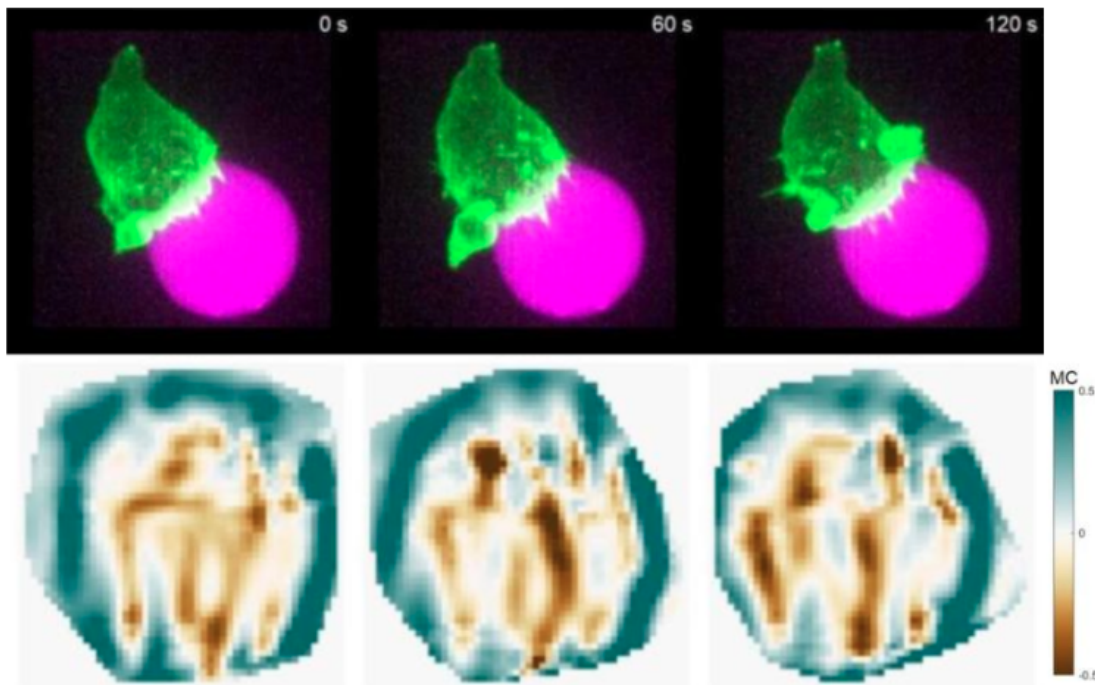


Figure 2.6: Active micropatterning by the T cell.

Top: A brief montage of a video, showing the same CTL-bead conjugate from Figure 2.4. Bottom: *En face* curvature map of the contact interface, demonstrating the changing synapse patterning over minute intervals.

sions/indentations (Figure 2.7). For these representative experiments, OT-I CTLs were fixed and then imaged while interacting with antigen/ICAM-1-coated hydrogels using the Vt-iSIM system. These metrics clearly distinguished synaptic contacts from unengaged particles (Figure 2.7).

Thus, CTLs actively micropattern their targets within the context of a compressive contact.

2.2.3 Actin remodeling fuels CTL mechanical activity

By measuring the T-cell actin intensity within 1 μm of the particle mask, we observed that many of the discrete topographical features were associated with F-actin-rich

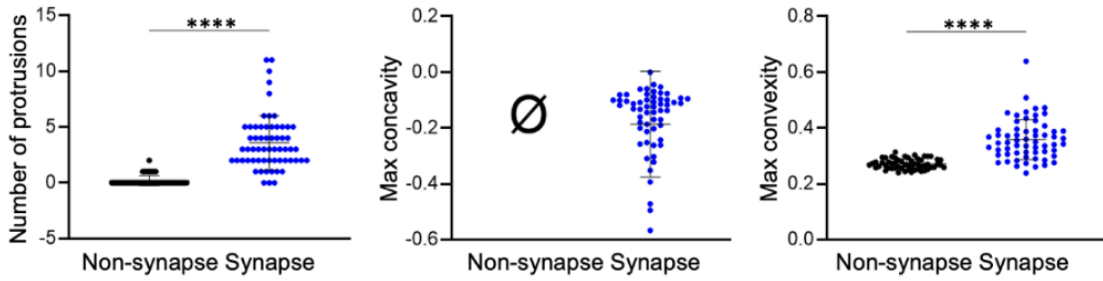


Figure 2.7: Topographical feature enumeration at the synapse.

Shown are the number of protrusions/indentations (negatively curved patches), the concavity of identified protrusions, and the convexity of identified reliefs (positively curved patches). Non-synapse controls lacked sufficient indentations for concavity scoring ($\emptyset$). **** denotes $P \leq 0.0001$, respectively, calculated by unpaired Welch's t-test. Error bars indicate SD. This dataset is representative of conjugates observed throughout the entire study. Figure panels match Figure S1E-F of [262].

structures (Figure 2.8). This agrees with previous work showing that CTL physical activity relies on local cytoskeletal remodeling [190]. We decided to confirm that actin was involved in the compression and establishment of protrusions—we allowed CTLs to form synapses on hydrogels for 30 min, then incubated Latrunculin A (LatA), CK666, or vehicle control dimethylsulfoxide (DMSO). We then fixed these cells and imaged them using diffraction-limited confocal microscopy.

As expected, we found that Latrunculin A (LatA), which inhibits actin polymerization by binding to monomeric G-actin, almost fully ablated target compression (Figure 2.8 B-F). LatA also markedly reduced the number and magnitude of surface indentations and reliefs. CK666, which inhibits branched actin polymerization through Arp2/3, induced defects in protrusion formation. Most likely, a combination of formin-driven and Arp2/3-driven actin polymerization is necessary for the full suite of mechanical activities. However, these results indicate that CTLs employ the F-actin cytoskeleton to form globally compressive synapses with dynamically undulating topography.

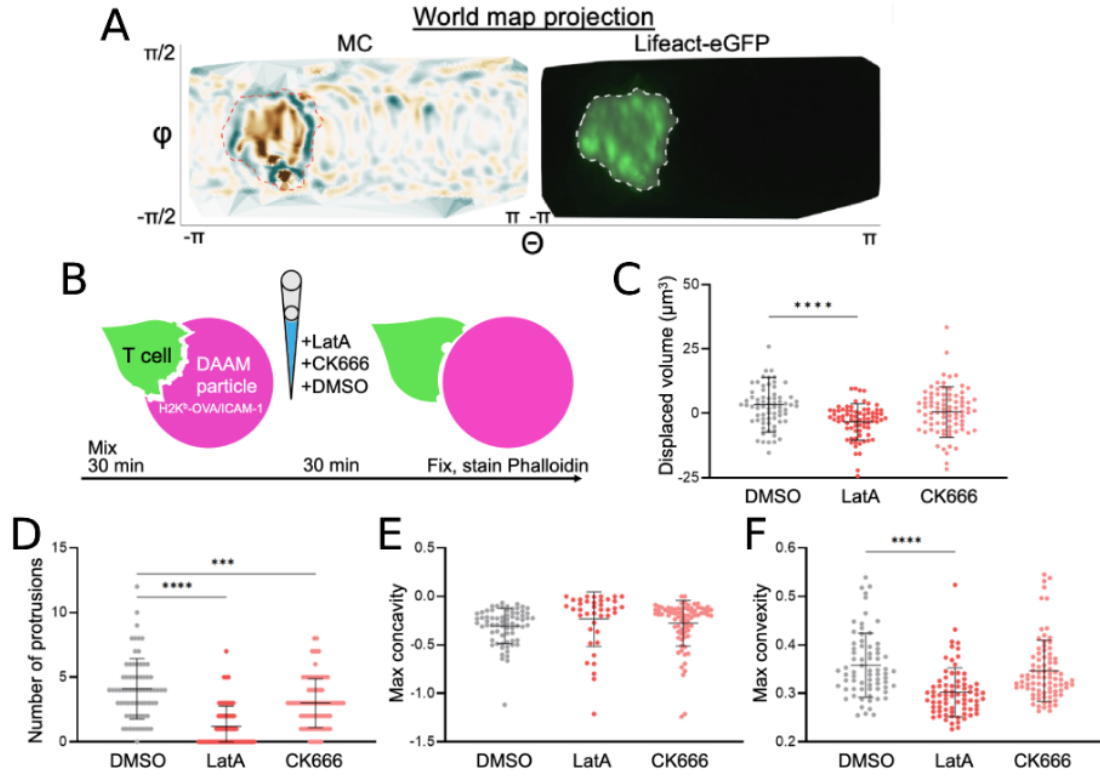


Figure 2.8: Involvement of actin in CTL mechanical activity.

A: By "unfolding" the 3-D triangulations into a world-map projection, the actin map and curvature map can be more readily segmented for downstream analysis (perforated outlines). B: Schematic showing the actin perturbation experiment. C-F: Compression strength and topographical feature enumeration of CTLs under different actin-perturbing conditions. ***, and **** denote $P \leq 0.0002$ and $P \leq 0.0001$, respectively, calculated by multiple t-testing with Tukey's correction. Error bars indicate SD. Panels A-B reflect data in Figure 1C of [262]. Data in panels C-F are representative of at least $n=2$ experiments.

2.2.4 Bumpy craters are also formed on *bona fide* target cells

Because target cells are viscoelastic, using them as force probes is experimentally difficult. A recent report suggested that CTLs wrap around target cells, seemingly contradicting our findings on hydrogel beads [274]. To rule out that the compressive activity we observed is not an artifact of the experimental system, we imaged conjugates of perforin-deficient (Prf1^{-/-}) OT-I CTLs and OVA-pulsed B16 melanoma cells by point-scanning confocal microscopy (Leica Stellaris 8). Prf1^{-/-} CTLs were used, as

rapid cytoskeletal contraction and synapse dissolution occurs within minutes of synapse formation with Prf1-replete CTLs, due to the induction of target cell apoptosis [270]. A custom pipeline was developed to generate smoothed contour lines across the entire cell mask, which we used to measure the degree of compression at the synapse relative to control non-synapse regions of the APC.

The synapses that we observed in these experiments were both dynamic and highly protrusive. The CTLs physically displaced the surfaces of their targets, forming globally compressive contacts with positively curved rims, predominantly negatively curved central domains, and undulating central topography (Figure 2.9). Relative to control regions of the target cell (Figure 2.9B, gray data), the CTL contact area (Figure 2.9B, red data) developed substantial negative concavities within the synapse. We quantified this using histograms (top row) and by geographically mapping the contour curvatures relative to the contact center (bottom row). The architectural similarities between these synapses and the contacts formed with the hydrogel particles indicated that the interfacial mechanics captured by the microparticle system are physiologically relevant.

2.3 Discussion

To our knowledge, this system is the first elastic, compliant 3-D system used to probe CTL contact mechanics [258]. In agreement with a few indirect studies demonstrating forward-directed stresses [233, 256, 216, 190]. Our initial studies established that the CTL synapse is a compressive crater through which CTLs actively micropattern the APC surface, leading to an undulating appearance with dynamically evolving and dissolving protrusions and reliefs. CTLs utilize F-actin polymerization to generate these compressive and protrusive activities. Notably, this pattern of actin-based activity—of an actin band

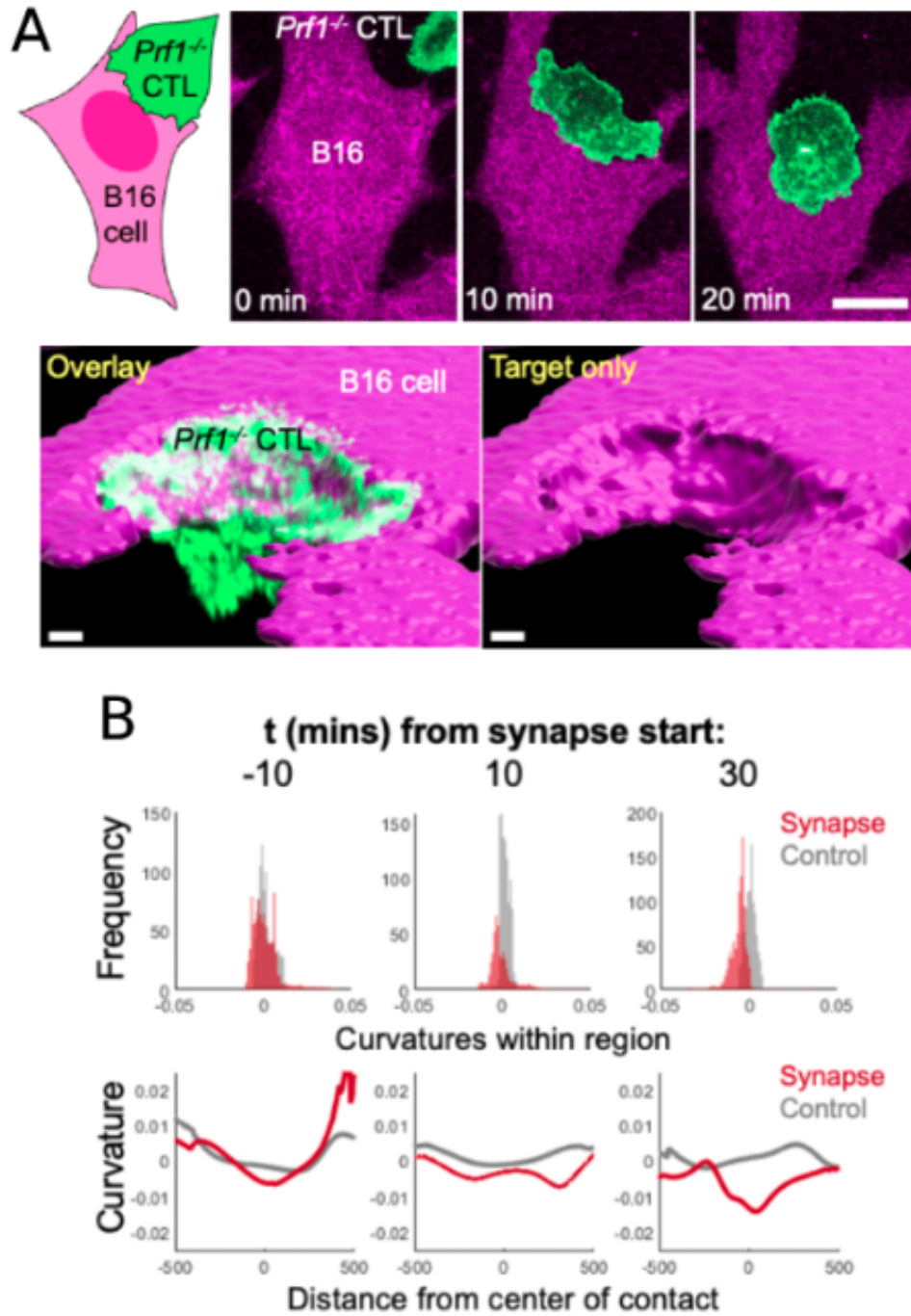


Figure 2.9: Measurement of compressions on *bona fide* APCs.

A: Top row shows screenshots of a Prf1^{-/-} Lifeact-eGFP-expressing CTL attacking a B16-OVA cell. Bottom row shows surface renderings of the contact area with (left) and without (right) the CTL. B: Contour curvatures about the B16 cells were calculated per slice, and the contact areas were manually delineated in order to calculate synapse-associated curvature distributions (top row) and the localization of curvatures along the contact (bottom row). The data are representative of n=9 individual observations made across n=2 experiments.

at the rim, with dynamic, finger-like protrusions in the middle—could be seen in a pioneering lattice light-sheet study of Lifeact-eGFP-expressing CTLs on antigen-pulsed EL4 target cells from 2015; however, no remarks on the actin-based fingers were made in this study [185]. We believe these fingers correspond to the protrusions that generate pockets of concavity on our hydrogel beads.

The experiments with Prf1^{-/-} CTLs against B16 APCs demonstrate that these forward-directed force exertions are the *bona fide* configuration of CTL synapses on APCs; it is possible that other studies demonstrating a globally convex, "wrapping" contact (akin to phagocytosis) may reflect idiosyncrasies of other experimental systems [274]. In our hands, CTLs cannot appreciably compress 1-kPa hydrogels, hence the use of 300-Pa beads for this study; it is therefore possible that the targets used in other studies were too stiff to be distended by the T cells, hence missing out on the compressive nature of the synapse.

2.4 Materials and methods

2.4.1 Cell culture, isolation, and retroviral transduction

All animal protocols used for this study were approved by the Institutional Animal Care and Use Committee of Memorial Sloan Kettering Cancer Center. CD8⁺ cytotoxic T lymphocytes (CTLs) were cultured from OT-I TCR-transgenic mice on the C57BL/6J background (Jackson Laboratories). Briefly, splenocytes from these mice were stimulated for 2 days at 37 °C and 5.0 % CO₂ with 150 nM of OVA 257-264 as cognate antigen. On the second day, activated lymphoblasts were purified by density gradient centrifugation and thereafter maintained in culture until day 7, or retrovirally transduced.

For experiments in which , CTLs were cultured from spleens of P14 TCR-transgenic mice (also on a C57BL/6J background) in the same manner, except that lymphocytic choriomeningitis virus (LCMV) glycoprotein residues 33-41 (gp33) was used as cognate antigen. CD4⁺ T_HCs were obtained in a similar manner, using splenocytes from 5C.C7 TCR-transgenic mice on a B10.A background, which were stimulated with 1 μ M of the cognate antigen MCC 88-103. All T-cell cultures were maintained in RPMI medium containing 10 % fetal bovine serum (FBS, VWR 97068-085) and 30 IU/mL human IL-2 (Roche).

For all live experiments, CTLs were made to ectopically express Lifeact-eGFP. Phoenix E (PhoE) cells were first transfected with expression vectors for Lifeact-eGFP [275] or Lamp1-GFP [276] together with packaging plasmids *via* the calcium phosphate method on the day of spleen harvesting. Ecotropic viral supernatants were then collected after 48 h at 37 °C, filtered through a 0.20- μ m sieve, and added to 1.5×10^6 lymphoblasts on day 2. These mixtures were centrifuged at $1400 \times g$ in the presence of polybrene (4 μ g/mL) at 35 °C for 2 hours. Cells were thereafter split 1:3 in complete RPMI, then grown, selected in puromycin, and maintained as stated above until day 7.

2.4.2 Hydrogel synthesis, derivatization, and peptide coating

For bead derivatization, the procedure below, adopted from the original MP-TFM paper [258], was found to be broadly applicable and flexible, as long as molecular orientation is irrelevant or can be accounted for *via* site-specific amination. For this work, two steps are required: one to covalently ligate streptavidin and fluorophores onto the "naked" hydrogels, then a second step to coat the streptavidinylated hydrogels with biotinylated peptides to induce cellular interactions.

Hydrogel beads were first streptavidinylated using amine coupling as previously described. Briefly, 4.0×10^7 beads were activated with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC, Thermo Fisher Scientific [TFS] 22980) and N-hydroxysuccinimide (NHS, TFS 24500) in 4-morpholinoethanesulfonic acid (MES) buffer at pH 6.0 with 0.1 % (v/v) Tween-20. Then, the buffer was exchanged into basic PBS (pH 8.0) with 0.1 % Tween-20 for a 2-hour conjugation with 12.0 μ M streptavidin (Prozyme, SA10) and 100 μ M fluorescent dye (e.g., Alexa Fluor 488 Cadaverine or Lissamine Rhodamine B ethylenediamine, TFS A30676 or L2424 respectively). Unreacted carboxy-NHS was quenched with excess ethanolamine for 30 min, and the resulting particles exchanged into PBS (pH 7.4).

For T-cell experiments, batches of 5.0×10^6 fluorescent streptavidinylated DAAM particles at a time were incubated for 2 hours in 1 mL PBS containing 500 nM tail-biotinylated ICAM1 (residues 28-485), and 7.5 nM of either (1) tail-biotinylated cognate pMHC complex or (2) biotinylated Armenian hamster α CD3 ϵ antibody (clone 145-2C11, Biolegend 100304). ICAM1 and pMHC proteins were expressed and purified as described [276]. This optimal density of antigen and ICAM1 was chosen based on the saturation of CD69 expression after incubation with functionalized particles (Figure 2.3). After incubation, the particles were spun down and washed thrice with PBS. In experiments wherein shotgun-biotinylated antibodies are used (e.g., 145-2C11 α CD3 ϵ), the coating orientations are random and cannot be assumed to be stimulatory on a per-molecule basis; hence, this matching coating concentration was validated independently by conjugate formation and Ca^{2+} flux imaging.

In general, for imaging-based assays, pMHC/ICAM1-coated beads were deposited and captured on 8-well imaging chambers (Ibidi μ -Slide 8 Well chamber) the day before assay (usually day 6) pre-loaded with α ICAM1 antibody (clone YN1/1.7.4, eBioscience,

TFS 13-0541-85). Glass chambers were coated directly with biotinylated poly-L-lysine (30 min), blocked with 1 % (m/v) BSA in PBS (60 min), coated with 10 mg/mL of streptavidin in PBS (60 min), then 1 μ g/mL of α ICAM1 in PBS (60 min). The bead density required per assay depended on bead size and assay preferences—for example, for high-resolution imaging, a sparser bead density of $0.9\text{--}1.5 \times 10^5$ beads/well was optimal, delivered in 200 μ L of PBS suspension. For Ca^{2+} flux imaging and flow cytometry assays, where a pseudo-monolayer of stimulatory beads was desired, density was increased 2- to 4-fold. Importantly, once the hydrogels are adhered onto the glass coverslip, they cannot be left to dry. Doing so will cause spreading and flattening of the hydrogels across the antibody-coated surface, precluding any imaging experiments. They will still stimulate immune cells, but they cannot be used for footprinting immune cell mechanical activity due to the ruined morphology and anisotropic elasticity.

2.4.3 Ca^{2+} flux imaging

For Ca^{2+} flux imaging, 2×10^5 pMHC/ICAM1-coated beads were deposited onto 8-well imaging chambers as just described. Day 7 CTLs from OT-I transgenic mice were loaded with 5 μ M fura2-AM for at least 20 min and deposited onto the particles. For microscopy, images were acquired at $\lambda = 510$ nm under excitation at both $\lambda = 340$ nm and $\lambda = 380$ nm. Images were acquired across 4 fields of view per well every 30 s. Population responses were analyzed using Slidebook software (3i) as follows: after image background correction, masks were generated using Otsu thresholding of the A_{340} image and size exclusion (8- μ m minimum). Then, T-cell events that overlapped with particle events for more than 2 frames were selected, and the $A_{340}/A_{380} \text{ } Em510$ MFI values collected and averaged alongside the matched fields of view. Finally, signals were normalized using the baseline value before the initial rise.

2.4.4 CD69 expression by flow cytometry

Cells were incubated at 37 °C with antigen/ICAM-1-coated beads for 5 hours, harvested, stained for CD69, and analysed by flow cytometry. Typically, 5×10^4 cells were deposited onto 1-kPa antigen/ICAM-1-coated hydrogels at a ratio of 1:2. Unstained cells and cells stained with an isotype control antibody were used as references for determining voltage settings. For determining the baseline CD69 signal, cells were incubated with uncoated beads; for the maximum potential CD69 signal, 20 ng/mL phorbol myristate acetate (PMA) was incubated directly with cells. CD69 expression levels on a per-cell basis (as quantified by the gMFI), as well as the proportion of CD69-expressing cells in the population, were taken and plotted. Measurements were taken on a BD FACSCalibur cytometer.

2.4.5 High-resolution imaging

For high-resolution imaging experiments, $1.0\text{--}1.5 \times 10^5$ pMHC/ICAM1-coated beads were deposited onto 8-well chambers. Prior to addition of T cells, particles were exchanged into complete RPMI by a series of well dilutions, taking great care to keep the particles submerged in fluid throughout. Wells with beads were then mounted onto an inverted microscope stage and temperature-equilibrated for 15-30 min, before depositing Lifeact-eGFP+ CTLs into the wells 0.5×10^5 at a time.

For fixation, an equal volume of pre-warmed 4 %(v/v) para-formaldehyde (PFA) in PBS, incubated for 15 min at 37 °C, and washed. Wells were blocked using Blocking Buffer [PBS with 1 %(m/v) goat serum and 0.5 %(v/v) Tween-20] for 1 hour, then stained using this buffer with 165 nM Alexa Fluor 488 or 568 Phalloidin (TFS A12379 or A12380, respectively) for 1 hour. Wells were washed by dilution in the blocking buffer, taking

care to keep the cell-particle conjugates fully submerged in fluid throughout. Afterwards, wells were washed by dilution into PBS.

Instantaneous structured illumination microscopy (iSIM) was performed on an Olympus IX-73 microscope equipped with a Vt iSIM scan head, an Optospin fast filter wheel (Cairn), a 60× 1.3-NA silicone oil objective, and a Hamamatsu Flash 4.0 v2 camera. For live-cell imaging, heating, humidity, and CO₂ levels were maintained using a WSKM-F1 stage top incubator (Tokai Hit). Images were acquired using MetaMorph software (Molecular Devices). Conjugates were identified morphologically by actin polarization towards the bead. Image stacks were captured using 0.3-μm z-sectioning (80–120 slices per stack). Alternatively, a Leica Stellaris 8 point-scanning confocal microscope was also used—in which case, the Zoom, Scan Rate, and other acquisition parameters LAS X software were adjusted such that the pixel size matched that on the iSIM as much as possible ($108 \times 108 \times 300nm^3$). Because of the method of hydrogel immobilization, for the most part, cells attack the beads side-on instead of landing on them from the top—this allows us to leverage the best resolution of the microscope along the x-y plane, and to maximize root-2 resolution gains on pixel reassignment confocal systems such as the iSIM.

2.4.6 Image processing and analysis

Initial image processing.

Before rendering the particles into triangulations, images were first cropped down to single cell-hydrogel conjugates. It was important at this stage to remove images in which hydrogels touch the borders of the images on the tops or bottoms, which was checked by re-slicing the images from the side on ImageJ and visually inspecting each stack.

Afterwards, the 3-D reconstruction of particles into triangulations was conducted using a previously described custom MATLAB script [258]. Briefly, individual microparticles were segmented by intensity thresholding on confocal z-stacks, then particle edges were detected using the 3-D Sobel operator and superlocalized by Gaussian fitting. This process yielded a set of i points $\mathbf{p}$, representing the edge coordinates of the particle. Each $\mathbf{p}$ could be represented as Cartesian coordinates

$$p_i = [p_{(i,x)}, p_{(i,y)}, p_{(i,z)}] \quad (2.1)$$

or as spherical coordinates, with the origin set to the particle centroid:

$$p_i = [p_{(i,\theta)}, p_{(i,\phi)}, p_{(i,\rho)}] \quad (2.2)$$

These point sets were triangulated using a convex hull approach to connect all edge coordinates without leaving gaps. At each node of the triangulated mesh, the first two principal curvatures (k_1 and k_2) were determined and then used to calculate mean curvature H :

$$H_i = (k_1 + k_2)/2 \quad (2.3)$$

Contact areas were identified as regions of interest (ROI) based on the MFI of phalloidin-stained F-actin in contact with the particle. The bounds of this ROI were defined by the theta and phi coordinates enclosing the F-actin-rich region, determined visually. After cell-contact demarcation, all edge coordinates within the ROI were isolated to define the contact area of interest for downstream analysis.

Estimation of deformation strength via deviations from sphericity.

