T cells maintain fluid motion in crowded environments

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Abstract

Migration within and between tissues is indispensable for T cell function. Naïve T cells must survey secondary lymphoid organs for their cognate antigen, and activated T cells traffic to sites of infection. Upon arrival into tissues, T cells traverse heterogenous microarchitectures dense with cells. Active matter physics, which studies the phenomena arising from large groups of moving particles, such as pedestrian traffic, demonstrates a phase transition from faster “fluid” motion to a “solid” state as density increases, with crowds reaching a jamming state that prevents motion. This holds true for cells studied, including epithelial cells growing in a monolayer where jamming may be key to preventing unwanted cellular extrusion. This however begs the question: how do T cells remain motile even in crowds?

We imaged T cells in one-dimensional microchannels to investigate whether they also experience jamming at increased cell densities. We found that T cells maintained rapid speeds regardless of cell density. To determine whether this was a T cell-specific effect or generalizable to other immune cells, we repeated our experiments with neutrophils. In contrast to T cells, neutrophils slowed with increased cell density and moved minimally in tight crowds. Additionally, our research suggested that T cell crowding may aid in the navigation of tight spaces. We found that in microfluidic devices with constrictions, groups of T cells were better able to traverse these obstacles than singlet cells. Our data suggests that T cells have evolved strategies to maintain fluid motion even in crowded microenvironments, and that these adaptations contribute to their effector capabilities in diverse tissues of the body.

Résumé

La migration au sein et entre les tissus est indispensable à la fonction des lymphocytes T. Les cellules T naïves doivent explorer les organes lymphoïdes secondaires à la recherche de leur antigène, et les cellules T activées se rendent sur les sites d'infection. À leur arrivée dans les tissus, les cellules T traversent des microarchitectures hétérogènes denses en cellules. La physique de la matière active, qui étudie les phénomènes découlant de grands groupes de particules en mouvement, comme la circulation des piétons, montre une transition de phase entre un mouvement "fluide" plus rapide et un état "solide" à mesure que la densité augmente, les amas atteignant un état de blocage qui empêche le mouvement. Cela vaut pour les cellules étudiées, y compris les cellules épithéliales qui se développent en monocouche, au sein desquelles un tel blocage peut être essential pour empêcher l'extrusion cellulaire non désirée. Cela soulève toutefois la question suivante : comment les cellules T restent-elles mobiles, même au sein d'amas ?

Nous avons imagé les cellules T dans des microcanaux unidimensionnels afin de déterminer si elles se bloquent également lorsque la densité cellulaire augmente. Nous avons découvert que les cellules T conservaient des vitesses rapides quelle que soit la densité cellulaire. Pour déterminer s'il s'agit d'un effet spécifique aux cellules T ou s'il peut être généralisé à d'autres cellules immunitaires, nous avons répété nos expériences avec des neutrophiles. Contrairement aux cellules T, les neutrophiles ralentissaient avec l'augmentation de la densité cellulaire et se déplaçaient peu dans les amas. En outre, nos recherches suggèrent que le rassemblement des cellules T peut faciliter la navigation dans les espaces restreints. Nous avons constaté que dans les appareils microfluidiques présentant des constrictions, les groupes de cellules T étaient mieux à même de franchir ces obstacles que les cellules isolées. Nos données suggèrent que les cellules T ont développé des stratégies pour maintenir un mouvement fluide même dans des micro-environnements encombrés, et que ces adaptations contribuent à leurs capacités d'effecteur dans divers tissus du corps.

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This thesis would not exist without the scientific and moral support of my colleagues and my community, and I want to express my deepest gratitude to you all.

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Author contributions

The ideas behind this work were conceived by Dr. Judith N. Mandl, Dr. Johannes Textor, and Dr. Daniel Parisi. Dr. Mandl and I designed the research. I performed the experiments with help from Dr. Jéremy Postat and Connie Shen. Dr. Postat and Dr. Mandl designed the molds for the microchannels. Data were analyzed by me, and Dr. Inge N. Wortel analyzed T cell speed by density and created the T cell fundamental diagram. The R script used for cell speed analysis was developed by Dr. Johannes Textor and Dr. Inge N. Wortel. Care of our animal colony was undertaken by Geneviève Perrault. Images were collected in the McGill University Advanced BioImaging Facility (ABIF). The manuscript was written and figures created by me with feedback and input from Dr. Mandl.

List of Abbreviations

APC – Antigen-presenting cell

Arp2/3 complex – Actin-related protein 2/3 complex

ATP – Adenosine triphosphate

BLT – Leukotriene B_4 receptor

CD – Cluster of differentiation

CIL – Contact inhibition of locomotion

CCL/CCR – C-C motif chemokine ligand/receptor

CTL – Cytotoxic lymphocyte

CXCL/CXCR – C-X-C motif chemokine ligand/receptor

DC – Dendritic cell

Dock8 – Dedicator of Cytokinesis 8

ECM – Extracellular matrix

EMT – Epithelial-to-mesenchymal transition

ENU – *N*-ethyl-*N*-nitrosourea

FRC – Fibroblastic reticular cell

GEF – Guanine exchange factor

GFP – Green fluorescent protein

HBEC – Human bronchial epithelial cells

IL – Interleukin

KO – Knock-out

LTB_4 – Leukotriene B_4

MAT – Mesenchymal-to-amoeboid transition

MDCK – Madin-Darby Canine Kidney

MHC – Major histocompatibility complex

Myo1G – Myosin 1G

NET – Neutrophil extracellular trap

NPF – Nucleation promoting factor

PDMS – Polydimethylsilicone

PGD_2 – Prostaglandin G_2

ROS – Reactive oxygen species

SLO – Secondary lymphoid organ

TCR – T cell receptor

Th2 – T-helper type 2

WASp – Wiskott-Aldrich syndrome protein

WAVE – WASp family verprolin homolog

WT – Wild-type

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Chapter 1: Introduction and Literature Review

1.1 Cell migration overview

Cell migration is essential for the survival of multicellular life. From the earliest stages of embryogenesis, cells migrate to establish the structures that will develop into all tissues and organs of the body, and migratory deficiencies in embryonic stem cells can be catastrophic to development [1, 2]. In the case of injury to the skin, sheets of keratinocytes collectively migrate into the wound bed [3, 4]. This allows them to begin the process of wound closure and repair of the damaged tissue, in order to restore an organism to homeostasis and health. However, cell migration is also a key factor in disease progression. Cancer cells' infiltration from the initial tumor site into the surrounding tissue is the first step towards metastasis [5]. As we will review in more detail later, cells of the immune system consistently survey the diverse tissues of the body for signs of infection or injury. Effective locomotion is necessary for leukocyte activation, sensing of infection or damage, and effector function when fighting pathogenic invasion.

1.1.1 The cytoskeleton

While migration is present across a wide range of biological processes and cell types, the basic mechanisms that allow for cellular locomotion are conserved. A cytoskeleton, whose gross structures are largely composed of actin and myosin, is present in all eukaryotic cells regardless of migratory capacity, and helps to maintain a cell's shape and rigidity. The primary "motor" of cell motion is the polymerization of actin filaments. As a cell polarizes to move in a given direction, actin continuously polymerizes into branched chains at the leading edge of the cell [6]. Branched actin filaments are subsequently broken down into monomers in a cyclical, "treadmill-like" pattern of assembly and disassembly [7].

Actin polymerization relies on specific salt concentrations in the cell and is facilitated by ATP hydrolysis [8]. The organization of the actin network as it assembles is regulated in a context-dependent manner by a diverse family of molecules. The

Arp2/3 complex, a group of 7 proteins [9], is responsible for nucleating new actin branches from the initial strand. Arp2/3 cannot induce actin nucleation on its own, and requires other proteins to bind and activate the Arp2/3 complex. Nucleation promoting factors (NPFs), including the Wiscott-Aldrich syndrome protein (WASp) family and WASp family verprolin-homologous protein (WAVE), associate with Arp2/3 in response to a wide range of intra- and extracellular stimuli in order to drive actin branching. As actin polymerization continues, expanding networks of branched filaments develop into the wide, wave-like lamellipodium that is characteristic at the leading edge of a moving cell.

1.2 Migration and the extracellular environment

While actin polymerization is necessary for cell migration, a cell must also couple intracellular forces with the external environment in order to physically propel itself forward. In traditional models of cell migration that focus on mesenchymal cells, this force transmission is described in the context of integrins, transmembrane proteins that associate both with the cytoskeleton and diverse extracellular matrix (ECM) ligands [10]. While many cells do rely on integrin-mediated adhesions coupled with cytoskeletal assembly to move forward, the interaction between a moving cell and its external environment is highly dependent on the physical attributes of the extracellular environment [11] and the type of cell.

Tissues of the body vary greatly in their architectural and mechanical properties, and this has profound effects on the migration of cells within and between these microenvironments (**Figure 1**). The skin is a very stiff tissue with dense networks of fibrillar collagen [12], posing a challenge for cells that migrate through a highly crosslinked network of ECM fibers. Neural crest cells in the *Xenopus* embryo have been shown to probe the stiffness of surrounding tissue and migrate along gradients of stiffness during development [13]. Cancer cells have been shown to migrate along collagen fibrils, using the structure to guide their path and invade tissues more efficiently [14]. It is important to consider cell migration as not just a phenomenon of each moving cell, but in terms of the interplay between cells and their surroundings.

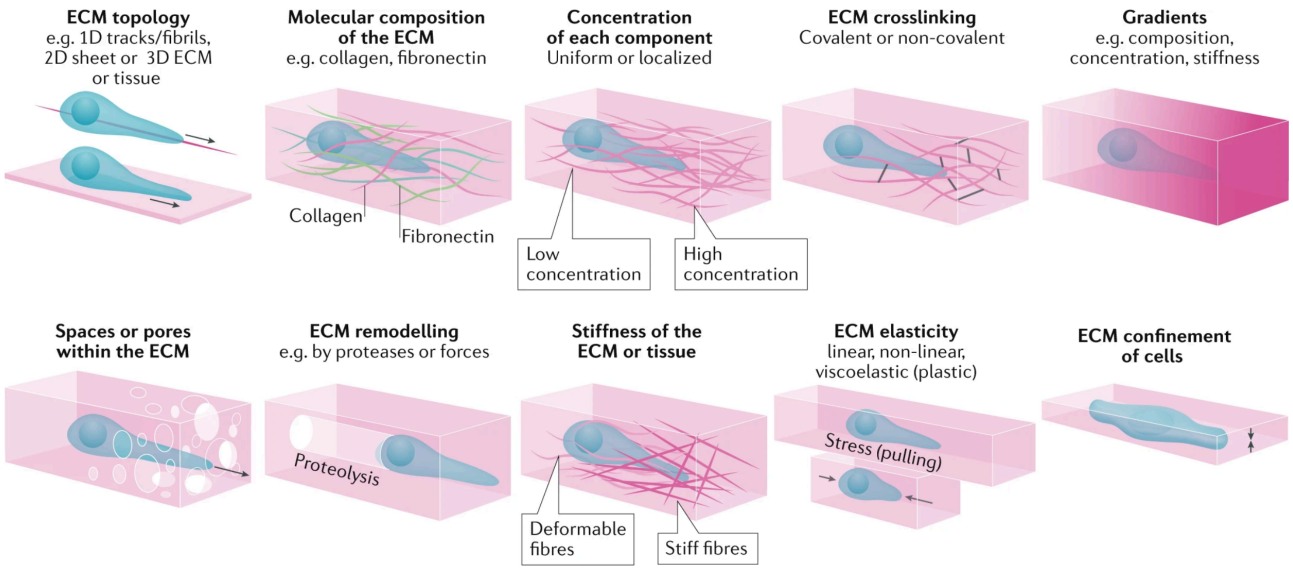


Figure 1. Diverse mechanical and chemical characteristics of the extracellular environment influence cell migration. Adapted from [15].

1.2.1 Modes of cell migration

The migratory strategy of a cell varies in response to biological context and the surrounding environment. The "classical" mode of migration that has been studied the most extensively is that of mesenchymal cells. Studies of fibroblasts migrating on 2-dimensional surfaces formed much of our initial understanding of the molecular mechanisms of cell migration. In this situation, cells move forward in a three-step cycle of protrusion of the leading edge via actin polymerization and branching, focal adhesions between cellular integrins and ligands on the underlying substrate, and myosin-mediated contraction at the trailing edge of the cell to move forward as adhesions are released [16]. However, upon further study of migration in the three-dimensional ECM more closely resembling the microenvironments cells usually face *in vivo*, it was found that this simple and cyclical migratory model was fairly specialized to the experimental context in which it was elucidated [17].

