The Role of ROCK Inhibitor in the Polarization of Human Natural Killer Cells

Batya Koenigsberg

B.A. Candidate, Cellular and Molecular Biology

Emily Mace, PhD

Mentor, Principal Investigator

Shira Eisman

Mentor, Graduate Student

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Barnard College of Columbia University, Department of Biology

Columbia University Irving Medical Center, Department of Pediatrics

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I. Introduction

Natural killer (NK) cells are a significant part of the human immune system and deficiencies in NK cells result in susceptibility to viral infections and cancer[1]. NK cells are part of the innate immune system that mount an immediate protective response before adaptive responses develop[2]. NK cells are distinct from T and B cells, given their unique expression of surface receptors and cytotoxic activity. NK cells secrete enzymes perforin and granzyme, which disrupt the target cell membrane and activate the target cell's "cell death" machinery. This mechanism serves to kill virally-infected or primary tumor cells[2,3]. To recognize a target, NK cells can identify a "missing self," cells without a critical amount of Major Histocompatibility Complex (MHC) class I surface proteins, and then lose "inhibition," or engagement of receptors that will prevent activation of cytotoxic processes, such as inhibitory killer immunoglobulin-like receptors (KIRs), and inhibitory CD94-NKG2A heterodimeric receptors that bind to the various MHC complex isoforms (i.e. classical and non-classical)[4]. This lack of MHC class I recognition is also accompanied by "activation" of NK cells, where signals to activate "killing activity" are mediated through the engagement of activating receptors such as NKG2D and NCRreceptors, and NKp80. Inhibitory receptors both sense the presence or absence of MHC class I proteins on a target cell, as well as other cell-surface ligands, and activating receptors are engaged by the appropriate target cell ligands, activating a signaling cascade, resulting in apoptotic activity of perforin and granzyme[5]. To mount an immune response, NK cells also secrete cytokines and chemokines to further stimulate other components of the host immune system. Other unique NK cell surface receptors include CD56, CD16, and CD62L. NK cells have been shown to have functional and phenotypic overlap with T cells, while having a complementary function to the T

cell response. NK cells have their own functional niche in the immune environment, as demonstrated by cases of patients with viral susceptibilities due to NK cell deficiencies[6].

Natural Killer cell development is characterized by six stages, where progenitor cells will progressively develop in each stage, displaying a characteristic combination of surface receptors marking that specific developmental stage (Figure 1)[7]. In bone marrow, CD34+ hematopoietic stem cells (HSCs), give rise to CD34+ hematopoietic progenitor cells (HPCs) . Stimulated by local cytokine signaling, cell-cell contacts, and extracellular matrix interactions, CD34+ HSCs will continue on to the following developmental stages in secondary lymphoid tissue (SLT), until they reach their terminally differentiated stage in peripheral (circulating) blood as NK cells, marked by the loss and acquisition of stage-specific cell surface markers and receptors[8]. In their terminally differentiated state, NK cells gain the expression of receptors CD94, CD16, CD57, and NKp80. In the peripheral blood, NK cells are found in a subset distinguished by the density of CD56. NK cells at the earlier stage of development have been found to contain a higher density of CD56, thereby known as $CD56^{bright}$ NK cells. Terminally differentiated NK cells in PB have a lower density of CD56, thereby known as $CD56^{dim}$ NK cells[4,5,9].

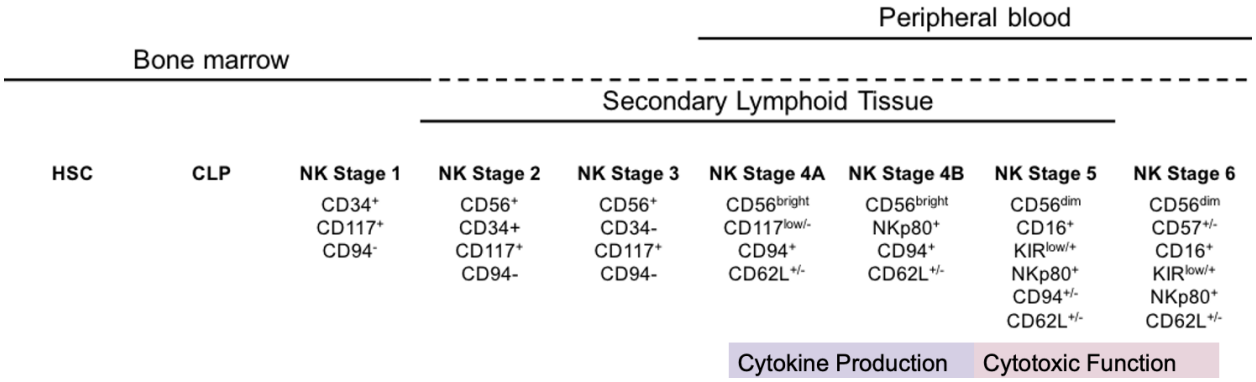

Figure 1: Schematic of Human NK cell development. *Made with Biorender, Freud, Immunological Reviews 2006 (Shira Eisman)*

Previous work has suggested that the transit of NK cell developmental intermediates between sites of NK cell maturation is mediated by a novel immune synapse, termed the developmental synapse (DS). The DS is an expanded definition of the immunological synapse, which defines the general cell-cell interactions between immune cells that arrests typical migration under flow conditions and forms a tight cell-cell junction, initiating and facilitating contact between an immune cell and an antigen-presenting cell (APC)[10,5]. In the context of human NK cell biology and function, an immunological synapse constitutes the signaling platform that directs secretion of lytic granules toward its target cell. This directed secretion of lytic granules is facilitated by the relative activity of activating versus inhibitory NK cell receptors, where the activity of the inhibitory NK cell receptors are surpassed by the activating receptors, leading to the formation of the immune synapse, a rearrangement of cytoskeletal and intracellular soluble factors that polarizes the NK cell toward its target[11]. The immunological synapse has been characterized by four criteria: adhesion, polarity, signaling, and resulting function[10]. The DS likewise adheres to these requirements for formation of a synapse, in the context of the contact between NK cells and developmentally supportive cells in the specific tissue region appropriate to that stage of development, ultimately promoting terminal maturation of NK cells[12]. Integral to the interaction between NK cells and their developmental niche is the stage-specific patterns of motility and migration. From their earliest lymphoid progenitor stages, T and NK cells receive chemical signals (i.e. chemokines) from surrounding cells in the specific developmentally supportive tissue environment and are further recruited by multistep adhesion cascades[13,14]. These adhesion cascades are characterized by interactions between cell adhesion molecule receptors on the progenitor cell surface and ligands on the developmentally supportive cells. Such adhesion molecule receptors, in the context of NK cell development include