As mentioned earlier, directly assessing bulk deformation stress from 3-D reconstructions proved difficult for two reasons: first, the bottom of the particle often flattened on

the glass imaging surface, creating a non-cellular indentation that would register as a nontrivial stress. Second, numerical calculation of precise force fields is computationally intensive (i.e., hours to days for small datasets of $n < 10$) and infeasible to accomplish for the large ($n > 1000$) data sets produced. Thus, we estimated bulk force by measuring the volumetric deformation from an idealized sphere along sampled planes parallel to the direction of T-cell attack.

We first re-aligned the particle in cartesian space so that the direction of maximum indentation was parallel to the x-y plane. We then sub-divided the contact area along z into 10–20 equally spaced bins. We isolated the edges within each bin and projected them onto the x-y axis, creating a cross-sectional point cloud of the object at that z-value resembling a deformed circle. To infer the coordinates of an unindented circle, we fit a circle to the edge coordinates *not in contact with the cell* (i.e., the unindented regions of the hydrogel) *via* the least-squares method (MATLAB *circle_fit* by Schmidt, 2012). For each z-bin, we calculated a deformed area by integrating the deviations from the fitted radius over the contact area. This area deformation was integrated over the entire ROI to determine the volumetric deformation (strain). Thus, indentation forces were measured as positive volumetric displacement and pulling forces were measured as negative volumetric displacement. We assumed the Young’s modulus of all particles to be identical and that all forces were within the linear regime of the strain-stress curve of polyacrylamide; thus, integrated volumes could be used to compare total force across different immune cell contacts. To estimate the deformation stress of craters induced by T cells, we developed a simplified model of Hertzian contact stress at the contact between two elastic spheres of radius R_{cell} and $R_{particle}$. Applied force was calculated as follows:

$$F = \frac{4}{3} E^* R^{1/2} d^{3/2} \quad (2.4)$$

where the effective radius R is defined by:

$$\frac{1}{R} = \frac{1}{R_{cell}} + \frac{1}{R_{particle}} \quad (2.5)$$

The effective elastic modulus E^* is defined by:

$$\frac{1}{E^*} = \frac{1 - \nu_{cell}^2}{E_{cell}} + \frac{1 - \nu_{particle}^2}{E_{particle}} \quad (2.6)$$

where ν is the Poisson's ratio of the hydrogel, E is the Young's modulus, and d is the total deformation. The Poisson's ratio value is specific to a material, and describes how much it expands laterally under axial compression (or how much it contracts laterally under axial tension). Because the T cell was the only source of applied force, we assumed it to be effectively rigid ($E_{cell} \rightarrow \infty$) and the deformation was calculated as the maximum depth of the indentation.

Topography mapping and watershed-based topographical feature identification.

Prior to feature picking, ROI points were projected into 2-D space *via* their spherical coordinates θ and ϕ . To enable comparisons between synapses of different sizes, we interpolated the mean curvature values H_i onto a 50×50 topography matrix M using the MATLAB *interp2* function. Within each topography matrix M , we identified protrusions as distinct areas of negative curvature with unique local minima. We first smoothed the data by interpolation into a 100×100 matrix, then applied a watershed transform using the MATLAB *watershed* function, using a curvature threshold of < 0 to remove non-protrusive local minima and pixels near the ridges of each watershed. Topographical protrusions were enumerated and their internal mean curvatures, maximum curvatures, areas, and centroids were measured using the MATLAB *regionprops* function. Topographical peaks were identified by inverting the sign of each H_i -value on the grids and repeating the above procedure, filtering with a positive curvature threshold of > 0 .

Measuring crater formation on target cells using contour curvatures.

For measuring the compressions by CTLs on B16 cells, B16s were first antigen-pulsed with OVA for 60 min, then stained for 30 min with either a cytoplasmic dye such as CellTrace Yellow or Far Red (TFS C34567 & C34564, respectively), or a membrane-targeted dye such as CellVue Maroon (TFS 88-0870-16). Prf1^{-/-} OT-I CTLs were transduced with Lifeact-eGFP. Segment masks were first generated for the B16 cells in ImageJ by Otsu thresholding, hole filling, and filtering by size (include only the largest contiguous object per frame). Afterwards, the ImageJ plug-in *Contour Curvature* [277] was used to generate contours line estimates of the shape at each slice. Synapse ROIs and non-synapse control ROIs were manually determined, and the contour curvatures plotted as histograms or as functions of distance from the contact center, also manually determined per frame.

CHAPTER 3

RATIONALIZING UNDULATION: INSIGHTS ON DEGRANULATION FROM A MINIMAL *IN SILICO* MODEL OF SYNAPSE DISTORTION

3.1 Background

3.1.1 T-cell degranulation through the cortical actin mesh

CTLs store perforin and granzyme in specialized secretory lysosomes known as lytic granules [278]. Upon target recognition and engagement, lytic granules traffic to the synapse, where exocytic fusion of these granules (degranulation) results in the directional release of perforin and granzyme into the intercellular space. High-resolution and super-resolution microscopy studies determined that these granules access the plasma membrane through gaps in the cortical F-actin mesh (Figure 3.1) [279, 181, 185, 38].

I contributed to recent work establishing that CTL degranulation is regulated by coordinated engagement of the TCR and integrins [276]. TCR/LFA-1 coengagement in *cis* was necessary for degranulation—more degranulation was elicited against beads with both pMHC/ICAM1 as opposed to coengagement in *trans* by separate beads, each with pMHC and ICAM1. Furthermore, on micropatterned substrates, granule exocytosis events (as measured by degranulation-dependent pHluorin-LAMP1 fluorescence) could be targeted, with increasing efficiency, to local spots of ICAM1, pMHC, or pMHC/ICAM1 (Figure 3.2). Furthermore, areas of degranulation were associated with active tugging forces, as measured by DNA-based force probes. Thus, actin-based activity does not always present barriers *per se* to degranulation; rather, it seems that a subset of actin associated with co-engaged TCR/integrins actually focuses the degranulation machinery

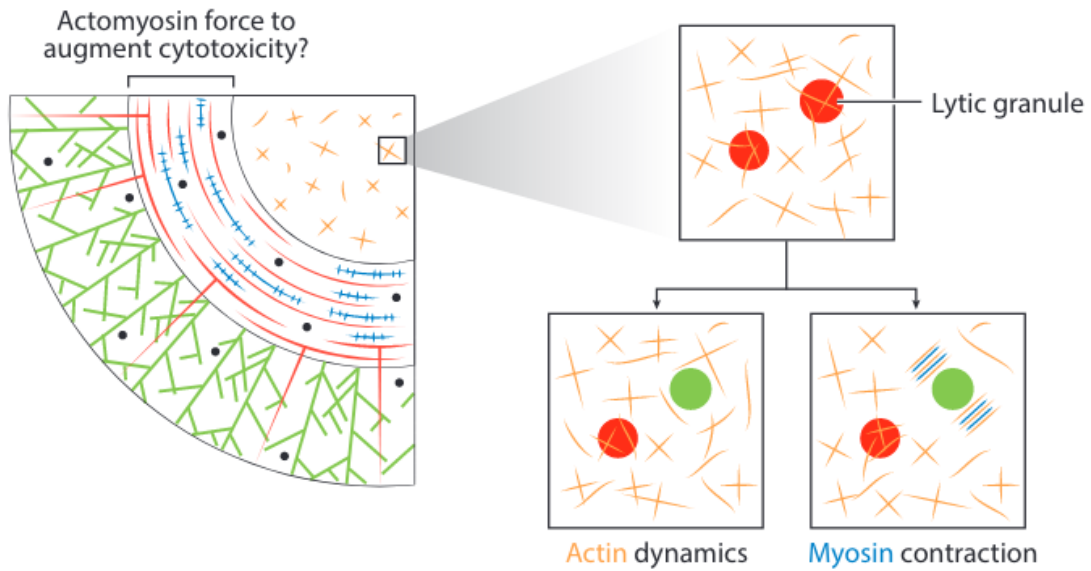


Figure 3.1: CTL degranulation through the cortical actin mesh.

Shown in green, red/blue, and yellow are different actin network architectures at the immune synapse—respectively, the dSMAC, pSMAC, and cSMAC. At the cSMAC, the thin cortical actin mesh serves as a steric barrier to granule exocytosis, such that degranulation occurs through actin remodeling or myosin activation, which are predicted to clear up space on this "granule penetrative domain" [279]. Figure reproduced from [38] with permission from the copyright holder.

towards the membrane, where perhaps at the actual areas of granule fusion, local actin clearance will be necessary [279].

Notably, in our assays, F-actin-mediated target distortion *via* protrusive "fingers" is not excluded from the contact center (e.g., Figures 13 & 15). In fact, this area was associated with the most topographically interesting F-actin-based dynamics. Therefore, the same synapse sub-zone on which the chemical output (lytic granules & exocytic machinery) must dock also has to accommodate the mechanical output (axially directed F-actin polymerization) of the activated CTL.

Taken together, these findings suggest that degranulation is somehow coordinated with physical activity—thus, CTLs might optimize the extent of mechanopotential *via* synaptic self-organization. In this section, we measure the correlation between

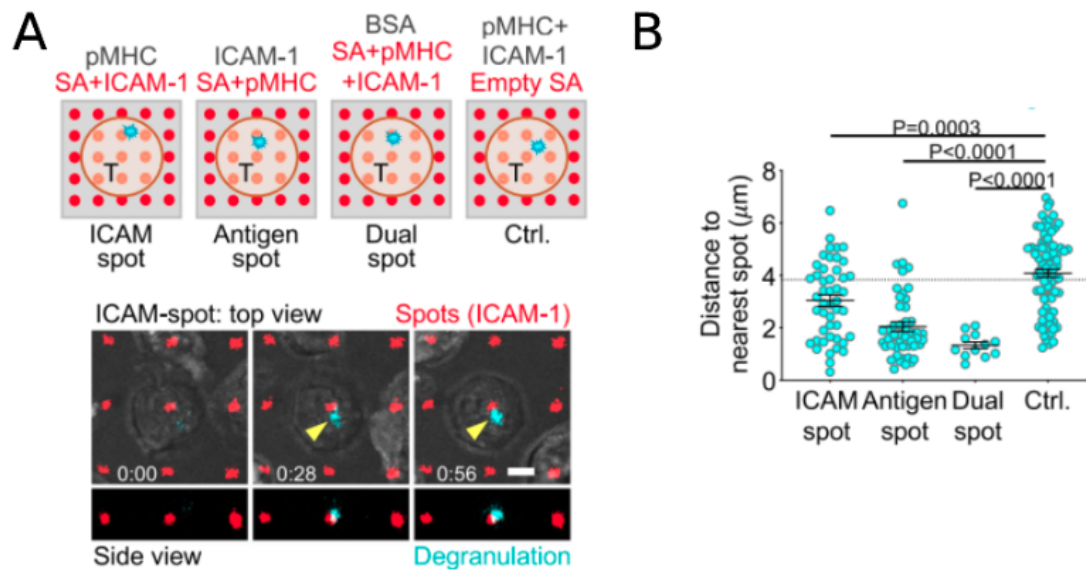


Figure 3.2: CTL degranulation is gated by engagement of the integrin-talin clutch.

A: On the top row, four types of micropatterned substrates were used to investigate the spatial focusing of degranulation machinery—(1) mixed streptavidin/ICAM1 spots on a pMHC background, (2) mixed streptavidin/pMHC spots on an ICAM1 background, (3) mixed streptavidin/pMHC/ICAM1 spots on a BSA background, and (4) non-stimulatory streptavidin beads on a uniform pMHC/ICAM1 background. On the bottom row, representative image of a pHluorin-LAMP1 degranulation event (blue spot, yellow arrow) overlaid onto micropatterned ICAM1 spots (condition [1]). B: Distances of granules to the closest spots were measured for all four conditions. I conducted a data simulation in Matlab to model the random distribution (dotted line), which matches the "non-focused" condition (4). P-values were calculated by one-way ANOVA with Tukey's correction. Error bars indicate SEM. Figure reproduced from [276] in accordance with the Creative Commons Attribution License CCBY—A is taken from Figure 4A-B, and B is taken from Figure 4C.

degranulation location and local topographical features (i.e., curvatures) on the target surface to gauge the extent of this coordination. Afterwards, with collaborators from the University of Tennessee at Knoxville, we developed a computational model based on continuum elastic theory model target surface distortion by CTL F-actin activity. We used this model to identify a fundamental mechano-chemical trade-off that characterizes T-cell cytotoxic efficiency [191, 276].

3.2 Results

3.2.1 CTL degranulation occurs at the undulatory crater floor of the synapse

We sought to visualize degranulation while CTLs manipulated the hydrogel beads in order to measure the extent of coordination between granule release and actin-based target distortion. OT-I CTLs were transduced with LAMP1-eGFP and then imaged with α CD3 ϵ /ICAM1-coated beads (Figure 3.3). Close examination of granule dynamics in the resulting time-lapse data revealed instances in which granules docked onto the synaptic membrane and then disappeared, indicative of exocytic fusion (Figure 3.3A-C). To measure the local mechanical environment of degranulation events, we collected the curvatures within 1 μ m of seventeen discrete degranulation events, and then plotted them on a radially collapsed representation of the population topographies (Figure 3.3D-E).

We observed a strong preference for degranulation at the crater floor, within an intermediate band excluding both the inner 20 % and outer 20 % of the synapse radius (Figure 3.3E, blue lines). This pattern was remarkable given that the furthest peripheral domain accounts for 36 % of total contact area. The surface curvature in degranulation spots varied: 11/17 (65 %) events occurred close to concavities on the crater floor, while 5/17 (29 %) were adjacent to convexities and 1/17 (6 %) occurred a flat areas. In almost every case, the degree of curvature within degranulation areas did not significantly deviate from the average curvatures observed at that specific radial location across the entire synapse, implying that the degranulation machinery does not specifically target local convexities or local concavities. Instead, these results link cytotoxic secretion to the

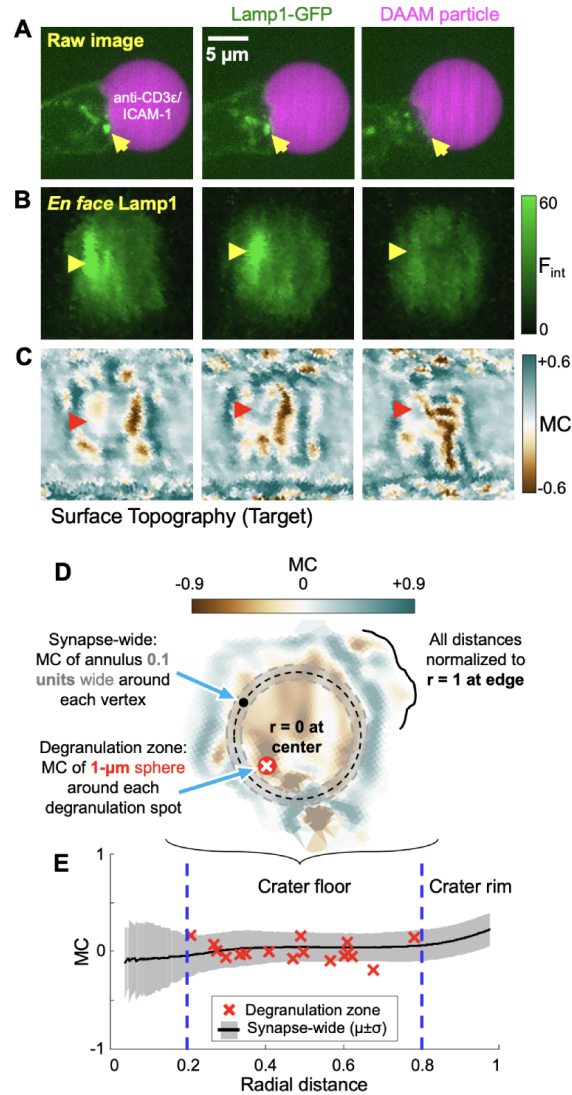


Figure 3.3: Mapping target topographies associated with degranulation.

A-C: Videos of LAMP1-eGFP-expressing CTLs engaged with α CD3 ϵ /ICAM1-coated beads were recorded and visually inspected to determine distinct degranulation events. D: We generated the curvature distribution vs. radial distance plot of each frame by generating annular bins that span ± 0.1 units from each vertex of the synapse, and collecting all the curvatures associated with this specific radial distance. The curve representing average synapse-wide curvatures is plotted as a black line with gray error bars representing mean $\pm$ SD. The degranulation spot's associated curvatures were defined by a 1- μ m distance filter about each coordinate. Panels A-C reflect data presented in Figure 2E-G of [262], panel D reflects Figure A (left panel) of the same publication, and panel E reflects Figure H of the same publication. Data in panel E were pooled from $n=1$ experiment.

undulatory crater floor as a whole, implying that the mechanical and architectural features of this synaptic subdomain might be configured towards potentiating cytotoxicity.

3.2.2 Synapse simulations generated by an energy minimization approach resembles real synapse topographies

Because synaptic forces might increase killing efficiency by physically sensitizing the target cell membrane to perforin-mediated lysis [191, 274], we speculated that the mechanical features of the inner crater of the synapse might allow CTLs to pair their chemical and physical outputs efficiently—specifically, dynamic F-actin-based micropatterning would be expected to generate a continuous distribution of concave and convex high-tension hotspots throughout the undulatory target surface.

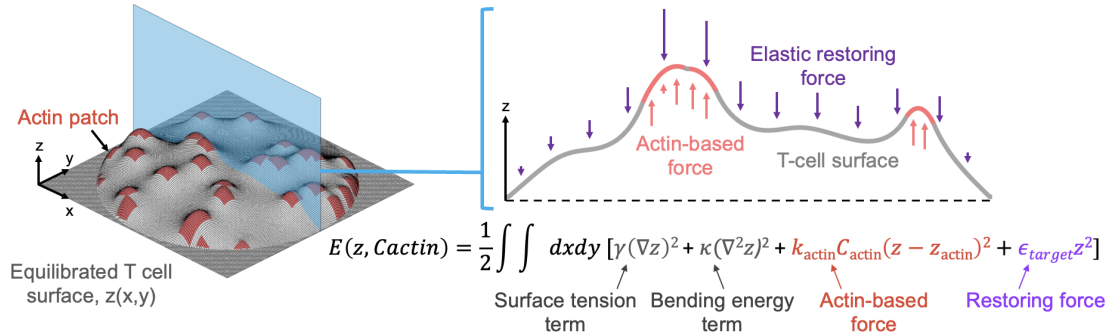


Figure 3.4: Model parameters for simulating synapse topographies by an activated CTL.

A simulated synapse interface is shown on the left, with a cross-sectional slice through the surface on the right, alongside the four key parameters of the model. This figure reflects Figure 7A of [262].

To explore this idea, we developed a minimal model of the synaptic crater floor *in silico*, depicted as a 10-μm region in contact with a deformable material representing the target (Figure 3.4). The CTL was parameterized by its surface tension and bending rigidity, while the target material was characterized by a rigidity of 300 Pa, matching the hydrogel

particles. Regions of protrusive F-actin were modeled as spring-like potentials pushing against the target surface, with F-actin cluster sizes ranging from 0.5–2 μm in diameter. These efferent cellular forces were opposed by elastic counterforces from the target material, as well as by energetic penalties for increasing the membrane surface area and local bending/curvature of the T cell membrane. Simulations were performed at various levels of F-actin coverage, spanning 6.4–51.0 % of the surface. During each iteration, F-actin clusters were distributed in a random spatial configuration, and the effective free energy of the system was minimized using a time-dependent Ginzburg-Landau approach [280].

The resulting topographies ranged from largely flat surfaces pock-marked with weak indentations to undulatory contours with pronounced hills and valleys, in large part qualitatively resembling the actual topographies measured by our assay (Figure 3.5A). Two indices of membrane distortion were calculated from the membrane simulations: the surface area of the distended membrane and the curvature variance across the entire synapse surface. Both of these membrane distortion indices increased with increasing F-actin coverage, as expected (Figure 3.5B). Interestingly, the degree of F-actin clustering also affected the topographical patterns that ultimately formed, and a higher degree of clustering seemed to induce patterns with more undulatory complexity at intermediate levels of actin "loading"/coverage (Figure 3.5C).

3.2.3 Degranulation and target membrane strain establish a fundamental trade-off for CTL activity

We hypothesized that the need to exert F-actin-dependent forces at the synapse must be balanced with the need to create actin-free sites for degranulation. We thus defined

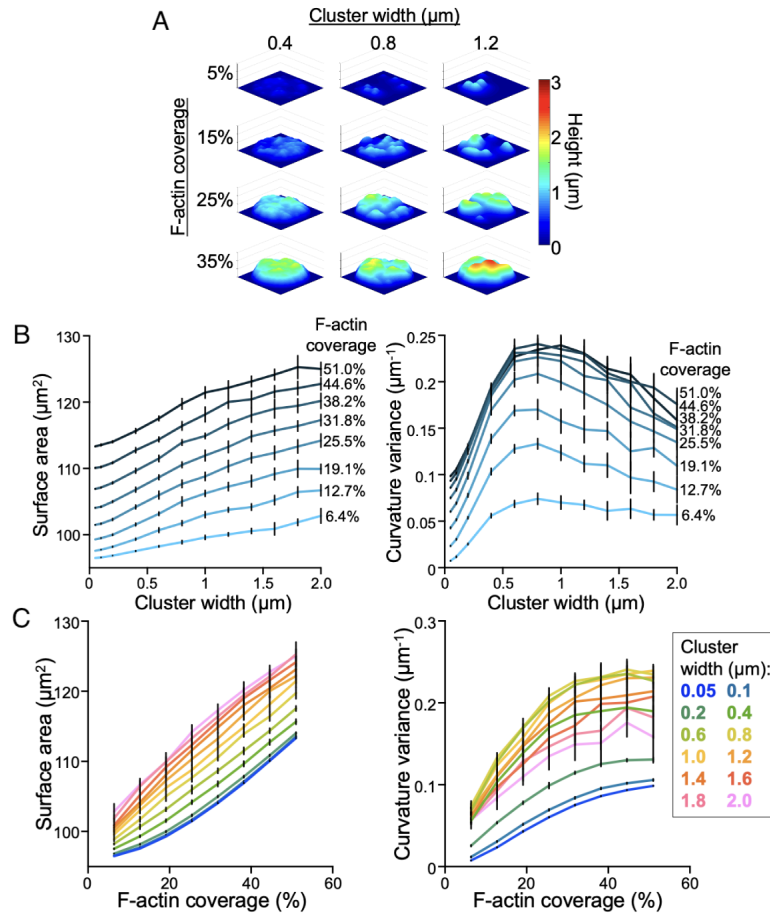


Figure 3.5: Synapse simulations resemble real topographical data.

A: Simulations of synapses in our model, shown from the side to emphasize individual protrusions. B-C: Two indices of membrane distortion can be calculated from the membrane simulations: the surface area of the distended membrane (Left), and the curvature variance across the entire synapse (Right). Error bars indicate SD, calculated from 10 replicate simulations. These data reflect Figure 7C and 7E of [262].

in each of our synapse simulations the degranulation-permissible area ("degranulatable area") as the total surface area situated at least 1 μm from the closest F-actin cluster (Figure 3.6). This parameter is similar to the "granule penetrative domain" described in [279].

Degranulatable area also increased with actin F-clustering (Figure 3.7), reflecting how the consolidation of protrusive activity leaves more accessible space within the synapse

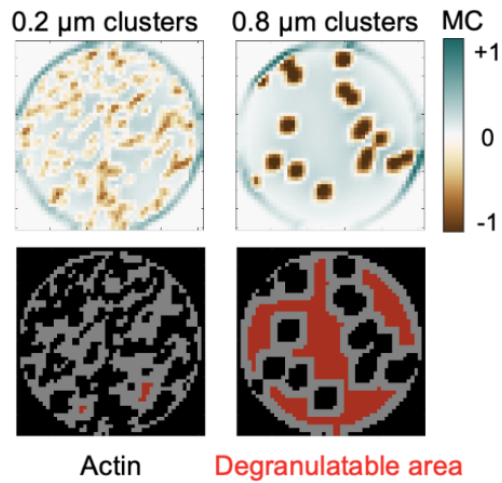


Figure 3.6: Definition of degranulation-permissible ("degranulatable") area, an index of chemical activity by the CTL.

Two synapse simulations are shown (top row) alongside their degranulatable area index (bottom row). At a given amount of synaptic actin coverage, F-actin clustering into small puncta (left) sterically hinder granules from docking onto the membrane; larger actin clusters (right) leave more membrane area free to accommodate degranulation machinery.

for granule docking and fusion. We plotted against either index of membrane distortion to define a conceptual phase space describing cytotoxic efficiency, i.e., how well a CTL's given mechanical configuration can co-optimize its chemical outputs (degranulatable area) and mechanical outputs (protrusive F-actin). Increasing F-actin cluster size shifted topographies upward and rightward in these plots, indicative of gains in overall efficiency. Remarkably, these gains appeared to level off for actin clusters larger than $\sim 1.2 \mu\text{m}$ in width, beyond which any additional additional F-actin clustering led to only marginal changes in curve position. Importantly, this threshold cluster size was reflected in the size distributions of real topographical indentations observed on particle data, implying that F-actin features of this size or larger enable CTLs to combine target cell distortion and perforin release in the most efficient way possible (Figure 3.8).

At the saturating limit of these curves, tradeoffs between degranulatable area and surface distortion are effectively minimized. Similar limiting contours were observed

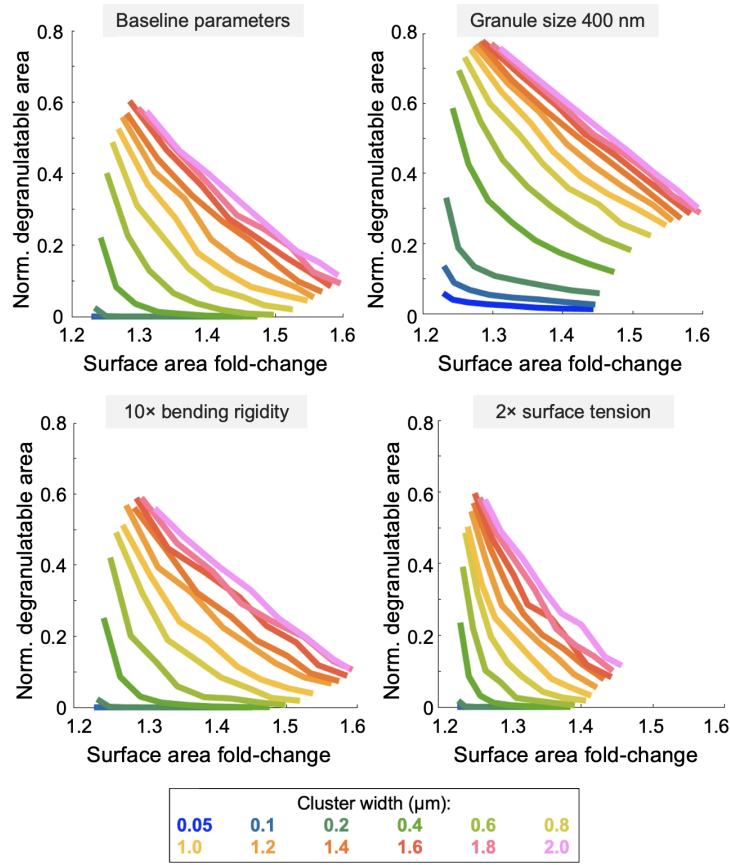


Figure 3.7: Mechano-chemical tradeoffs in CTL activity.