In a 3-dimensional microenvironment, mesenchymal migration still relies on integrin-mediated adhesions, but, particularly under confinement, they are used more sparingly than they are on flat coverslips [18, 19]. A microenvironment containing a dense, crosslinked ECM poses a migratory challenge for cells, as ECM fibers can often cross to form tight pores that obstruct directional migration. Cells migrating in the mesenchymal mode rely on proteolytic degradation of the extracellular matrix in order to clear a path through the ECM [20]. Because 3-dimensional migration relies on the speed of ECM remodeling in this scenario, mesenchymal cells migrate quite slowly, with fibroblasts moving at a speed of approximately 0.5 micrometers per minute [21].

By contrast, many faster cell types, including highly invasive cancers and the cells of the immune system, migrate via amoeboid migration. As the name suggests, amoeboid migration is characterized by rapidly changing cell shapes. Rather than degrading fibers with proteases, amoeboid cells traverse dense matrices by drastically deforming their bodies and nuclei to squeeze through tight pores [22]. While mesenchymal cells physically carve a path for themselves through the ECM, amoeboid cells probe the width of pores with both the cytoplasm and nucleus to preferentially migrate along the “path of least resistance” [23]. Additionally, amoeboid cells, including dendritic cells and neutrophils, can use WASp to polymerize cortical actin that pushes orthogonally against collagen fibers in a mechanosensitive fashion and temporarily dilates ECM pores as they migrate [24, 25].

Another important distinction between these modes of migration is that amoeboid cells are not reliant on integrin-mediated focal adhesions for their movement. In a complex 3-dimensional matrix, immune cells that lack all functional integrins migrate identically to wild-type cells [26]. Adequate confinement and a complex substrate are necessary for this non-adhesive migration, as T cells lacking Talin can migrate as well as wild-type cells, but not along a smooth surface or on a 2-dimensional coverslip [27]. Integrins are necessary for T cell adhesion to vascular endothelial surfaces for extravasation [28], and are used by T cells as they crawl along the network of fibroblastic reticular cells (FRCs) in the lymph node [29]. The context-based plasticity of migration strategy is also shown in the mesenchymal-amoeboid transition (MAT) undergone by invading cancer cells [30], and the phenomenon of mesenchymal cells

adopting an “amoeboid” migration style in a confined, low-adhesion extracellular environment [19]. This adaptation to different microarchitectures allows for immune and cancer cells to migrate within and between diverse organ tissues, an ability unique to these groups.

1.3 Cell migration and immunity

The cells of the immune system work around the clock to keep us healthy. As we go about our daily lives, these cells patrol the vasculature and tissues, constantly surveying for antigen or injury so that they can rapidly initiate processes of inflammation or tissue remodeling. The range, speed, purpose, and pathway of migration varies by cell type, and is closely linked to the immunological function of the migrating cell. As an example, dendritic cells (DCs) cover wide swaths of anatomical space in order to regularly sample antigen and present it on their surface to adaptive immune cells in the lymph nodes. Their highly deformable and mechanosensitive nuclei [23], alongside their ability to probe and dilate narrow ECM pores to enter lymphatic circulation [31], are key contributors to their ability to patrol the parenchyma of a diverse repertoire of tissues and to bring antigen back to the lymph node for presentation and potential initiation of the adaptive immune response [32]. In contrast, neutrophils move at extremely high speeds to sites of insult and recruit numerous other neutrophils, quickly forming a swarm in the early stages of inflammation. T cells, the star players of this thesis, show a variety of migratory strategies based on their effector function.

1.3.1 T cells

Throughout their stages of development and activation, T cells tune their migratory strategy to their current function. Naïve T cells, upon exit from the thymus, migrate with the ultimate goal of encountering an antigen-presenting cell (APC) that is presenting an antigen on its major histocompatibility complex (MHC) that complements the structure of their unique T cell receptor (TCR). As there are estimated to be between 10^6 and 10^8 different TCR sequences in a human body, often with only a handful of other T cells expressing each TCR, the probability of a T cell encountering its cognate antigen

is vanishingly small [33]. Attempting to find this rare match means that naïve T cells consistently circulate between secondary lymphoid organs (SLOs) to scan peptides presented on the MHCs of APCs and to receive IL-7 and interactions with self-peptides for prolonged survival [34, 35].

In this extreme needle-in-a-haystack search, naïve T cells prioritize a migration pattern that scans a wide area through the SLOs, while taking enough time to scan each peptide-MHC complex for a potential match. This process is not homogenous among T cells [36, 37], with CD4+ naïve T cells circulating rapidly through each lymph node and spending a large proportion of their circulation time in direction interaction with peptide-MHC on dendritic cells, while CD8+ T cells spend twice as long in each lymph node and circulate more slowly overall [38]. Chemotactic cues do also play a role in the search process of naïve T cells; after infection, naïve CD8+ T cells have been shown to upregulate CCR4, allowing them to be drawn to the CCL3- and CCL4-rich sites of interactions between CD4+ T cells and APCs in the lymph node and increasing their likelihood of activation [39]. Additionally, CCL22 secreted by DCs in the lymph node has been identified as an important step for an adequate regulatory T cell T_{reg} response, preventing mice from experiencing excessive levels of inflammation following vaccination [40].

Through intravital two-photon microscopy of T cells in the lymph node [41] and mathematical models incorporating existing imaging data to simulate naïve T cell searching behaviour [42], the migratory process of naïve T cells has been described as a random walk [43] with topographical guidance from the stromal network of FRCs [44, 45]. In order to facilitate their guidance of T cells through SLOs, FRCs secrete multiple chemokines that attract T cells, including CCL19, CCL21 [46], and autotaxin. While chemotaxis is crucial for naïve T cells to maintain their baseline motility and speed in the lymph node [47], chemokines do not contribute directly to the observed randomness of a naïve T cell's exploratory migration. Instead, there may be inherent cytoskeletal features of a naïve T cell that specialize them for the job of "wandering" through a broad area in lymphoid organs. Myosin 1g (Myo1g) acts as a "turning motor" in naïve T cells, allowing it to take a winding path with consistent turns and enhancing its ability to successfully meet the APC carrying its cognate antigen upon infection [40]. This intrinsic

propensity for turning, coupled with variable speed and directional persistence [37, 48], allows naïve T cells to effectively explore a wide area and increase their chance of becoming activated and differentiating into an effector T cell.

The meandering motility of naïve T cells is contrasted by the more directional, guided migration of activated T cells. This is due to the different roles of T cells in the immune response based on their developmental stage. Once they encounter their cognate antigen, recently-activated T cells must enter specific regions of the SLOs to complete the process of differentiation and activation. These newly activated T cells upregulate a variety of chemokine receptors that include CXR3, CCR3, CCR4, CCR5, CCR6, and CCR9, with variation based on the anatomic location of the lymph node and T cell lineage [49]. To aid in the switch in migratory strategy, in the early stages of an immune response, FRCs downregulate the chemokines that help guide naïve T cells [46]. Recently activated CD8+ T cells also upregulate CXCR3, which allows them to traffic to inflammatory sites within SLOs and increases the likelihood that they differentiate into cytotoxic effector CD8+ T cells [50].

Once they are antigen-experienced and differentiated into their effector groups, T cells become competent to exit the lymph node and seek peripheral tissues that are experiencing infection or injury by following chemotactic gradients secreted by other cells of the immune system [51-53]. Upon lymph node egress, effector T cells can exit the vasculature with the help of chemokines and integrin ligands presented on the surface of endothelial cells [54]. Chemokines released at the site of insult attract T cells as they navigate the tissue ECM, stimulating and guiding their rapid 3D migration [55]. APCs precede T cells in the early inflammatory response at the site of infection, and TCR signaling based on re-recognition of cognate peptide-MHC provides a potent “stop” signal that allows effector T cells to stop migrating through the tissue once they reach their destination [56].

Further underlining the importance of effective immune cell migration for health is the number of profound immunodeficiencies that have been found to arise from migratory defects. A cohort of children with repeated infections and life-altering immunodeficiencies were found to all be deficient in Dedicator of Cytokinesis 8 (Dock8-KO) [57]. This guanine exchange factor (GEF) is only expressed in cells of the immune

system, and has been shown to be an important regulator of cytoskeletal rearrangement in this highly motile group of cells. Specifically, Dock8 helps leukocytes to maintain their shape and structural integrity when moving through complex microenvironments [58].

1.3.2 Neutrophils

As the first responders of the immune system, neutrophils are among the fastest cells in the body, as well as being the most abundant immune cell type. They have been observed to exceed 20 micrometers per minute in speed during chemotactic migration [59], eclipsing other fast amoeboid cells of the immune system, including activated T cells. Their rapid trafficking to the site of immunological insult is important for early pathogen control, with defensive responses that include neutrophil extracellular traps (NETs), reactive oxygen species (ROS), and degranulation [60, 61].

At steady state, neutrophils live in the circulation, trafficking through the blood vessels as long as there are no signs of danger. From the earliest stages of infection or damage, various cell types at the site of insult release myriad molecular warning flags, including CXCL8, ATP, and H_2O_2 [62-65]. What follows is a complex interplay between activated endothelial cells that express adhesion ligands [66] and neutrophils themselves, which upregulate integrins within minutes of chemoattractant detection in order to roll along the endothelium and begin the process of extravasation, exiting the vasculature to navigate the tissue, following chemoattractant gradients towards the site of immunological insult [67, 68]. To augment the speed of this already-rapid response, a reservoir of neutrophils has been found to preferentially reside in pulmonary capillaries for the fastest possible response to lung damage or infection [69]. “Pioneer” neutrophils that reach the site of infection early recruit large numbers of other neutrophils to amplify the inflammatory response, and this “swarming” behaviour will be discussed in section 1.6, “Collective behaviour of T cells and neutrophils”.

Further evidence for neutrophils’ adaptation for speed can be found in the appearance of the cells themselves. When looking at a neutrophil under a microscope, one of the most obvious characteristics is its oddly-shaped nucleus– ring-shaped in mice, and multi-lobed in humans, described as a “string-of-pearls” shape. Even though amoeboid cells outrun their mesenchymal counterparts easily, the nucleus is the largest

and least deformable organelle within a given cell [70], posing a challenge as cells squeeze through gaps in endothelial layers and dense tissue matrices [71, 72]. The elongated, lobular nuclei of neutrophil have long been hypothesized to play a role in their function [73]. Recently, human neutrophils with more nuclear lobes have been shown to navigate narrow pores in vitro more easily than those with simpler nuclear shapes [74].

1.4 Collective cell migration

For multicellular organisms, cell motility alone is not sufficient for development or survival– groups of cells must also be able to coordinate their movement and move together. Collective cell migration is required for the large-scale tissue remodeling processes that occur during embryonic development [75], and has been shown to play key roles in developing the morphology of the neural crest, trachea, and mammary ducts [76-78]. The epithelial-to-mesenchymal transition (EMT), the process by which the cells of a stationary epithelial layer change into a group of collectively motile mesenchymal cells, is an important topic in the study of cancer metastasis, as groups of moving cancer cells are involved in the dissemination of many cancers [79-81]. Rather than simply being a group of independently motile cells that happen to be in close proximity, collectives of migratory cells are regulated by complex physical and biochemical cell-matrix and cell-cell interactions, and in fact often migrate more efficiently than individual cells [82]. It is important to emphasize that the bulk of research on collective cell migration has overwhelmingly focused on groups of adherent mesenchymal cells, and that this portion of the literature review focuses on these cell types.

In a traditional model of collective mesenchymal cell migration, individual cells take on “leader” or “follower” roles based on their relative position in the group, but these roles are plastic and can change over time, spontaneously or due to microarchitectural changes in the extracellular environment [83, 84]. As cells tend to collectively migrate in a sheet, these groups have a “front line” of leader cells rather

than an individual cell at the helm. Leader cells tend to more closely resemble solo cells in their migratory pattern; they are the only cells in a migrating sheet that have a true leading edge characterized by Arp2/3-mediated actin polymerization and a wide lamellipodium [85]. Due to their position, leader cells respond more strongly to chemokines [86] and form more integrin-mediated adhesions with the ECM than follower cells [87], such that they effectively determine the directionality and speed of the group's migration. They are also crucial for the migration of the collective through complex 3-dimensional microenvironments, as leader cells undergo the lion's share of proteolytic ECM remodeling [88], clearing an initial path for subsequent cells.