P-selectin and PSGL1, chemokine receptors such as CXCR4/CXCL12, and integrins LFA-1/ICAM-1 and VLA-4/α. Following migration and adhesion, integrins taking part in the adhesion process mediate "arrest" of the migratory lymphoid progenitor cells, which become tethered to their developmentally supportive surface through interactions between these integrin-ligand pairs, namely LFA-1/ICAM-1, VLA-4/VCAM, and VLA-4/fibronectin. Upon arrest and tethering, integrins will cluster to the site of arrest to further facilitate attachment of the developing cell to the supportive environment[8]. Additionally, it has been shown that $CD56^{bright}$ NK cells demonstrate an accumulation of CD56, CD62L, and F-actin at uropod-like structures when "tethered" to developmentally supportive stromal cells (EL08.1D2). This finding suggests that the uropod plays an important role in facilitating cell-cell contact constituting the DS[12].

During integrin-mediated arrest, developing lymphocytes have been found to consistently adopt a specific, dynamic, morphology, consisting of a broad, flat leading cell edge, and a narrow, foot-like, trailing projection at the cell end. The leading edge of the cell is known as the lamellipodium, and the trailing end of the cell is known as the uropod. This changed morphology is accompanied by numerous changes to the cell's internal architecture[2]. F-Actin, an important cytoskeletal subunit, displays differential morphology in the lamellipodium versus the uropod[15]. In the leading edge of the lymphocyte, actin will form two-dimensional branched networks, while undergoing a dynamic process of actin assembly and disassembly, aided by small soluble proteins such as WASp, the Arp 2/3 complex, and RhoA GTPases. The uropod is rich in microtubules, spindle-like proteins emanating from the microtubule organizing center (MTOC), which also localizes to the cell rear when polarized[16]. Unlike at the leading edge of the cell, where F-actin undergoes extensive, dynamic branching, F- actin forms bundles oriented along

the axis of migration in the uropod. Formation of these bundles is aided by the activation of myosin II, which associates closely with F-actin, forming linear bundles (generally known as stress fibers). The combined activity of myosin II and actin is also accompanied by the redistribution of surface receptors such as ICAM-1, CD43, CD44, PSGL1, and CD95 at the uropod when polarized.

The redistribution of surface receptors and the formation of uropod- associated actomyosin bundles are underpinned by internal signaling occurring in the uropod. Motile leukocytes initiate internal signaling in response to either contact with antigen-presenting cells (APCs), or in response to chemotactic gradients, transient cues in the form of a diffusible chemical signal secreted in the environment, for the purpose of directing cells for a short burst of migration[17]. These chemotactic gradients may be composed of chemokines, cytokines, bacterial peptides, or complement-derived peptides[16], and are received by specific G-Protein Coupled Receptors (GPCRs) such as CXC chemokine receptors, initiating an internal signaling cascade. Specifically, the ligand binding to the receptor will cause a conformational change in the receptor, resulting in the dissociation of the receptor-associated heterotrimeric G-proteins on the inner membrane of the cell. This is followed by an increase of effector enzymes, activating signaling pathways resulting in biological changes in the cell[18]. Of interest is the pathway regulating actin polymerization at the leading and trailing edge of the cell. This pathway is characterized by the activity of Rho-family GTPases, effector proteins whose activity depends on their binding to GTP[16]. The activity of GTPases of the Rho family result in the polarized activity of actin at the poles of the cell, as well as integrin clustering via activation of ERM proteins. GTPase RhoA regulates myosin II contractility, and is thereby directly responsible for contractile activity at the cell rear, which is essential for cell motility[16]. RhoA is activated by the

conformational change of the GPCR following interaction with a ligand. This activates two proteins: formins, which facilitates the construction of parallel actin bundles, and Rho-dependent coiled-coil containing protein kinase (ROCK), an effector protein. The activation of ROCK is essential to the construction of parallel actin fibers and their association with activated myosin II, which allows the parallel actin bundles to contract, thereby forming stress fibers. The formation of these stress fibers via actomyosin contraction is essential for cell motility, as the development of the uropod is essential for net advance of leukocytes across developmentally supportive surfaces in their developmental niche. At the leading edge of the cell, Rac-GTP is the active Rho-family GTPase, activating WASp family proteins (such as WAVE/SCAR) and PAK1. WASp family proteins activated by Rac-GTP then activate the actin-related protein 2/3 (Arp2/3) complex, which serve as branching actin nucleators. Activation of PAK1 leads to phosphorylation of protein filamin, which allows it to act as an actin-cross linker. Activation of PAK-1 also results in phosphorylation of proteins regulating myosin II activity, myosin heavy chain (MHC) and myosin light chain kinase (MLCK). Phosphorylation of MHC and MLCK inhibits both proteins, leading to increased myosin II disassembly, decreased myosin activity, and thereby decreased contractile activity as well as less stress fiber formation at the trailing edge of the cell. Meanwhile, the increased activity of proteins that support the assembly of branched actin webs lead to increased protrusion at the leading edge of the cell, resulting in the development of a lamellipodium[16].