"Cytotoxic efficiency" was plotted for the simulations as: degranulatable area vs. surface area, both normalized to the original circular area. Actin cluster width values were swept from 50 nm–2 μm (blue to pink curves). The same overall phase behavior is seen across plots for larger granules, stiffer membranes, and more tense membranes.

in simulations using different surface resistance parameters and granule size thresholds (Figure 3.7), suggesting that these tradeoffs represent a fundamental property of the cytotoxic process. Relationships of this kind are the defining feature of a Pareto frontier [281], a concept used primarily in economics and engineering to describe the set of maximally efficient solutions to a multi-objective optimization problem. Our simulations indicate that F-actin protrusions of sufficient size enable CTLs to access their own set of maximally efficient cytolytic configurations.

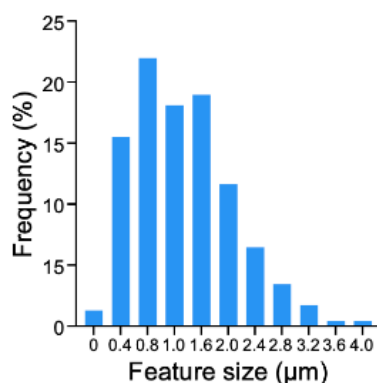


Figure 3.8: Protrusion sizes of real CTL-hydrogel conjugates.

The protrusions of CD8+ OT-I CTLs were measured on stimulatory hydrogels, and the protrusion sizes were measured using the watershedding approach introduced in Chapter 1. The mean hovers around $\sim 1 \mu\text{m}$ in diameter, which falls well within the saturable region of the "cytotoxic efficiency" phase space plots.

3.3 Discussion

Is cytotoxicity a physicochemical optimization problem that CTLs solve? *In silico* simulations allowed us to explore this question: by modeling F-actin-based forces against a surface with inherent resistance to compression, bending, and stretching, we were able to generate topographies that grossly resembled our experimental observations and revealed key insights about killing efficiency. One is that consolidating protrusive F-actin into clusters increases target distortion per unit of F-actin *while simultaneously leaving more plasma membrane free for degranulation*. Such a "win-win" relationship, as seen in our cytotoxic efficiency plots, could represent an evolved strategy to maximize mechanopotential by CTLs on target APCs. According to our simulations, the incremental gains in cytotoxic efficiency diminish beyond cluster widths of $\sim 1 \mu\text{m}$; this implied that there is little need to build larger structures within the crater floor than this, at least if cytolytic mechanopotential is the major or only consideration. Remarkably, this matched the average feature size of actual F-actin-based protrusions on the 300-Pa beads.

The saturable behavior of the cytotoxic efficiency phase space is interesting, as it describes a Pareto frontier defining the set of all "maximally efficient" mixtures of F-actin coverage and clustering. The existence of multiple comparably efficient synaptic configurations is intriguing, given that the molecular composition of a CTL, in particular its cytotoxic proteins, can vary widely depending on its differentiation state and whether it has recently killed [282, 283, 284]. It is therefore tempting to speculate that a CTL at a given moment selects the most Pareto-efficient mechanical output pattern given its current biochemical situation. Alternatively, a CTL's mechanical activity could also remain at a dynamic steady-state, continuously distending the target membrane in an undulatory fashion regardless of the amount of lytic granules it currently has ready for deployment.

Fundamental limitations of our model validation are that (1) the experimental system does not have a mobile membrane, and (2) our imaging approach is unable to visualize the actin mesh associated with the "actin-cleared" cSMAC [38]. Future work could employ super-resolution modalities on hydrogels and target APCs alike to better spatially correlate lytic granules with actin foci, the thin cortical actin mesh, and areas of target distension.

In all, these simulations indicated that forming an undulatory pattern of target surface distortion holds benefit for CTL function. In the next chapters, motivated by the insights from this theoretical exercise, I describe our attempts to link not only a CTL's compressive strength to cytolytic capacity, but also the topographical patterns they engender on the target surface. This necessitated the development of novel pattern analysis approaches, which we believe help us rigorously define the cytolytic *mechanotype* from Chapter 1, as a compressive crater with undulatory and radially asymmetric complexity.

3.4 Methods

3.4.1 Cell culture and retroviral transduction

Lamp1-eGFP was introduced to CTLs *via* retroviral transduction in a manner similar to Lifeact-eGFP (see Chapter 2.4.1).

3.4.2 Image processing and analysis

Tracking and mapping degranulation events.

For calculating the curvatures associated with degranulation spots, movies of Lamp1-eGFP-expressing CTLs interacting with α CD3 ϵ /ICAM1-coated beads were imaged by point-scanning confocal microscopy (Leica Stellaris 8). Because of the speed limitations of this system (at best $\sim 3\text{--}4\times$ slower than the iSIM) and the need to image 70–120 slices per frame (in order to properly sample the hydrogels at proper Nyquist coverage), LAMP1-eGFP+ granule docking and vanishing over time was used as a measure of degranulation, instead of pHluorin-LAMP1-eGFP fluorescence, which decays quickly due to lateral diffusion on the CTL membrane. Movies were first visually inspected on ImageJ to identify discrete degranulation events, confirmed by the "unfolded" world projections of the bead triangulations in $r - \theta - \phi$ space. Degranulation spots were denoted in this representation as a $\theta - \phi$ coordinate pair (p_{degran}), and curvatures associated with a "degranulation spot" were defined as any vertices along the shape that fell within a 1- μm spherical shell about the closest real-data coordinate to p_{degran} .

To generate a radial representation of the synapse and properly map the locations of the degranulation spots, a radial distance (d_{rad}) was calculated for each vertex within the synapse ROI, defined as the Euclidean distance in $\theta - \phi$ space from the synapse centroid, normalized to the edge of the cell contact passing through that point (d_{max}).

$$d_{rad}(p_i, c) = \frac{\sqrt{\sum_{j=\theta, \phi} (c_{i,j} - p_{i,j})^2}}{d_{max}} \quad (3.1)$$

This way, each degranulation spot was associated not only with a mean curvature value, but with a radial distance value d_{rad} , where $d_{rad} = 0$ at the contact center and $d_{rad} = 1$ everywhere along the contact perimeter. The average curvatures of the entire synapse at a given radial distance was calculated by taking all vertices within $d_{rad} \pm 0.1$ and averaging them.

3.4.3 Design of the minimal model

We considered a circular region of the T-cell surface, 10 μm in diameter, in contact with a deformable elastic material. The shape of the T-cell surface was given by the function $z(x, y)$, which represented the penetration distance of the surface into the target material as a function of Cartesian coordinates x and y . We assumed the initial contact region was flat and that the T cell surface was anchored at $z = 0$ along the boundary of the domain. To capture key physical attributes of the system, we utilized the following free energy functional [285]:

$$E[z, C_{actin}] = \frac{1}{2} \int \int dx dy [\gamma (\nabla z)^2 + \kappa (\nabla^2 z)^2 + k_{actin} C_{actin} (z - z_{actin})^2 + \epsilon_{target} z^2] \quad (3.2)$$

The first two terms in the integral are associated with the T-cell surface and represent energetic contributions from surface tension (γ) and bending rigidity (κ). These terms

are a standard form of the Helfrich Hamiltonian in the small-gradient approximation; they penalize surface area and curvature of the membrane, respectively. The third term corresponds to energetic contributions from actin-associated protrusive processes at the membrane. The function $C_{actin}(x, y)$ represents the spatial distribution of actin-based protrusive forces. We assumed that the forces were governed by a harmonic potential that did not depend strongly on z ; to satisfy this assumption, we chose z_{actin} to be large compared to typical deformations (z). The final term corresponds to the restoring force imposed by the elastic properties of the target particle. We assumed the material was uniform and remained in the linear elastic regime, and thus ϵ_{target} was constant.

We then characterized the most probable equilibrium shape of the T-cell surface by minimizing the free energy of the system with respect to $z(x, y)$. We used the time-dependent Ginzburg-Landau approach [280] to describe the time evolution of $z(x, y)$:

$$\frac{\partial z}{\partial t} = -M \frac{\partial E}{\partial z} = -M [-\gamma \nabla^2 z + \kappa \nabla^4 z + k_{actin} C_{actin}(z - z_{actin}) + \epsilon_{target} z] \quad (3.3)$$

We started from a flat surface and let the equation approach steady state. The parameter M is a phenomenological constant associated with membrane relaxation that does not affect the steady-state solution. For numerical implementation, we discretized the membrane into a square lattice with lattice spacing $\Delta x = \Delta y = 50nm$. The shape of the membrane was determined by numerically solving the governing differential equation using a forward finite difference scheme for the time derivative and a central finite difference scheme for the spatial derivatives. We used a time step of 0.001 s, which was sufficiently small to ensure numerical stability. The numerical scheme was iterated forward in time until the membrane shape approached steady state, which we classified using the threshold of $10^{-7}nm$ for the total change in z , summed over all lattice sites, for an update step.

A central objective of the computational modeling was to characterize how the shape of the T-cell surface was impacted by the spatial distribution of actin-generated forces. As such, we systematically varied the total coverage and organization of protrusive actin forces. The coverage was varied from 6.4–51.0 % of the surface area. For each surface coverage, we varied the degree of clustering of protrusive regions. The regions ranged from single lattice sites (i.e., $50 \times 50 \text{ nm}^2$) to clusters of 40×40 lattice sites ($2 \times 2 \mu\text{m}^2$). The clusters of sites were placed at random until the specified surface coverage was reached. If clusters overlapped, the overlap region counted only once toward the total surface coverage; if part of a cluster was outside of the circular simulation domain, only the portion inside of the domain counted toward the total coverage. Given a distribution of actin protrusions $C_{actin}(x, y)$, we then determined the shape of the T-cell surface that minimized the free energy. The following physically-motivated parameters were used in our simulations: $\gamma = 2 \text{ pN/nm}$, $\kappa = 400 k_B T$, $M = 5000 \text{ nm pN}^{-1} \text{ s}^{-1}$, $\epsilon_{target} = 5 \times 10^{-5} \text{ pN/nm}^3$, and $z_{actin} = 2 \times 10^4 \text{ nm}$ [280, 285, 286]. Based on the specified distribution of protrusive actin forces, we set $k_{actin}C_{actin} = 1 \times 10^{-5} \text{ pN/nm}^3$ where actin was present; otherwise, it was zero. In addition to varying actin coverage and organization, we tested the impact of parameters by varying surface tension (γ), bending rigidity (κ), strength of actin-generated forces (k_{actin}), and stiffness of the target medium (ϵ_{target}).

Simulated synapses were analyzed using an adapted version of the primary analysis pipeline. To resemble the data structures of real data, the simulated lattices were first downsampled to a 50×50 lattice with 200-nm step-sizes, and the lattice nodes were converted to Cartesian coordinates. These points were then re-triangulated into a surface using the MATLAB *delaunay* function. We computed mean curvature at each point on the surface and extracted features and curvature distributions as in Chapter 2. To calculate degranulatable area, the lattice defining the locations of simulated F-actin clusters was used to define a 50×50 binary map, with 0s denoting empty space and 1s denoting pixels

containing actin or pixels outside of the 10- μm synaptic circle. We then convolved this with a 5×5 box blur kernel (representing a 1- μm diameter granule) using the MATLAB *conv* function. Degranulatable area was defined as the sum over all areas with a value of 0 after convolution.

CHAPTER 4

LINKING SINGLE-CELL PATTERN ANALYSIS WITH
CYTOLYTIC POTENTIAL *IN VITRO* (I) AND *IN VIVO* (II)

4.1 Background I

4.1.1 Expanding the concept of mechanical output: borrowing functions from ophthalmology and telescopy to capture *topographical patterning* by CTLs

So far, the analysis methods we developed so far for measuring CTL mechanical output are (1) volume displacement, (2) identification of topographical reliefs and indentations, and (3) radial mapping of curvatures throughout the synapse. The first indicates compression strength while the latter two indicate some measure of topographical patterning; however, they fail to capture any information about the exact spatial distributions of discrete topographical features within the synapse. A method to parameterize the intensity and spacing of reliefs and indentations would be ideal, and possibly allow us to distinguish mechanical patterns that associate with cytotoxic activity.

Fortunately, there exists a set of orthogonal mathematical functions defined about the unit circle called the *Zernike circle polynomials* (hereafter "Zernike polynomials") that is reasonably well-suited to mapping our topographical patterns (Figure 4.1). Introduced by Frits Zernike, the Nobel Physics Laureate who developed phase-contrast microscopy, their mathematical properties make them widely applicable to the analysis of circular signals and phenomena—e.g., the determination of microscope image aberrations (because the

objective aperture is a circle), wavefront detection for correcting atmospheric distortions in telescope images (for the same reason), and even the diagnosis of corneal/lens aberrations in ophthalmology [287, 288, 289, 290, 291].

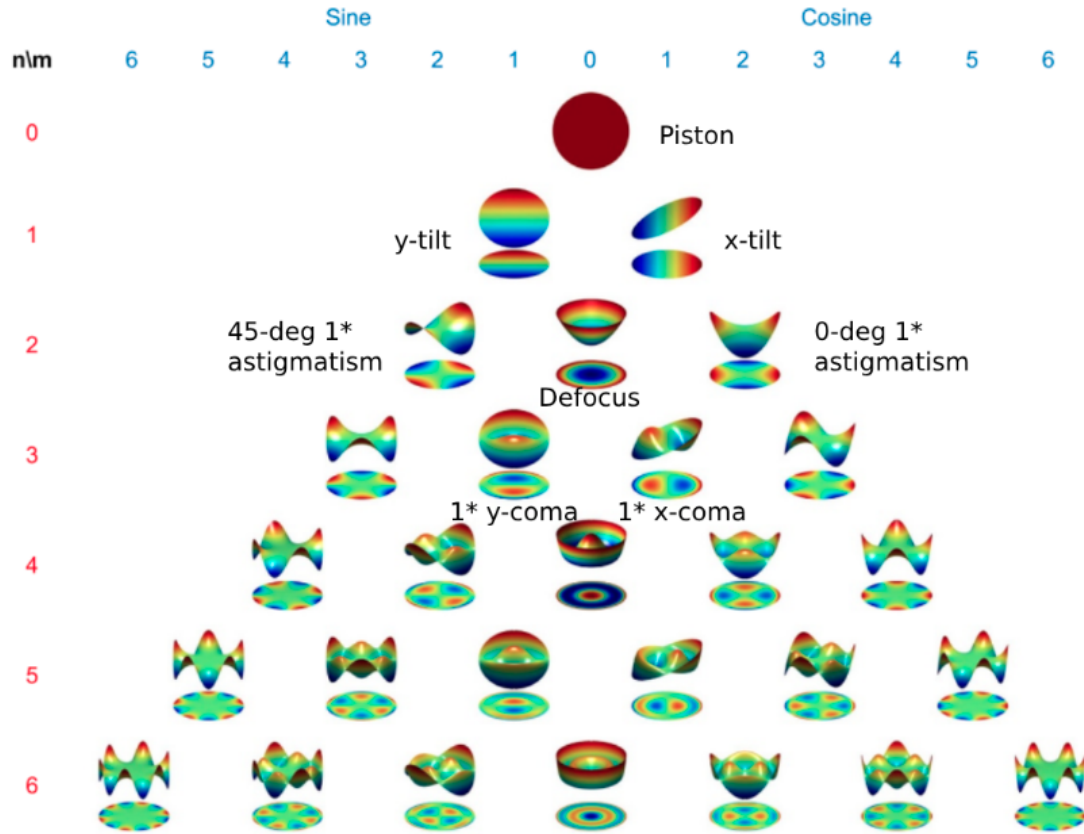


Figure 4.1: Zernike polynomials, arranged according to the Noll index convention.

A pyramidal listing of the Zernike polynomials from radial degree $n = 0$ to $n = 6$. Some of the conventional names for these functions from the literature on lens aberration are included. Figure modified from [290] in accordance with the Open Access policy of the Journal of Optics.

The major optical application of Zernike polynomials is in wavefront fitting for microscopy or telescopic applications. Because the Zernike polynomials form an orthonormal basis (i.e., they are perpendicular unit vectors with respect to each other), any arbitrary wavefront defined over a unit circle can be represented as a linear combination of Zernike polynomials (Figure 4.2). The expansion coefficients in this linear combination are independent from each other, and each Zernike polynomial except for the first "piston"

function have a mean value of zero across the entire circle. This process is similar to the Fourier-analytic decomposition of arbitrary waveforms, except our basis functions are the Zernike polynomials. Such a method can be used to represent our surface curvature patterns as equivalent spatial frequency spectra.

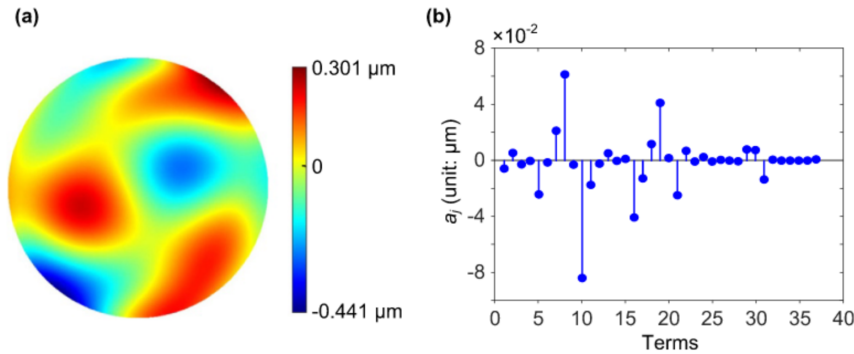


Figure 4.2: Wavefront fitting *via* a linear combination of the Zernike polynomials.

(a): A sample wavefront about a unit circle, which could represent optical aberrations in a microscope set-up, atmospheric disturbances in a telescope image, or the lens distortions in someone's eye. (b): The equivalent representation of this wavefront as a Zernike expansion. Here, 37 coefficients are used to encode the information in the wavefront. Figure reproduced from [290] in accordance with the Open Access policy of the Journal of Optics.

We utilize this method to parameterize our single-cell topographical patterns, develop a simple pattern sorting algorithm using principal component analysis (PCA), and intuit from the major axes of variation the patterns that distinguish CTLs in different experimental conditions.

4.1.2 Actin regulators and T-cell cytotoxicity

The concept of mechanopotential arose from findings on the relationship between a CTL's ability to exert force on its targets and its ability to kill target APCs [191]. Key to this was the discovery of the inhibitory effects of the PIP_3 phosphatase PTEN on CTL force exertions: PTEN loss strengthens forces *via* elevated PIP_3 levels, which enhances

the synaptic recruitment and activation of the Rac1 GEF complex DOCK2-ELMO1 [151]. PTEN-knockdown (KD) CTLs exerted stronger forces on micropipette-based measurements, and killed APCs better than PTEN-replete cells. With no dramatic changes observed in TCR signaling, cell polarization, or degranulation, the argument was made that mechanical force potentiates the biochemical activity of secreted perforin-1 at the synapse, with target membrane distension increasing the probability of perforation [191]. Prf1 activation requires Ca^{2+} binding [292], while its subsequent oligomerization & pore formation are modulated by lipid composition of the membrane, which determine membrane lipid order and charge [293]. It is possible that local membrane stretching could also facilitate Prf1 binding by changing the lipid packing density and nanoscale topography of the membrane [294].

Later work demonstrated that the same relationship between force and cytotoxicity was also governed by the actin NPF WASp, which is responsible for the generation of synaptic forces through central protrusions [190]. WASp-knockout (KO) cells exerted weaker forces biased towards the periphery by TFM micropillar measurements; they also killed to a lesser extent despite degranulating more. Thus, through two separate actin-regulatory pathways, mechanical force seems to enhance T-cell-mediated cytotoxicity independently of T-cell activation or even secretion of cytotoxic molecules. Having described in Chapter 2 the overall mechanical configuration of CTL synapses, and having described in Chapter 3 the potential pro-cytolytic benefits of such a mechanotype, in this chapter we attempt to find correspondences between specific mechanical outputs of CTLs to various indices of killing capacity—known *in vitro* cytolytic phenotypes and *in vivo* differentiation states. Specifically, we hypothesized the existence of a cytolytic *mechanotype*—a specific pattern of mechanical output associated with cell-mediated cytotoxicity.

4.2 Results I

4.2.1 WASp, WAVE2, and Talin contribute distinctly to CTL mechanical output

We reasoned that comparing the mechanical output patterns of CTLs lacking key force-generating proteins against wild-type controls would expose mechanical features necessary for killing. We initially focused on perturbing cytoskeletal forces genetically *via* CRISPR-KO of WASp, WAVE2, and talin. Talin, as described earlier, is necessary for clutch engagement and integrin-mediated bidirectional force transduction; loss of talin leads to degranulation and killing defects [276]. Thus, we expected talin-KO cells to exhibit impacted force generation in the hydrogel system. On the other hand, previous work has shown that WAVE2 affects synaptic force exertion in a manner different from WASp: it appears to drive peripheral force generation, and WAVE2 loss does not appear to appreciably weaken force exertions or impact cytotoxicity *in vitro* [190]. Thus, WAVE2-KO served as an interesting comparator to WASp-KO in our system.

Talin, WASp, and WAVE2 were depleted from Cas9-expressing OT-I CTLs by retroviral transduction of targeted sgRNAs, and the resulting cells were mechanically profiled alongside non-targeting control CTLs (sgNT). Fixed samples were used to facilitate comparison of hundreds of synapses imaged at roughly the same time point (30–45 min) after activation (Figure 4.3). Talin deficiency markedly weakened compressive strength and protrusion formation, while loss of either WASp or Talin impaired the generation of convex reliefs within the synapse. WAVE2-deficient CTLs did not exhibit these defects, eliciting particle distortions that were statistically indistinguishable from control cells by all these metrics. Thus, we re-established in our system the connections

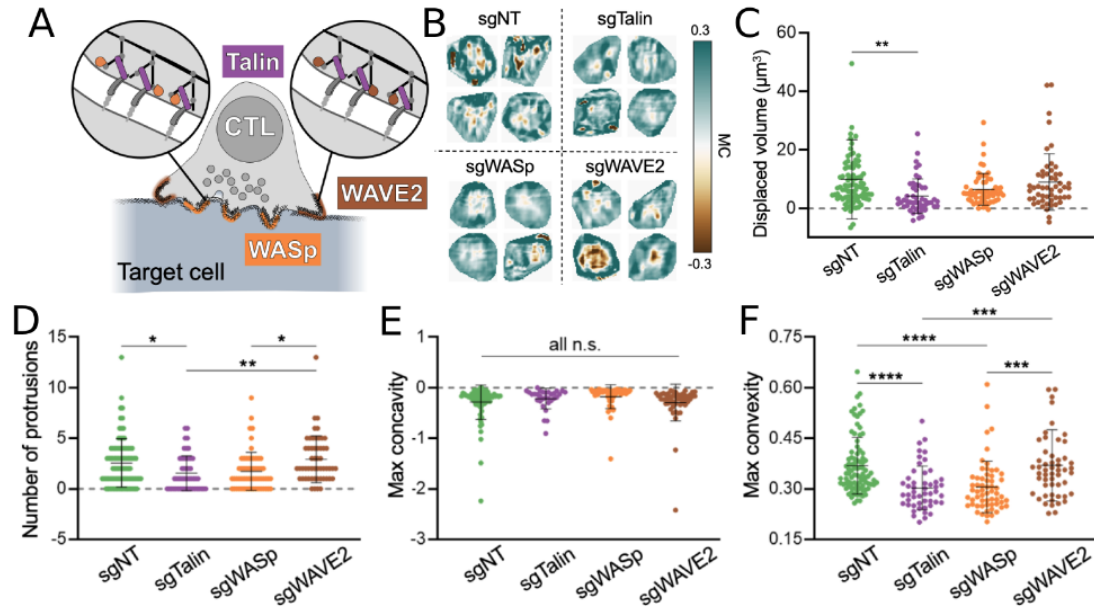


Figure 4.3: Mechanical defects of CTLs after genetic perturbation of force regulators.

A: Schematic of the roles WASp, WAVE2, and talin play in the immune synapse of CTLs. B: Representative quartets of topographies elicited on hydrogels by these CTLs. C: Measurement of displaced volume on the hydrogels. D-F: Enumeration of protrusions, concavity of protrusions, and convexity of reliefs on topographies induced by these CTLs. $n_{sgNT} = 89$, $n_{sgTalin} = 50$, $n_{WASp} = 60$, $n_{WAVE2} = 54$. *, **, ***, and **** denote $P \leq 0.0332$, $P \leq 0.0021$, $P \leq 0.0002$, and $P \leq 0.0001$, respectively, calculated by multiple t-testing with Tukey's correction. Error bars indicate SD. Data are representative of $n=2$ independent experiments. Panels A-C reflect Figure 3A-C, and panels D-F reflect Figure SA-C, in [262].

between mechanical outputs and the known cytolytic impact (or lack thereof) across these three genetic perturbations.

4.2.2 A modified Zernike transform successfully sorts single-cell topographies by similarity

We then developed an approach to express our synapse topographies, represented by mean curvature maps of the hydrogel surfaces about a unit circle, as linear combinations of the Zernike polynomials with the corresponding expansion coefficients defining a

spatial frequency spectrum (Figure 4.4A). We soon realized that it became necessary to combine azimuthally related Zernike coefficients into a set of rotationally degenerate indices: there was no inherent reason to privilege the x-tilt function over the y-tilt, for example, which would have been the case if a given topography were rotated by 90° (Figure 4.4B). Therefore, we reduced the full Zernike spectrum into a rotationally degenerate Zernike spectrum by "combining" the expansion coefficients for functions that were simple rigid-body rotations of each other (i.e., those related by $n \pm m$). To do this, we employed a simple quadrature sum (the Pythagorean formula), which no doubt discards some information. Other approaches exist, such as the incorporation of information from the Zernike moments, which are unaffected by rotational transformations of the waveform [290], or perhaps calculating a phase angle between the "paired" coefficients for $n \pm m$ (e.g., as $\theta = \tan^{-1} \frac{a_{n+m}}{a_{n-m}}$). However, the quadrature sum method was sufficient for generating spectra that were insensitive to the rotation of individual patterns (Figure 4.4C)

We used this approach to analyze the single cell patterns of CRISPR-KO and sgNT control synapses, alongside topographies of unengaged particles ($n = 314$ total). A rotationally degenerate Zernike pattern spectrum (hereafter "Zernike spectrum") was calculated for each synapse topography, after which the entire data set was subjected to PCA (Figure 4.5). On this PCA plot of Zernike spectra (Z-PCA), unperturbed surfaces—"non-synapse" controls drawn from the same fields of view as the real data—formed a tight, PC1-low cluster. CTL topographies, on the other hand, distributed to varying extents along both PC1 and PC2. We binned the data into quartiles by PC1 value to avoid arbitrarily defining distinct pattern categories based solely on visual appearance. After splitting the dataset into PC1-quartiles, each bin appeared to contain similar patterns while contrasting against the other three, validating the utility of this method for pattern sorting. Contacts in Quartile 1 (Q1) were globally convex, reflecting the uncompressed state;

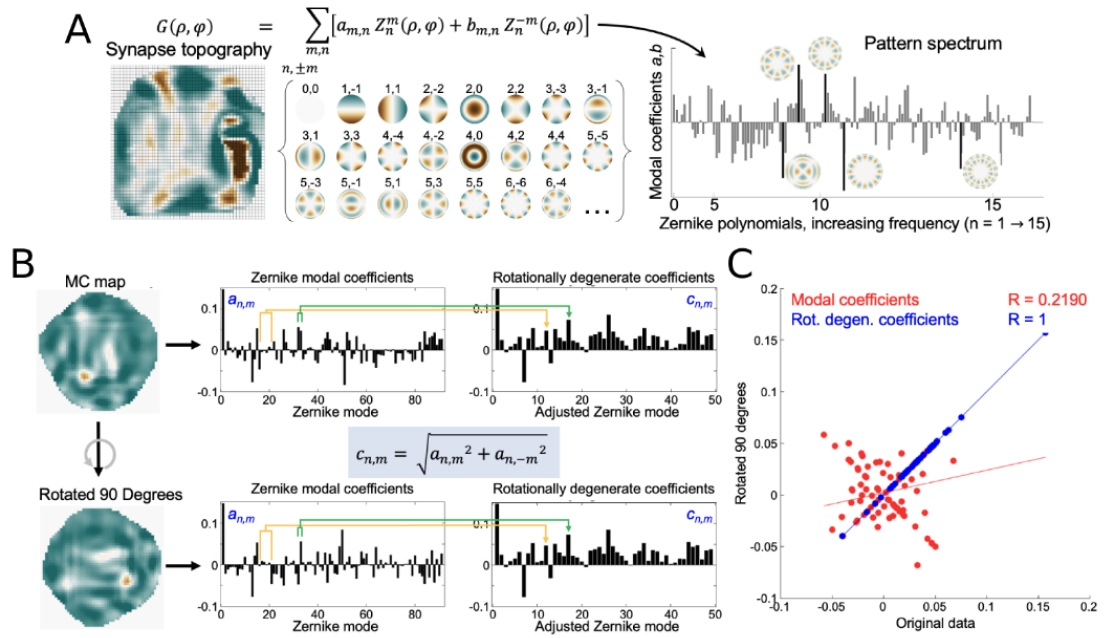


Figure 4.4: Representing individual synapse topographies as rotationally degenerate Zernike spectra.