The migratory strategy of a follower cell in a migrating collective is more specialized to this context. Their position behind the vanguard necessitates that they do not form a leading edge or form large, stable focal adhesions to drive their forward migration. All cells in a migrating group of mesenchymal cells are linked to their neighbours via cadherin cell-cell adhesions, and the tensile forces generated by polarized leader cells stimulate their followers to polarize in the same direction [89-92] (**Figure 2**). Follower cells also play an active role in the overall persistence and efficiency of collective cell migration, largely through contact inhibition of locomotion (CIL). This phenomenon, described in microscopic studies of motile cells beginning in the mid-20th century, describes how cells tend to polarize away from other cells upon making membrane contacts [93]. The strong cell-cell contacts between follower cells and leading cells are important for stabilizing the forward polarization of the leading edge, as CIL predicts that cells on the front edge will polarize away from other members of the group [94, 95].

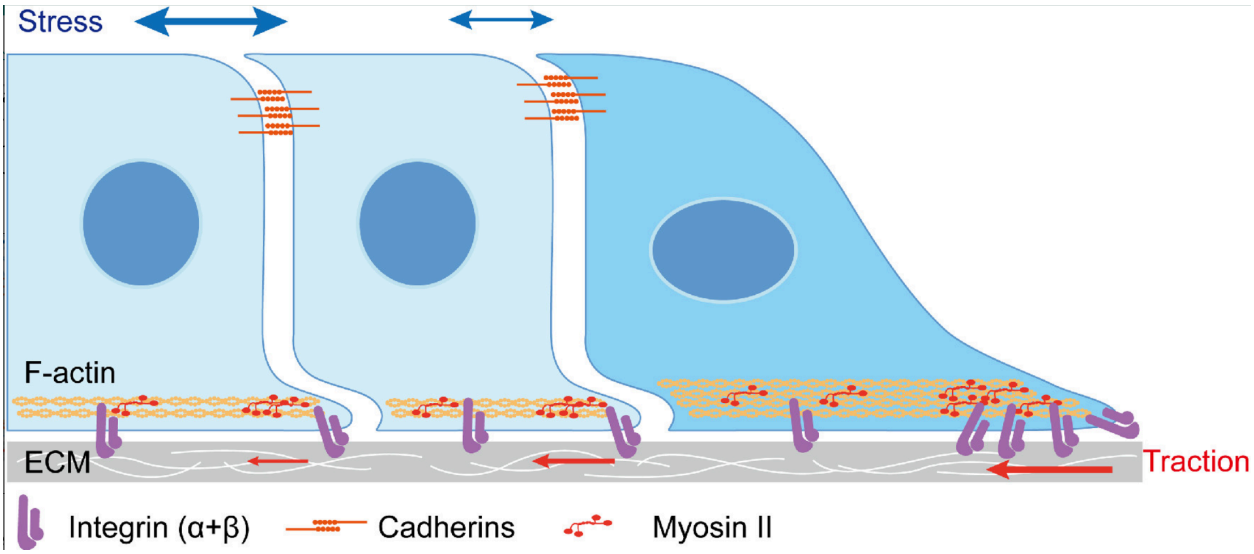


Figure 2. Tensile forces and cell-cell junctions link leaders and followers in collective epithelial cell migration. Leader and follower cells are shown here in a lateral view. A row of leader cells form lamellipodia at the leading edge and generate new focal adhesions with the ECM. As they protrude forward, cadherin-mediated cell-cell junctions transmit tensile forces to follower cells, stimulating their polarization in the same direction. Adapted from [91].

1.5 Active matter physics

Though this thesis focuses on the migratory behaviour of immune cells, the experiments therein were inspired by the field of active matter physics. This field focuses on the phenomena that arise in large groups of individual agents, in which each individual agent or object is independently moving [96]. Given the "active" nature of the systems studied by active matter physicists, predictions and analytical methods in the field are frequently applied to systems in the life sciences [97]. Some biological models used in active matter physics research include flocks of animals and the molecular elements of the cytoskeleton as they assemble and disassemble [98, 99].

A general prediction in this field is the phase transition, often visualized as a fundamental diagram (**Figure 3**). As the density of agents in a given space increases, the speed of each agent is expected to decrease [100]. These distinct phases are often described with the analogy of the molecules of different phases of matter; a system consisting of freely moving, less dense agents is said to have "fluid motion", and the

denser, less motile systems are described as having “solid motion”. As the increase in density continues, it eventually reaches a threshold at which jamming occurs; the agents can no longer move, as they are physically trapped into their position by the neighbours crowding around them [101].

These predictions have been shown to be consistent with observations of density and migration differences within a variety of real biological systems. For example, human pedestrians decrease in speed with increased crowd density and eventually jam to the point of preventing motion, both in open floor-plans and in evacuation situations where large numbers of people attempt to move through a bottleneck [102, 103]. Importantly, phase transition and jamming have also been shown to occur in migratory mesenchymal cells, altering the shape and speed of cells and potentially playing a direct biological role in guiding morphogenesis during early vertebrate development [104-106]. However, this is not a hard and fast rule, and certain models, including ants, can avoid jamming and maintain a “fluid” style of motion even at high densities [107].

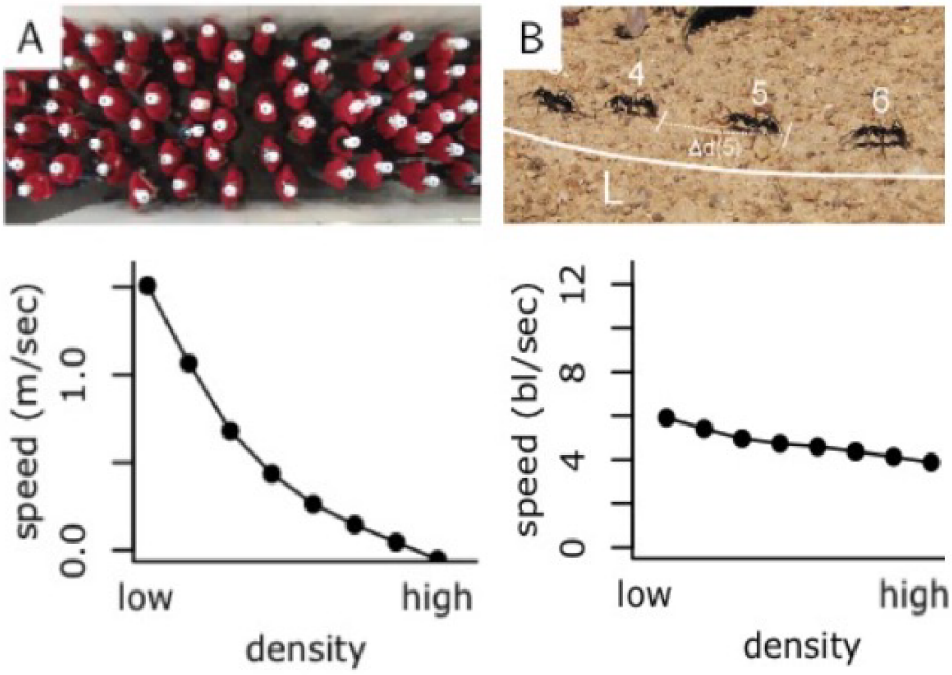


Figure 2. Examples of fundamental diagrams. (A) Human pedestrians experience a phase change and eventually jam with increased density. (B) Ants are tolerant to jamming, and do not experience phase changes even at high densities. Adapted from a grant proposal by Dr. Johannes Textor.

1.6 Collective behaviour of T cells and neutrophils

Though the body of research on immune cell migration is continually growing, these studies overwhelmingly focus on defining the migratory characteristics of individual cells. In vivo, however, T cells and neutrophils are rarely acting alone. As the adaptive immune response mounts following infection, the lymph node swells appreciably, due in part to massive T cell proliferation [108]. Effective T cell migration is necessary to complete the process of differentiation and to exit the lymph node to fight infection, and these processes must be achieved in an organ that is densely packed with T cells and other immune cells [50, 51]. However, little is known about the physical interactions and adaptations governing T cell migration in tight crowds.

While migration-specific studies of groups of T cells are limited, T cells have been shown to work cooperatively with other T cells in a variety of immunological contexts. CD8+ T cells, or cytotoxic lymphocytes (CTLs), have recently been found to work together to amplify their killing capacity. Multiple CTL attacks on virus-infected cells was demonstrated to increase the likelihood of death [109], and *in vitro* tumour-killing experiments have shown CTLs recruiting other CTLs to gather and collectively attack cancerous growths [110]. Cooperation between T cells also appears to be necessary for activation in response to antigens; T cells bound to peptide-MHC on APCs sense the IL-2 secreted by neighbouring activated T cells, and proliferate only beyond a certain threshold of IL-2 concentration [111]. This quorum sensing is hypothesized to be a strategy to avoid autoimmunity by requiring multiple IL-2 secreting peers to be nearby before a T cell can proliferate. Additionally, T cells in juvenile and adult T cells have recently been shown to migrate in a coordinated loop through the body as a potential immune surveillance mechanism in the absence of lymph nodes [112]. This tessellated lymphoid network contains T cells moving in a rapid, directed migratory pathway while maintaining seemingly stable contacts with other T cells, providing an interesting example of collective T cell migration. However, no similar phenomenon has yet been observed in mammalian models.

Contrary to the fairly recent focus on cooperative T cell activity, it has long been understood that neutrophils recruit other neutrophils in massive numbers to the site of

infection or injury. Upon arriving at the site of insult, an initial group of “pioneer” neutrophils begins to release the lipid mediator leukotriene B_4 (LTB_4), which is known to be a potent driver of neutrophil chemotaxis [113, 114]. Neutrophils experience large influx of calcium upon interacting with microbial products, and calcium signaling propagates outward to other neutrophils, facilitating LTB_4 production and clustering [115]. As other neutrophils respond to primary and secondary chemoattractant signals, the response amplifies further until a densely localized swarm of neutrophils forms [116, 117]. Though neutrophil recruitment and paracrine chemotactic signaling is well-studied, neutrophil swarms tend to be fairly stationary, and more explicit evidence or potential mechanisms of cooperative neutrophil migration in vivo are still lacking. Groups of neutrophils migrating chemotactically through complex microenvironments have been shown to coordinate their motion by preferentially entering a different pore at a bifurcation than the preceding cell [118]. This is expected to be due to changes in fluid flow and local chemotactic gradients caused by the preceding neutrophil blocking the channel at the bifurcation. Potential sensing of these minute local changes may allow neutrophils to coordinate their group migration and avoid bottlenecks as they migrate into complex tissue microenvironments.

1.7 Hypothesis and objectives

Active matter physics and evidence from other migratory cell types suggest that T cells would experience slowed migration and jamming at high densities. However, the fact that T cells in healthy hosts regularly encounter microenvironments full of densely packed cells and still successfully perform their migration-intensive immunological functions suggests that this may not be the case. We hypothesize that T cells have evolved cell-intrinsic mechanisms that allow them to maintain high speeds and resist jamming even at high cell densities. Furthermore, we predict that cooperative migration may alter how T cells interact with obstacles in the external microenvironment, and that collective movement may even be helpful in specific migratory contexts.

Given these hypotheses, we had the following **three aims**:

(1) determine whether T cells experience phase transition or jamming at increased cell densities.

(2) to interrogate the effects of collective migration on the 'decision-making' of T cells as they are navigating through complex environments.

(3) to compare the migratory behaviour of crowded T cells with crowded neutrophils, to begin to ask whether crowding behaviours are T-cell specific or generalizable to a broader population of motile immune cells.

Chapter 2: Materials and Methods

Mice

LifeAct-nTnG mice were generated by crossing LifeAct-GFP mice [119], shared by J. Burkhard at the University of Pennsylvania, with $Rosa^{nT\text{-}nG}$ mice [120], purchased from Jackson Laboratories (stock #023035). $Dock8^{-/-}$ mice [121] were generated by GenOway. All mice used for experiments were between 6 and 12 weeks of age and on a B6/J background. Mice were housed with ambient temperatures of 18-24 ºC and 30-70% humidity, on a light-dark cycle of 12 hours. Animal housing, care and research were in accordance with the Guide for the Care and Use of Laboratory animals and all procedures performed were approved by the McGill University Facility Animal Care Committee.