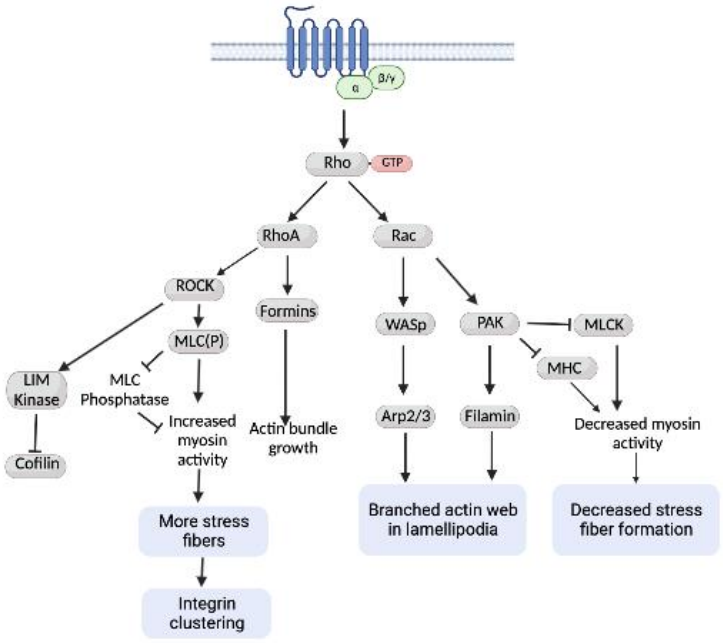

Figure 2. Schematic of the canonical Rho-GTP-ROCK pathway. Divergent RhoA and Rac signaling results in stress fiber formation and integrin clustering in the trailing edge, and branched actin network formation in the lamellipodium (*Mol. Bio. of the Cell, 2015, Created on Biorender)*

In addition to the divergent activity of Rho-GTPases at the cell poles, receptor and integrin clustering facilitates the formation of the DS. Multiple studies have shown that several cell surface markers, receptors, and integrins are localized to the uropod during leukocyte (including NK cell) migration, including ICAM1, ICAM2, ICAM3, CD44, CD43, and PSGL-1[15]. The localization of these molecules to the uropod is due to the activity of ERM proteins (Ezrin-Radixin-Moesin), whose transient activation and inactivation are mediated by GTPases and phosphoinositides. Immediately before polarization, phosphorylated ERM proteins are found at the cell front, attached to adhesion receptors. Then, in response to a chemoattractant, protein

phosphatases PP2A and PP1 transiently inactivate the ERM protein, causing it to detach from the inner face of the adhesion receptor. Then, phosphatidylinositol 4,5 bisphosphate (PIP_2), phosphorylated by phosphoinositide-3-kinase (PI3K), will convert ERM proteins to an active form, allowing it to re-bind to the adhesion receptors on the inner face of the plasma membrane. The ERM proteins will re-bind to several adhesion receptors in clusters, aided by actomyosin, complexes of myosin and actin fibers. The myosin proteins will bind to each other in a head-head tail-tail manner, thereby bringing the adhesion receptor-ERM-actomyosin complex together in a cluster[15]. The mechanism through which the adhesion receptor-ERM-actomyosin complex is redistributed to the cell rear is unknown. In a display of signaling crosstalk and a positive feedback loop, downstream kinases that are already active, such as ROCK, phosphorylate ERM proteins, and active ERM proteins facilitate the binding of RhoA (upstream of ROCK) to GTP, thereby converting it to its active form, further facilitating the activation of ROCK, resulting in formation of stress fibers in the leukocyte[15].

ROCK plays an essential role in receptor clustering and actomyosin bundle formation. Therefore, it exerts significant influence on the formation of the DS, particularly in the concentration of uropod-associated receptors and surface markers, such as ICAM, CD44, and β_1 integrin, and in migration following the period of arrest characteristic of the DS. The importance of ROCK as a downstream RhoA effector is further demonstrated by ROCK inhibitors. Interestingly, ROCK proteins have their own autoregulatory inhibitory domains within their amino-terminal kinase domain, by toggling between an “open” and “closed” conformation, allowing the kinase domains to be exposed for phosphorylation. ROCK proteins also have other “natural” inhibitors, small GTP-binding proteins Gem and Rad. ROCK proteins have been found in two main isoforms in mammals, *ROCK1* and *ROCK2*, and each isoform has been

subsequently found to have different, yet related downstream targets. ROCK2 is active in inhibition of myosin light chain phosphatase (MLCP) activity. while ROCK1 phosphorylates LIM kinases, promoting actin filament assembly. Additionally, ROCK1 and ROCK2 are differentially expressed in mammalian tissue, where ROCK1 is more generally expressed, and ROCK2 expression is localized to cardiac and brain tissues[19].

The widespread expression of ROCK proteins throughout mammalian tissue is indicative of its essential function. Indeed, as a downstream target of Rho, ROCK has a necessary role in regulation of cell polarity and migration. Aberrations in ROCK expression have been shown to alter cellular functions related to maintenance of polarity, migration, and chemotaxis. Increased ROCK activity has been observed in tumor metastasis, and constitutively activated ROCK has been shown to promote tumor invasion[19]. Therefore, it is evident that increased ROCK expression has roles in increased cell motility and migration, possibly providing malignantly-transformed cells with the mechanism for increased motility, resulting in an ”enhanced” migratory phenotype. Conversely, decreased ROCK expression has been demonstrated to play a role in decreased motility and migration. It has been shown that invasion of rat hepatoma cells and migration of metastatic breast cancer cells are inhibited by overexpression of dominant-negative ROCK constructs, or using the ROCK inhibitor Y-27632[19]. Additionally, ROCK inhibitor fasudil has been used to reduce endothelial activation and eosinophil adhesion *in vitro*, as well as allergic inflammation in the lungs of mice with sickle cell disease. ROCK inhibitor was able to inhibit endothelial-leukocyte interactions, thereby reducing vascular inflammation[20]**.** These findings serve as evidence toward ROCK inhibitor’s role in decreasing cell motility and migration. ROCK directly phosphorylates LIM kinase and myosin light chain kinase, inhibiting cofilin-mediated actin filament disassembly, and promoting myosin

activity. Additionally, ROCK phosphorylates and inactivates myosin light chain phosphatase, preventing the breakdown of the myosin light chain kinase. Any disruption of these functions of ROCK would prevent the sufficient development of actomyosin bundles, which are integral for cell rear detachment during migration. It has been shown that the Rho-ROCK pathway is essential in the regulation of the process of uropod detachment and of rear release[21]. Therefore, inhibition of ROCK may lead to disrupted uropod formation, as it is unable to lift from the plane of the cell body.