A: Each synapse topography can be represented as a linear combination of the Zernike polynomials up to a pre-defined radial degree n (number of "sets" of polynomials describing a given frequency level). B: Rigid-body rotation of individual topographies leads to a shift in the spectra due to azimuthally degenerate functions $n \pm m$. Therefore, we combined these paired coefficients *via* a simple quadrature sum approach. C: A scatterplot of the expansion coefficients of a test pattern before and after a 90° rotation. The original expansion coefficients (red) correlate poorly before and after rotation, but the azimuthally combined versions are robust to the transformation. Panel A reflects data in Figure 4A-B, and panel B reflects Figure S3A-B, of [262]. These are demonstrative data of the method, and are taken to represent the observations at large throughout this study.

Q2 comprised shallow craters with weak indentations; Q3 contained strong, finger-like protrusions; and Q4 comprised deep craters or claw-like contacts with sharp indentations.

In general, features appeared stronger and more numerous as PC1 increased. Although wild-type and mutant CTLs were not neatly separated by this analysis into sharp clusters, they clearly distributed differently along the PC1 axis (Figure 4.5C and Figure 4.6): majority of sgNT and sgWAVE2 cells occupied Q3 and Q4, whereas sgWASp and sgTalin populations were about evenly split between the lower half and the upper half of PC1 values.

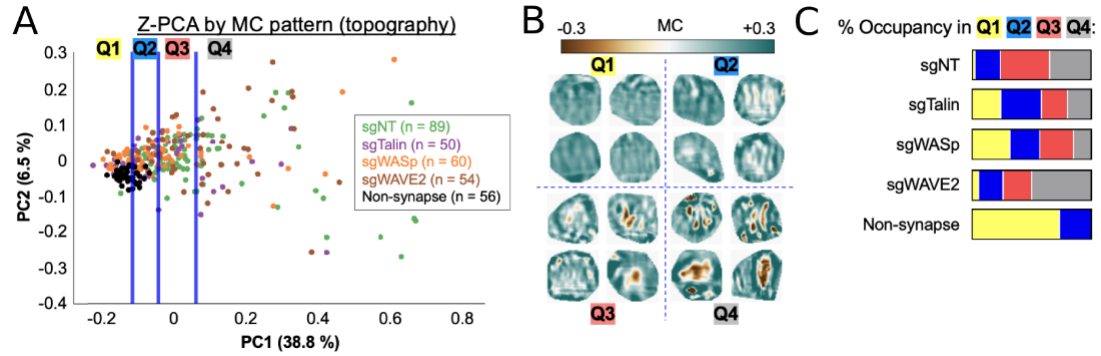


Figure 4.5: PCA of Zernike spectra (Z-PCA plot) facilitates pattern sorting.

A: The Z-PCA using mean curvature patterns as input. PC1, the major axis of variation ($\sim 6\times$ more than PC2), was split into quartiles to avoid the determination of pattern categories *a bene placito*. B: Representative patterns from each of the four quartiles. C: The disproportionation of each genotype (and controls) into the four pattern categories reveals differing population tendencies to exhibit stronger or weaker pattern types. Panels A-C reflect data in Figure 4C-E of [262]. These data were pooled from single-cell data from $n=1$ independent experiment.

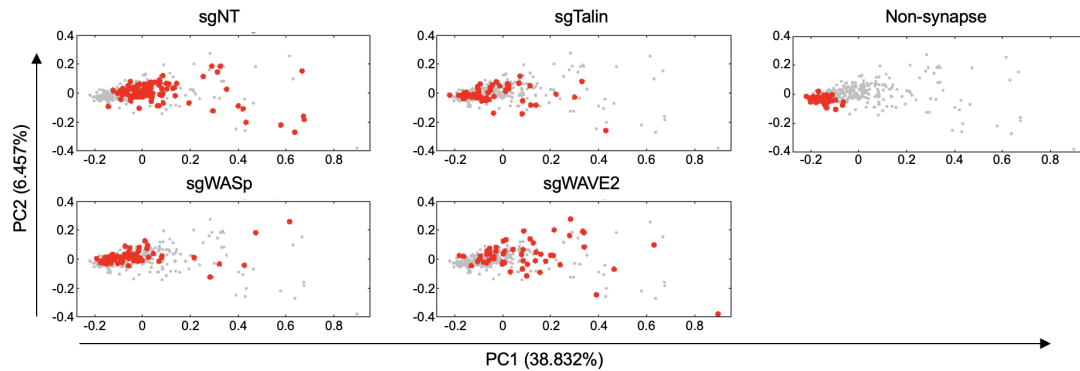


Figure 4.6: Z-PCA plot, with cell-type-specific galleries highlighted.

The same Z-PCA plot as in Figure 4.5 is shown, with each topography type (i.e., cell type) highlighted separately. Data reflects those of Figure S3C of [262]. These data were pooled from single-cell data from $n=1$ independent experiment.

In agreement with this, the CTL subtypes with unperturbed cytotoxic potential according to recent *in vitro* work (sgNT and sgWAVE2 CTLs) on average exhibited larger PC1 values than CTLs with lower killing capacity *in vitro* (Figure 4.7A), further linking mechanical patterning to cytolytic capacity.

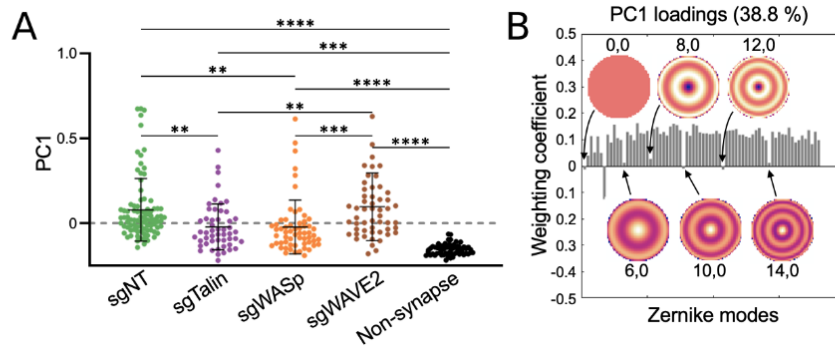


Figure 4.7: PC1 encodes information on radial asymmetry.

A: Comparison of PC1 values across the five topography data types. sgTalin and sgWASp exhibit lower PC1 values compared to sgNT and sgWAVE2 cells. *, **, ***, and **** denote $P \leq 0.0332$, $P \leq 0.0021$, $P \leq 0.0002$, and $P \leq 0.0001$, respectively, calculated by multiple t-testing with Tukey's correction. Error bars indicate SD. B: The loadings of each Zernike pattern reflect their relative contributions to the major axis of variation among the topographies. Key absences of concentric functions were noted. Panels A-B reflect Figure S3D-E in [262]. These data were pooled from single-cell data from $n=1$ independent experiment.

We then inspected the PC1 loadings to determine which patterns, if any, made major contributions to the pattern variance in this dataset. Surprisingly, we did not see enrichment of certain patterns but rather roughly equivalent contributions from low- and high-frequency Zernike modes alike, with the conspicuous exclusion of concentric functions. Thus, we concluded that PC1, which accounts for a plurality (38.8 %) of the pattern variation among the topographies in this dataset, appears to capture both radial asymmetry (absence of concentric functions) and topographical complexity (contributions from low- and high-frequency patterns). To us, this suggested that the capacity to induce complex, asymmetrical undulations on the target surface could be a key feature distinguishing cytotoxic CTLs from killing-deficient mutants.

4.2.3 Spectral pattern complexity distinguishes killing-competent from perturbed CTLs

In an attempt to parameterize this concept of *undulatory pattern complexity* (i.e., measure "undulatory-ness"), we leveraged, by analogy, the Fourier analysis of songs or pictures, in which low-frequency information corresponds to the overall *gestalt* of a sound or image, while increasing amounts of high-frequency information add crisper detail and sharpness to the reconstructed signal. We reasoned that a smooth and relatively flat synapse topography, even uniformly strong synapses resembling a giant concave bowl, would require only a few Zernike polynomials to accurately reconstruct; on the other hand, an undulating pattern with alternating patches of concave and convex features could only be captured using a larger set of Zernike polynomials (Figure 4.8).

Thus, we defined a Spectral Complexity index reflecting the rate by which Zernike reconstructions converge to the original data. Importantly, this metric is sensitive not only to local feature intensity but also to spatial variation among local features: uniformly strong or weak synapses both scored low in spectral complexity relative to patterns in which concave and convex features alternate. Spectral complexity strongly correlated with PC1 (Figure 4.9A), suggesting that it also captures radially asymmetric, undulating topography. Importantly, wild-type CTLs exhibited higher spectral complexities than their WASp- and Talin-deficient counterparts, while sgWAVE2 cells were indistinguishable from sgNT CTLs (Figure 4.9B). This further substantiated a connection between undulatory complexity and cytolytic capacity, and gave us a quantitative handle on the topographical complexity of a synapse.

Collectively, these results establish a strong association between cytolytic potential and the induction of complex, asymmetric patterns on the target surface, and suggest that

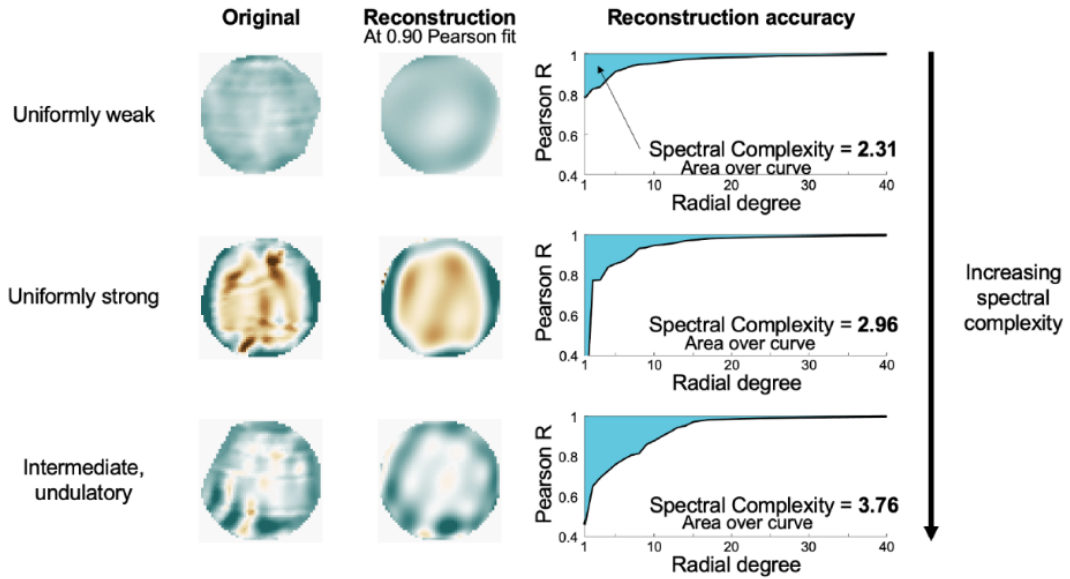


Figure 4.8: Definition of spectral complexity.

Three synapses are compared: a uniformly weak synapse, a uniformly strong synapse, and a synapse that is intermediate in strength but whose features are spaced out in an undulatory fashion. The amount of high-frequency information in the pattern is highest with the undulatory synapse, as measured by the areas over the curve of reconstruction accuracy (Pearson R) vs. Radial degree n . These data reflect those in Figure S4A in [262]. These are demonstrative data of the method, and are taken to represent the observations at large throughout this study.

this interfacial architecture is critical for optimal killing. Interestingly, spectral complexity correlated only weakly with particle compression at a single-cell level (Figure 4.10), indicating that the two metrics measure fundamentally distinct mechanical properties. Furthermore, they suggest that compressing the target surface into a crater and imposing undulating topography within it are two independent aspects of the CTL mechanical output and mechanotype, potentially regulated by distinct biochemical mechanisms.

4.3 Discussion I

Herein we described a novel method to analyze synapse topographical patterns, by expressing them as linear combinations of orthonormal functions defined about the

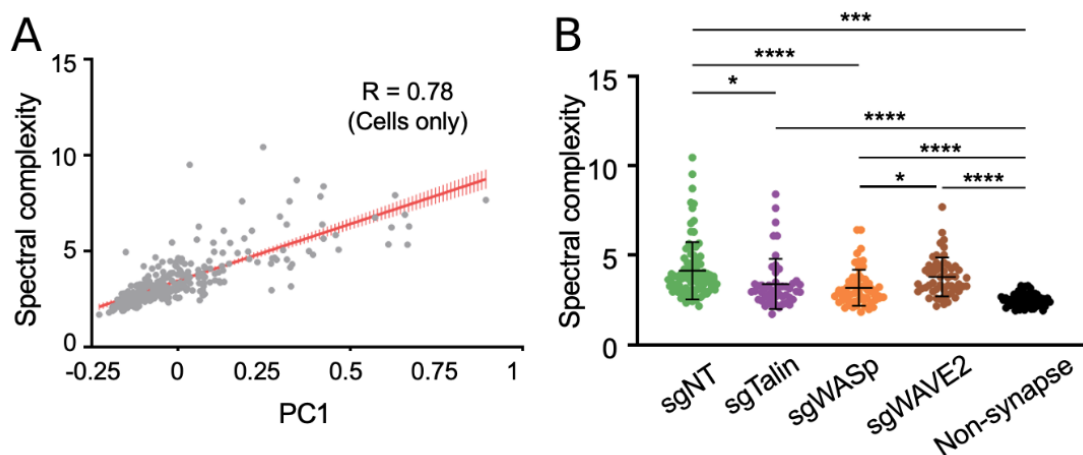


Figure 4.9: Spectral complexity as an index of CTL mechanical output.

A: For the current dataset, spectral complexity was plotted against PC1 score, which reflects radial asymmetry and (visual) complexity of the patterns. Pearson's R is shown, and the regression line (red) is plotted with bars denoting the 95 % confidence interval. Non-synapse control synapses were not included. B: Spectral complexity measures for the various synapse topographies. *, ***, and **** denote $P \leq 0.0332$, $P \leq 0.0002$, and $P \leq 0.0001$, respectively, calculated by permutation testing with Benjamini-Hochberg correction for multiple hypothesis testing. Error bars indicate SD. Panel A reflects data from Figure 4G, and panel B reflects data in Figure S3F, in [262]. These data were pooled from single-cell data from $n=1$ independent experiment.

unit circle. Although there have been prior efforts to apply Zernike polynomials to biological image analysis [295], the utility of this approach has been limited by the fact that Zernike modes have a fixed angular orientation. Consequently, the spectralization process interprets patterns related by simple rotations to be fundamentally distinct. Combining coefficients into a rotationally degenerate representation enabled us to circumvent this problem and effectively compare complex topographies lacking an intrinsic radial orientation.

The first useful application of our method was in generating feature vectors amenable to PCA. Spectral decomposition of the kind used here represents an alternative and complementary approach to algorithms based on a supervised classifier [296]. While both strategies can cluster image-based data in an unbiased manner, spectral representations and PCA together have the advantage of yielding physically interpretable axes of separation

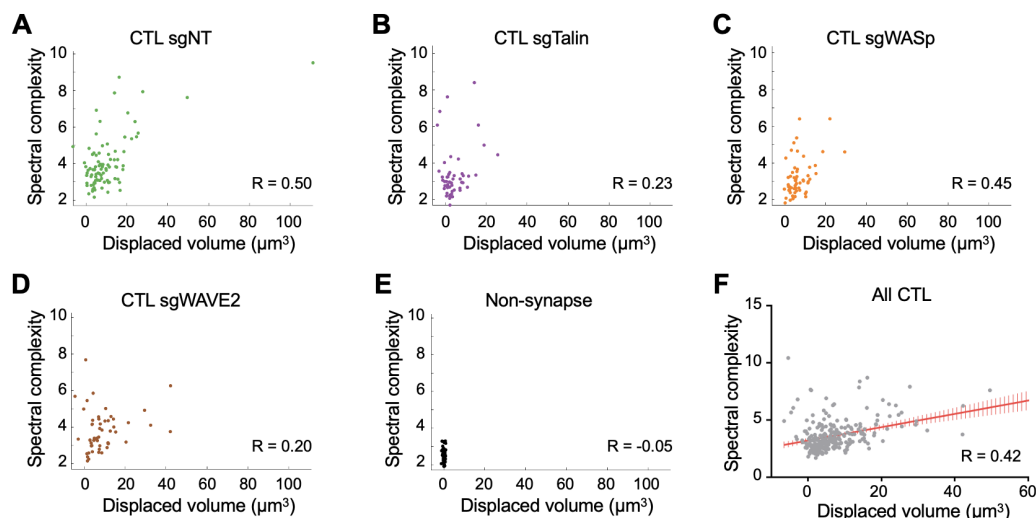


Figure 4.10: Independence of spectral complexity and compression strength.

The various panels show cell-type-specific (A-E) and pooled (F) correlations between the two metrics of spectral complexity (topographical "undulatory-ness"/pattern complexity and deformation volume (compression strength). Across the board, correlations are poor. Pearson's Rs are shown, and the regression lines (red) are plotted with bars denoting the 95 % confidence intervals. Panels A-F reflect data in Figure S4B-G in [262]. These data were pooled from single-cell data from $n=1$ independent experiment.

(as seen in the PC loadings), thereby potentially providing more mechanistic insight than non-linear embedding methods (e.g., t-distributed Stochastic Neighbor Embedding [t-SNE] or Uniform Manifold Approximation and Projection for Dimension Reduction [UMAP]). The other application we developed from the Zernike spectral decomposition of synapse topographies was a spectral complexity metric, which reflected both intensity and the undulatory distribution of topographical features along the synapse area. This enabled us to directly compare populations of synapses from each other in a quantitative manner that preserved spatial patterning information.

Our methods should be applicable to any data set containing images of roughly circular dimensions. Moving forward, we expect that the thoughtful pairing of orthogonal basis sets (such as Zernike polynomials) with appropriate data types will find broad applicability in the analysis of cellular patterning. As imaging data sets become larger

and more complicated, methods for interpreting image complexity and heterogeneity will be critical for extracting biologically meaningful information from spatial patterns of topographies, as in this Chapter, or biomolecules, as in Chapter 6.

These methods of encoding spatial patterning information into meaningful numbers were first applied to the analysis of cytoskeletal perturbations on CTL mechanical activity. To our knowledge, this experiment provided first-in-kind observations on the effects of WASp and talin loss on mechanical patterning of the immune synapse. Not only did these synapses become "simpler" than control (sgNT) synapses *via* the spectral complexity metric, but we were also able to demonstrate *population-level tendencies* of these two genotypes to disproportionate more into weaker, simpler pattern categories using the Z-PCA method (Q1-2 of the Z-PCA). In spite of the single-cell heterogeneity present in this dataset, this method allowed for the measurement of population-level patterning phenotypes and shifts.

One interesting finding was that, on a single-cell level, compression strength (deformation volume) and spectral complexity did not correlate appreciably. A proportional relationship would have perhaps pointed to shared regulation, i.e., strong cells can both compress and micropattern their targets strongly, while a negative correlation would have implied a division of labor amongst the F-actin machineries, i.e., cells choose between compression and micropatterning. (We know the latter is not exactly true, as we have already discussed numerous representative examples of T cells exhibiting both abilities.) The lack of correlation implies that individual CTLs can do both, either, or neither—and thus, these two activities may be regulated by distinct actin-based mechanisms. It is possible that the microtubule cytoskeleton contributes to one of these activities (perhaps compression), or that distinct actin pools are responsible for establishing bulk axial forces

and smaller palpations across the contact (e.g., PIP₃-templated actin and WASp-mediated actin foci, respectively).

Finally, defects in patterning concorded well with the known cytolytic defects of sgWASp and sgTalin cells; in contrast, sgWAVE2 cells demonstrated no measurable difference in pattern composition or spectral complexity. Thus, we were able to establish a connection between cytotoxicity and the ability to induce complex, undulatory patterns of distortion on target surfaces. We subsequently aimed to substantiate this connection in at least three more biological contexts.

4.4 Materials and methods I

4.4.1 Cell culture and imaging

For depleting WASp, WAVE2, or Talin in CTLs, lymphoblasts for transduction were isolated from OT-I TCR-transgenic mice containing an additional Cas9-knockin allele [297] (Jackson Labs #026179), also on the C57BL/6J background. PhoE cells were transfected with expression vectors containing guide RNAs (gRNAs) targeting these genes [190, 276], and the resulting viral supernatants used to transduce lymphoblasts on day 1-2 with sgRNAs subcloned into an optimized MRIG [298] or LEP (Addgene # 111160) backbone. These cells were then selected using puromycin or zeocin, and validated by flow cytometry or western blot. Cells were used at day 7 for high-resolution imaging, with α CD3 ϵ /ICAM1-coated beads.

4.4.2 Image processing and analysis

Generating Zernike spectra of topographies for principal component analysis

Zernike polynomials are orthogonal to each other over a unit circle. Thus, any function on a unit circle $f(r, \theta)$ can be expressed as a linear combination of Zernike functions, in a manner similar to a 1-D Fourier Transform:

$$f(r, \theta) = \sum_{n=0}^{\infty} \sum_{m=-n}^n a_{n,m} Z_n^m(r, \theta) \quad (4.1)$$

where $a_{n,m}$ is the modal/expansion coefficient associated with the Zernike polynomial of the n th radial degree and m th azimuthal order, Z_n^m . Notably, the Zernike polynomials comprise an infinite set of orthogonal functions, so to numerically calculate the modal coefficients, we truncated the set to a pre-defined N radial degrees (sets of polynomials):

$$f(r, \theta) = \sum_{n=0}^N \sum_{m=-n}^n a_{n,m} Z_n^m(r, \theta) \quad (4.2)$$

We used $N=15$ for most analyses as this generated a faithful reconstruction of topographies while avoiding overfitting (Figure 4.11).

To numerically compute the modal coefficients for a 50×50 topography matrix M , we removed all data outside of the unit circle, linearized the remaining data into a discretized function vector $F(r_i, \theta_i)$, and then computed the Zernike polynomials up to N degrees on the gridded data point locations $Z(r_i, \theta_i)$ using the MATLAB *zernfun* function [299]. This yielded the following overdetermined system of linear equations:

$$F(r_i, \theta_i) = \sum_{n=0}^N \sum_{m=-n}^n a_{n,m} Z_n^m(r_i, \theta_i) \quad (4.3)$$

which we solved using the MATLAB left matrix inversion operator ($\backslash$), producing Zernike modal coefficients $a(n, m)$. Critically, while the degree n and absolute value of the order m define the shape and frequency of the Zernike polynomials, the sign of m determines their

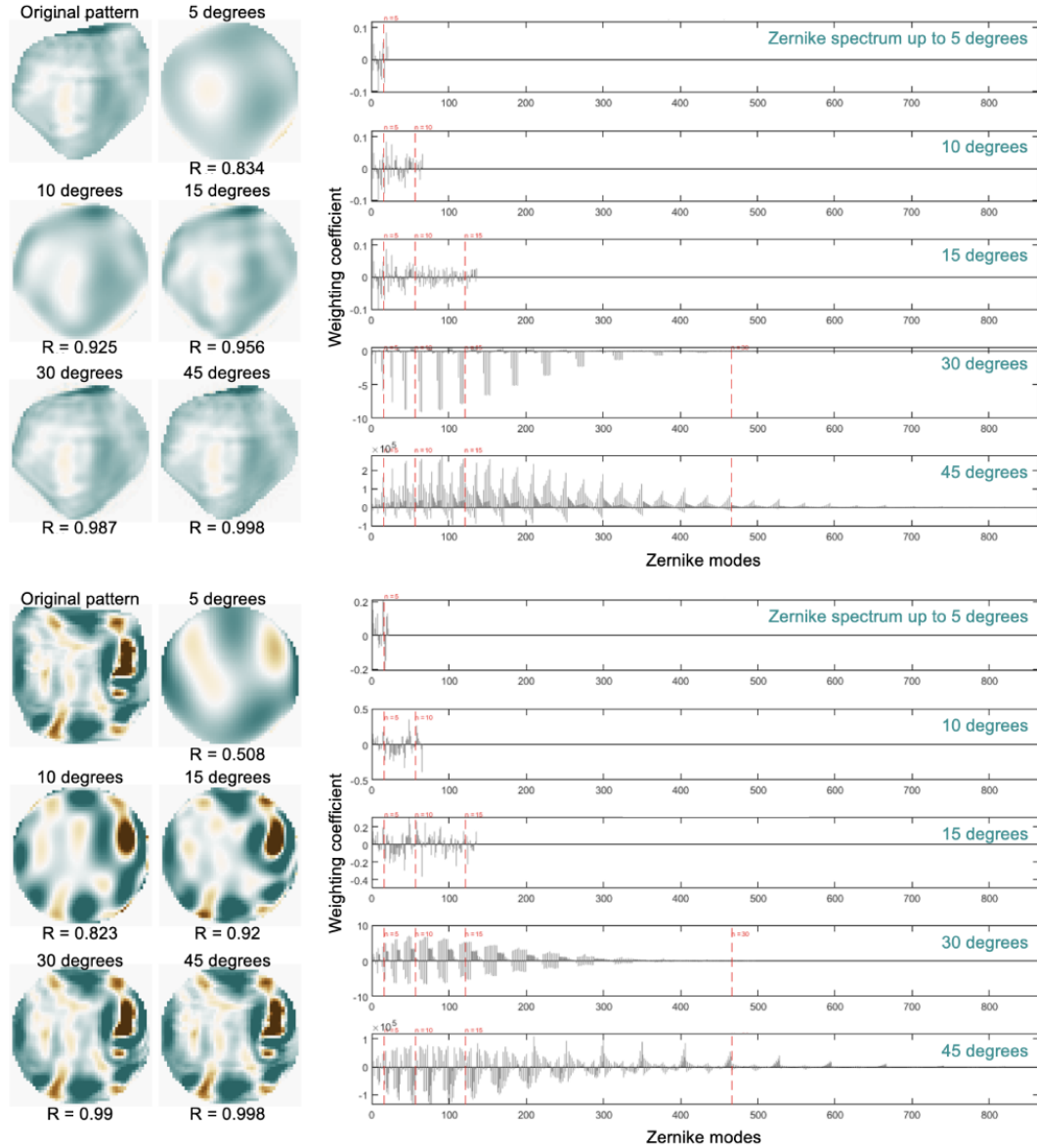


Figure 4.11: Zernike spectra and reconstructions of two synapse topographies.