Isolation and culture of primary immune cells

For T cell isolation, peripheral lymph nodes (inguinal, axillary, brachial, and cervical) and spleens were harvested, crushed through a 70 µm filter, and rinsed with complete RPMI (10%, 1% penicillin/streptomycin, 1% L-glutamine, 1% HEPES buffer, 1% sodium pyruvate, 1% non-essential amino acids, 0.1% 2-mercapto-ethanol 1000X solution). Red blood cells were lysed with ACK lysis buffer (Life Technologies) for 3 minutes, then cells were washed with complete RPMI. Cells were quantified in a 1:10 dilution with Trypan Blue, and manual counting of Trypan Blue-negative cells was performed using a hemacytometer. Total T cell isolation was performed using a StemCell EasySep Mouse Total T Cell kit. For in vitro T cell activation, 96-well plates were coated with α-CD3 (Biolegend) at 3 µg/mL in PBS and incubated for 2 hours at 37 ºC, and isolated total T cells were resuspended at 2.5 x 10^6 cells/mL in complete RPMI supplemented with α-CD28 (Biolegend) at 2 µg/mL and cultured in the coated plates. T cell cultures were maintained at 37 ºC and 5% CO_2, and T cells were stimulated on day 2 with carrier-free recombinant mouse IL-2 (BioLegend) at 20 ng/mL in complete RPMI. Migration assays were performed on day 4 of T cell culture.

For neutrophil enrichment and isolation, femurs were harvested and bone marrow was flushed with a total of 10 mL complete RPMI using a 25G needle in a 5 mL syringe. Cells were counted with Trypan Blue as described above. Total neutrophils were enriched and isolated using a StemCell EasySep Mouse Neutrophil Enrichment Kit, and were not further isolated into subpopulations. Neutrophils were not exogenously

stimulated or activated, other than potential stimuli received by their isolation and handling. Migration assays were performed on the same day as neutrophil harvest and enrichment.

Microfluidic devices

Microfluidic devices [122] were prepared and assembled by mixing and adding polydimethylsilicone (PDMS) (RTV615, Momentive Performance Materials) into custom-designed moulds manufactured by 4DCell. Air bubbles were removed with a vacuum chamber, then PDMS was cured at 80 ºC for 1 hour. Microfluidic devices were then unmoulded using isopropanol, cut into the proper configuration for each experiment, and microchannels were cleaned with ethanol in an ultrasonic cleaner for 2 minutes. Microchannels and glass-bottomed dishes (WPI Fluorodish, 30 mm) were then plasma cleaned for 2 minutes on high intensity (Harrick Plasma) and irreversibly bound by assembly after plasma cleaning and incubation at 50 ºC for 1 hour. Prior to migration assays, channels were plasma cleaned on high intensity for 5 minutes and coated with a 1:100 solution of fibronectin bovine plasma (Sigma-Aldrich) in PBS for 1 hour at room temperature. After incubation, channels and dishes were washed 3 times with PBS and 3 times with complete RPMI without phenol red.

Migration assays and image acquisition

After enrichment and isolation (neutrophils) or isolation, activation, and expansion (T cells), cells were resuspended in complete RPMI without phenol red at a final concentration of 1 x 10^8 cells/mL. To determine cell viability, 1 drop of NucRed Dead 647 (ThermoFisher) was added to the neutrophil suspension. Media was aspirated from the dishes and cells were loaded into one (constriction and forked microchannels) or both wells (straight microchannels). No chemoattractant was used. Microchannels were incubated for 1-4 hours at 37 ºC, 5% CO_2 before imaging, and these conditions were maintained during live cell imaging. Migration time-lapse images were acquired using a Zeiss Axio Observer widefield microscope with a 20X Plan Apochromat air objective (NA = 0.80). Brightfield, GFP, and tdTomato channels were

acquired, along with Cy5 for neutrophil viability, and images were acquired at 10 second (for forked microchannels) or 20 second intervals.

Analysis

Cell tracks were generated using Imaris (Oxford Instruments) and speeds were quantified in R using the CellTrackR package [123]. Cell contacts and outcomes at constrictions or 4-way junctions were manually analyzed, and static images were generated using Fiji (ImageJ). Data were visualized and statistically analyzed using Prism 8 (GraphPad), and $P < 0.05$ was considered significant.

Chapter 3: Results

3.1 There is no detectable decrease in T cell speeds with increased cell density

In order to analyze T cell migration in a reductionist, 1-dimensional model, we loaded activated murine T cells into moulded PDMS straight microchannels [122] with a height of 5 μm and a width of 6 μm. This layout allows for rapid migration while preventing the cells from bypassing one another, such that the cells move through the channels in a single-file line. Cells were loaded into both ends of the microchannels in order to maximize collisions and crowding, and cells were imaged with widefield microscopy (**Figure 1A**). Cells in each time-lapse were manually coded as either “solo cells” migrating separately from others or “trains” of cells migrating while maintaining contact with one another (**Figure 1B**).

The average speed of each cell over the course of a 90-minute time lapse was computed, with regards to whether the cell was manually counted in the “solo” or “trains” category. While the average speed of individual cells covered a broad range from nonmotile cells to speeds exceeding 20 μm/minute, the speeds of all cells moving alone and all cells moving in a train overlapped significantly, without notable differences in the mean speed of each group (**Figure 1C**). Then, the cells in the “solo” and “trains” category were pooled to find the average cell speed in each category across multiple experiments. While there was some variation in the overall cell speed from experiment to experiment, the mean time lapse speeds were not significantly different between solo T cells and T cells moving in a group (**Figure 1D**). Average cell speed by cell and by movie was within a range 10 and 20 μm/minute, which is consistent with expected T cell speeds from the existing literature [41]. When the T cell speeds in these experiments were analyzed across a range of densities, shown as a fundamental diagram, there was no decrease in speed, even when the cells were migrating in more dense groups (**Figure 1E**).

3.2 Neutrophils have significantly decreased migratory speeds at higher cell densities

Our first set of experiments showed fairly clearly that T cells are capable of maintaining their speeds even at high densities in 1-dimensional microchannels. However, it was not known whether this may be a T cell-intrinsic effect, or one that is generalizable to other populations of immune cells, or even to any cells that migrate in the amoeboid fashion. We chose neutrophils as a comparison population with which to replicate the 1-dimensional migration assays, because they are another group of highly motile immune cells that are known for recruiting other neutrophils and forming large, amplifying swarms in vivo [124]. As the bone marrow contains neutrophils at various stages of maturity and activation, we understood that the isolated neutrophil population would be heterogeneous [125], but did not further isolate neutrophils in order to have a more straightforward comparison to our sorted total T cells, also a heterogeneous population of cells.

Time-lapses of neutrophils migrating in 6 μm or 5 μm microchannels were qualitatively quite different than those of T cells migrating in one dimension. While T cells maintained consistent speeds regardless of whether they were moving alone or in contact with other T cells, neutrophils migrated very rapidly when alone but formed large trains that stayed relatively stationary within the microchannels (**Figure 2A**). As expected from the literature, individual neutrophils exceeded 30 μm/minute in speed [59], while individual T cells rarely moved faster than 20 μm/minute [41]. When comparing the average speed of each cell in a representative time-lapse of T cells and neutrophils migrating in 6 μm microchannels, there was a markedly different distribution between the two populations of cells. While T cell speeds were evenly distributed around an average of approximately 10 μm/minute, the majority of neutrophils moved under 6 μm/minute, with a smaller group of individual cells reaching the high speeds characteristic of neutrophil migration (**Figure 2B**).

Later experiments demonstrating neutrophil migration in 1-dimensional microchannels were performed using 5 μm microchannels instead of the 6 μm width used with T cells and early neutrophil experiments. Due to their smaller size and lobed nucleus that facilitates migration through narrow spaces [74], we found that neutrophils were able to bypass one another in the 6 μm microchannels. This effect caused the large groups of neutrophils to swirl around one another in the channels, making cell

tracking impossible and making movies qualitatively dissimilar to those of T cells. In the 5 μm microchannels, neutrophils could not pass other cells and formed “trains” that more closely resembled the existing time-lapses of T cells in 6 μm microchannels. Importantly, this did not change the observed differences between T cell and neutrophil migration in a group. In the 5 μm microchannels, trains of neutrophils migrated more slowly than individual neutrophils (**Figure 2C**), forming large, fairly stationary aggregates of cells in the microchannels.

3.3 T cell trains are more likely than individual T cells to pass through constrictions in 1D microchannels

Next, we aimed to determine how collective behaviour may impact the manner in which T cells approach external migratory challenges. We took time lapse images of T cells moving in 6 μm microchannels with 2 μm constrictions (**Figure 3A**). As T cell morphology is already noticeably elongated in the 6 μm channels, this narrow constriction forces the cells to deform and squeeze their nucleus into an irregular shape, risking DNA damage and even nuclear rupture and cell death as a result of mechanical stress [126]. After testing the migratory assays on a variety of constriction widths from 1.5 to 4 μm, we chose to use the 2 μm width, as cells were capable of passing through the constriction and few cells died, but the 2 μm width was a challenging enough squeeze that the majority of cells turned away instead of moving through the constriction.

From the time lapse movies we acquired of T cells moving through microchannels with 2 μm constrictions, we again manually divided the cells into groups based on their interactions with other cells. Cells that were alone for the entire time they were in contact with the constriction were classified as “solo”. Cells that were touching other cells as they approached and made contact with the constriction, and maintained contact with other cells throughout their interaction with the constriction, were classified as being in “trains” (**Figure 3B**). Of note, each cell in a train was counted individually, as many trains had some cells pass through the constriction while the others eventually retreated.

The outcome of each cell's interaction with the constriction was also manually coded (**Figure 3C**). If the cell successfully moved through the constriction to the other side of the microchannel, the event was classified as a "pass". If the cell entered into the constriction but then withdrew and reversed directions back toward the entry port, the event was classified as a "retreat". If the cell ruptured while inside the constriction, visible by morphological changes and dimmed fluorescence, the event was classified as "death", though this event occurred very rarely in these assays. Importantly, we only counted events in which the cell clearly passed through the other side of the constriction or retreated away from the constriction, eliminating the possibility of a "stuck" event.

As expected, individual T cells retreated from the constrictions a majority of the time. For the T cells that were classified as solo cells, they passed through the 2 μm constriction in approximately 29% of events, retreated in 69% of events, and died in 2% of events (**Figure 3D**). However, contact with other T cells greatly increased the likelihood that a cell would successfully pass through the 2 μm constriction. T cells that were classified as part of a train passed the constriction in 55% of events, retreated after attempting to enter the constriction in 43% of events, and died in 2% of events. These assays demonstrated that T cells moving as a group were more than twice as likely to pass through a narrow confinement than T cells moving alone.

The outcome of T cells' attempt to traverse a 2 μm constriction was shown to be strongly related to whether the cell was in contact with other T cells. To determine the extent of this potentially helpful crowding effect, we next examined whether crowding may also impact the speed at which T cells pass through these obstacles. To accomplish this, we determined how long each cell remained inside the constriction in the process of squeezing through the narrow pore. For all "pass" events, we tabulated the number of frames in which a cell was physically in contact with the constriction, and used this to determine the dwell time of each cell that successfully crossed to the other side. There was no significant difference in dwell time between solo T cells and trains of T cells, with T cells in both groups taking an average of approximately 6 minutes to move through the 2μm constriction before continuing on in the microchannel (**Figure 3E**). Thus, while crowding may facilitate the passage of T cells through a tight

constriction, it does not impact the actual speed at which the cell moves through the pore.

3.4 T cell trains are more likely than individual T cells to enter the narrower path size at a 4-way junction

After showing that crowding does increase the likelihood of T cells passing through a constriction in a straight microchannel, we continued to a more complex assay. As they do not proteolytically degrade the extracellular matrix to clear a path, T cells must interact with the space in front of them to map out a “path of least resistance”. This process causes the cell to deform and probe into pores with both the cytoplasm and nucleus, comparing the width of the nucleus to the width of the pores to avoid tighter squeezes that have the potential to cause DNA damage [23].

We performed T cell migration assays on microchannels that begin as 6 μm straight channels before the cells reach a fork, where the microchannel splits into 4 different pathways. Our time lapse movies showed that as T cells reached the junction in the microchannel, they probed into multiple pores before moving forward, as expected for amoeboid-migrating cells (**Figure 4A**). In this design of microfluidic devices, the pores have different widths, with two of the pores being a comfortable 6 μm wide and the other two being 4 μm wide, requiring greater cell deformation to pass through (**Figure 4B**).