The role of the Rho-ROCK pathway has been well-characterized for generally migratory cells. The Rho-ROCK pathway has also been elucidated in the context of leukocytes. However, it has emerged that in leukocytes, the uropod has functional significance as an important structural unit, the site of cell signaling, and in its facilitation of important cellular processes such as intercellular contact and apoptosis. Additionally, the uropod has been shown to be associated with myosin II and actomyosin distribution in the trailing edge of leukocytes[15]. In leukocytes, the mechanistic link between rearwards distribution of actomyosin bundles and adhesion receptors has been linked to Rho activity. Therefore, the Rho-ROCK pathway has been implicated in uropod formation in leukocytes, an essential mechanism of cell polarization, the precedent to migration. However, the role of ROCK as a Rho effector has not been well-characterized in Natural Killer cell biology. The importance of the Rho-ROCK pathway in developing proper migratory phenotypes demonstrates the need to elucidate the role of these processes for natural killer cells, especially in a developmental context. Migration as well as arrest and conjugation of developing NK cells are crucial for proper formation of the DS.

Given the impact of ROCK on two functions that are essential to the formation of the DS, I sought to investigate the role that ROCK plays in facilitating essential components of the

developmental synapse: polarization and receptor clustering. Thus, I isolated fresh human NK from healthy donor peripheral blood, and plated them on an adherent fibronectin surface. The isolated NK cells were treated with the chemokine IL-15 a to induce polarization, and were then treated with ROCK inhibitor to disrupt the function of ROCK, with cells treated solely with IL-15 serving as a control. We imaged the resulting fixed cells using confocal microscopy, and scored the resulting images as "polarized" or "non-polarized" based on the localization of CD44 and polarized morphology to indicate the presence of a uropod (i.e. a thinner "trailing edge" with a greater distribution of CD44 than the rest of the cell). We found that based on both sheer number of polarized cells, as well as a percentage of cells in each condition, there was a greater frequency of polarized cells in the untreated (IL-15 only) condition, based on the localization of CD44 and polarized morphology. Additionally, we observed several unusual phenotypes in the samples treated with ROCK inhibitor, which serve as the basis for further hypotheses and exploration of the Rho-ROCK pathway in facilitating the DS.

II. Results

ROCK inhibitor interferes with polarization and CD44 clustering

NK cells were validated by flow cytometry. The isolated sample was validated as 68.5% NK cells (Figure 3). Isolated NK cells were prepared for imaging by confocal microscopy as described in Methods. Initial visualization of cells by microscopy suggested that NK cells treated with ROCK inhibitor generally demonstrated decreased polarized morphology and CD44 clustering. We found that out of 19 individual captured cells in the untreated condition (IL-15), 11 cells were scored as polarized, and out of 57 individual captured cells in the treated condition (IL-15 and ROCK inhibitor), 4 cells were scored as polarized (Table 1, Figure 4A). We

additionally quantified the frequency of cells scored as polarized across each condition. We found that the frequency of cells scored as polarized in the untreated condition was 0.57 (57% of cells in the untreated condition samples). In the treated condition, the frequency of cells scored as polarized was 0.07 (7% of cells in the treated condition samples, Figure 4B). This finding demonstrates that ROCK-inhibitor interferes with IL-15-induced polarization of NK cells.

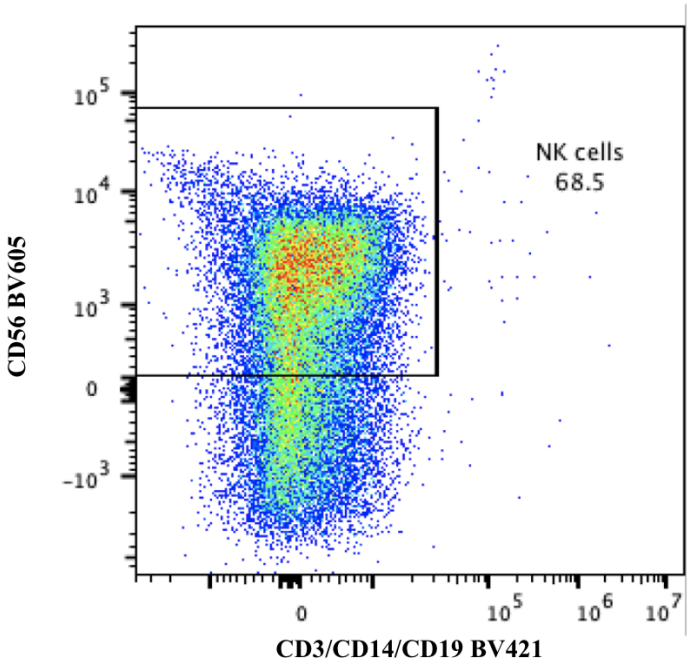

Figure 3. Flow validation of NK cell population. The isolated NK cell suspension was validated using flow cytometry. Gating for CD56 (BV605) + and Lin- (CD3, CD14, CD19, BV421) cells demonstrated that the isolated population was 68.5% NK cells.