Shown are two synapse topographies ("original pattern") alongside reconstructions spanning various levels of accuracy, based on the use of varying numbers of radial degrees for the wavefront fitting. Note the relative independence of the expansion coefficients from $n=5$ to $n=15$, compared to the development of a periodic waveform by $n=30$. We thus chose $n=15$ for this work; even the relatively complex pattern at the bottom was accurately ($R = 0.90$) reconstructed with 15 sets of polynomials. These data reflect those in Figure S10 in [262]. These are demonstrative data of the method, and are taken to represent the observations at large throughout this study.

azimuthal rotation. Thus, the relative weighting of coefficients $a_{(n,m)}$ and $a_{(n,-m)}$ only provide rotational information. We assumed that force exertion patterns are rotationally degenerate, i.e., two patterns that are identical except for their azimuthal (*en face*) rotation are functionally identical. Thus, we reduced the modal coefficients to an azimuthally degenerate set of coefficients C by calculating the quadrature sum of coefficients with the same degree and absolute order:

$$C_{n,m} = \sqrt{a_{n,m}^2 + a_{n,-m}^2} \quad (4.4)$$

Notably, rotational information is lost at this step, and thus the original pattern $F(r_i, \theta_i)$ cannot be reconstructed from C . We used the azimuthally degenerate spectral coefficients C as feature vectors in principal component analysis using the MATLAB *pca* function. Each 50×50 gridded synapse M_i was transformed into coefficient vector C_i , then the vectors were concatenated into a matrix with each column corresponding to one synapse and each row corresponding to the index of C . The output principal component scores, loadings, and explained variance were then truncated to the first two principal components and visualized in scatter plots.

Defining a metric for spectral complexity

Using modal Zernike coefficients $a_{n,m}$, we can approximately reconstruct the original data:

$$F_{reconstructed}(r_i, \theta_i) = \sum_{n=0}^N \sum_{m=-n}^n a_{n,m} Z_n^m(r_i, \theta_i) \quad (4.5)$$

The correlation between the original data $F_{original}(r_i, \theta_i)$ and the reconstructed estimate $F_{reconstructed}(r_i, \theta_i)$ increases with N asymptotically (Figure 4.8):

$$\lim_{N \rightarrow \infty} F_{reconstructed}(r_i, \theta_i) - F_{original}(r_i, \theta_i) = 0 \quad (4.6)$$

While this necessarily holds true for all synapses, the exact shape of this curve can only be determined empirically and is dependent on the underlying frequency information & complexity of the data. For example, very simple patterns with low frequency undulations can achieve near-perfect reconstruction with relatively few Zernike polynomials, while complex high frequency undulations require higher order polynomials to achieve the same reconstruction accuracy. We defined a spectral complexity parameter based on the Zernike transform as the area over the curve of a given topography reconstruction plot of Pearson's correlation coefficient (R) vs. radial degree (N). By this definition, the simplest synapses would have a negligible area and spectral complexity, while synapses with more high-frequency information and greater underlying complexity would require a higher N and thus span more area before converging to $R = 1$.

We first computed modal coefficients as described above iteratively, increasing from $N = 1$ to $N = 40$. At each iteration, the computed coefficients were multiplied by their respective Zernike functions to compute $F_{reconstructed}(r_i, \theta_i)$. Pearson's R between $F_{reconstructed}(r_i, \theta_i)$ and the discretized topography matrix $F_{original}(r_i, \theta_i)$ was calculated using the MATLAB *corr2* function. The spectral complexity was calculated as the integrated sum of the R-N plot, calculated using the MATLAB *trapz* function, subtracted from $N_{max} = 40$ (which is equivalent to a perfect reconstruction $R = 1 \times N_{max}$). For the statistical analysis of spectral complexity values across samples, we used permutation testing to assess statistical significance; this was done in order to avoid making assumptions of a uniform p-value distribution for the null hypothesis. In brief, to compare experimental groups, we used bootstrap resampling to generate 105 random permutations of the samples in each group, and computed the absolute difference in mean between permuted groups as the test statistic to generate an empirical null distribution. The measured difference in means ($|\Delta\mu_{actual}|$) between experimental groups was computed and p-value was calculated as the proportion of the null distribution greater than $|\Delta\mu_{actual}|$. For experiments with

multiple comparisons, False Discovery Rates (FDR) were computed using the Benjamini-Hochberg method and differences between experimental groups was deemed statistically significant for $FDR < 0.05$.

4.5 Background II

4.5.1 The course of T-cell activation and differentiation during an infection

We next wished to investigate whether the mechanical indices that distinguish effective from incompetent CTLs *in vitro*—compression strength, spectral complexity, and population pattern compositions—would also delineate functionally distinct CD8+ T-cell differentiation states in a physiological context. Over the course of a Lymphocytic Choriomeningitis Virus (LCMV) infection [300], unactivated (CD44-/CD62L+) naive CD8+ (T_n) cells in secondary lymphoid organs differentiate into two functional states: activated (CD44+/CD62L-) KLRG1+/CD127- short-lived effector CTLs (SLECs, T_{eff}), which home to sites of inflammation and kill infected host cells, and KLRG1-/CD127+ memory precursor cells (MPECs) (Figure 4.12, [301]). By the resolution of infection, antigens become scarce and SLECs/ T_{eff} s die by apoptosis (contraction); however, various subsets of MPEC-derived memory cells T_{mem} remain [302], long-lived populations with reduced cytolytic activity but with the sustained ability to regenerate the T_{eff} pool upon restimulation with antigen. In cases where T_{eff} cells fail to clear the pathogen, chronic antigen exposure compounded by lack of CD4+ help induces a hyporesponsive, "exhausted" (T_{exh}) phenotype characterized by weak lytic function, poor cytokine output,

dampened respiration [303], and uniquely recalcitrant epigenetic & transcriptional landscapes [304, 305].

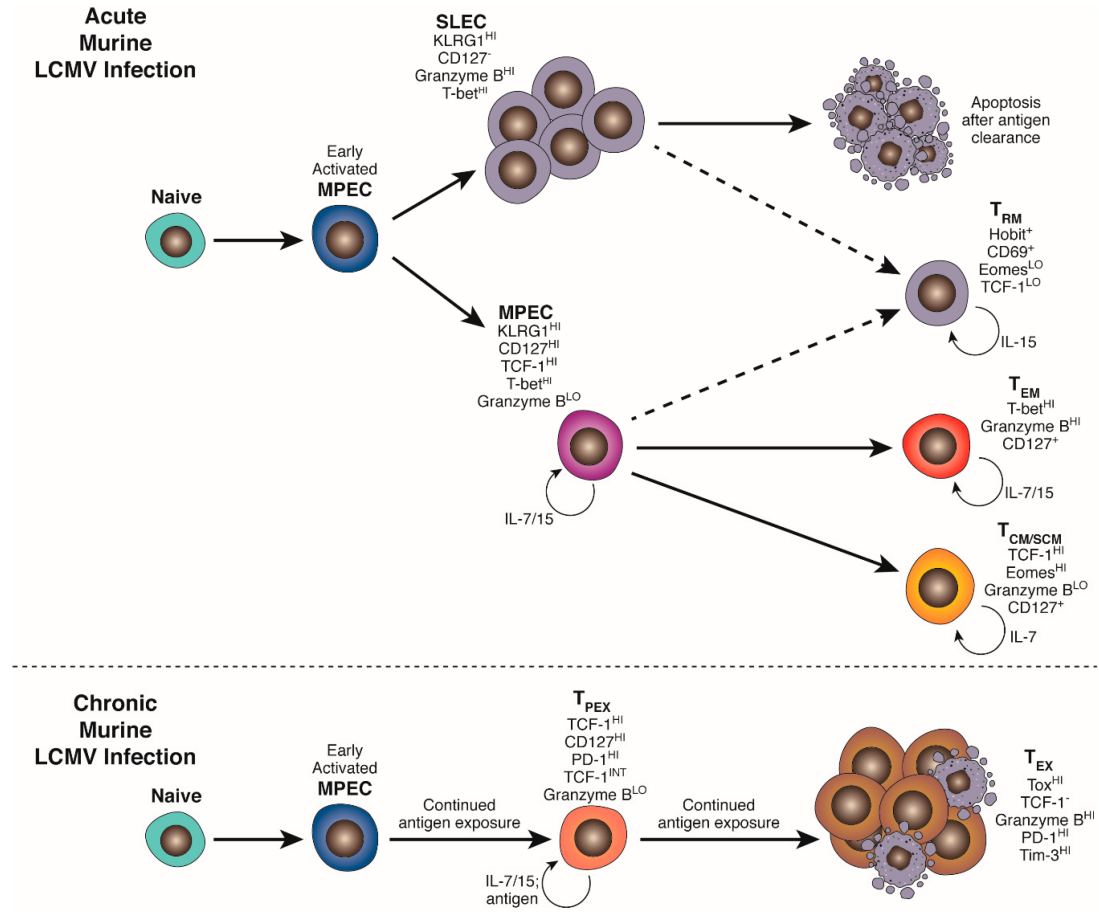


Figure 4.12: Differentiation landscape of CD8+ T cells.

Shown are various T-cell differentiation states over the course of two types of LCMV infections relevant to this section, acute and chronic. Relevant phenotyping or functional markers are listed where applicable. The Armstrong strain of LCMV (LCMV-A), which is normally cleared by recipient mice within 8 days, induces a distinct pathway of differentiation from the chronic Clone-13 strain of LCMV (LCMV-C113), which remains disseminated at high viral titers for months post-inoculation. Figure reproduced from [301] in accordance with the Creative Commons Attribution License CCBY.

Interestingly, recent two-photon intravital imaging work on tumor tissue slice cultures documented that T_{exh} cells migrate more actively than non-exhausted T_{eff} cells (Figure 4.13). Motility measured by imaging was shown to be proportional to PD-1, CD38, and CTLA-4 expression. In a mouse tumor model that delivers chronic antigen stimulation

to CTLs, two populations of CTLs were transferred—one batch 4 days before imaging, and the other 14 days before imaging. On the day of imaging, the 14-day-resident CTLs were highly motile in comparison to the 4-day-residents, implying that chronic antigen stimulation was associated with an enhancement of motility or a dampening of their ability to form synapses upon encountering antigen. The latter is intriguing, as recent work demonstrated that PD-1 engagement depressed multiple indices of synaptic mechanical output: total synaptic F-actin intensity (especially at the periphery/dSMAC), cell spreading, and T-cell shape changes associated with synapse formation (i.e., they were much more spherical instead of polarized) [156]. Together, these findings suggest that there exists an aspect of T-cell exhaustion that is mechanical. We thus sought to isolate and mechanically profile four prototypical subsets of CD8+ T cells in our system— T_n , T_{eff} , T_{mem} , & T_{exh} —and test whether the cytolytic mechanotype was associated with a T cell’s functional differentiation state.

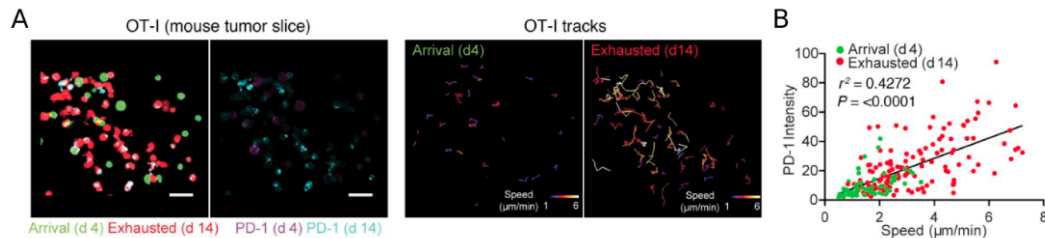


Figure 4.13: Comparison of motility between T_{eff} and T_{exh} in a tissue slice culture.

Serial adoptive transfers of CTLs on day -4 and day -14 prior to imaging were carried out in mice harboring PyMT-OVA-expressing tumors, to induce T-cell exhaustion. A: Comparing the tracks of T_{eff} correlates (day 4) and T_{exh} correlates (day 14) in the same field demonstrates the extent of motility exhibited by the latter (or the lack of cessation in response to antigen). B: Plotting PD-1 intensity against speed shows a degree of proportionality between the two. Figure modified from [306] in accordance with the Open Access policy of the Journal of Clinical Investigation—panels

A-B reflect data in Figure 4E-G of the publication.

4.6 Results II

4.6.1 LCMV infection establishes cohorts of naive, effector, memory, and exhausted T cells *in vivo*

We utilized adoptive P14^{tg} transfers into C57BL/6J mice and subsequent LCMV infections in order to generate cohorts of T_n, T_{eff}, T_{mem}, and T_{exh} cells for *ex vivo* isolation and mechanical profiling (Figure 4.14). P14 T_n cells, whose TCR recognizes the LCMV glycoprotein antigen residues 33-41 (H2D^b-gp33), differentiate into T_{eff} 8 days post-LCMV-A infection and eventually into T_{mem} 40 days post-LCMV-A. We set up another cohort of mice to receive LCMV-Cl13 and an α CD4 depletion antibody in order to remove T-cell help; under such conditions, adoptively transferred P14 cells exhibit a strongly T_{exh} phenotype after 40 days. Transfers and infections were timed such that on the day of harvest, all four types of CD8+ T cells could be isolated from spleens by a two-step process of (1) negative magnetic enrichment for CD8+ T cells, followed by (2) fluorescence-activated cell sorting (FACS).

In vivo induction of the four differentiation states was confirmed by flow cytometry (Figure 4.15): T_n were CD44-/CD62L+, T_{eff} were CD44+/CD62L-/CD127-/KLRG1+, T_{mem} were CD44+/CD62L-/CD127+/KLRG1-, and T_{exh} were PD-1^{high}/TIM3+. Four cohorts of mice were prepared and randomly distributed into three batches each for cell isolation, pooling, and analysis. Because the cells were coated with full-length antibodies during the sorting process, they did not interact with antigen/ICAM1-coated beads in our first attempt (not shown). In the subsequent attempt reflected in this study and in [262], therefore, the isolated cells were incubated with α CD3 ϵ /ICAM1-coated particles for 60

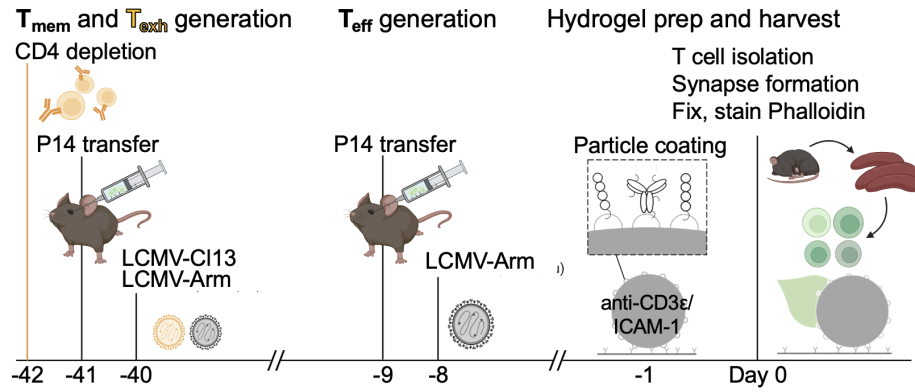


Figure 4.14: Experimental design for mechanical profiling of different T-cell differentiation states.

Protocol for *in vivo* differentiation of CD8+ T_{mem} , T_{exh} , and T_{eff} cells expressing the P14 TCR. All three cell types, together with naive P14 T cells, were isolated concurrently and imaged together with hydrogels coated with α CD3 ϵ and ICAM1.

mins (the extended time to allow recovery from sorting), PFA-fixed, and imaged on the Vt-iSIM (n = 944 cells in total).

4.6.2 *Ex vivo* measurements of compression strength and spectral complexity follow the course of infection

Volumetric analysis and feature identification revealed a hierarchy of compressive strength in the order: $T_n < T_{mem} \approx T_{exh} < T_{eff}$ (Figure 4.16A). T_n cells formed the weakest compressions and induced the shallowest topographical features, concave and convex alike (Figure 4.16B-C). T_{eff} cells, on the other hand, generated the strongest particle distortions, which is consistent with prior studies showing that T_{eff} mount stronger and faster killing responses than any of the other subsets [307, 308, 282, 309]. T_{mem} and T_{exh} exerted intermediate levels of compressive activity, but differed markedly in their capacity to create discrete indentations in the target surface, with T_{exh} cells

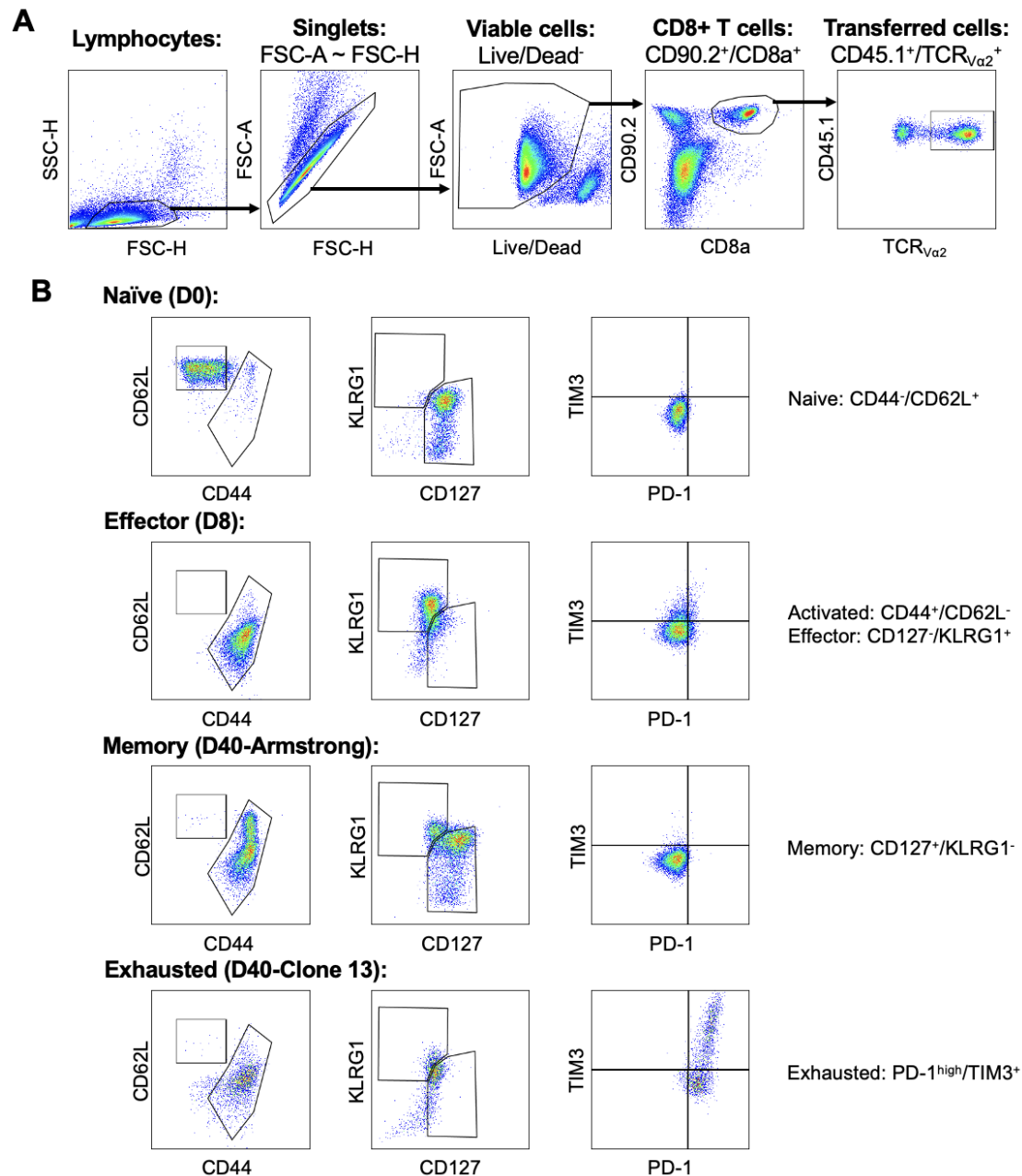


Figure 4.15: Purification and immunophenotyping of *in vivo* differentiated CD8⁺ T cells.

A: Sorting strategy to extract CD45.1⁺ P14 T cells from pre-enriched splenocytes. B: Representative phenotyping of the four T-cell differentiation states. The leftmost panels distinguish naïve from antigen-experienced T cells; central panels distinguish terminal effectors (SLECs) from naïve and memory-like cells; the rightmost panels identify exhausted cells. Panels A-B reflect data in Figure S6A-B in [262]. Data are representative of the entire sorting experiment, conducted n=1 time across n=3 pooled biological replicates.

generating fewer protrusions than any other subset, even T_n cells (Figure 4.16D). This result suggested that T-cell exhaustion encompasses not only transcriptional and secretory responses, but also biomechanical output at the synapse.

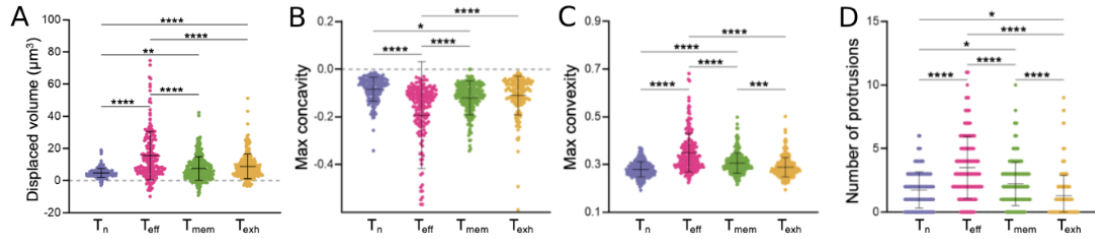


Figure 4.16: Comparison of T-cell compression strength and topographical features across the four differentiation states.

A-D: Deformation strength by displaced volume, concavity of protrusions, convexity of reliefs, and number of protrusions across T_n , T_{eff} , T_{mem} , T_{exh} . *, **, ***, and **** denote $P \leq 0.0332$, $P \leq 0.0021$, $P \leq 0.0002$, and $P \leq 0.0001$, respectively, calculated by multiple t-testing with Tukey's correction. Error bars indicate SD. Panel A reflects data from Figure 5B, and panels B-D reflect data from Figure S7A-C, in[262]. Data are pooled from $n=1$ experiment utilizing $n=3$ sets of pooled biological replicates.

To examine mechanical patterning, synapse topographies from the four subsets ($n = 944$ in all) were subjected to Z-PCA analysis (Figure 4.17A). The results recapitulated those of our prior comparison of wild-type and mutant CTLs, in that topographies again spread as a continuum along a PC1 axis that specifically excluded concentric Zernike functions (Figure 4.17B). The data was once again binned into four PC1-quartiles, and representative topographies within these pattern categories scaled with curvature intensity and variation. Looking at the pattern category composition of the four data types (differentiation states), we saw that the vast majority of T_n cells occupied the PC1-lowest regions Q1–2, whereas most T_{eff} cells fell into the PC1-highest Q4 (Figure 4.17C, gray). T_{mem} cells displayed an intermediate distribution that spread evenly between Q2–4, while T_{exh} cells straddled Q1–3. The leftward PC1 shift of T_{exh} cells relative to their T_{mem} counterparts lent further support to the idea that T_{exh} cells are mechanically

hypofunctional. In agreement with these population-level tendencies, T_{eff} cells exhibited the highest spectral complexities, followed by $T_{mem} > T_{exh} > T_n$ (Figure 4.17D).

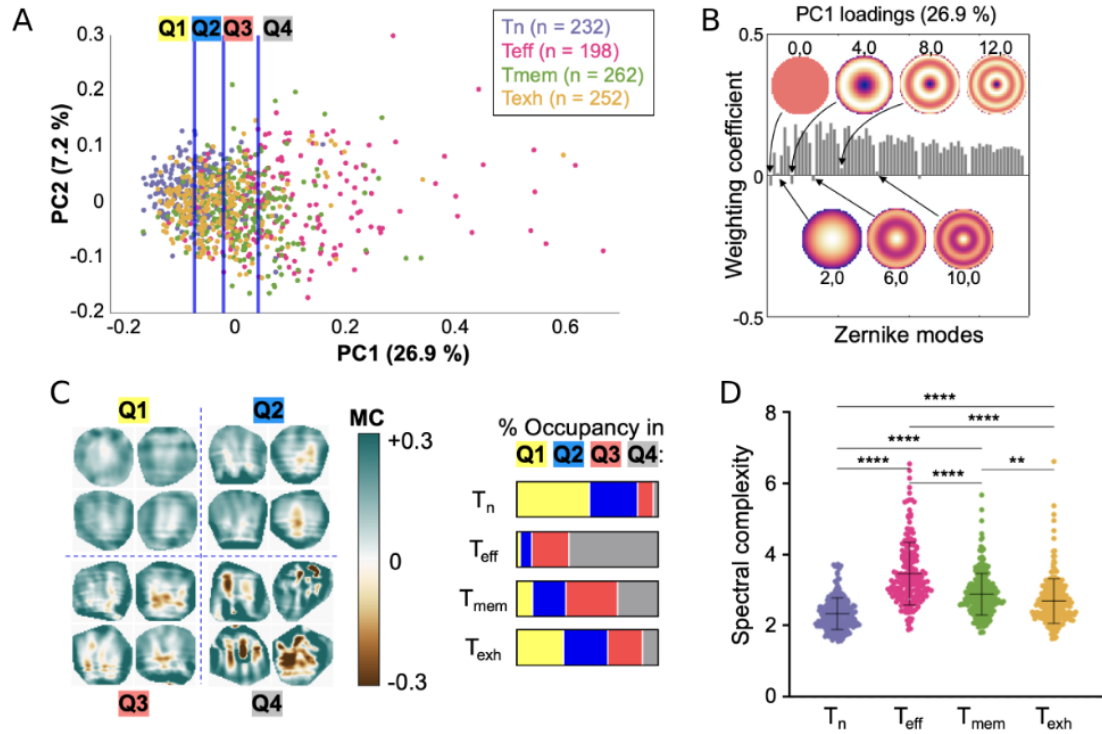


Figure 4.17: Comparison of T-cell topographical patterning across the four differentiation states.

