Interrelating Mechanical Behavior and Calcium Bursts in Human Neutrophils

Key Terms: *mechanotransduction, micropipettes, chemotaxis, phagocytosis, β_2 integrin*

Introduction: Future medical innovation will require a detailed knowledge of the causal sequences of events in biological processes. Currently, much of the understanding of these processes comes from correlative studies, whereas cause-effect relationships are less often explored. For instance, in immune cells, several signaling pathways are associated with dramatic, global bursts in cytosolic calcium concentration, but it remains unclear which pathways trigger the calcium burst and which depend on it. In human neutrophils, these bursts are correlated with several mechanically demanding processes, including β_2-integrin-mediated cell arrest[1], the onset of active cell spreading on immobilized IL-8[2], and the acceleration of β_2-integrin-mediated phagocytosis[3]. On the other hand, my prior work in the Heinrich Lab has shown that pure (i.e. adhesion-free) complement-mediated chemotaxis neither causes nor requires such global calcium surges[4] (see Fig. 1B). We further demonstrated that unphysiologically high levels of chemoattractant can cause calcium bursts, but contractile forces stalled or even reversed pseudopod formation in such cases. These findings imply a close connection between calcium bursts and mechanical behavior. **The purpose of this project is to examine the cause-effect relationship between changes in cytosolic calcium concentration and mechanical responses of human neutrophils to chemotactic and phagocytic stimuli on a single-cell basis.**

Background: Store-operated calcium entry (SOCE) is considered the dominant mechanism for calcium bursts in human neutrophils. In this paradigm, ligation of certain receptors, such as G-protein coupled receptors (GPCRs), triggers a signaling cascade that leads to the depletion of intracellular calcium stores (usually via IP_3 production). This prompts a calcium influx from the extracellular space through channels such as Orai1. However, our own findings and several earlier studies indicate that this view of SOCE is incomplete, as GPCR ligation can cause chemotaxis without triggering a calcium burst[4,5]. Furthermore, shear force on high-affinity β_2-integrins is known to mediate calcium influx[1], which implies that mechanotransduction is important for SOCE in neutrophils. It also remains largely unclear which cellular activities depend upon the elevated calcium levels after store release and calcium influx. Calcium bursts often precede F-actin-mediated spreading[1-3], but this connection is not fully understood.

I hypothesize that β_2-integrin-mediated mechanotransduction is key to inducing a global calcium signal in human neutrophils, which controls a mechanistic switch between two distinct modes of cytoskeletal organization and dynamics. I will primarily use single-cell, single-target micropipette experiments (Fig. 1A) to quantify aspects of the mechanical response (e.g. cell morphology, cortical tension, or surface area) while monitoring intracellular calcium concentration using a calcium-sensitive dye (e.g. Fluo-4 or Fura-2). This will be supplemented by data from other biophysical experiments, as well as mathematical modeling.

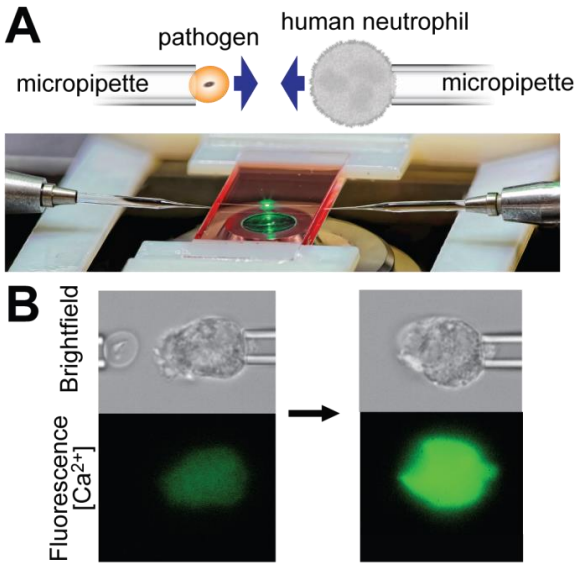

Fig 1. A: Experimental setup. B: Neutrophil shows a calcium burst during phagocytosis, but not during pure chemotaxis[4].