There were two possible configurations of the 4 microchannels extending from the junction. In one of the layouts, termed Design 1, the two 4 μm pores were next to one another on one side of the junction, and the two 6 μm pores were also adjacent to one another (**Figure 4C**). In this design, all possible combinations of pore width (4 μm or 6 μm) and turning angle (wide or narrow turn from the original straight channel direction) are present at the 4-way junction. In the other configuration, termed Design 2, the two outermost pores are those that are 6 μm wide, with the 4 μm pores being in the center (**Figure 4D**). The directional persistence of a moving cell has been shown to be mathematically linked to its speed [127], such that a fast-moving cell such as an activated T cell would be predicted to avoid making extreme temps and attempt to

migrate in the straightest possible path. As such, this design required cells to prioritize either avoiding a sharp turn or avoiding a narrow pore at the 4-way junction.

As in the constriction experiments, all quantitative data were collected by manual annotation of time-lapse movies. Cells were classified as “solo” cells if they were not making contact with any other cells at the point where the microchannel diverged into 4 separate pores. To be classified as a member of a “train”, cells needed to be touching another cell as they reached the split in the microchannel. Then, the width of the pore chosen by the cell was noted, either 4 μm or 6 μm. As discussed, T cells often probe into multiple pores before continuing forward. Therefore, an event was only counted if the cell fully entered a certain pore and continued migrating beyond the 4-way junction.

In the junction Design 1, physical interactions between T cells appeared to modulate the outcomes at the 4-way junction (**Figure 4C**). Solo T cells entered the narrower 4 μm pore in only 21% of events, instead entering the wider 6 μm pore 79% of the time. In contrast, T cells moving in trains entered the 4 μm pore with a frequency of 39% and entered the 6 μm pore in 61% of events. The fact that T cells in a train were nearly twice as likely to enter the narrower pore at a 4-way junction provides another useful piece of evidence towards the potential beneficial effects of T cell crowding in more challenging extracellular environments.

The exact configuration of the 4-way junctions proved to have a profound impact on the distribution of paths taken by T cells. When moving through junction Design 2, the overall likelihood of choosing a narrow pore was increased in both trains and individual T cells compared to Design 1. This supports the idea that rapidly migrating cells tend to avoid turning at wide angles, as these cells preferentially migrated through a narrower 4 μm pore over a 6 μm pore that required a sharper turn to enter. While more experimentation would be needed to determine the degree to which moving T cells prioritize avoiding turns versus avoiding constrictions, it is interesting that this configuration resulted in a nearly 50% split, meaning that cells entered each pore at the same frequency (**Figure 4D**). This occurred regardless of whether the cells were moving alone or maintaining contact with other T cells while they moved into the forked microchannel.

Among the trains of T cells at the 4-way junction, we aimed to determine whether groups were more likely to stay together and enter the same pore or to spread out into different paths. We re-examined the trains of T cells to determine whether a given cell entered the same pore as the immediately preceding cell (with which it had been in contact), or entered any of the other 3 available pores. These analyses were pooled from both configurations of the 4-way junctions, as we simply wanted to determine whether cells follow one another at the split. We found that approximately 60% of cells entered a different channel than the cell it had been following, and approximately 40% entered the same channel. This shows a potential preference for cells to enter the same channel as their predecessor, as a truly random decision would result in 25% of cells entering the same channel as the one before.

3.5 T cells at the back of trains experience diminished crowding-based differences in migratory decision-making

The preceding analyses categorized cells into “solo” or “trains” based on whether they were physically in contact with other cells in the microchannels. However, one of the key differences between our cells of interest and the mesenchymal cells whose collective migration has been studied more extensively is that immune cells migrate in a more protrusive manner, generating minimal tensile forces in a sufficiently confined microenvironment [25]. As mesenchymal cells coordinate their collective migration through the transmission of tensile forces at cell-cell interfaces [82], we hypothesized that immune cell collective migration would be fundamentally different, due to the altered balance of “pulling” versus “pushing” force. This prediction was especially relevant when analyzing how collective migration may help T cells to pass obstacles in their microenvironment. Given our observations and the knowledge that immune cell migration is protrusive, we re-analyzed our data to begin interrogating whether groups of T cells were “pushing” their through constrictions.

If T cells were pushing forward against their immediate neighbour to facilitate migration through tight squeezes, then the cells at the back of a train would not experience the same effects as the cells ahead of them. Thus, we manually re-categorized the WT T cells from our existing migratory assays (**Figure 5A**). “Solo” cells

remained the same—cells that were not in contact with other cells during the event of interest. However, the rearmost cell of any train was analyzed in a separate category, called “last in train”. The remaining cells of any train, all of which had another cell immediate behind it and potentially pushing it forward, were categorized as “followed”.

In the simplest of our in vitro migratory challenges, the 2 μm constriction, the outcomes of the last cells in trains closely mimicked those of solo T cells (**Figure 5B**). T cells that were followed by another T cell passed through the constriction in 61% of events, an even higher rate than all T cells in trains. Concordantly, the last cells in each train passed through 2 μm constriction in 33% of events. This frequency was much closer to the 29% pass rate seen in solo T cells than it was to the other members of a train of T cells.

Finally, we re-analyzed the time lapse movies of T cells migrating through the 4-way junction microchannels. In junction Design 1, solo T cells entered the 4 μm pore in 21% of events and T cells that were followed by another cell entered the 4 μm pore in 44% of events, over double the frequency (**Figure 5C**). Meanwhile, the last cell of a train showed an intermediate rate of entry into the 4 μm pore at 29% of events. Junction Design 2 again resulted in a fairly even split between 4 μm and 6 μm pores in WT T cells (**Figure 5D**). This occured regardless of whether the cell was alone, followed, or the last in a train. Solo T cells entered the 4 μm channel in 52% of events, the last cells in a train entered the 4 μm channel in 52% of events as well, and followed T cells entered the 4 μm channel in 54% of events. While in this case, the last cells in a train do not resemble solo T cells as closely as they do in the constriction microchannel design, this still points to a potential forward-pushing effect that helps T cells in microchannels to move through tight spaces.

3.6 T cells deficient in Dock8 have different crowding outcomes

We next considered how perturbations to the overall migratory phenotype of T cells could impact their group interactions and decision-making in the microchannels with 4-way junctions. First, we repeated the migration assay using T cells deficient in Dedicator of Cytokinesis 8 (Dock8-KO). Dock8 is a guanine exchange factor (GEF) expressed exclusively in immune cells (**Figure 6A**). It was first discovered in a clinical

setting, when a group of children with severe immunodeficiencies and recurrent viral infections were found to be deficient in Dock8 [57]. Further research determined that Dock8 is important for cytoskeletal regulation as immune cells migrate through complex microenvironments, and that Dock8-KO leukocytes become entangled in ECM fibers and eventually shatter [58]. The central role of immune cell navigation in the Dock8-KO phenotype, alongside the potential impacts of this immunodeficiency on host defense, caused this protein to be an important topic of study in our lab group [121].

While entanglement and cytothripsis result in a severe migratory impairment for Dock8-KO T cells in 3D collagen matrices, unpublished data from our lab has shown that, in 1-dimensional microchannels, Dock8-KO T cells migrate at higher speeds than WT T cells (**Figure 6B**). Given their higher speed, we predicted that Dock8-KO T cells may exhibit different probing behaviour at a 4-way junction than WT T cells, potentially entering narrow pores at a higher rate if they fail to take the adequate time to determine pore widths. Additionally, we aimed to determine the degree to which the T cell crowding phenotype we had seen thus far is specific to WT T cells.

In the 4-way junction microchannel Design 1 previously described, Dock8-KO T cells exhibited a surprising distribution of decisions. Solo Dock8-KO T cells entered the 4 μm pore in 31% of events, which is comparable to the frequency of WT solo T cells (**Figure 6C**). However, Dock8-KO T cells in a train were slightly less likely to enter the narrow pore than solo cells were, entering in 23% of events. Given WT T cells exhibited a major increase in 4 μm pore entry when they were moving as a train, this is a striking difference. Similar to WT T cells, the results for junction Design 2 were ambiguous in Dock8 KO T cells, regardless of whether they were migrating alone or as part of a train (**Figure 6D**).

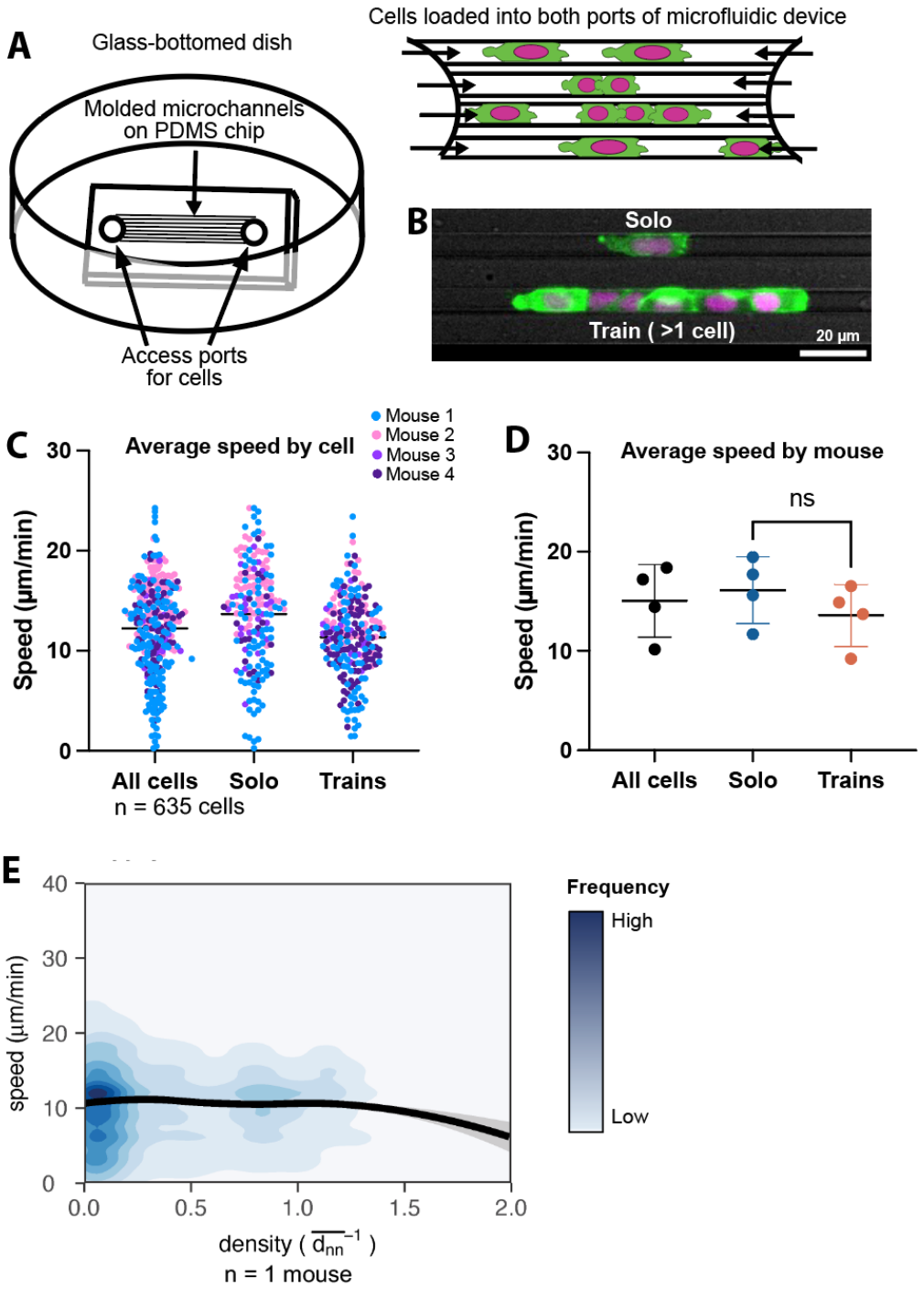


Figure 1. There is no detectable decrease in T cell speeds with increased cell density. (A) Schematic of experimental setup. Moulded PDMS microchannels were bound to a glass-bottom dish, then LifeAct-Rosa$^{nT\text{-}nG}$ cells were loaded into punched ports in the PDMS chip and imaged via widefield epifluorescent microscopy. (B) Representative image of solo T cell and train of T cells in a 6 µm microchannel. Scale bar is 20 µm. (C) Average speed of individual cells across a 90-minute time lapse. Colour of points differs by mouse/experiment. Data represent n = 635 cells from n = 4

mice. (D) Average speed of all cells from each movie/mouse. Statistical analyses were performed using a two-tailed T test. (E) Representative fundamental diagram, courtesy of Inge N. Wortel.