A: Z-PCA based on synapse topography Zernike spectra. Data points were separated into quartiles (divided by the blue lines) for downstream analysis. B: Loadings of PC1, with emphasis on the bottom seven contributors, all of which resemble concentric patterns. C: Representative topographies from the four quartiles, and the pattern category composition of each cell type. D: Spectral complexity comparison. ** and **** denote ≤ 0.0021 and $P \leq 0.0001$, respectively, calculated by permutation testing with Benjamini-Hochberg correction for multiple hypothesis testing. Error bars indicate SD. Panel A reflects data from Figure 5C, panel B reflects data from Figure S7F, panel C reflects data from Figure 5D-E, and panel D reflects data from Figure 5F, in [262]. Data were pooled from n=1 experiment encompassing n=3 pooled biological replicates, n=944 cells in total.

Although this ordinal trend in spectral complexity matched that of compressive strength exactly, the two parameters again did not correlate on a single-cell level (Figure 4.18). This further supported the interpretation that topographical complexity is decoupled from overall force exertion. We conclude that the capacity to create complex, undulatory surface patterns within the context of a compressive synaptic crater distinguishes cytotoxic

T_{eff} cells from non-cytotoxic (T_n) and weakly cytolytic (T_{mem} & T_{exh}) cells, strongly suggesting that this mechanotype promotes cytolytic function *in vivo*.

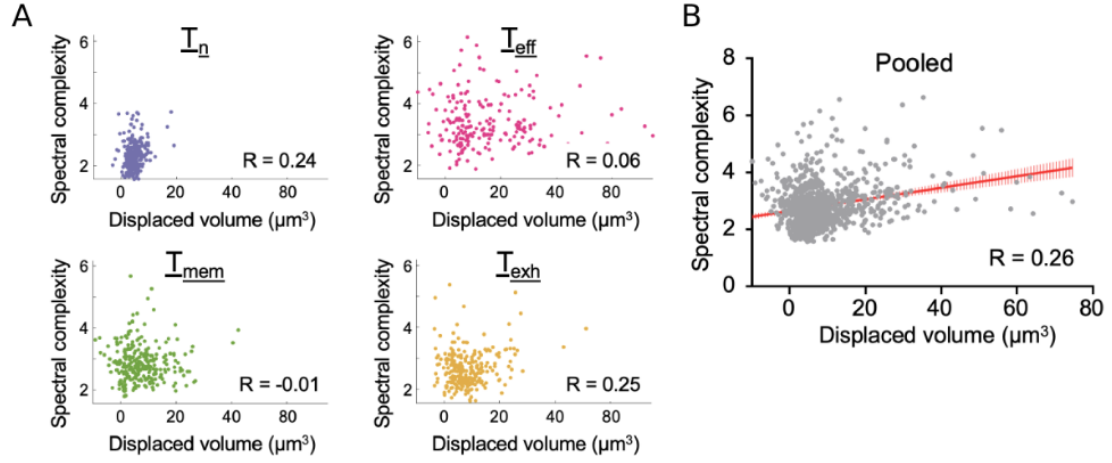


Figure 4.18: Independence of strength and pattern complexity in this dataset.

A: Scatter plots relating net particle compression to spectral complexity for topographies elicited by the four differentiation states. B: Scatter plot relating net particle compression to spectral complexity for all topographies in this data set (with Pearson R). Regression line (blue) is plotted with bars denoting the 95 % confidence interval. Panels A-B reflect data from Figure S7G-K in [262]. Data reflect a pooled population of $n=944$ cells from $n=3$ pooled biological replicates.

4.7 Discussion II

Earlier, we showed that compression strength and topographical pattern complexity (or undulatory-ness) independently distinguish wild-type and WAVE2-KO CTLs (both of which are killing-competent *in vitro*) from WASp-KO and talin-KO CTLs (both of which are killing-deficient *in vitro*). In this set of *ex vivo* mechanical profiling experiments, we were able to demonstrate that these two parameters also track with four distinct T-cell differentiation states throughout an infection: the antigen-naïve, short-lived effector, post-infection memory, and exhausted states. To our knowledge, this is the first time that synapse quality *via* mechanical output has been shown to reflect cellular state in such a manner.

That T_{exh} cells imparted weaker and less undulatory force exertion patterns than T_{mem} cells strongly suggests that defective mechanical output is an integral part of the exhaustion program. The basis for this lack of strength remains to be seen, but transcriptomic studies of T-cell exhaustion have documented widespread changes in gene expression that affect the composition of the cytoskeleton, as well as metabolic pathways that fuel its dynamic remodeling [283, 310]. F-actin dynamics are particularly costly from an energetic standpoint, accounting for half of cellular ATP consumption [311, 312], and perhaps attenuating these dynamics could enable T_{exh} cells to survive in nutritionally restricted contexts like the tumor microenvironment. However, certain models of T_{exh} cell generation have demonstrated enhanced motility of $PD-1^{high}$ cells [306]; indeed, nutrient-starvation-induced pro-migratory behaviors have been documented in cancer cells [313, 314] and bacteria [315]. Thus, rather than being subjected to such dire nutritional demands as to preclude all cytoskeletal activity, perhaps the specific energetic demands to transition from random locomotion to synapse formation are not met. In line with this, PD-1/PD-L1 ligation has been shown to impact T-cell synapse quality and cytoskeletal architecture independent from its canonical signaling activity [156].

The T-cell exhaustion program may therefore involve multiple distinct pathways that impinge on F-actin-based activity; whether the purpose is to bias them towards unproductive locomotion, inhibit productive synapse formation, or reduce cytolytic efficiency by repressing mechanopotential remains an exciting question for future work to tackle.

4.8 Materials and methods II

4.8.1 Murine infection model

One cohort of mice was separately used to synchronously generate each of the CD8+ differentiation states on the harvest day. For generating antigen-naïve T_n cells, P14^{tc^r-tg} mice backcrossed onto the C57BL/6J background were directly used on the day of harvest. For generating short-lived T_{eff} cells and T_{mem} cells, 1×10^4 P14 cells were first adoptively transferred into recipient C57BL/6J intravenously. The next day, these mice were infected with 2×10^6 pfu LCMV-A per mouse intraperitoneally. The T_{eff} cohort was infected eight days before, and the T_{mem} infection was executed forty days before the final harvest.

For the T_{exh} generation, CD4+ T cells were first ablated using a α CD4 depletion antibody (InvivoMab clone GK1.5), delivered intraperitoneally into the mice. The day after, 1×10^4 P14 cells were transferred as in the T_{eff} cohort. One day later, 2×10^6 pfu LCMV-Cl13 were injected into the mice similarly. The harvest was timed to be forty days after the infection, as in the T_{mem} -generating cohort.

4.8.2 Cell isolation and T-cell phenotyping

On the day of harvest, mice were sacrificed and splenectomized. Spleens were pooled and split into three batches each. Total CD8+ T cells were first enriched from the spleens using the Dynabeads Untouched Mouse CD8 Cells Kit (TFS 11417D) according to the recommended protocol. Afterwards, cells were split into two pools: (1) a majority pool for FACS was stained with live-dead/CD90.2/CD8a/CD45.1/TCR $V_{\alpha 2}$, and (2) a small

aliquot for phenotyping was stained with CD44/CD62L/KLRG1/CD127/TIM3/PD-1. FACS was conducted into pure FBS using a BD FACS Aria Cell Sorter. Afterwards, recovered P14 cells were counted, transferred into complete T-cell media containing hIL-2 (30 U/mL), and pipetted into 8-well chambers containing α CD3 ϵ /ICAM1-coated hydrogels. Cells were allowed to form synapses for 45–60 mins, fixed, and imaged as previously described.

CHAPTER 5

THE LIMITS OF ONTOGENY: DEFINING SYNAPTOCYTES AND PHAGOCYTES ON THE BASIS OF FORM

5.1 Background

Having shown that the mechanotype of killing-competent CTLs tracks with maximally cytolytic T cells in two different contexts, we considered the relationship between mechanical output and cellular ontogeny more broadly. The wealth of research on immune synapses has revealed that CD4+ and CD8+ T cells each harbor a few synaptic idiosyncrasies, in spite of their close ontogeny [316]: relative to conventional CD4+ T_H Cs, CD8+ CTLs form more dynamic, meta-stable "kinapses", while T_{regs} form longer-lived, hyperstable synapses. Little comparative anatomy work has been done in this regard, but these observations may point to cytoskeletal differences that would reflect in mechanical output (i.e., strength and/or patterning).

Some authors have likened phagocytosis to synapse formation due to some shared signaling pathways, cytoskeletal features, and organelle polarization events (Figure 5.1, [317, 318]). The functional endpoints of these two processes are very different, however, implying that they might be distinguishable by their interfacial mechanics [258]. However, as in the case of CD4 and CD8 effector cells, a direct head-to-head comparison between phagocytic and synaptic contacts has not yet been carried out.

Thus, we elected to compare the mechanical outputs of (1) functionally distinct, ontogenically related cell types—CD4+ and CD8+ T_{eff} cells—and (2) functionally distinct, ontogenically unrelated cell types—CD8+ T_{eff} cells (CTLs) and bone-marrow-derived macrophages (BMDMs). We hypothesized that differences would be muted

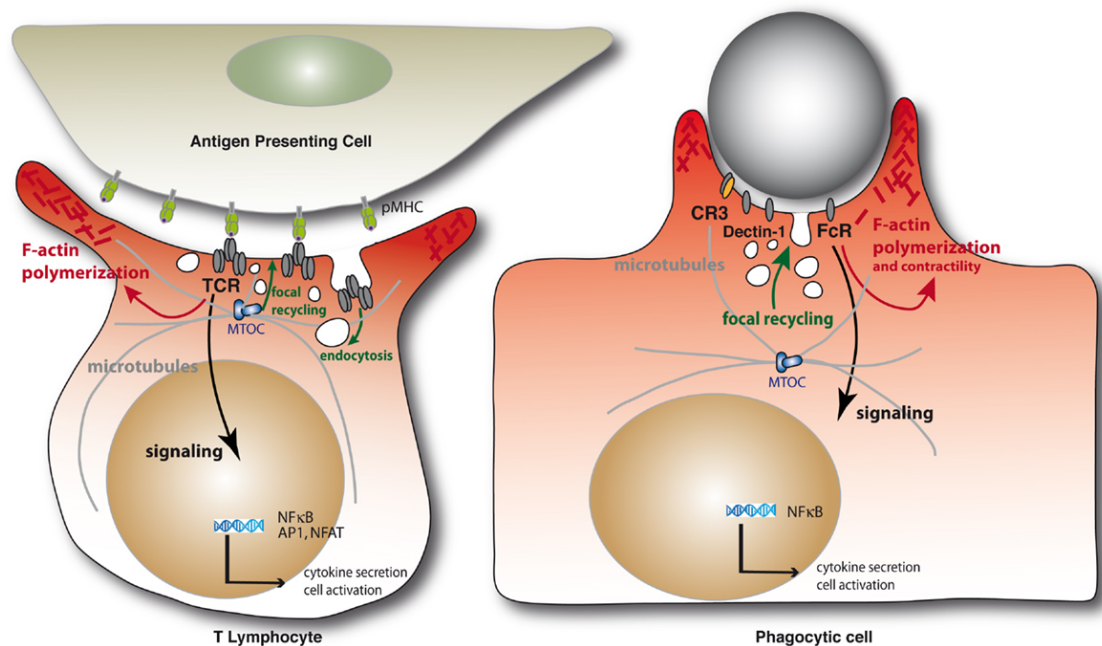


Figure 5.1: Comparison between T-cell synapses and macrophage phagocytic contacts.

This figure emphasizes similarities between synapsis and phagocytosis on the basis of actin and the tubulin polarization, endocytic pathways, and transduction to NF- κ B transcription. Reproduced from [317] in accordance with the Creative Commons Attribution License CC BY.

between the two effector T-cell subsets, and major synapse-defining features would be highlighted in the comparison against phagocytic contacts.

5.2 Results

5.2.1 Cytolytic synapses are stronger and more complex than non-cytolytic synapses

To compare CD8⁺ OT-1 CTLs and CD4⁺ helper T_HCs, we cultured OT-I T cells in parallel with CD4⁺ T cells expressing the class-II-MHC-restricted 5C.C7 TCR (bearing the IE^k-MCC antigen). Measurements were performed using the same preparation

of α CD3 ϵ /ICAM1-coated particles to circumvent potential artifacts arising from the differential TCR-pMHC binding affinities (Figure 5.2A). T_H Cs formed crater-like contacts that were similar to CTL synapses in terms of their overall structure (convex rim, concave floor), suggesting that the compressive configuration is a general feature of T-cell contacts. However, CTLs exhibited significantly stronger mechanical outputs across all measurable parameters (Figure 5.2B-E). First, CTLs displaced more of the hydrogel volume, indicating stronger compressions. They also induced deeper and more numerous indentations and steeper curvatures in general on the hydrogels than did CD4+ cells.

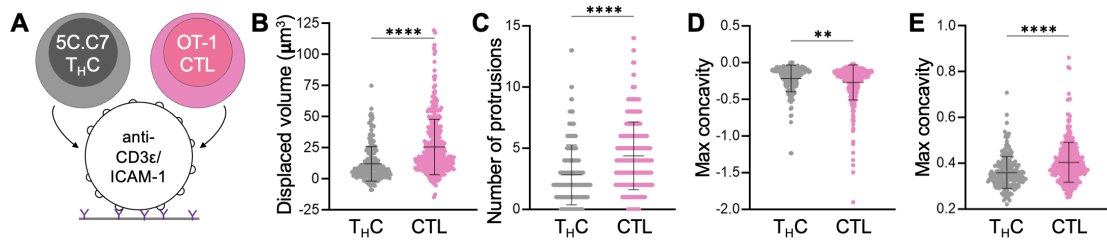


Figure 5.2: Comparison of CD4+ and CD8+ mechanical outputs during synapse formation.

A: Schematic demonstrating the measurement of synapse mechanical outputs against the same batch of α CD3 ϵ /ICAM1-coated particles. B-E: Quantification of particle compression by volume displacement, number of discrete protrusions, the concavity of protrusions, and convexity of reliefs across the synapses. $n_{CD4} = 217$, $n_{CD8} = 331$. ** and **** denote $P \leq 0.0021$ and $P \leq 0.0001$, respectively, calculated by unpaired Welch's t-test. Error bars indicate SD. Data are representative of $n=3$ independent experiments. Panels A-E reflect data from Figure S5A-E in [262].

This increased penchant for compression by CTLs could be seen more clearly in a discretized version of the radially collapsed synapse representation from Figure 3.3E (Figure 5.3A-B). Here, we divided the synapse radially into twenty discrete shells emanating from the contact center ($r = 0$) to the edge ($r = 1$), and then in each of the vertical columns further plotted the frequencies of various curvatures (ranging from around -0.5 to +0.7) represented in each radial shell. By doing so per cell (Figure 5.3B) and averaging across all replicates of a cell type, each data type (e.g., CD4+, CD8+) was associated with a cell-type-specific radial distribution matrix of deformations, expressed as probability densities (PD). Subtracting the map of one cell type from another

(Figure 5.3C) allowed us to note in this *difference map* of radial curvatures which specific mechanical activities were enriched in one cell type over the other: it revealed, for example, that CD4+ T_H Cs were enriched all throughout the synapse in weakly concave curvatures that resemble unindented spheres more closely; on the other hand, concave curvatures closer to the center and relatively strong convex curvatures at the periphery were associated with CD8+ CTLs. These not only confirmed the displaced volume and local topographical feature enumerations, but highlighted that even amongst effector T cells, CTLs are distinguished by their specific ability to form compressive craters containing protrusions and reliefs. While T_H Cs can as well, they do so to a lesser extent.

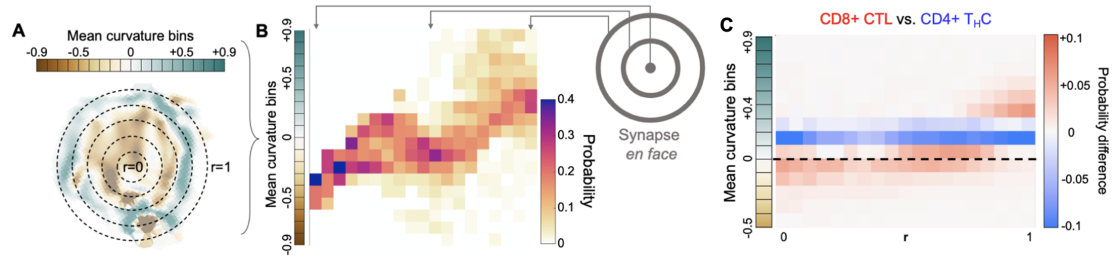


Figure 5.3: Difference map of topography as a function of radial location for CD4+ and CD8+ T_{eff} cells.

A: Schematic of the process. Briefly, all the vertices of each synapse were assigned radial distances as previously described (see Chapter 3.4.2, Equation 3.1). Then, each vertex was assigned into one radial bin out of twenty (r from 0 $\rightarrow$ 1), and one curvature bin out of twenty (from -1 $\rightarrow$ +1, in increments of 0.10 curvature units). B: Matrix representation of the data. The abscissa represents the radial distance, while the ordinate represents the curvature bin, and the color represents the frequency of that particular x-y "box" within the radial shell. Seen this way, the columns are histograms of curvature, here truncated to the range -0.9 $\rightarrow$ +0.9 within radial shell r . C: Difference map showing the δPD of CD8 - CD4 populations. Panels A-B are reproduced from Figure 2A-B of [262], while panel C reflects Figure S5F of the same source. Data are representative of $n=3$ independent experiments.

In terms of patterning, CTLs also appeared to induce superior distortions overall compared to T_H Cs (Figure 5.4). In the Z-PCA analysis of this dataset ($n = 548$ total), data was spread along a PC1 axis that again represented complexity and radial asymmetry. Dividing the PCA plot into terciles along PC1 (T1–T3) identified compressive synapses with a gradient of claw-like protrusive behavior. Lytic and non-lytic topographies were

present in all three regions, implying that no single functional subtype of T_{eff} synapse exclusively induces strong, complex topographies on their targets. However, we found that almost half of the T_HC synapses fell into the PC1-low region T1, while only a quarter of CTL contacts did so. CTL synapses also exhibited significantly higher spectral complexity values than their T_HC counterparts, indicating that CTLs impart stronger and more complex distortion patterns than T_HCs . This further substantiates the link between cytolytic activity and topographical complexity, even when comparing effector T cells differentiated in parallel.

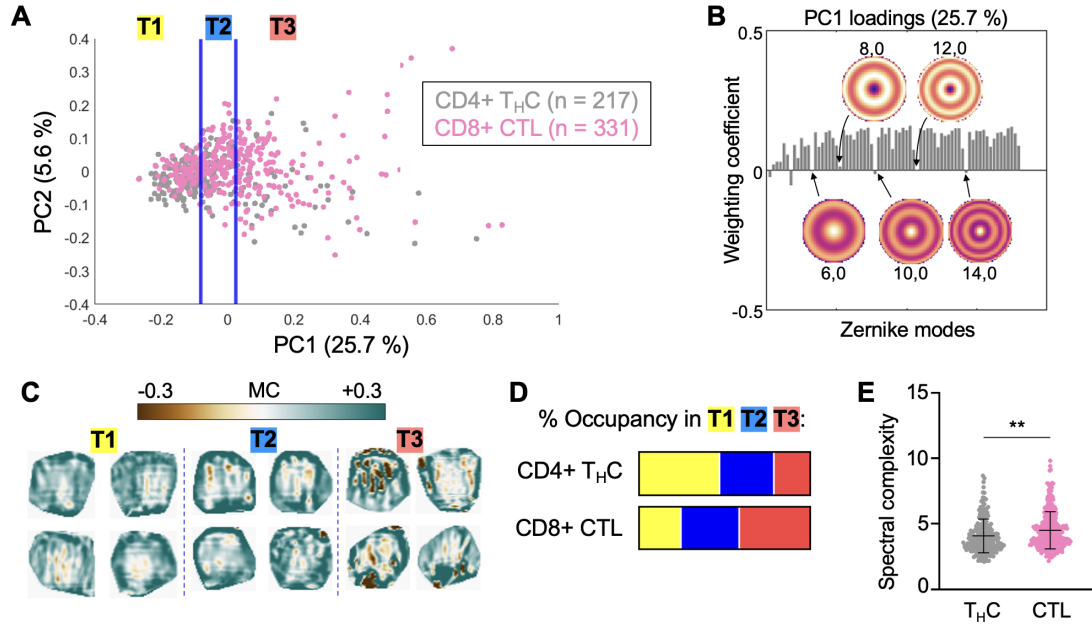


Figure 5.4: Comparative patterning studies of CD4+ and CD8+ T_{eff} cells.

A-B: Z-PCA of both subsets, with PC1 loadings diagram. C-D: Representative topographies from each tercile, alongside the population disproportions of each cell type into the three pattern groups. E: Spectral complexity metric between CD4+ and CD8+ cells. ** denotes ≤ 0.0021 , calculated by permutation testing with Benjamini-Hochberg correction for multiple hypothesis testing. Error bars indicate SD. Panels A-B reflect data in Figure S5G-K of [262]. Data are pooled from all cells in n=1 biological replicate, and are representative of n=3 independent experiments.

5.2.2 Primary macrophages form protrusive, undulatory contacts that are globally tensile

We next examined the mechanical output pattern of phagocytosis, and note what mechanical output patterns may distinguish it from synapsis. We therefore prepared both IgG- and α CD3 ϵ /ICAM1-coated hydrogels from the same 300-Pa preparation, and mixed them with BMDMs and OT-I CTLs, respectively (Figure 5.5A). BMDMs were fixed at 15 mins, as phagocytic cup closure occurred with high frequency beyond this point. Upon first glance, the *en face* macrophages appeared to induce strongly protrusive contacts just like CTLs (Figure 5.5B). However, cross-sectional views revealed that these phagocytic contacts were globally tensile, pulling at the center of the phagocytic cup (Figure 5.5C). This bore out in the quantification of compression strength *via* volume displacement, wherein macrophages exhibited a negative displaced volume, suggesting *volumetric extension* along the contact rather than compression (Figure 5.5D). This indicated either a pulling force or an axial bulging of the hydrogel induced by squeezing along the sides.

This pattern of mechanical activity, which is consistent with ongoing engulfment, was essentially opposite to the global compression seen in CTL synapses. To quantify this observation, we once again generated difference probability densities of the curvatures arranged from center to periphery (Figure 5.5E): whereas CTL plots sloped upward (blue), indicating a tendency for cratering and compression, BMDM plots sloped downward (red), reflecting central tugging and peripheral squeezing. BMDMs displayed more numerous and deeper protrusions than CTLs (Figure 5.5F-H), but appeared to organize these structures in a fundamentally different way: Indeed, some individual macrophage topographies exhibited strong concavities that were focused on the periphery (Figure 5.5B, lower pair), akin to the phagocytic "teeth" previously described on similar hydrogels that mediate lateral pinching [259]. In other instances, protrusions were evoked closer to the

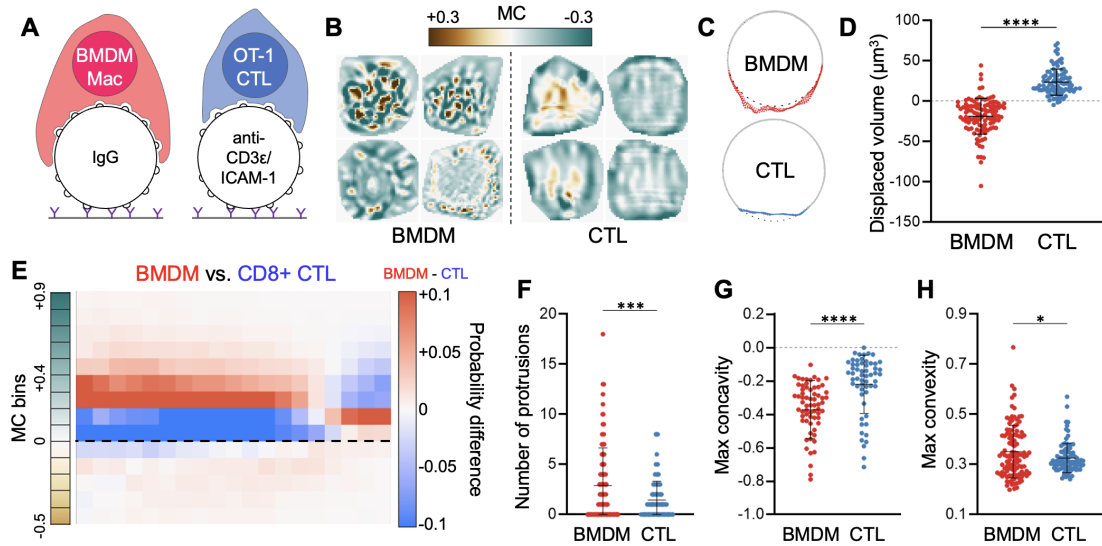


Figure 5.5: Metrics of strength and local topographical feature generation of BMDMs and CD8+ CTLs.

A-B: Schematic of the experiment, and representative topographies from each cell type. C: Representative cross-sections emphasizing the tensile nature of the phagocytic contact, vis-a-vis the compressive contact of the CTL. D: Quantification of displaced volume, where BMDMs induce "negative compression" relative to CTLs. E: Difference PD of radial curvature distributions, plotted as BMDMs - CTLs. F-H: Quantification of protrusion number and feature steepness of protrusions and reliefs. $n_{BMDM} = 109$, $n_{CTL} = 96$. *, ***, and **** denote $P \leq 0.0332$, $P \leq 0.0002$, and $P \leq 0.0001$, calculated by unpaired Welch's t-test. Error bars indicate SD. Panels A-H reflect data in Figure 6A-H of [262]. Data are representative of $n=2$ independent experiments.

center of the contact but did not alter the convex tone of this region (Figure 5.5B, top pair). Collectively, these results indicate that phagocytosis and synapsis are fundamentally distinct, and in some ways opposite, forms of immune-mechanical activity.

Z-PCA appeared to cleanly separate phagocytic cups from CTL synapses (Figure 5.6A). As with previous datasets, PC1 contained contributions from most Zernike modes but excluded key concentric functions, indicating that even among BMDMs and CTLs, complexity and radial asymmetry can distinguish individual patterns from each other. Accordingly, for both cell types, topographies with strongly undulating protrusive character occupied PC1-high regions, while interactions with more muted variations were PC1-low. Interestingly, in this dataset PC2 segregated CTL synapses, which were

overwhelmingly PC2+, from the largely PC2- BMDM contacts. PC2 loadings included sizable contributions from two particular low order, concentric Zernike modes reflecting global convexity (2,0) and concavity (4,0) (Figure 5.6B-C), which together distinguished the globally compressive synapses from tensile phagocytic cups.