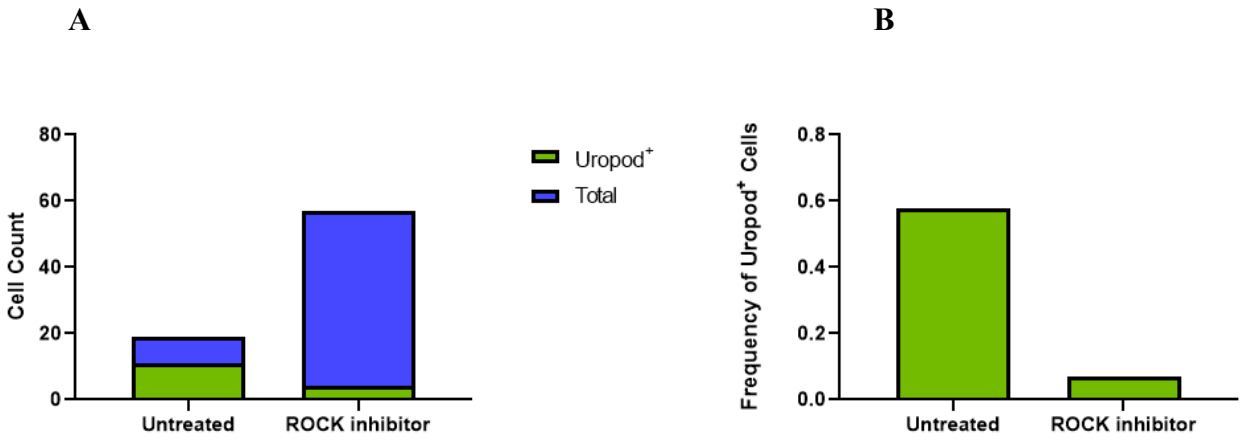

Figure 4. Counts of cells with a clear uropod treated with ROCK inhibitor versus untreated population. [A] Total cells with a clear uropod as a portion of total cells. In the untreated condition, 11 out of 19 cells displayed a clear uropod, whereas 4 out of 57 cells treated with ROCK inhibitor displayed a clear uropod. [B] Frequency of cells with a clear uropod in the treated versus untreated population. NK cells in the untreated population displayed a greater percentage of polarized cells.

	Untreated (IL-15)	*Treated (IL-15 + ROCK inhibitor)*
Cell count *(including overlaps)*	*21*	*92*
Cell count (*individual cells*)	19	57
Uropod$^+$ cells	11	4

Uropod$^-$ cells	8	53

Table 1. Tabulated results of total Uropod$^+$ and Uropod$^-$ NK cells in the Untreated (IL-15 only) condition and the Treated (IL-15 + ROCK inhibitor), as depicted in Figure 3. Only individual cells (i.e. non overlapping and distinct cells) were counted.

ROCK-inhibitor treatment induces aberrant phenotypes restricted to the trailing edge of NK cells

In addition to assessing polarization based on CD44, additional morphological abnormalities were produced by the inhibiting ROCK in NK cells. Noted first was the typical "polarized" versus "non-polarized" morphologies, based on the localization of CD44 to the uropod as well as the identification of a "thinner" trailing edge at the cell rear. "Non-polarized" cells were assessed as having an even distribution of CD44 and an absence of a thin projection at the cell rear (Figure 5A and 5B). Throughout the treated condition samples were thin projections at the rear edge of the cell, which were crossed with the same thin projection at the rear edge of an adjacent cell, often crossing at the "stalk" between the cell body and the thin projection (Figure 5C). These cells were not scored as "polarized," because these cells displayed a consistently even distribution of CD44 around the cell body, and the thin projection at the cell rear made a unique form of intercellular contact, whereas typical NK cells are able to be captured on a single-cell basis. Additionally, NK cells treated with ROCK inhibitor demonstrated a unique phenotype of "feathery", or "spiky" cell trailing edges. Like the crossed NK cell trailing edges, these spiked trailing edges were not scored as "polarized", due to the lack of clustered CD44 at the uropod and a trailing cell edge (Figure 5D).