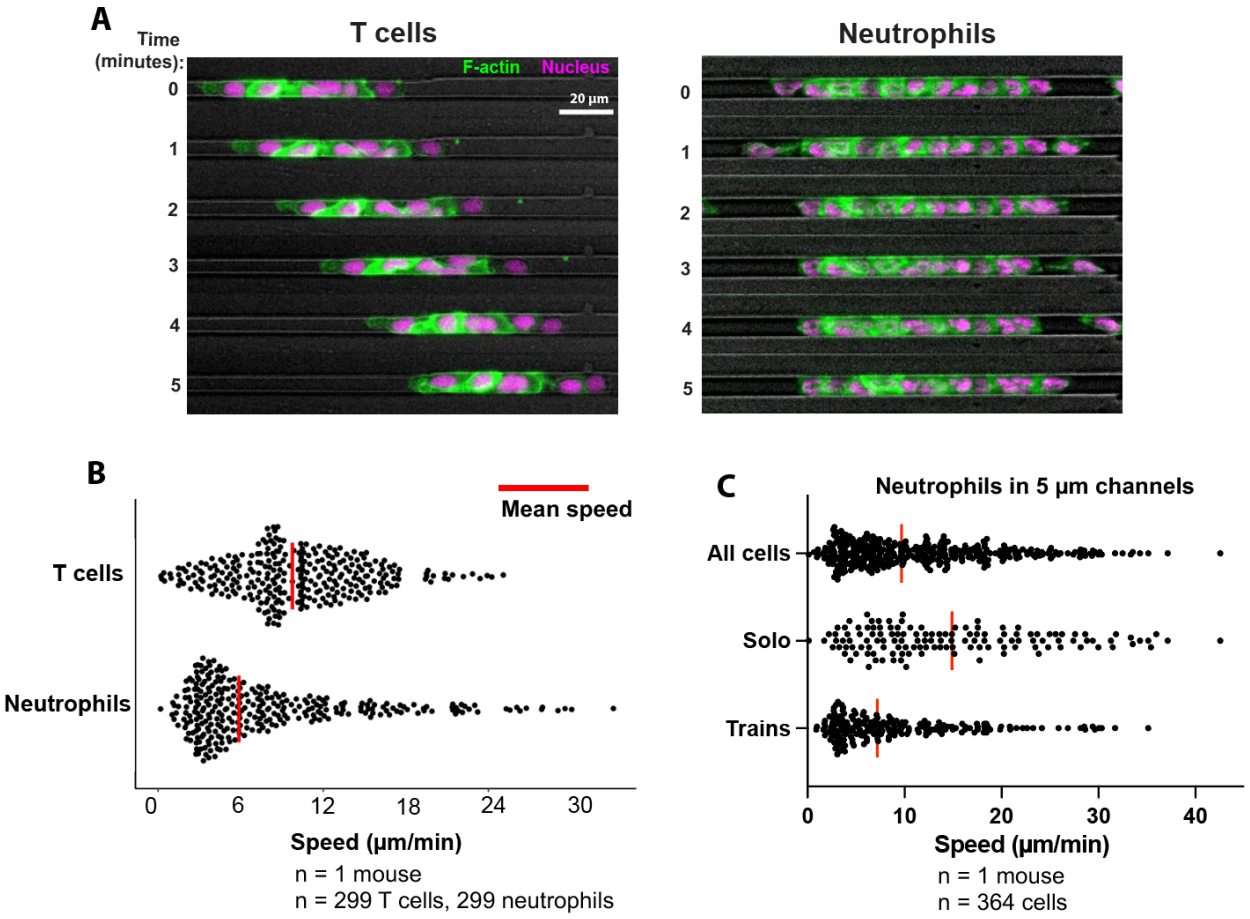


Figure 2. Neutrophils have significantly decreased migratory speeds at higher cell densities (A) Representative widefield images of trains of T cells and neutrophils. Scale bar is 20 μm. (B) Comparison of average T cell and neutrophil speeds in 6 μm microchannels. Each data point represents the average speed of 1 cell over a 90-minute time lapse. Data are from one representative experiment/mouse and include n = 299 T cells and neutrophils each. (C) Average speed of neutrophils in 5 μm microchannels. Data are from one representative experiment/mouse and include n = 364 cells.

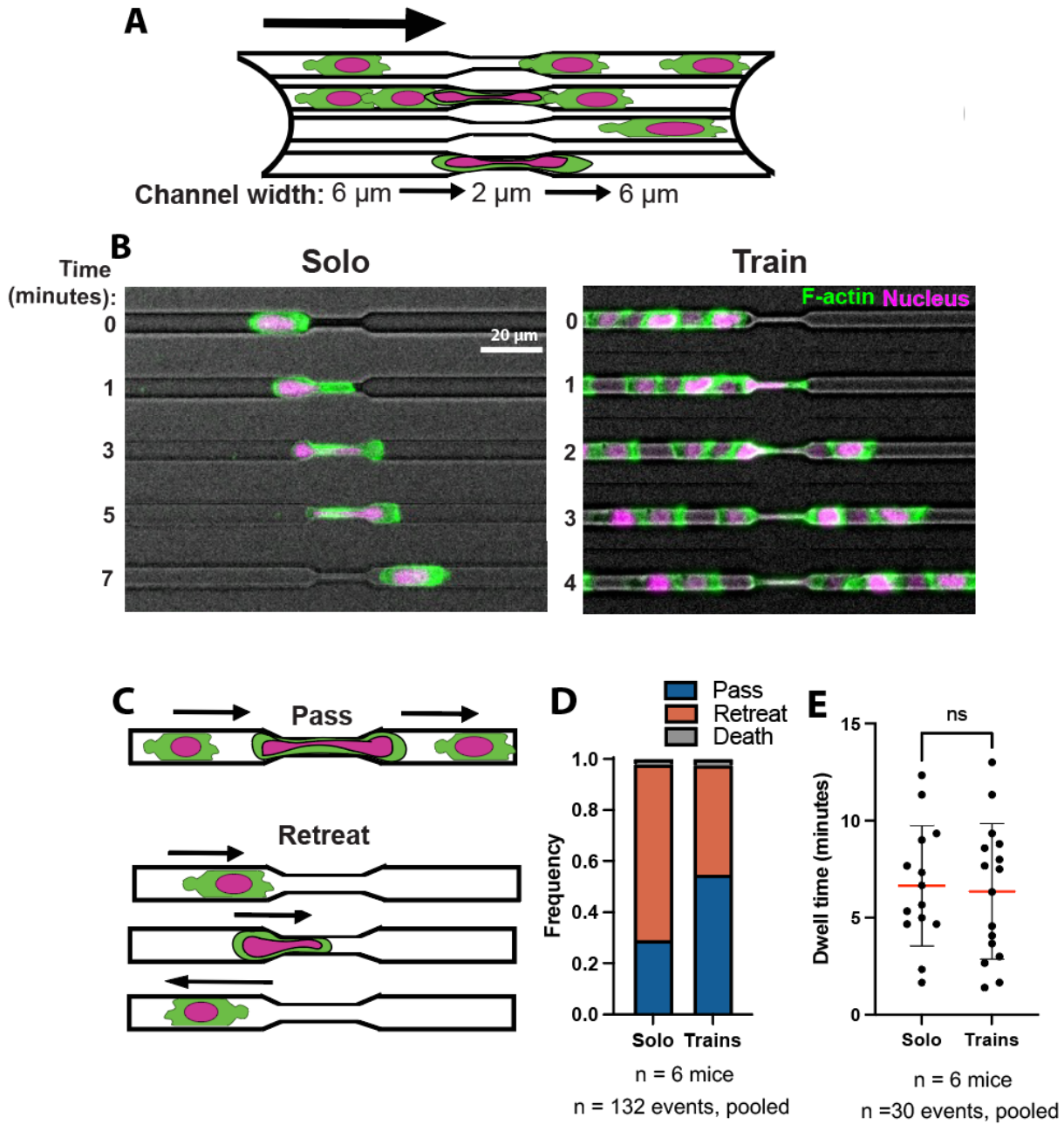


Figure 3. T cell trains are more likely than individual T cells to pass through constrictions in 1D microchannels. (A) Schematic of constriction microchannel design. Cells are loaded into one access port and migrate through 6 μm wide microchannels with a 2 μm wide constriction in the middle. (B) Representative widefield images of an individual T cell and a train of T cells passing through a 2 μm constriction. Scale bar is 20 μm. (C) Schematic of pass/retreat events when annotating time-lapses of T cell migration through constriction. Events are notated as “pass” when the cell moves through the constriction, “retreat” when the cell probes into the constriction but

reverses its migratory direction, and “death” when the cell ruptures inside the constriction. (D) Frequency of each event for solo cells and trains. Data represent n = 132 events pooled from n = 6 experiments/mice. (E) Time spent in contact with constriction for T cells that successfully passed. The average dwell time for all passing cells was taken for each train. Data are pooled from n = 6 experiments/ mice.

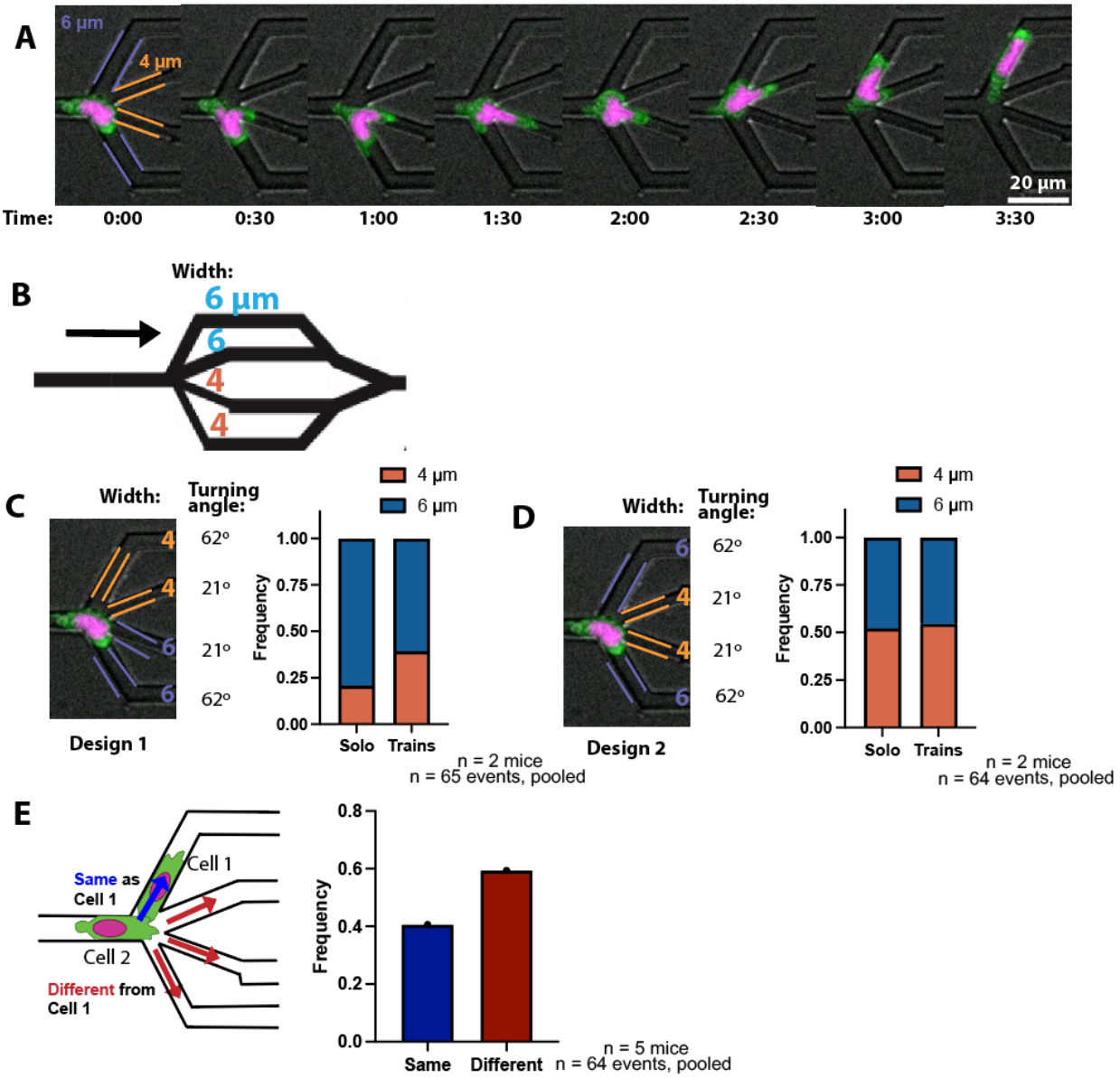


Figure 4. T cell trains are more likely than individual T cells to enter the narrower path size at a 4-way junction. (A) Representative time lapse of a T cell probing the pores of a 4-way junction microchannel. Scale bar is 20 µm. (B) Design of 4-way junction microchannel. Cells migrate down 6 µm microchannels from an access port and meet a 4-pronged fork with pores of variable size. (C) Rates of entry into each width of pore for solo T cells and trains of T cells in junction Design 1. Data are representative of n = 2 experiments/mice and n = 65 events (D) Rates of entry into each width of pore for solo T cells and trains of T cells in junction Design 2. Data are representative of n = 2 experiments/mice and n = 64 events. (E) Frequency that a given cell in a train entered the same or different pore as the immediately preceding cell. Data are representative of n = 5 experiments/mice and n = 64 events.