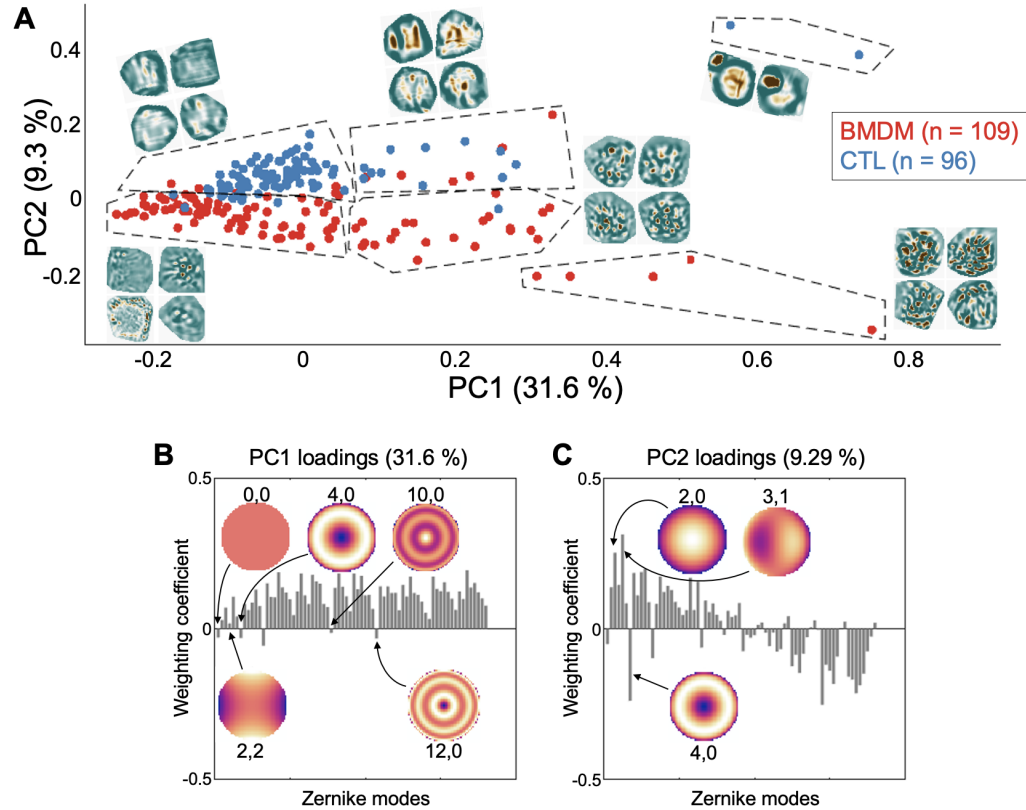


Figure 5.6: Spectral pattern analysis of BMDMs and CTLs.

A: Z-PCA of BMDMs and CTLs, with manually "gated" regions and representative topographies from those regions overlaid. B-C. Loadings diagrams for PC1 and PC2, respectively. For PC1, minimally contributing patterns are highlighted; for PC2, large contributors are highlighted. Panels A and B-C reflect data from Figure 6I and Figure S8 in [262], respectively. Data are pooled from all cells of n=1 biological replicate, and are representative of n=2 independent experiments.

Thus, even by using curvature-based patterning analysis, the globally convex (tensile) character of BMDMs distinguished their cell-cell contacts from that of the globally concave (compressive) CTL synapses. Taken together with the earlier set of measurements, these results demonstrate that in contrast to T-cell synapsis, BMDMs strongly manipulate the target surface within a globally tensile configuration that accentuates the convexity of

target cargo. However, they too, dynamically micropattern their targets *via* finger-like protrusions that may be F-actin-driven. Our observations on BMDMs reduced the likelihood that the compressive nature of T-cell contacts, which we have consistently seen across all CD4+ and CD8+ T cells measured, was simply an artifact resulting from cell spreading on a soft, spherical particle. Thus, the marked differences between these two mechanotypes strongly suggest that they do not result inevitably from close contact and adhesion, but rather reflect the distinct functions of these interfaces.

5.3 Discussion

5.3.1 A plurality of immune mechanotypes

In this section, we leveraged two more mechanotype comparisons to investigate how ontogeny determines the mechanical outputs of immune cells. By comparing CD4+ and CD8+ T_{eff} s, we established that compression strength, sharp topographical features, and undulatory complexity are associated more with cytolytic activity than with T-cell help. These two cell types, functionally distinct but derived from a common progenitor and in approximately similar activation states, nevertheless exhibited consistently measurable differences in mechanical output. Thus, we suggest that the extent to which a T cell can mechanically compress a target surface, as well as distort it in an undulatory fashion, reflect a T cell's cytotoxic capacity.

An interesting scenario with which to pit ontogeny against function would have been the inclusion of CD4+ CTLs [319]. Indeed, we profiled TRP1^{tc^r-tg} CD4+ CTLs in parallel with the CD4+ T_H C and CD8+ CTL experiments, and gathered evidence favoring neither ontogeny nor function exclusively (not shown)—CD4+ CTLs exhibited

intermediate levels of target compression and similar spectral complexities as CD8+ CTLs. However, these cells were not included in the final datasets, because the kinetics of cell activation, growth, and differentiation of the TRP1 cells were different from those of the conventional CD4+ and CD8+ T_{eff} s.

On the other hand, comparing the BMDMs to CTLs was motivated by some differences that we previously noted [258]. This initial study focused on validating the hydrogel system using different cell types rather than making any cell-type comparisons, however—thus, we embarked on the first systematic, head-to-head comparison between phagocytosis and synapsis against the same preparation of 300-Pa beads. BMDMs were stronger and more protrusive than any CTL subset queried thus far; however, they did not compress their targets, instead maintaining an extensive, tensile contact in spite of their strongly protrusive character. Indeed, other work in our lab has demonstrated that this is a *bona fide* configuration adopted by macrophages on soft particles, and not simply an intermediate phagocytic state or a reflection of phagocytic failure [264]. It was rather remarkable that loadings from the Z-PCA would allow us to differentiate phagocytosis and synapsis on the basis of *global* convexity or concavity, given that mean curvature is a local surface metric. Along with our measurements of displaced volume and the differential radial curvature maps, these results confirm that T-cell synapsis is characterized by its compressive character—and among synapses, the extent of compression and/or undulatory complexity engendered on the target surface are functional metrics of a cell’s cytolytic capacity.

Future work ought to profile other functional subsets of T cells in parallel, such as CD4+ T_{reg} s, CD4+ T_H Cs of different polarizations, and perhaps once again attempt to include TRP1 CD4+ CTLs. Effects of pro- and anti-inflammatory cytokines on CD8+ CTL mechanics would also be of interest, in order to see whether cytolysis-associated mechanical output could be tuned by a cell’s local *milieu*. For macrophages, investigating

the effects of different phagocytosis-inducing surface molecules on resulting contact mechanics would also be illuminating, as macrophages impose non-phagocytic, synaptic interactions with other cells (e.g., CD4+ T_HCs). Indeed, one tantalizing possibility, considering results from a parallel project in our lab [264], is that a mechanical checkpoint for the phagocytosis of soft targets exists in order to instruct macrophages to attempt synapse formation before engulfment.

5.3.2 Cell size as a determinant of contact mechanics: a brief, neutrophilic detour

These results together raised an important question: is cell size a major determinant of contact mechanics? BMDMs are orders of magnitude larger than T cells, and by extension, the hydrogel probes used in this work; does this predispose them to wrapping around targets instead of compressing them? T cells, on the other hand, are usually much smaller than the target APCs with which they form synapses; does this predispose them to pushing into their targets instead of wrapping around them?

Neutrophils make an interesting test case for answering this question, as they are approximately T-cell-sized phagocytes. Indeed, we attempted to profile Hoxb8-differentiated neutrophils against the same 300-Pa batch of IgG-coated hydrogels; however, across multiple attempts, they did not attempt to phagocytose the beads (not shown). Because the same neutrophils readily engulf 3-kPa beads, it is possible that target rigidity represents a mechanical checkpoint for neutrophil commitment to phagocytosis. However, before giving up on this question, future work should attempt to override this reluctance with higher concentrations of IgG, as has been very recently shown in BMDMs [264].

5.3.3 Preliminary exploration of cytoskeletal taxonomies within the immune system

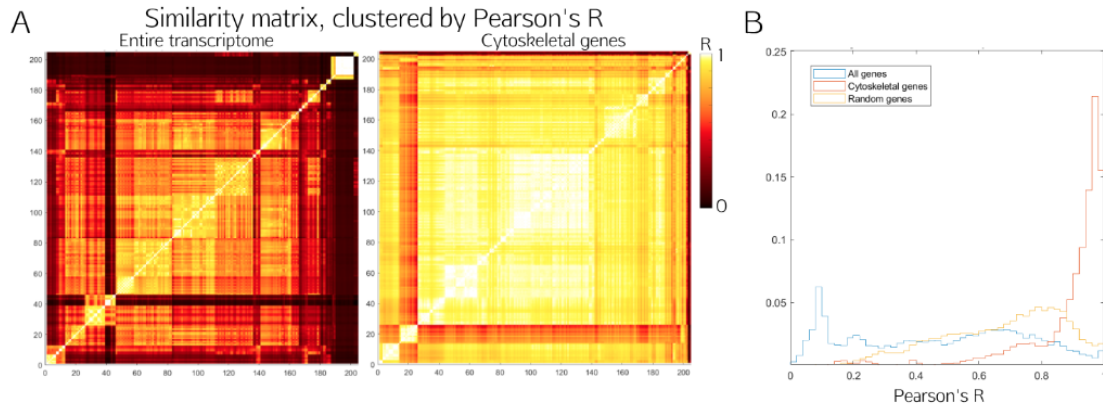


Figure 5.7: Similarity analysis of the ImmGen transcriptomes dataset.

A: Across the sample space, pairwise inter-sample Pearson's R values were calculated based on the entire transcriptome (left) or a subset representing the cytoskeletal genes (right). Samples were then grouped by R value to reveal blocks of cell types that associated with each other based on the gene set queried. B: Distribution of pairwise Pearson's R values as a probability histogram. Curves are shown based on all genes, cytoskeletal genes, and randomly selected genes in a size-matched bin with the cytoskeletal gene set. Data processing and cleanup were performed by Alex Settle, while I determined the analysis approach.

My data show that functional sub-specification of immune cells, even across ontogenically related subsets (i.e., CD4+ and CD8+ T_{eff}), is associated with differences in the capacity for eliciting specific patterns of mechanical output. On the other hand, ontogenically distinct subsets (i.e., CD8+ T cells and macrophages) elicited quite different kinds of contacts, in keeping with their functions. Thus, we wondered if cytoskeletal differences exist on the transcript level across immune subsets. We queried the ImmGen RNA-sequencing database of 86 FACS-sorted cell populations, which spanned stromal, B-, T- ($\gamma\delta$, $\alpha\beta$, activated), monocytic, stem, dendritic, and phagocytic cell subsets (GSE109125, [320]). Interestingly, grouping samples into similarity *via* a simplistic k-means clustering approach revealed how little variation immune cells exhibit based on

cytoskeletal gene expression compared to the entire transcriptome or a size-matched gene set of random genes (Figure 5.7).

This was highlighted in a comparison of the inter-sample Pearson's R within each of 28 distinct gene sets, linearly scaled by subtracting the inter-sample Pearson's R of a randomly chosen, size-matched gene set (Table 5.1). By this metric, only four gene sets exhibited positive values: '*actincytoskeleton_actomyosin*', '*cellcycle_cellcyclephase*', '*cytoskeleton_septin*', and '*lamellipodium*'. Notably, three out of four of these explicitly refer to cytoskeletal gene subsets, further implying that cytoskeletal genes are more transcriptomically convergent than the average gene ontology term/set of terms.

Does this imply that there is a limited number of ways to (physically) blueprint a cell, or that within this relative parsimony of expression patterns, translational and post-translational regulatory diversifications are the *de facto* control points of cellular "hardware"? Either way, future studies are warranted to systematically explore the relationship between morphotypical diversity, cytoskeletal gene expression patterns, and cytoskeletal protein expression patterns across the immune system [321].

5.4 Materials and methods

5.4.1 Cell culture, hydrogel derivatization

OT-I CTLs were cultured as previously described. BMDMs were differentiated over a 7-day period from C57BL/6J bone marrow isolates using L929-conditioned media, as previously described [322].

Table 5.1: Gene set comparison according to inter-sample Pearson's R.

Listed are different gene ontology terms and their corresponding inter-sample Pearson's Rs according to the transcriptomic data of the ImmGen database. These R values were corrected by subtracting the inter-sample Pearson's R of a randomly selected, size-matched gene set. Values beyond ± 0.2 are highlighted in green (+) and red (-), while those between 0.1–0.2 are highlighted in yellow.

Gene set	Pearson (actual - size matched random set)
'actincytoskeleton_actomyosin'	0.233877
'apoptosis'	-0.3016
'autophagosome'	0.005067
'cellbody'	0.045182
'cellcycle_cellcyclephase'	0.232005
'centriole_centrosome'	0.017777
'cisgolgi'	-0.09534
'corticalactin'	0.022745
'cytoskeleton_septin'	0.214602
'endocyticvesicle_snare'	-0.08567
'endolysosome_lysosomallumen'	-0.31703
'glycolysis'	0.119903
'humantranscriptionfactorsCCBRUToronto'	-0.25666
'intermediatefilament'	-0.04021
'ipafinflammasome_lps_nadphoxidase_nlrp3inflammasome_ros'	-0.25356
'lamellipodium'	0.204023
'microtubule'	0.152033
'nheirepair_nucleotideexcisionrepair'	-0.32081
'nuclearbody_nuclearenvelope_nucleusorganization'	-0.07908
'phagocyticcup_vesicle_membrane_phoreassemblysite_lysome'	-0.05781
'precatalyticspliceosome_prespliceosome'	0.150294
'proteinsecretion_ribosome'	-0.07549
'tcrcomplex'	-0.31159
'tnfasignalingnfkb'	-0.33496
'ubiquitinliqase'	-0.01102
'voltageKchannel_voltageNachannel'	-0.12234

For macrophage experiments, batches of 5.0×10^6 fluorescent streptavidinylated hydrogel particles at a time were incubated with 500 nM biotinylated mouse immunoglobulin G (IgG, Jackson ImmunoResearch 015-000-003). After all coatings, beads were washed > 3 times by centrifugation at $12,000 \times g$ and resuspension in clean PBS, and then used for imaging experiments within 3 days. Because murine IgG was shotgun-biotinylated, the coating orientations are random and cannot be assumed to be stimulatory on a per-molecule basis as the pMHC molecules. The capacity of these coating concentrations to stimulate phagocytosis was empirically determined by flow cytometry and microscopy [263, 264]. For high-resolution microscopy, $5\text{--}7.5 \times 10^4$ day-7 cultured macrophages were deposited onto 8-well chambers directly and left to attach and acclimatize overnight. The next day, $0.5\text{--}1.0 \times 10^5$ IgG-coated particles were deposited onto them, and conjugates were PFA-fixed after ≤ 15 min and stained as previously described.

After imaging, BMDM contacts were processed in the same way as the CTL contacts (see Chapter 2.4.6), with the only change imposed being an additional filter for the "progression of contact", to account for the forward-directed evolution of the contact towards full engulfment. By visual inspection of the world-map ($\theta - \phi$) projections, it was found that beyond 40 % engulfment, macrophage phagocytic cups could not be anymore segmented using the same methodology as CTL synapses. Therefore, only contacts within 15–40 % engulfment were included in this analysis, which included 109 cells.

5.4.2 Radial profiling

After cell-contact demarcation and radial distance assignation of each synapse image (d_{norm} , see Chapter 3.4.2), each vertex was assigned into a radial distance bin $\mathbf{r}$ (from 0 to 1, in 0.1 distance steps) and a curvature bin $\mathbf{c}$ (from -1 to +1, in 0.1 curvature steps).

Then, for each of the 20 radial bins, the relative frequencies in each curvature bin were calculated. The final probability density (PD) map of radial curvature profiles was plotted as a 3-D matrix, with $x = \mathbf{r}$, $y = \mathbf{c}$, and $z = \text{frequency}$. Population-wide PD maps were calculated by calculating the average PD maps of a given cell/data type, and difference PD maps were calculated by subtracting one population-wide map from another.

CHAPTER 6

MODULATING THE CYTOLYTIC F-ACTIN ARCHITECTURE

6.1 Background

6.1.1 Cell-intrinsic and cell-extrinsic influences on synaptic actin architecture

While T-cell activation is mechanosensitive across a large range of rigidities [243, 244, 245, 47, 246], within a more limited physiological range of 2–25 kPa, CD4+ and CD8+ T cells exhibit different degrees of mechanosensitivity. Specifically, mouse CD8+ T cells display a dampened differential response to rigidity relative to their CD4+ counterparts based on CD69 expression, CD25 expression, and proliferation [248]. Earlier, I suggested that this could reflect a greater need for co-stimulation by CD4+ T cells in general, or reflect a functional benefit to cytolytic immune cells adapting a mechanically agnostic disposition. However, *in vitro* differentiated CTLs do exhibit mechanosensitive degranulation [248] and cytokine secretion [250, 251], which contribute to the selective eradication of stiffer tumor cells (e.g., those with cytoskeletal mutations enhancing their spreading and colonization properties) [323, 250]. Thus, it appears CTLs are able to sense and interpret rigidity information of APCs *via* the synapse, presumably through integrin-mediated mechanotransduction and actin-based activity. In this Chapter, we investigate how stiffness can act as a cell-extrinsic determinant of synaptic cytoskeletal (F-actin) architecture.

In the search for a cell-intrinsic factor that could impact synaptic architecture, we leveraged previous work on PI3K and PTEN in CTLs [151]: PI3K enhances T-cell

signaling through Akt activation, but also contributes to actin assembly at the synapse by recruiting the Rac1 GEF DOCK2/ELMO1 to PIP₃ on the inner leaflet. PI3K is naturally antagonized by the PIP phosphatase PTEN, which acts as a general tumor suppressor in many cell types, including T cells [324]. Because of its inhibitory effects on actin templating, PTEN-KO CTLs demonstrate enhanced spreading, synapse formation, axial force exertion, and cytotoxicity *in vitro* [191]. Thus, we reasoned that this specific perturbation would be a viable approach to attempt a gain-of-function measurement on our hydrogel system (Figure 6.1).

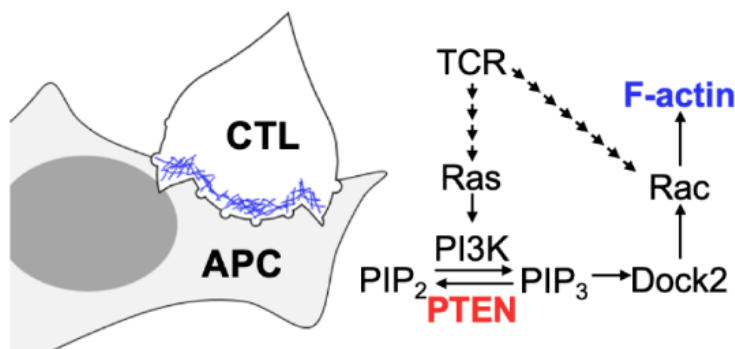


Figure 6.1: PI3K and PTEN balance in driving synaptic F-actin polymerization.

A simplified diagram of the TCR signaling pathway is presented, highlighting the involvement of PIPs in F-actin assembly at the synapse.

6.2 Results

6.2.1 PTEN-KO CTLs display enhanced cytolytic and mechanical activities

We generated PTEN-KO OT-I CTLs using CRISPR-Cas9 methodology in a manner similar to the generation of WASp-, WAVE2-, and talin-KO CTLs. Because PTEN acts

as a tumor suppressor, sgPTEN CTLs expanded more aggressively in culture than sgNT CTLs, eventually outgrowing the minority of untransduced cells to reach near-100 % sgPTEN+ by day 5. Killing assays were conducted by mixing OVA-pulsed EL4 target cells with sgNT- or sgPTEN-transduced Cas9+ CTLs. Cells were incubated for 3-4 hours and assayed for viability using morphological metrics (FSC/SSC) on a Beckman Coulter CytoFLEX flow cytometer. To measure degranulation, CTLs were co-incubated with antigen/ICAM1-coated hydrogel beads for 1.5 hours in the presence of excess α CD107a-eFluor660 (eBioscience clone 1D4B). Compared to control cells, sgPTEN CTLs exhibited a greater capacity to kill OVA-pulsed target cells while simultaneously releasing fewer lytic granules—a possible indication of enhanced cytolytic mechanopotential (Figure 6.2).

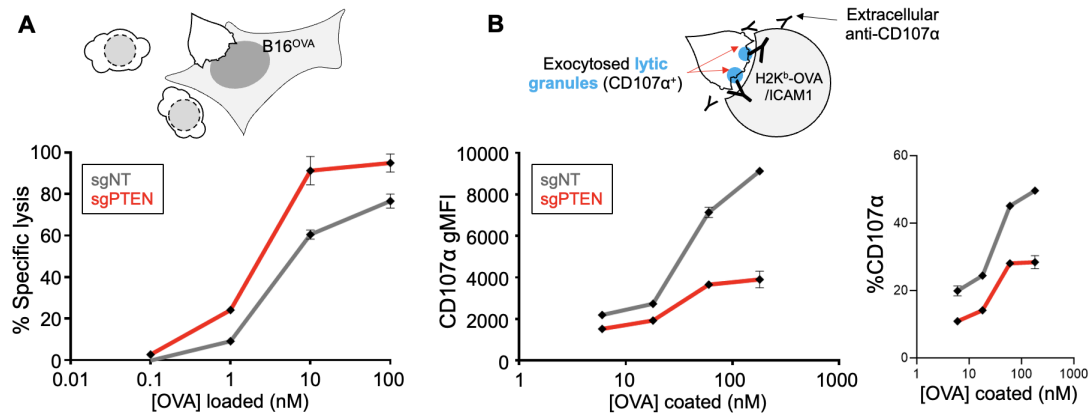


Figure 6.2: Cytotoxicity and degranulation of sgPTEN CTLs.

A: EL4 cells were pulsed with OVA and incubated for 3-4 hours with sgNT or sgPTEN cells, after which cytotoxicity was measured by flow cytometry. Specific lysis was calculated relative to the wells containing OVA- EL4 cells. B: 300-Pa hydrogels were coated with H2K^b-OVA/ICAM1, and incubated with both genotypes of CTLs for 1.5 hours with lysosomal staining antibody. Left graph shows the intensity levels by gMFI of externalized CD107a, while the right graph shows the % CD107a+ cells per condition, gated against a fluorescence-minus-one control. Error bars indicate SD. Cytotoxicity data are representative of at least n=2 independent experiments, while degranulation data are representative of n=2 independent experiments containing n=3 biological replicates per curve.

sgNT and sgPTEN cells were then mechanically profiled in parallel, by presenting them with the same preparation of H2K^b-OVA/ICAM1-coated beads (Figure 6.3). Both cells generated similar numbers of protrusions on their targets and similarly steep curvatures across these features, but sgPTEN cells compressed target hydrogels to a greater extent and also generated more convex reliefs throughout the synapse.

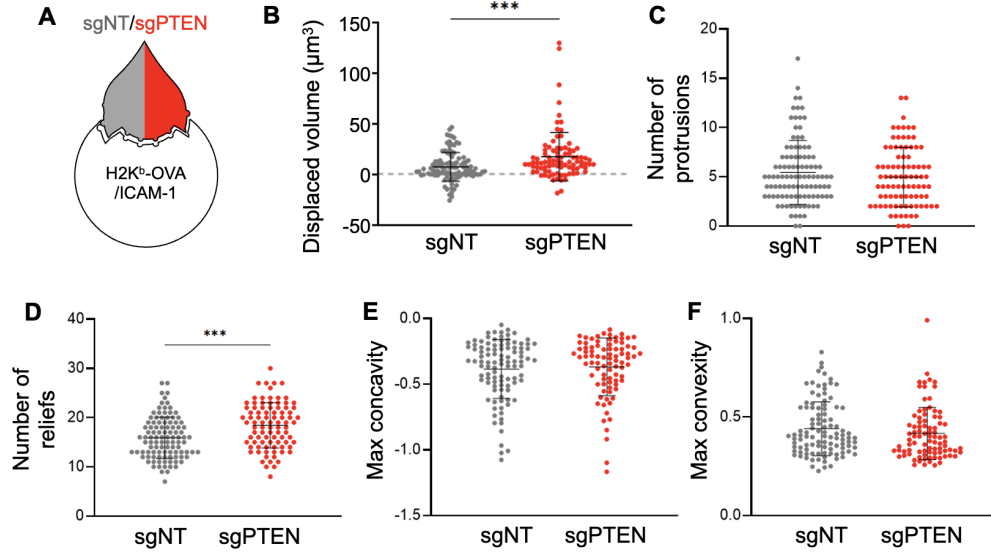


Figure 6.3: Comparison of compression strength and topographical feature generation by sgPTEN CTLs.

A: Schematic of the experiment. B: Comparison of displaced volume. C-F: Number of protrusions and reliefs, as well as concavity and convexity measurements of local topographical features. $n_{sgNT} = 104$, $n_{sgPTEN} = 89$. *** denotes $P \leq 0.0002$, calculated by unpaired Welch's t-test. Error bars indicate SD. Data are pooled from $n=3$ biological replicates.

In terms of topographical patterning, little difference was seen (Figure 6.4): sgPTEN and sgNT cells largely occupied similar regions of the Z-PCA plot, and while sgPTEN spectral complexity appeared to trend upward, no statistical difference was measured between the groups. These results suggested that sgPTEN CTLs exhibit enhanced mechanical activity through increased target compression rather than a qualitative change in target micropatterning.

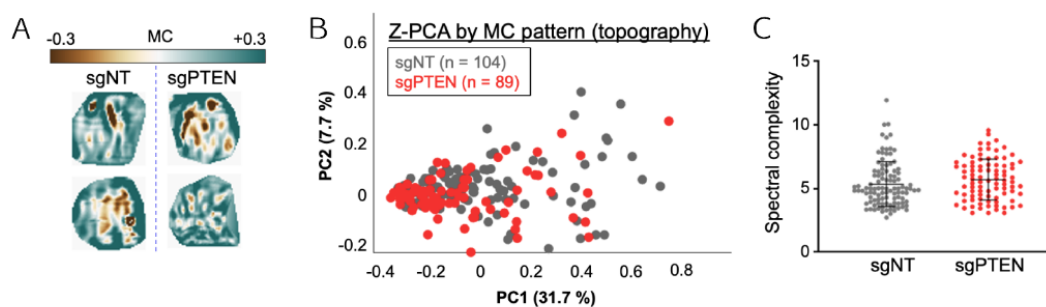


Figure 6.4: Comparison of topographical patterning by sgPTEN CTLs.

A: Representative topographies generated by sgNT and sgPTEN cells on 300-Pa hydrogels. B-C: Z-PCA plot and spectral complexity measurements. Data are pooled from n=3 biological replicates.

6.2.2 Applying the Zernike transform to Phalloidin maps reveals an emergent cytoskeletal pattern

In order to determine if synapse architectural changes could be induced by (1) the genetic perturbation of PTEN (cell-intrinsic) or (2) the rigidity of the target hydrogel (cell-extrinsic), we imaged four batches of cell-hydrogel conjugates, all fixed and stained with Phalloidin: sgNT and sgPTEN cells interacting with 300-Pa and 3-kPa beads (Figure 6.5A). This time, we used the actin intensity patterns of the CTLs, extrapolated onto a similar 50×50 grid as the topographies, as the inputs for conducting a Z-PCA analysis (Figure 6.5B). We noticed on this plot that the PC1-high region was populated by a large proportion of sgPTEN cells against 3-kPa beads (red Xs), suggesting a particular actin pattern was enriched in this conjugate combination.