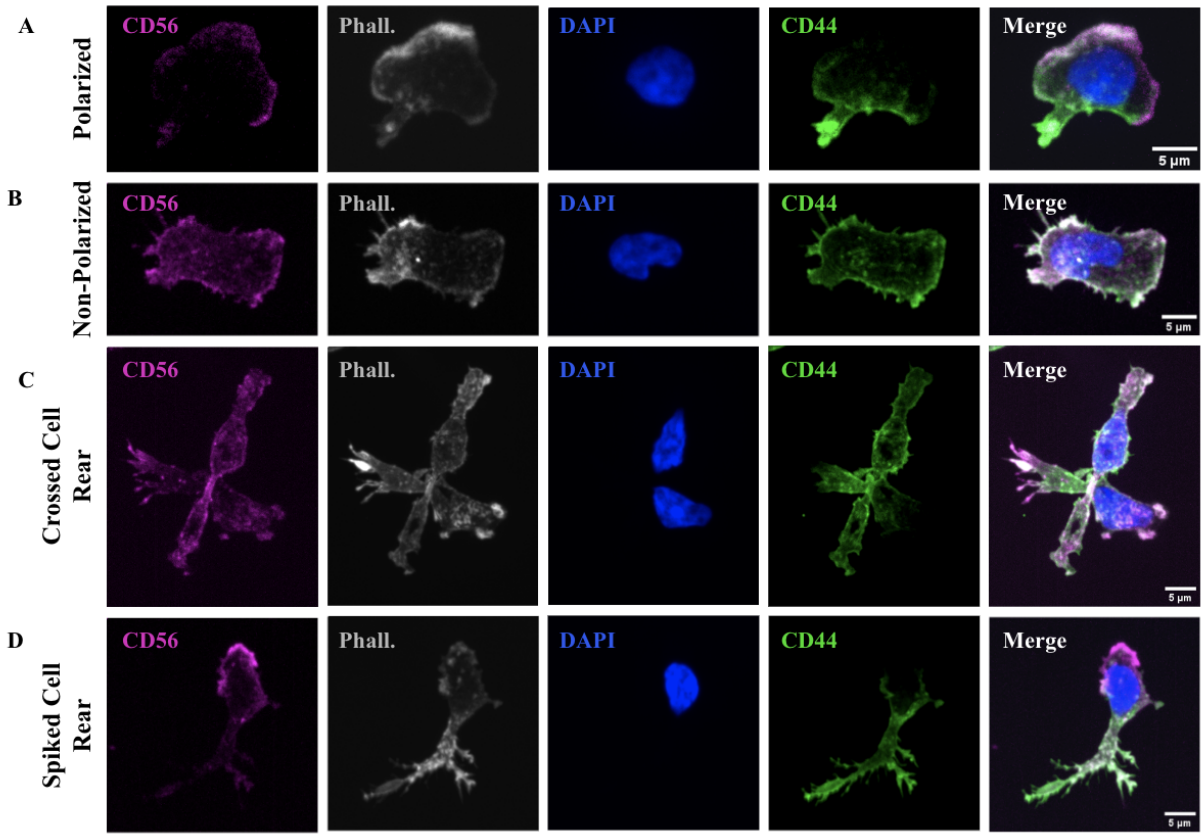

Figure 5. Select examples of polarized, non-polarized, and other notable phenotypes. [A] Example of a polarized cell, based on morphology and CD44 localization (untreated). [B] Example of a non-polarized cell (untreated). [C] Example of a notable phenotype, "crossed" trailing edge projections (treated). [D] Example of a notable phenotype, "spiked" cell rear projections (treated).

III. Discussion

The Rho-GTPase and Rho-dependent kinase (ROCK) are integral to the migration of motile cells, including metastatic cancer cells and migrating leukocytes. Thus the activity of Rho-GTPase and ROCK are of interest to understanding the cellular and molecular dynamics of

NK cell development. While the role of ROCK, as an essential kinase in cellular cytoskeletal polarization mechanisms, the understanding of its role in NK cell biology and NK cell polarization, especially in a developmental context, is incomplete. Thus, I sought to characterize the role that ROCK plays in the polarization of human NK cells. NK cells were isolated from healthy donor blood and plated on adherent fibronectin surfaces[23]. The isolated NK cells were treated with IL-15 to induce polarization, and a "treatment group" consisted of NK cells treated with both IL-15 and ROCK inhibitor to determine the role of ROCK inhibitor in the polarization of NK cells. In treating NK cells with ROCK inhibitor, cells treated with both IL-15 and ROCK inhibitor formed uropods less frequently, both in terms of amount of uropod-displaying cells as well as on a per-cell basis, calculated as frequency.

The observed and quantified behavior of NK cells treated with ROCK-inhibitor was consistent with hypotheses for the expected behavior of NK cells with aberrant ROCK function. ROCK is an immediate downstream kinase of Rho, a GTPase essential for the formation of a thinner, trailing edge of the cell, which can both adhere to and/or detach from a developmentally supportive surface and scan the environment for chemotactic cues. This trailing edge is also characterized by the localization and redistribution of adhesion receptors on the cell surface, where adhesion receptors that are evenly distributed around the leukocyte membrane are re-localized to the uropod through an actomyosin-based mechanism. Clearly, these two processes consist of a mechanistic link through functional ROCK activity, and both cooperate in the formation of a uropod, characterized by both morphology and the localization of specific adhesion receptors and cell surface markers that have been consistently demonstrated to be enriched at the uropod[15,24]. Thus, the decreased frequency of polarized NK cells treated with ROCK-inhibitor indicates that the improper function of ROCK disrupts both the formation of

actomyosin bundles, as well as the redistribution of adhesion receptors from the general cell body to the uropod. I hypothesize that this occurred in a two-fold manner. ROCK has been demonstrated to regulate actomyosin bundle formation due to activating myosin light chain kinase (MLCK) formation as well as LIM kinase formation, allowing F-actin fibers to gain contractile activity. Simultaneously, ROCK will phosphorylate ERM proteins at their carboxyl termini and activate the guanonucleotide exchange factor (GEF) DBL by recruiting it to the plasma membrane[25], which serves to further activate the Rho-GTPase, the upstream activator of ROCK, further stimulating actomyosin bundle formation. These actomyosin bundles formed by ROCK activation of LIM kinase, MLCK, and inhibition of myosin light chain phosphatase, are the basis for the mechanism that clusters the adhesion receptors together, mediated by activated ERM proteins. While it remains unclear precisely how myosin II regulates ERM-mediated redistribution of adhesion receptors in motile cells, it is well-established that rearwards redistribution of adhesion receptors as well as myosin activity is localized to the cell rear[26,27]. Prior work has shown that ROCK activity is necessary for detachment of the trailing edge of the cell in T-cells[28]. Thus, in the NK cells analyzed in the present study, disrupting ROCK function may have led to the inability of an NK cell uropod to lift off from the adherent surface, possibly by eliminating the contractile activity that allows the cell rear to detach from the surface. Typically, the contractile activity of actomyosin bundles will "overcome" the strength of the attachment of adhesion receptors to ligands at the cell rear. In the present observations, where cells more often failed to form a clear uropod when treated with ROCK inhibitor, uropod-associated markers, such as β_1 integrin, was able to maintain its adhesion to fibronectin[23], the adherent surface on which the cells were plated, and thus the uropod could not achieve its typical "detached" phenotype. Additionally, a cell was scored as being polarized by the

localization of CD44 at the cell rear. As cells treated with ROCK inhibitor also displayed a more even distribution of CD44 around the cell body, as opposed to being concentrated to the uropod, disruption of ROCK has interrupted the Rho-ROCK mediated mechanism of receptor clustering and relocalization to the cell rear, as has been described to occur in leukocytes. Although the mechanism by which actomyosin complexes facilitate ERM-associated adhesion receptor relocation to the cell rear is not well-characterized, the role of ROCK in both these processes is evident as demonstrated by the frequent failure of NK cells treated with ROCK inhibitor to both form a detached trailing edge and to re-localize essential adhesion receptors and surface markers.