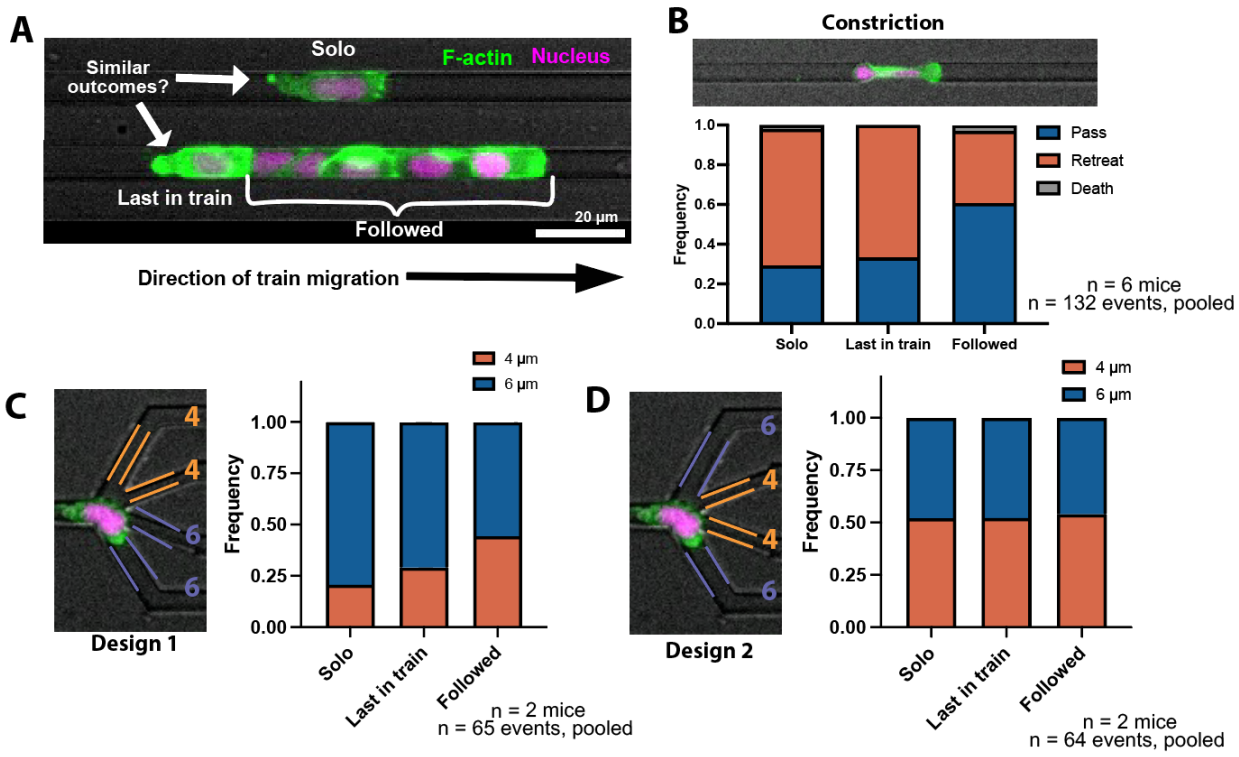
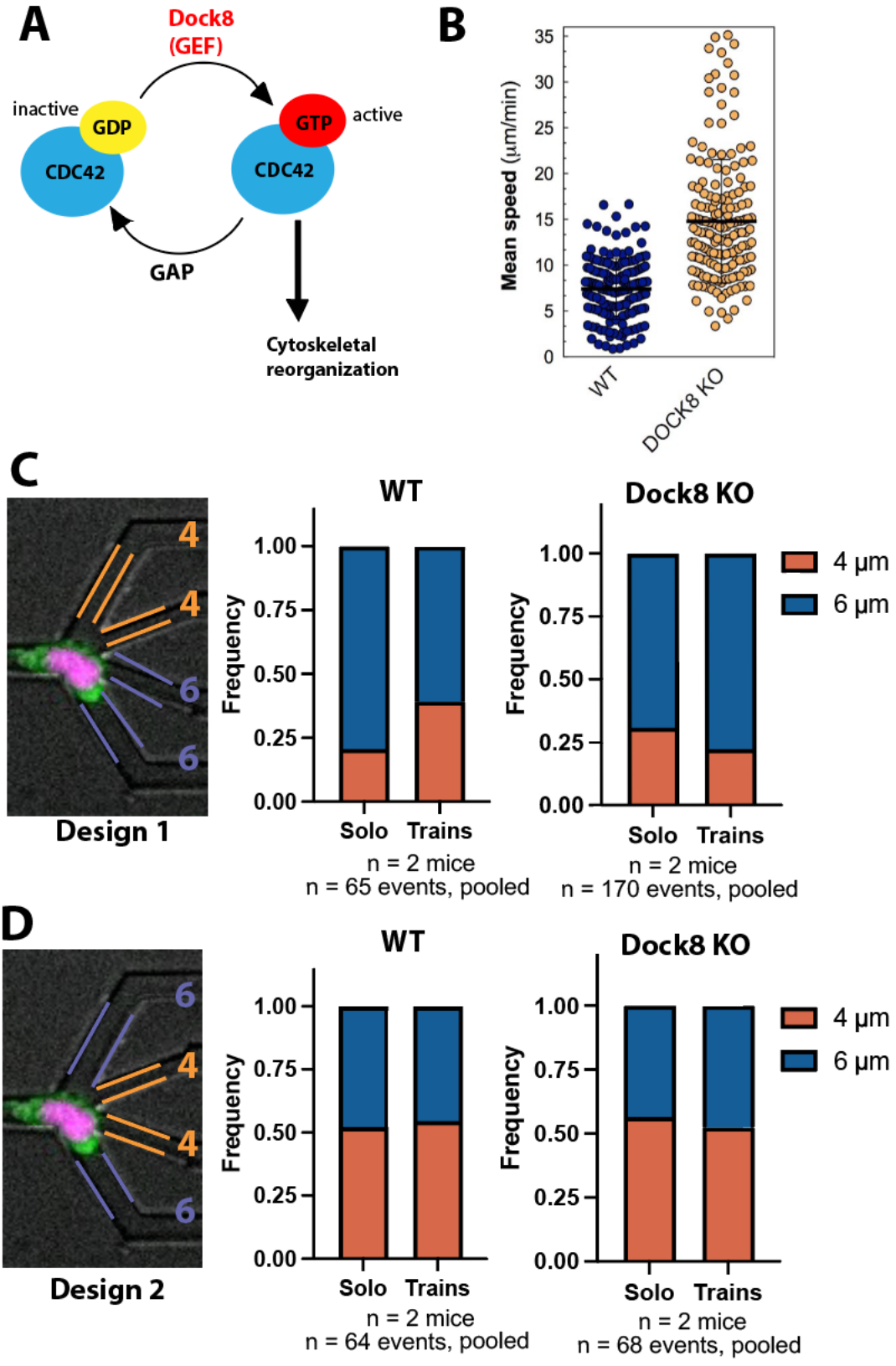


Figure 5. T cells at the back of trains experience diminished crowding-based differences in migratory decision-making. (A) Representative image of solo T cell and a train of T cells 6 μm microchannel, identifying the last cell in the train separately from the cells followed by another. Scale bar is 20 μm (B) Frequency of pass, retreat, and death events of T cells in 2 μm constrictions, separated into solo cells, the last cell of a train, or a cell followed by another. Data are representative of n = 132 events pooled from n = 6 experiments/mice. (C) Rates of entry into each width of pore at a 4-way junction in the "fair" junction configuration, separated by solo cells, the last cell of a train, or a cell followed by another. Data are representative of n = 2 experiments/mice and n = 65 events. (D) Rates of entry into each width of pore at a 4-way junction in the "forced choice" junction configuration, separated by solo cells, the last cell of a train, or a cell followed by another. Data are representative of n = 2 experiments/mice and n = 64 events.


A
Dock8
(GEF)
inactive
GDP
CDC42
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active
CDC42
GAP
Cytoskeletal
reorganization
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Mean speed (μm/min)
35
30
25
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15
10
5
0
WT
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C
4
4
6
6
Design 1
WT
Dock8 KO
Frequency
1.00
0.75
0.50
0.25
0.00
Solo
Trains
4 μm
6 μm
n = 2 mice
n = 65 events, pooled
n = 2 mice
n = 170 events, pooled
D
6
4
4
6
Design 2
WT
Dock8 KO
Frequency
1.00
0.75
0.50
0.25
0.00
Solo
Trains
4 μm
6 μm
n = 2 mice
n = 64 events, pooled
n = 2 mice
n = 68 events, pooled

Figure 6. Migratory mutations alter outcomes of groups of T cells at 4-way junctions. (A) Diagram of the GEF activity of Dock8. (B) Speeds of WT versus Dock8 KO T cells, courtesy of Connie Shen. (C) Rates of entry into each width of pore for WT and Dock8 KO T cells in junction Design 1. Data are representative of n = 2 experiments/mice per genotype, n = 65 events for WT T cells, and n = 170 events for Dock8 KO T cells. (D) Rates of entry into each width of pore for WT and Dock8-KO T cells in junction Design 2. Data are representative of n = 2 experiments/mice and n = 64 events for WT T cells, and n = 2 experiments/mice and n = 68 events for Dock8-KO T cells.

Chapter 4: Discussion

Active matter physics typically predicts that as the density of moving objects increases, the speed of each agent will decrease until they reach a critical density, at which jamming occurs [101, 128]. We showed that, in one-dimensional microfluidic devices, T cells can migrate at a fast speed whether alone or in contact with other T cells. Additionally, T cell speed remained consistent across a range of cell densities. This suggests that T cells can avoid the expected phase change and jamming that frequently accompanies increasing densities, and that indeed occurs in dense crowds of epithelial cells [105].

When questioning why this ability to maintain a faster, fluid-like migratory state at high densities might be present in T cells but not in other cell types, the biological role of each cell type studied and the specific migratory context exemplified by the experiment(s) is important to consider. Early cell migration research was carried out using adherent cells on a 2-dimensional substrate, which inherently biases cells toward the classical protrusion-adhesion-retraction cycle of mesenchymal cell migration, and decades of subsequent cell migration studies have used this model, with relatively recent shifts towards developing 3-dimensional in vitro models that more accurately mimic in vivo microenvironments [18, 129]. As such, the existing body of work that addresses the effects of density on cell migration is limited to epithelial cells growing in a monolayer. While a variety of models, including MDCK cells and HBECs, have been shown to jam in vitro, epithelial cells growing in a monolayer use strategies for locomotion and collective coordination that are distinct from T cells' amoeboid, 3-dimensional migratory behaviours [104, 130, 131]. As epithelial cells form cell layers and physical barriers in vivo, tightly regulated, leader-and-follower collective migration is important for maintaining the structural organization of tissues during wound repair [3, 4].