Aim 1. Uncover specific mechanical or biochemical cues that are necessary and/or sufficient to induce calcium bursts. Before micropipette experiments, human neutrophils will be treated with an actomyosin inhibitor (e.g. latrunculin A, cytochalasin D, blebbistatin) or a β_2-integrin (LFA-1 or Mac-1) blocking antibody. Our analysis of the calcium response will reveal the roles of the respective molecules in calcium burst induction. I will also use reflection interference contrast microscopy (RICM) to measure the contact areas of neutrophils spreading on glass with a known ligand density, determining if a threshold of engaged receptors can trigger a calcium burst. Application of a measurable force on β_2 integrins on a neutrophil using atomic force microscopy (AFM) will allow me to explore whether force can directly stimulate a calcium burst or if there is a synergistic effect between force and number of integrins engaged.

Aim 2. Characterize the mechanical and morphological changes that require calcium store release and/or calcium influx. I will conduct micropipette experiments after depleting extracellular calcium with EGTA or emptying internal calcium stores with thapsigargin. In similar experiments, I will block IP_3-dependent store release using a PLC inhibitor (U73122), or use murine neutrophils with a deficient calcium influx ($Orai1^{+/-}$ or $Orai1^{-/-}$, collaboration with Dr. Scott Simon, BME Dept.). These experiments will indicate the relative importance of calcium store release and calcium influx for mechanical changes such as elevated cortical tension or surface area expansion. I will also collaborate with Dr. Soichiro Yamada (BME Dept.), using confocal microscopy to simultaneously image actin arrangement and calcium concentration in a neutrophil-like cell line (PLB-985) transfected with GFP-actin and loaded with Fluo-4. I will assess actin structure and dynamics following calcium bursts in these cells.

Aim 3. Incorporate global calcium signaling into an established computational model of neutrophil phagocytosis. I will collaborate with Dr. Samuel Walcott (Math Dept.) to build on the computational model developed by Herant et al.[6], which accurately describes the phagocytic behavior of neutrophils. The model predicts a key role for cytoskeletal membrane anchors, connections that form between integrins and F-actin via adaptor proteins. The assembly of these complexes is associated with elevated calcium levels[1,3]. I will incorporate the calcium-dependence of cytoskeletal membrane anchor strength into the model and leverage this revised model against the phagocytic behavior of neutrophils in the above experiments.

Intellectual Merit: My three years of experience with micropipette experiments and quantitative data analysis have prepared me well for this important work. This project will fill a fundamental knowledge gap regarding one of the most dramatic signaling events in the life of a neutrophil. In addition, clarifying the sequence of molecular events leading from receptor engagement to calcium burst to protrusive force generation may elucidate similar mechanisms in other cells.

Broader Impacts: An improved quantitative and mechanistic understanding of immune cells will strengthen the foundational knowledge for novel medical treatments such as immunotherapy. With an understanding of the cause-effect relationship between calcium signaling and immune cell motility, new therapeutic targets for immunodeficiencies and autoimmune diseases could also be identified. Furthermore, because calcium bursts are easily detectible and are strong indicators of immune cell activation, my research may inform the development of future diagnostic tools. I will include undergraduate students in this project, share findings in publications and at international conferences, and inform the public by creating and sharing videos online (www.youtube.com/heinrichlab).

References: [1] Dixit, N. et al. (2011) *J Immunol.* 187(1):472-481 [2] Beste, M.T. et al. (2015) *Ann Biomed Eng.* 43(9):2207-2219 [3] Dewitt, S. and M.B. Hallett (2002) *J Cell Biol.* 159(1):181-189 [4] Francis, E. and V. Heinrich (2017) Revision under review. [5] Laffafian, I. and M.B. Hallett (1995) *J Cell Sci.* 108(10):3199-3205 [6] Herant, M. et al. (2011) *PLoS Comput Biol.* 7(1):e1001068