In addition to the quantitative conclusions reached, the present study also demonstrated several notable phenotypes of the NK cells in response to treatment with ROCK inhibitor. A prominent phenotype includes the trailing edges of the NK cells forming what appears to be a uropod, highly consistent with a morphologically typical uropod. Interestingly, these NK cells make contact with adjacent cells, and their trailing edges cross with each other, forming what appears to be overlapping uropods. Another prominent phenotype that consistently appeared in the confocal images was cells displaying spikes, or a feathered appearance at the cell rear. The cells displaying these notable phenotypes were restricted to the class of NK cells treated with ROCK inhibitor. Thus, these phenotypes are considered to suggest sequelae of ROCK disruption, and may be indicative of aberrant NK cell activity in the presence of a typical inducing agent, such as IL-15. The specific NK cell activity incurred by the “overlapping” cell trailing edges and the spikes at the cell rear requires further investigation. However, the overlapping cell trailing edges have also been observed in CD56-knockout cells (Mace, unpublished data). The similarity between the observations in two conditions conferring different molecular disruption of NK cell function hints at a common point of convergence in the downstream targets of both CD56 and

Rho-GTPase/ROCK. In CD56-knockout cells, non-receptor tyrosine kinase-2 (Pyk2) has been shown to be a critical downstream signaling intermediate of CD56, mediating both NK cell cytotoxic function as well as Cdc42, a small-protein GTPase downstream of Rac, activity, ultimately impacting actin branching through recruitment of WASp-family proteins and the Actin-related protein 2/3 (Arp 2/3) complex, which serves as a branching nucleator for the protruding leading cell edge[29]. As both in the CD56 knockouts and the NK cells treated with ROCK inhibitor, disrupted signaling polarity results in the specific phenotype of crossed cell rears as a particular form of intercellular contact. The basis and consequences of this phenotype is a promising avenue of further investigation.

The spikes and feathery projections at the NK cell rear may also be explained by disrupted ROCK function due to treatment with ROCK inhibitor. On an adherent surface, the uropod is in part characterized by its ability to detach and lift away from its substratum. This allows the leading edge of the cell to move forward, and the uropod to act as a "wind vane" for the rest of the cell, directing migration[2,30]. This detachment occurs after initial adherence of the cell to the substratum, mediated by integrins and adhesion receptors. However, due to ROCK inhibition, actomyosin bundles cannot be formed to facilitate the contractile activity essential to the function of the uropod. While this process is disrupted, the inhibition of ROCK leads to Rac activity uninhibited by RhoA activity, feeding an overactive cycle of actin remodeling at the leading edge of the leukocyte. This is aided by the inability of RhoA to continually inhibit Rac toward the cell rear. RhoA acts as the GTP-Activating Protein (GAP) of Rac at the cell rear, hydrolyzing the GTP molecule associated with Rac, thereby inactivating it, allowing RhoA and its downstream targets to be the dominant active agent at the cell rear. However, as previously described, RhoA plays a role in phosphorylation of ERM proteins, which once activated, recruit

the GEF DBL, which serves as the GEF to RhoA, further amplifying the RhoA-ROCK pathway. Once ROCK is inhibited, the pathway is no longer able to further mediate formation of actomyosin bundles for contractile activity and protrusion of the uropod[25]. Thus, actin remodeling continues at the leading edge, an essential step in forward motion in cell migration. However, due to the perturbed Rho-ROCK pathway, the cell cannot effectively detach itself from the substratum. Due to the forward force from the leading edge, these connections between β1 integrin and fibronectin at the cell rear may be torn off, causing remnants of β1 integrin remaining on the fibronectin surface. While β1 integrin was not studied here, CD44 serves as a useful clue to the localization of β1, as they both are uropod markers.

The present study suggests that the Rho-ROCK pathway may play an expansive and integral role in the NK cell uropod, but the mechanistic link between receptor clustering and the Rho-ROCK pathway presently remains uncharacterized. While hypotheses and previous work supports that phosphorylated ERM proteins coupled with adhesion receptors are shuttled to the cell rear by an actomyosin-based mechanism, the specifics of that mechanism, how the receptors become clustered and relocalized is still unknown. Additionally, a broader question remains of how Rac-Rho signaling polarity is maintained within NK cells to promote uropod and leading edge formation at distinct poles of the cell and how those signals are systematically maintained and/or dismantled in Nk cells. The present work indicates that ROCK, thereby the Rho-ROCK pathway, is integral for maintaining that polarity at the trailing edge, especially with respect to proper formation of the uropod[25]. However, the specific modulation of the signaling pathways dictating front and rear polarized characteristics in terms of a “signaling gradient” remains to be investigated. Finally, how these processes are relevant NK cell maturation and development,

especially though different developmental stages and with respect to the role of the uropod in NK cell development may inform future investigations into the ROCK-Rho pathway in NK cells.

IV. Conclusions

In conclusion, I have treated human NK cells with ROCK inhibitor to investigate its role in inducing polarization in NK cells, compared to NK cells treated solely with IL-15. In the untreated condition (induced to polarize by IL-15), there were a greater number of cells that were scored as polarized based on CD44 and morphology than in the treated condition, based both on simply the number of polarized cells in each condition as well as a percentage of the cells in each condition.The images also revealed unique phenotypes for cells treated with ROCK inhibitor compared to untreated cells. I observed cells with spiky/feathery projections, and I observed cells with crossed projections at a location in the cell body from which a uropod would typically project. Based on these observations, I hypothesize that ROCK inhibitor disrupts the development of a clear uropod, due to the integral role it plays in receptor clustering and in formation of actomyosin bundles that characterize the thin projection of the uropod. Based on the absence of this thin projection as well as "clustered" CD44 observed in the non-polarized cells, I propose that ROCK inhibitor has a role in disrupting these two processes. The cells treated with ROCK inhibitor are not able to make those receptor clusters, and are therefore unable to form the tether that has been shown to be an integral part of the period of "arrest" that has been shown to initiate the DS. With respect to the spiked projections, I propose that they are present in part due to the connections that were able to form between fibronectin and beta 1 integrin, but were torn off as the lack of actomyosin due to disrupted ROCK function prevents the uropod from lifting and detaching from the surface. This work has implications for future studies investigating the

signaling gradient that defines polarization and formation of the developmental synapse between developing NK cells and developmentally supportive cells.

VI. Materials and Methods:

I isolated NK cells from human peripheral blood, and used fixed-cell confocal imaging to determine the differential effects of IL-15 and ROCK inhibitor on NK cell polarization. The fixed-cell confocal images were analyzed using FIJI image analysis software.