While epithelial cells migrate within a certain organ or tissue, immune cells are among the only cells that are capable of traveling between different peripheral tissues of the body, adapting their shape, speed, and pathways in response to the structural parameters of their current microenvironment [132]. Furthermore, T cells are required to

navigate through densely packed secondary lymphoid organs in order to undergo necessary processes of activation and differentiation, and subsequently to navigate through diverse organ parenchyma to perform their effector functions at the site of immunological insult. Avoiding jamming and phase transitions in crowds would then be a highly adaptive strategy for T cells, given the anatomical and physiological contexts in which they operate. While the use of one-dimensional microfluidic devices is an intentionally reductionist approach to the complex question of T cell collective migration, confirming that T cells are resistant to jamming in vitro is the first step to untangling the 3-dimensional interactions and biological mechanisms underlying this phenomenon.

Having seen that T cells are tolerant to crowding in 1-dimensional in vitro migration assays, we then interrogated the migration of neutrophils in similar crowding experiments. Neutrophils were chosen as a comparison population due to their high speeds [59] and tendency to perform their immunological functions in large, amplifying crowds. While solo-migrating neutrophils reached higher speeds than solo-migrating T cells, occasionally exceeding 30 μm/minute, we observed that crowds of neutrophils, as well as the individual cells in these crowds, moved much more slowly (generally below 6 μm/minute). This was true both in 6 μm microfluidic devices, where neutrophils could bypass one another in the channels, and 5 μm microfluidic devices, where they formed "trains" that more closely resembled the trains of T cells observed in the preceding experiments.

While these migratory assays were observational and did not address potential biological mechanisms, it is possible that paracrine chemoattractant signaling is responsible for the differences between the crowding behaviours of neutrophils and T cells. A hallmark of neutrophil activity at the site of insult is swarming, a well-defined process in which "pioneer" neutrophils call other neutrophils to amplify the early inflammatory response [66, 114, 117, 124]. Importantly, neutrophils both secrete the chemoattractant LTB_4 and express leukotriene B_4 receptors type 1 and 2 (BLT-1 and BLT-2, respectively), allowing both autocrine signaling to create and respond to local chemokine gradients and paracrine signaling for longer-range chemotaxis and swarm amplification [113-116, 133-136]. In contrast, activated T cells cannot call out to fellow activated T cells in a comparable manner. T cells do use paracrine signaling in multiple

contexts; IL-2 signaling is important for activation and effector or memory differentiation of CD8+ T cells, and prostaglandin D_2 (PGD_2) is secreted by recently-activated Th2 cells to recruit and facilitate activation of naïve T cells [137, 138]. However, there are no known chemotactic ligand/receptor pair expressed by activated T cells, such that activated CD3- and CD28-activated T cells alone would not be expected to cluster or swarm in the same way as neutrophils. Both groups of cells were suspended in complete RPMI without exogenous chemokines for the migratory assays. However, neutrophils may have secreted LTB_4, forming chemokine gradients that recruited other neutrophils into growing clusters, potentially explaining observed differences in the two populations.

Another important difference between the group behaviours of T cells an neutrophils is the contexts in which they experience crowding. T cells encounter crowds throughout their lifetime, and effective navigation of densely packed lymphoid organs is crucial for their activation and differentiation. Thus, they must be able to not only form crowds, but move through and escape these crowds via efferent lymphatic vessels to eventually enter the vasculature and reach diverse tissues [51, 139]. Historically, neutrophils were thought to swarm at the site of insult in early stages of inflammation, and then quickly die via apoptosis and be cleared away by macrophages. Subsequent research has demonstrated that a significant portion of neutrophils undergo reverse migration, exiting the inflammatory site into the vasculature [140-142]. While this shows that neutrophil collective migration is not as “terminal” as previously assumed, imaging studies of this phenomenon have shown that neutrophils at the edge of the swarm polarize and exit the site of insult first, with central cells continuing to move somewhat randomly, presumably until they reach the edge of the swarm and can sense chemokine gradients more effectively. The collective mechanisms and processes of collective neutrophil and T cell migration are still poorly understood, but the fundamentally different contexts and functions of these migratory events could explain the different outcomes of our in vitro assays. In the future, studies of the effects on crowding of other motile immune cells including dendritic cells and monocytes should be undertaken to explore how immunological function and collective migration intertwine.

After confirming that T cells, but not neutrophils, maintain their speed regardless of density in one-dimensional microchannels, we experimented with more complex microchannel designs to examine emergent behaviours when groups of T cells encountered migratory obstacles. The amoeboid mode of migration, employed by T cells alongside other leukocytes and malignant cells, earned its name from the highly deformed, rapidly changing cell shapes that are observed when these cells move through a complex 3-dimensional microenvironment. Because they largely forgo the use of metalloproteases to degrade the ECM, amoeboid cells can dramatically deform their cell body and nucleus to fit through small pores. However, this process is not without risks; excess compression and deformation of the nucleus can cause migrating cells to accumulate DNA damage or experience rupturing of the nuclear envelope [126, 143]. To reduce mechanical stress incurred while migrating through challenging environments, immune cells have been observed to compare their nuclear size to that of pores and determine a “path of least resistance” [23], and to polarize actin orthogonally from the cell membrane via WASp to transiently push apart ECM fibers and widen pores [25].

In our constriction assay, T cells did not have multiple options to choose a path of least resistance; they could either squeeze through a 2 μm constriction or turn back in the direction from which they came. While WASp-mediated actin patches can help to widen constrictions in a collagen matrix, our PDMS microchannels are too stiff for a cell to dilate. Given the challenging conditions of this migration assay, it is potentially unsurprising that a majority of individual T cells retreated instead of passing through a constriction. In contrast, when T cells were moving a train, they became more likely to pass through the tight squeeze than to retreat.

One potential explanation for this difference between individual and collective T cell motion could simply be that the cells were physically pushing each other forward. T cells, particularly in confined environments, rely heavily on protrusions and blebbing at the leading edge and use integrin-mediated adhesions sparingly if at all in their normal migration [15, 144]. It is then possible that the protrusion of the leading edge of a cell could be pushing against the back of the next cell ahead, physically moving the cell and providing extra force that may propel a cell forward through a constriction instead of

reversing directions. Along with or instead of brute force, T cells may be responding to protrusions from the cell behind them in a mechanosensitive manner. T cells express a variety of mechanosensitive ion channels, including Yap and Piezo1, that have been shown to profoundly modulate their immune function in response to their microenvironment [145-148]. Another potential explanation for T cells' increased likelihood to continue through constrictions when in contact with other T cells is a mechanosensitive response to the protrusive forces of other T cells that drives the cell forward. Whether mechanotransduction plays a role in this observed effect, and which specific pathways may be involved, remains to be investigated.

In a similar vein, contact inhibition of locomotion (CIL) is another potential explanation for why a T cell in a train would be more likely to continue forward through a migratory obstacle. This phenomenon, which has been observed in migratory cells for decades, is known to be important for collective cell migration in epithelial cells that migrate in sheets with a more rigid leader-follower organization [93]. While leader cells carry the primary responsibility of polarization with a lamellipodium at the leading edge and proteolytically degrading the ECM to clear a path, contacts with follower cells at their rear help to stabilize polarization of leader cells and keep the collective moving in the same direction [82, 91, 149, 150]. At these cell-cell interfaces, CIL prevents leader cells from forming backward protrusions or reversing directions, such that the sheet of cells continues forward with the help of follower cells. Amoeboid migration is distinct from mesenchymal migration in numerous ways, but the general mechanics of cell migration are conserved, such as actin polymerization being the driving force of motion and myosin-mediated contractility being an important aspect of motility. As such, CIL, which has been observed to be important for the dynamics of collective mesenchymal cell migration, should also be studied for its effects on immune cell migration. The potential mechanosensitive or physical response to pushing, as well as CIL, also provide a strong potential explanation for why cells at the back of trains were not more likely to enter constrictions than individual T cells.

When we increased the complexity of the migratory assay by imaging T cells making directional decisions at a 4-way junction in the microfluidic device, the results in turn became less straightforward. In the junction Design 1, where cells could turn at a

wide or narrow angle and through a 4 μm or 6 μm pore, T cells moving together were more likely to enter the narrow pore than the T cells moving alone, though this still occurred in a minority of counted events. As discussed with the constriction microchannel design, it is plausible that if a cell probed into a channel, pushing or CIL from the following cells would have driven it to commit to that pathway rather than probing more thoroughly. However, this fails to explain the lack of difference between solo T cells and trains of T cells in the junction Design 2. In this experimental design, it appears that cells became more likely to enter the 4 μm pore due to the fact that the 6 μm pore required them to turn at a more extreme angle. More thorough testing of how individual T cells balance directional persistence with probing variable pore sizes is likely warranted before the effects of crowding in this context can be understood.

Collective immune cell effects at migratory junctions have been addressed in a specific context, in which it was demonstrated that pairs of neutrophils following a chemotactic gradient are more likely to split into separate pores in a bifurcated microchannel than to follow an immediately preceding cell [118]. The effect was strong, such that even when the second pore of a bifurcation was narrower, neutrophils still avoided following a cell with which they had been making contact. In contrast, we showed that T cells migrating at a 4-way junction were slightly more likely to follow the cell ahead of them than they would be by random chance (40% likelihood of entering the same pore versus 25% if random).

One major difference between these experiments is that the T cells in our assays were migrating spontaneously instead of following a chemotactic gradient, and the neutrophil study showed that neutrophils locally disrupt chemokine gradients for the cells behind them, which likely played a key role in migratory decision-making. In narrow channels, the movement of neutrophils was also shown to disrupt the hydraulic resistance, potentially providing an impetus for subsequent cells to enter the alternate channel via barotaxis. As a 6 μm microchannel is somewhat confining for migrating T cells, the potential disruption of fluid flow is an important consideration for further study of T cell migration at migratory junctions.

Repeating the 4-way junction migration assays using Dock8-KO T cells further emphasized the myriad variables affecting T cell collective behaviour, particularly in a

complex microchannel design. Dock8 has been shown to be an important regulator of cytoskeletal coordination during immune cell migration through complex 3-dimensional microenvironments [58]. While the effects were modest, we showed that in the “fair choice” microchannel design, Dock8-deficient T cells were slightly less likely to enter the narrower 4 μm pore as a group than they were alone. Faulty cytoskeletal coordination would be expected to have broad-ranging effects on all aspects of cell migration, and is likely to affect how cells physically interact with other cells. Aberrant cytoskeletal rearrangements may cause groups of Dock8-KO T cells to navigate migratory obstacles in unusual ways. More broadly, understanding how defined migratory defects affect collective T cell behaviour can be an useful step towards better understanding the mechanisms underlying these behaviours in healthy T cells.

Summary and Conclusions

T cells are among the most motile cells in the body, circulating between lymphoid organs when naïve and navigating between diverse tissues once they are activated. The existing body of work on T cell locomotion is squarely focused on the behaviours and mechanisms of individual T cells. Large groups of self-propelled agents are generally expected to slow down and jam at increased densities, and this prediction has been demonstrated to hold true in studies of epithelial monolayers. However, knowing that T cells must successfully make their way through lymph nodes densely packed with other T cells in the process of activation, we hypothesized that T cells have evolved mechanisms to navigate crowds efficiently and avoid jamming. In this thesis, we showed that, in 1-dimensional microfabricated devices, T cells maintain consistent speeds and do not jam regardless of whether they are migrating alone or as a crowd. In contrast, neutrophil speeds slowed significantly upon crowding despite their rapid individual migration, indicating that these effects may be T cell-specific rather than broadly applicable to immune cells.

Though T cell speed was unaffected by crowding, we showed a situation in which crowding does impact T cell migratory behaviour. In microchannels with a narrow constriction, T cells moving as a group passed through the constriction nearly twice as frequently as individual T cells did. In microchannel designs in which cells reached a 4-way junction of variable pore size, we showed that T cells were more likely to enter a narrower pore when they were moving as a group than when they were migrating alone. However, we also found that these crowding effects are altered in different 4-way junction configurations that require cells to prioritize turning angles versus pore width. The changes in migratory decision-making were most pronounced in T cells that were followed by another cell, suggesting a potential role for physical or mechanosensitive responses to other cells pushing from behind. Additionally, T cells deficient in Dock8, a known regulator of cytoskeletal rearrangement, demonstrated different decision-making at the 4-way junctions, alone and as a crowd. Further investigation is needed into the mechanisms that control how T cells migrate as a group in both simple and complex microenvironments.

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