Indeed, by splitting this Z-PCA into terciles (T1–T3), we found that the PC1-high T3 contained mostly annular actin patterns, resembling the concentric, SMAC-like organization of actin on supported lipid bilayers or target cells (Figure 6.6A). This peripheral focusing of F-actin was not likely to be indicative of an initial spreading phase,

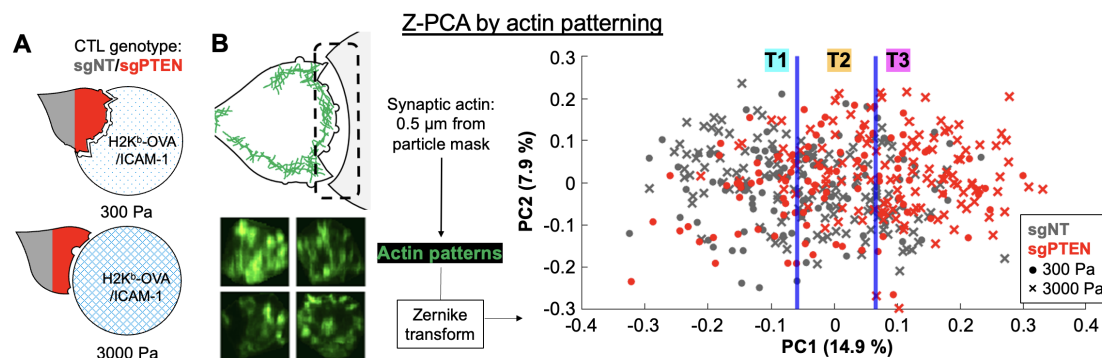


Figure 6.5: Sorting actin intensity patterns by Z-PCA.

A: Schematic depicting sgNT and sgPTEN cells imaged against 300-Pa and 3-kPa hydrogels for this dataset. B: For each cell-hydrogel conjugate, the "synaptic actin" signal within 0.5 μm of the hydrogel mask was taken, max-normalized, and used as the input for the Zernike transform and Z-PCA (right). Data are pooled from $n=3$ biological replicates, $n_{\text{sgNT},300\text{Pa}} = 104$, $n_{\text{sgPTEN},300\text{Pa}} = 89$ (same pair as in the previous two figures), $n_{\text{sgNT},3\text{kPa}} = 121$, $n_{\text{sgPTEN},3\text{kPa}} = 133$.

because 30–45 mins is typically sufficient time for both types of CTLs to degranulate and kill. As expected from this interpretation of PC1, its loadings reflected the presence of two ring-like patterns: Zernike circle polynomial (2,0), with which PC1 scaled positively, and Zernike circle polynomial (4,0), with which it scaled negatively. In addition, by measuring the population distributions of the four types of conjugates into T1–T3, we noted the presence of annular actin patterns under all conditions, but this was especially apparent in sgPTEN cells confronted with the relatively stiff 3-kPa targets (Figure 6.6B).

We developed a metric to directly measure the degree of central or peripheral focusing of the F-actin signals across a synapse. "Actin centralization" was calculated by dividing the synapses into "outer" and "inner" regions, and comparing their MFIs (Figure 6.7A). Because of the way the synapse boundaries are initially selected, we first excluded the outermost 10 % of the data. Then, we divided the outer and inner zones such that the area of the inner circle was twice that of the outer annulus. By this measure, we confirmed that the regions T1–T3 defined on the basis of PC1 value indeed separated data by along a central-to-peripheral axis, with T1 being the most centrally focused and T3 being the most

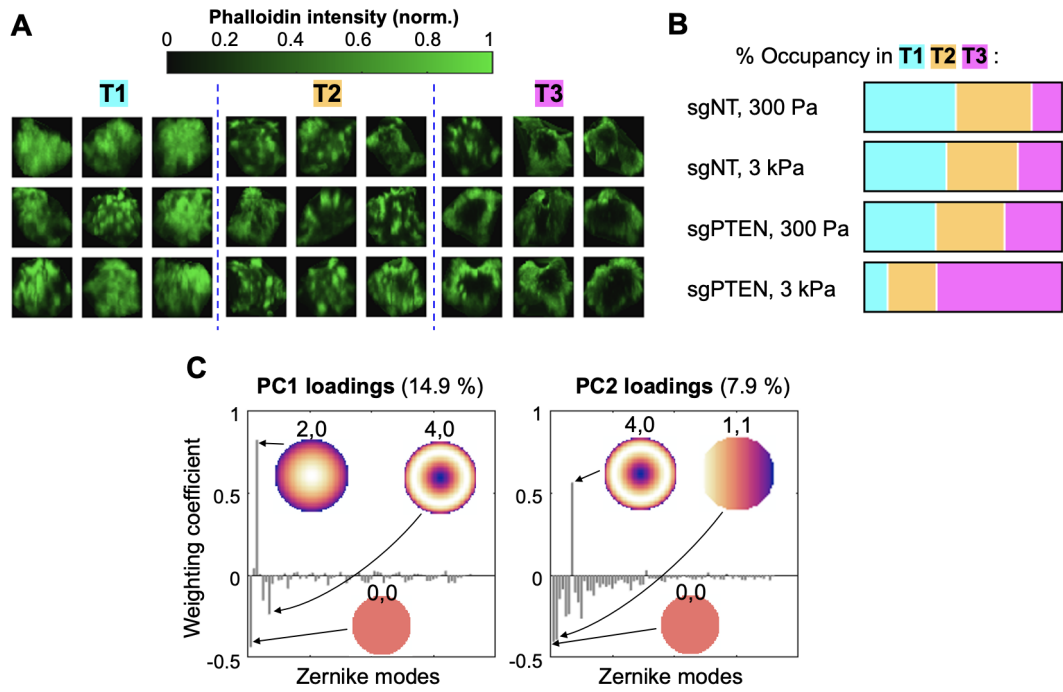


Figure 6.6: An emergent, annular pattern of actin is generated by PTEN-KO cells.

A: Representative actin signals from each tercile. B: Population distribution into each of the three terciles T1–T3. C: PC1 and PC2 loadings. Data are pooled from n=3 biological replicates.

peripherally focused, annular set of patterns. Interestingly, against both target rigidities, sgPTEN cells exhibited more peripheral/annular F-actin focusing. Furthermore, while sgNT cells did not exhibit any difference in actin centralization between the soft and hard beads, sgPTEN cells formed annular F-actin architectures against the harder beads, indicating a degree of mechanosensitivity to the actin patterning at the synapse.

Because sgPTEN cells have the capacity to form larger synapses than normal CTLs [151], we reasoned that a smaller, stiffer APC might lead a CTL to impose annular actin patterning on it. We tested this by comparing actin centralization elicited by the same batch of wild-type CTLs against 300-Pa, ~13- μ m beads and 1000-Pa, ~9- μ m coated with H2K^b-OVA & ICAM1 (Figure 6.7B). Indeed, against the smaller, stiffer population of beads, actin centralization decreased significantly, indicating that wild-type CTLs also access different actin-based architectures depending on the properties of their targets.

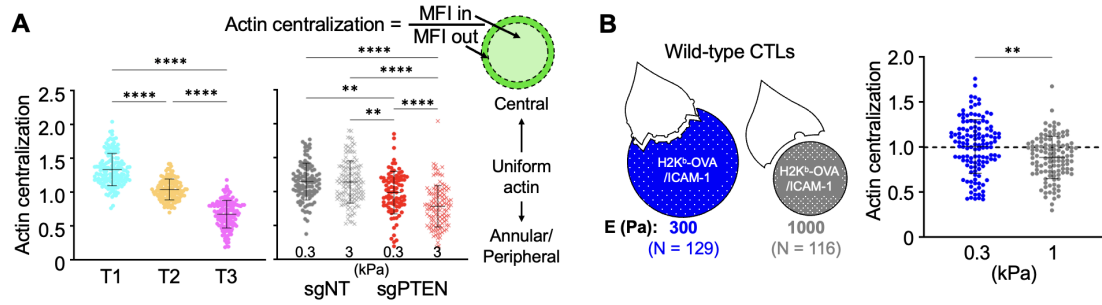


Figure 6.7: Actin centralization measured as a function of cell-intrinsic and cell-extrinsic features.

A: Actin centralization measurements of topographies in the different terciles of the Z-PCA plot (left), as well as of each of the four types of conjugates measured (right). Colors on the left plot reflect the color-coding scheme in Figure 6.6A-B, while the colors and symbols on the right plot reflect the Z-PCA plot in Figure 6.5B. ** and **** denote $P \leq 0.0021$ and $P \leq 0.0001$, respectively, calculated by multiple t-testing with Dunnett's T3 correction. Error bars indicate SD.

B: Actin centralization measured between wild-type CTLs encountering a large, soft bead and a small, hard bead. ** indicates $P \leq 0.0021$, calculated by unpaired Welch's t-test. Error bars indicate SD. Data are pooled from $n=3$ biological replicates.

Unfortunately, the synthesis of 9- μm 300-Pa particles proved technically challenging, precluding the direct uncoupling of size effects from rigidity effects in this experiment.

6.3 Discussion

In this final section, we explored cell-intrinsic and cell-extrinsic approaches to modulating the F-actin-based synaptic architecture of CTLs. We first leveraged a genetic PTEN-KO approach to enhance CTL synapse mechanical output, based on previous work that showed increased axial force exertion [191] and enhanced synapse formation by such cells [151]. In our hands, sgPTEN CTLs compressed hydrogel beads better and elicited deeper protrusions on them, although mechanical patterning remained largely unchanged. It is possible that the PTEN-KO phenotype is driven by enhanced WAVE2 activity, which would be expected to influence compression more than complexity; genetic

perturbations that increase WASp activity might selectively enhance spectral complexity without dramatically changing target compression.

We imaged sgNT and sgPTEN cells interacting with relatively soft (300 Pa) and stiff (3000 Pa) beads in parallel, then submitted not only the topographies, but also the actin intensity patterns to Zernike spectral decomposition and pattern sorting by PCA. In this manner, we not only demonstrated that the pattern sorting method we developed is generalizable beyond the synapse topographies used since Chapter 4, but also that the methodology we developed could be used to discover biomolecular patterns (e.g., annular F-actin) that functionally differentiate the imaged cells. We leveraged this knowledge to show that even wild-type CTLs could be coaxed to change their F-actin organization by presenting them targets with distinct mechanical characteristics.

This begs the question: what purpose might be served by a shift to annular, SMAC-like actin upon encountering smaller and stiffer targets? One possibility is that annular actin represents a synaptic adaptation that maximizes degranulatable area over target strain, in the language of Chapter 3. However, this is contradicted by our finding that sgPTEN cells generally secrete fewer lytic granules than their PTEN-replete counterparts. In fact, the enhanced target compression and protrusion exhibited by sgPTEN cells suggests that they can kill targets better in spite of depressed degranulation, perhaps through enhanced mechanopotential of perforin-1 [191]. This would suggest that the "actin-free" regions in the annular synapses still somehow preclude degranulation; indeed, super-resolution imaging of T-cell synapses by others has revealed that the cSMAC is not "clear" of actin *per se*, but rather contains a thin cortical meshwork of F-actin that precludes aberrant granule fusion [279, 38]. Perhaps, annular F-actin imposes global stretching of the target membrane *in lieu* of local protrusions (see "Shear traction force" distributions in [258]).

Nevertheless, it was rather remarkable that changing target mechanical properties (rigidity & relative size) could induce the adoption of an annular actin pattern by even wild-type CTLs. Future work is necessary to disambiguate the effects of target size and rigidity, either through side-by-side comparisons of CTLs against soft/small, soft/large, stiff/small, and stiff/large hydrogels, or by the measurement of CTL F-actin architectures against target cells pre-seeded on hydrogel substrates across a range of rigidities. This final set of experiments also brings forth the possibility that the relative sizes of CTL and the target APC help determine the ultimate configuration adopted by the intercellular conjugate; just like size differences between leukocyte and target could facilitate macrophage cupping over targets and CTL pushing into targets, so might more subtle size differences between CTLs and their targets determine the extent of central and peripheral F-actin focusing they establish at the synapse.

6.4 Materials and methods

6.4.1 Cell culture

Cas9+ CTLs were cultured as in Chapter 4.4.1 and transduced with sgNT or sgPTEN plasmids. Our sgPTEN construct was subcloned into an MSCV backbone containing eGFP, facilitating confirmation of guide RNA expression by flow cytometry.

6.4.2 Killing and degranulation by flow cytometry

For cytotoxicity assays, RMA-S cells were pulsed with OVA at different concentrations for 1 hour, then washed 2-3 $\times$ with PBS. In the meantime, sgNT and sgPTEN OT-I CTLs

were stained with cytosolic dye and then co-incubated with the OVA-pulsed targets for 3-4 hours. Cells were then harvested and analyzed for viability by flow cytometry.

For the degranulation assay, B16 cells were seeded 8000/well into 96-well plates in triplicate. They were pulsed with OVA for at least 1 hour on the day of the assay, and then washed out 2-3 $\times$ with PBS. Before co-culture with OT-I CTLs, α CD107a antibody was added to the cells for a final concentration of 1:100 with the OT-I cells (eBioscience 1D4B, eFluor 660 conjugate). Co-cultures were left to incubate for 1.5 hours and then harvested for analysis by flow cytometry.

6.4.3 Actin centralization measurement

For the analysis of actin centralization (annularity), data structures containing the vertex data (downstream of synapse ROI selection) were first filtered to remove the outermost 10 % of d_{norm} data. Then, the outer and inner zones were split according to a radius value that would render the inner circle's area twice that of the outer annulus ($r_{boundary} = 0.7348$). Vertices were then binned into inner or outer areas, their MFIs calculated, and converted into the relevant ratio: $actin_{centralization} = \frac{MFI_{in}}{MFI_{out}}$.

CHAPTER 7

OUTLOOK

7.1 The concept of an immune mechanotype

Work on B cells [325, 326, 268, 269] and macrophages [259, 260, 264] suggests that intercellular contacts formed by immune cells are mechanically active and driven by directed cytoskeletal rearrangements. Although it is clear that immune synapses both sense and impart mechanical forces, precisely how these forces are organized and the extent to which this organization controls immune function remained poorly understood. In tackling this gap, the current thesis work set three major goals: (1) develop a new tool to interrogate synapse mechanical output, (2) design novel analyses to extract information on T-cell strength and mechanical patterning from images, and (3) investigate the links between indices of mechanical output and CD8+ T-cell function (cytotoxicity). By adapting the hydrogel microsphere synthesis developed by Vorselen and colleagues [258] to enable T-cell experiments beginning in December 2018, we made the first-in-kind observation that the T-cell synapse is a compressive, dynamically micropatterned interface. Specifically, we found that T-cell cytotoxicity is closely associated, both *in vitro* and *in vivo*, with a cell's capacity to distort target surfaces into compressive synaptic craters containing complex, wavelike undulations. This undulatory property, which we quantified using the amount of high-frequency information in the synapse topographies (spectral complexity), associated closely with cytolytic capacity across multiple cell-type comparisons. Macrophage contacts, in contrast to the T-cell synapses, evoked a tensile configuration consistent with engulfment. What our battery of comparisons make clear is that the globally compressive mechanotype exhibited by T cells was not the inevitable consequence of building a mechanically active, F-actin-based interface. Indeed,

macrophages exert stronger protrusive forces and more extensive particle distortion than do CTLs, yet they do not form crater-like contacts. These results imply that immune cell-cell interactions occupy a broad and variable biomechanical landscape. The precise location of a given contact within this landscape likely depends on factors like cell size, actin availability, the energetic budget for F-actin remodeling, cytoskeletal composition, and the relative rigidities of the immune cell and its partner. Collectively, our results strongly suggest that the precise mechanical disposition of an immune cell-cell interaction reflects its specific function—this we term a *biomechanical signature* or *mechanotype*.

Evidence for the importance of the cytolytic mechanotype. Three lines of evidence in this work collectively suggest that the cytotoxic mechanotype described herein is required for optimal killing: (1) the results of our comparative profiling studies, (2) our imaging studies of degranulation, and (3) our analysis of the *in silico* simulations of synapse topographies. First, the formation of a topographically complex synaptic crater was closely associated with lytic potential across three sets of comparisons: (a) killing-competent (e.g., sgPTEN, sgWAVE2) and killing-incompetent (e.g., sgWASp, sgTalin) cytoskeletal mutants, (b) CTL differentiation states generated *in vivo* during a model viral infection, and (c) cytotoxic and non-cytotoxic T-cell subsets. In these three sets of comparative studies, compression strength and spectral complexity were linked to cytolytic function, although interestingly, the two never correlated well on a single-cell level. A proportionality between them would have pointed to shared regulation (strong cells can both compress and micropattern their targets strongly, while weak cells can do neither); a negative correlation would have implied, on the other hand, a division of labor amongst the F-actin machineries (cells choose between compression and micropatterning). The independence of mechanical outputs from each other not only implies that CTLs can utilize one or the other strategy to effectively potentiate its killing, but also that the

regulation of axial compression and target micropatterning could be driven by distinct cytoskeletal mechanisms. It is possible that the microtubule cytoskeleton contributes to these activities as well, perhaps compression—pilot work I conducted on nocodazole treatment suggested that synaptic compressions are restrained in part by microtubules (not shown). Indeed, microtubules also appear to co-regulate the perinuclear "actin cloud" in migrating DCs that sets a natural limit on the size of the lamellipodial F-actin pool at the cell front ([327] and Michael Sixt, personal communication). It is also possible that distinct actin pools establish the bulk axial forces and the smaller palpations throughout the synapse (e.g., PIP₃-templated lamellipodial actin and WASp-mediated foci, respectively [38]).

Degranulation was localized exclusively to the inner floor of the synaptic crater, where most of the undulatory surface activity is generated on targets. Our findings are consistent with imaging-based studies of degranulation position [328, 279, 181, 185] and with prior work implicating synaptic force exertion in the potentiation of perforin-1 activity [191, 190]. Recently, perforin-1 was shown to preferentially bind and oligomerize in convex membranes, where packing defects between phospholipids would presumably accommodate the interdigitation of its transmembrane helices [274]. Given the predilection of perforin for membrane convexity, it is noteworthy that the CTLs do not degranulate into the peripheral rim of the synapse, which is highly enriched in positive curvature. Any gains in cytotoxic potency realized by peripheral release of perforin would be likely offset by the risks of leaking this toxic protein out of the synapse, where it could damage innocent bystander cells. Inducing undulatory topography at the synaptic crater floor, on the other hand, could generate a series of local convexities that sensitize the target membrane to perforin-1 activity within the "safe zone" formed by the integrin "gasket". Hence, by generating undulatory distortions at the inner regions of the synapse,

CTLs can target degranulation to these zones [276] and effectively potentiate their killing responses without sacrificing host security.

Finally, computational simulations of protrusive distortion at the crater floor strongly suggested that the cytolytic mechanotype enables CTLs to maximize killing efficiency. *In silico* simulations allowed us to delve into the architecture of the cytolytic synapse as an optimization problem between its chemical and mechanical outputs, i.e., between a CTL's capacity for degranulation (chemical) and its ability to potentiate the released perforin-1 through target membrane straining (mechanical). The concordance between the optimal protrusion size for achieving this balance found by simulation and the distribution of protrusion sizes found against 300-Pa beads in real data suggests that CTLs indeed cluster F-actin into protrusive "fingers" to navigate this fundamental tradeoff. A saturable, "win-win" relationship of the sort seen in our cytotoxic efficiency plots seen in Chapter 3 suggests that focal clustering of synaptic actin [38] could represent an evolved strategy to maximize cytolytic efficiency *via* optimal mechanopotential. It is also tempting to speculate that cytoskeletal bundling at the synapse could protect CTLs from the effects of perforin-1 on their own membranes, by reinforcing the membrane at its own most convex locales.

Advances in signal pattern analysis *via* Zernike expansions. Instrumental to all these efforts was the successful application of spectral analysis to parameterize and arrange hundreds of single-cell force exertion patterns by similarity. The relatively simple algorithm we designed for this purpose—essentially, *take a circular representation of the data, convert each pattern into a frequency spectrum, render the spectrum rotationally invariant, then use the spectra as a feature vector for PCA*—allowed us to better understand the major role of radially asymmetric patterning in the cytolytic mechanotype. The other major development from the Zernike expansions was a spectral complexity metric

that reflected both feature intensity and undulatory distribution of these features across synapses; this enabled us to directly compare individual synapses from each other without discarding information on the geographical distributions of their features.

There are numerous advantages to the use of linear embedding methods such as PCA over more attractive visualizations such as UMAP or t-SNE, where distances in plot space are essentially meaningless [329]. In our Z-PCA plots, distances between data points could be interpreted as a metric of relative similarity or difference. This allowed us to correctly interpret, among other things, that PC1 in Chapter 6 (Figure 6.5B) reflected an actin centralization-to-annularity axis. Our use of PC1-quantiling to divide synapses into pattern category bins facilitated a robust method to compare population pattern compositions throughout the work; doing so exposed population-level tendencies in mechanical patterning in spite of the great deal of single-cell heterogeneity present in these datasets. Indeed, our methods should be applicable to any data set arranged roughly in circles. Moving forward, we expect that spectral renderings of appropriate data types and pattern categorization by linear embedding methods (or perhaps machine-learning) will expand our repertoire of analyses for cellular patterning studies.

A plurality of intercellular contacts. Even among T-cell synapses, a variety of different morphotypes have been found [316], such as multi-focal synapses [188, 330], hyper-stable synapses [331], or meta-stable kinapses [192, 332]. It is possible that the defective mechanical outputs by T_{exh} cells we measured could reflect a separate functional state with its own associated mechanotype, rather than a failure to execute the cytolytic mechanotype. Thus, careful mechanical profiling of different T-cell subsets, perhaps in different cytokine environments and at different activation levels, alongside proteomic profiling of their cytoskeletal proteins, may be a valuable source of insight for future work. Among B cells, there also exists a variety of synaptic morphotypes formed with APCs

depending on whether they are naive or activated, at the marginal zone or germinal center [326]. In this work, we contrasted the cytolytic T-cell mechanotype from the phagocytic BMDM mechanotype; still other mechanotypes might exist, corresponding to B-cell antigen extraction, the macrophage/DC side of the immune synapse, T_{reg} function, or others. While immune cell subsets are normally identified using specific surface markers, master regulator transcription factors, or characteristic cytokines, our results suggest that they might likewise be related or distinguished by their interfacial mechanotypes.

The role of cell size in mechanotype specification. It could be argued that relative differences in cell size between cellular partners determines the ultimate topology of the contact—i.e., whether it is globally convex or concave, that is, tensile or compressive. Indeed, T cells are among the smallest immune cells, while macrophages are larger than most cells they interact with; perhaps, encoded into cell-size specification during development is also a size-imposed restriction of the kinds of intercellular configurations a given leukocyte subtype can engage in. Beyond this, even among cells of a given type, natural heterogeneities in cell size may also predispose a certain fraction of the population to form different types of contacts, or to elicit characteristic mechanical outputs to different degrees. In the case of CTLs, for example, the largest sub-population of clones generated during an infection would have the smallest tradeoff between mechanopotential and degranulation-permissible area; they might also be the strongest cells of the given population, and the best killers. Indeed, PTEN-KO cells are slightly bigger than wild-type cells by about 1 μm (not shown); this may also underlie their tendency to form annular F-actin architectures on stiff targets.

Mechanical adaptations. Interestingly, we found that the F-actin architectures of CTLs can be changed using cell-intrinsic and cell-extrinsic approaches. Specifically, we were able

to induce annular F-actin ring formation by deleting PTEN in CTLs, which enhances their compressive strength. By observing the effect that target rigidity had on PTEN-KO F-actin patterning, we realized that presenting wild-type CTLs with smaller, stiffer targets likewise coaxed them to adopt ring-like actin configurations on the targets. Future work will be necessary to disentangle the roles of target size and rigidity in this determination.

This result, however, still begs the question of what benefit ring-like actin patterning could present for the CTL. Our computational model of synapse distortion did not account for annular F-actin organization; it was a model of a protrusive, undulatory crater floor based on the preponderance of this pattern on 300-Pa hydrogels. Annular patterning would theoretically maximize degranulatable area, if the "actin-free" regions seen on this level of diffraction-limited imaging were truly permissible for degranulation as is. In this case, would a ring-like F-actin pattern represent a CTL's choice to degranulate more at the expense of target surface distortion? Our findings on sgPTEN cells, which tended to form more annular synapses, contradicts this notion as they degranulate to a significantly lower level than wild-type CTLs. Perhaps, an annular synapse imposes global membrane stretch all along the target surface—this would be a viable strategy to maximize mechanopotential when targets are too stiff to poke or too small to compress (e.g., 1000-Pa, 9- μ m hydrogels). Simulations accounting for annular F-actin distributions will be necessary to update our model.

It is very tempting at this point to suggest that a CTL integrates information on target mechanical properties such as size or rigidity before adopting the optimal F-actin-based architecture for maximizing cytolysis. Of course, single T cells are likely not so "intelligent" in the manner this heuristic might suggest. However, given a large enough population enough time during any given infection, even a completely random exploration of the configuration space of all possible F-actin architectures will lead to

those resembling the mechanotype we described making non-trivial contributions to host protection. Our findings suggest that CTLs are at least more intelligent than the monkeys of the infinite monkey theorem, a *Gedankenexperiment* which roughly states that given an infinite amount of time, even a monkey banging randomly on a keyboard will be able to reproduce the complete works of Shakespeare, or of Dostoevsky, or of Kundera.

7.2 Future directions

High-throughput mechanotyping. Two types of follow-up studies will greatly inform our understanding of the cytolytic mechanotype, as well as of immune mechanotypes in general. The first of these is the development of a high-throughput force-profiling methodology to complement our information-rich but low-throughput approach. Indeed, cell-scale tension reporters that are amenable to a flow cytometry format have recently been developed [333]. Such tools will be necessary for reading out force generation phenotypes (gain or loss of function alike) in the context of targeted cytoskeletal screens or whole-genome screens of CTL mechanical activity. After initial hit identification and validation, our low-throughput, high-content method can then be used to provide mechanistic detail on specific mechanical contributions to single-cell strength, patterning, or both. The effects of various cell activation inputs on force generation could also be queried initially using this sort of approach, as can the effects of checkpoint blockade treatment on T_{exh} cell mechanical defects.

Extending the mechanotype concept into one of *biomechanical taxonomy*. As suggested earlier, profiling different leukocytes under the influence of different cytokines or activation states would be an interesting exploration of the idea that the immune system is likewise organized into discrete mechanotypic patterns. For example, different

T-cell subtypes could be profiled in parallel (e.g., CD4+ CTL vs. T_HC vs. T_{reg}), or the same T-cell subtype under different cytokine polarizations (e.g., T_{H1} vs. T_{H2} vs. T_{H17}), or perhaps ontogenically different leukocytes with shared function (e.g., BMDMs vs. BMDCs, or CD8+ CTLs vs. NK cells). In particular, the effects of different TLRs on DC mechanics would be worth investigating, as a dramatic F-actin phenotype switch has already been reported for DCs upon LPS treatment, but to the best of my knowledge, many other routes of TLR stimulation remain unexplored.

7.3 Concluding remarks

Taken together, the novel mechanical observables we described in these studies help to illuminate the enigmatic biophysical dimensions of immune cell activity. Deciphering the molecular principles underlying interfacial mechanotypes could inform our understanding of immune cell-cell interactions more generally, and open up new avenues for the selective modulation of these contacts in both health and disease. I can only hope that future research will find inspiration in the advances made during my PhD work, going further and faster than I.

—MDJ

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