NK Cell enrichment isolation and treatment:

NK cells were enriched from human peripheral blood using RosetteSep human NK cell enrichment cocktail (STEMCELL Technologies). 50 µL of RosetteSep human NK cell enrichment cocktail was added per every 1 mL of peripheral blood collected, then incubated at room temperature for 20 minutes. The sample was rinsed using 1X PBS and layered on top of density gradient medium Ficoll-Paque in a 50 mL conical tube for density centrifugation at 1200 *g* (rcf), with minimum acceleration and deceleration at room temperature (24ºC) for 20 minutes. The enriched NK cells were collected, placed in new conicals, and PBS was added to the harvested cells to fill the conical tube, and the cells were washed by centrifugation at 1250 rpm for 6 minutes, with maximum acceleration and low deceleration. The top layer was decanted and the cells were resuspended in 2 mL R10 media. The cells were then examined for their viability and density in suspension. To accomplish this, 20 µL of resuspended cells were mixed with 20 µL ViaStain™ AOPI staining solution (Nexcelom) in a 96-well round-bottom microwell plate. The mixture was added to a SD100 cell counting chamber (Nexcelom), and the cell counting chamber was placed into the Cellometer Auto 2000, and a count for immune cells (low red blood

cells) was performed. The percent viability, live cell count, and concentration were recorded. 200 µL of the cell suspension was reserved for the flow validation step. To prepare the enriched cells for the fixing and staining protocol, the volume of cells needed to reach a target count of 150,000 NK cells per plate were determined using the cell concentration determined from the previous step. Using data from previous work, the effective concentration of IL-15 was determined to be 1:1000, and the effective concentration of ROCK inhibitor was determined to be 50 µM. To make a 1:1000 solution of IL-15, 2 µL of IL-15 (Peprotech) was diluted with 2 mL of R10 media. To prepare a 50 µM solution of ROCK inhibitor, 2.25 µL of ROCK inhibitor (Y-27632, Stemcell) was diluted in 250 µL of R10 media. The enriched cell suspension was placed into eppendorf tubes of even volume, 200 µL, and the eppendorfs were centrifuged at 330 xg (rcf) for 5 minutes. The supernatant was discarded, the remaining cells were resuspended in the prepared media and treatment mixture, and incubated overnight at 37°C and 5% CO_2.

NK cell fixing and staining

During the incubation period, the adherent surfaces were prepared by plating fibronectin from bovine plasma (Sigma) on 22 x 22 mm glass coverslips (Corning, 7.5 µg/mL). A hydrophobic pen was used to mark the barrier in which the fibronectin was plated, and the coverslips were incubated for 2 hours at 37°C and 5% CO_2. Following overnight incubation, CD56 antibody (AlexaFluor-647, Biolegend, 1:100) was added to the eppendorfs containing the incubated purified cells, and incubated for 20 minutes at 37°C and 5% CO_2. Staining with CD56 before fixing and permeabilizing allowed for extracellular staining. The fibronectin coat was then removed, and washed three times with 1X PBS. After washing, 150 µL of each NK cell suspension was added to the coverslip inside the hydrophobic barrier, and were then incubated

for 1 hour at 37°C and 5% CO_2. After incubation, 100 µL of the mixture on the coverslips was removed, and 100 µL of 8% PFA (Paraformaldehyde, EMS) was added to each coverslip, incubated for 20 mins at room temperature, and was then washed 2 times with 1X PBS. 200 µL of BD cytofix/cytoperm buffer (Fisher Scientific) was added to the coverslips for 20 mins at room temperature, followed by Superblock blocking buffer (Thermo Fisher) for 45 minutes at room temperature, then washed twice with 1X PBS. Fluorophore-conjugated antibodies for CD44 (AF-488, Biolegend, 1:100) and Phalloidin (AF-568, Biolegend, 1:100) diluted in staining buffer 15% FBS in PBS with 0.01% Triton) were added to the coverslips and incubated for 1 hour at room temperature, then washed with staining buffer. DAPI (Sigma, 1:100) was then added for 3 minutes before mounting. ProLong™ Glass Antifade Mountant (without DAPI, Thermo Fisher) was used to mount the coverslips onto microscope slides (Fisherbrand™ Premium Superfrost™, Fisher Scientific). Slides were cured for 24 hours at room temperature. To prepare for imaging, the coverslips were sealed onto the slides and dried. Images were acquired using a Zeiss Spinning Disk confocal microscope at 100X and exported to FIJI for data analysis

Flow Cytometry Validation

After enrichment, NK cells were resuspended in 1X PBS for flow cytometry. The NK cell suspension in PBS was labeled with antibodies to validate the proportion of NK cells in the enriched fraction. Antibodies included lineage markers (CD3, CD14, CD19, BV421, Biolegend, 1:100), and other NK cell stage-specific markers (CD94 APC, CD16 PECF594, CD56 BV605, CD57 BV510, Biolegend), and a live/dead cell stain (Live/Dead Zombie NIR™, Biolegend, 1:100). Flow cytometry was performed on a Novocyte Pantheon flow cytometer. In addition to

selecting fluorochrome channels for the selected antibodies, FSC - H (forward scatter - height) and SSC - H (side scatter - height) channels were selected for flow cytometry validation. A compensation control matrix was created by using IgG-binding beads (Thermo) to provide a single color stain for each used fluorochrome. The compensation calculation was applied to the cytometry acquisition software for flow cytometry validation of enriched NK cells.

Image and Data Analysis

Image analysis was performed using FIJI image analysis software. In each image, each single cell was captured, marked, and enhanced using image analysis tools to make each marker distinct. Polarization was scored as "yes" or "no" based on morphology and an assessment of the relative distribution of CD44 (uropod marker) in the cell body versus the uropod.
Each single cell was counted as a tally of overall cells, and polarized cells were considered a portion of the whole cell count. "Percentage of polarized cells" were quantified as a percentage of a whole. The percentage was calculated as the number of cells with clear uropods divided by the number of total cells in that condition. The cell counts were compared across each condition, namely untreated and treated with ROCK inhibitor.

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