RICE UNIVERSITY

UNDERSTANDING RECEPTOR-MEDIATED NK CELL ADHESION AND MOTILITY THROUGHOUT DEVELOPMENT

by

BARCLAY LEE

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE

DOCTOR OF PHILOSOPHY

APPROVED, THESIS COMMITTEE

Michael Diehl, Chair
Associate Professor, Bioengineering

Emily Mace
Assistant Professor, Pediatrics
Columbia University

Robert Raphael
Associate Professor, Bioengineering

James McNew
Professor, Biosciences

HOUSTON, TEXAS
December 2019
ABSTRACT

UNDERSTANDING RECEPTOR-MEDIATED NK CELL ADHESION AND MOTILITY THROUGHOUT DEVELOPMENT

by

BARCLAY LEE

Human natural killer cells originate from hematopoietic stem cells and can be differentiated in vitro through coculture with EL08.1D2 cells, a developmentally supportive stromal cell line. It is thought that these stroma support NK cell maturation by providing signaling cues and structural support that the stem cell would normally experience within tissue in vivo. These cues from the local microenvironment induce changes in cell motility and adhesion to guide cell fate. Although these signals are well studied in T and B lymphocytes, the full extent of these interactions is currently not well understood for NK cell development.

In this dissertation, I describe changes in NK cell motility and adhesion that occur throughout development. Specifically, I show that developing NK cells undergo continuous changes in cell migration velocity and displacement that define their differentiation from precursor to functionally mature cell. In addition, I present extracellular matrix (ECM) derived from EL08.1D2 stroma as an alternate substrate for supporting NK cell development. This cell-derived matrix is composed of naturally secreted ECM components from the stroma and contains expected ECM components such as collagen and fibronectin. I show that this cell-derived matrix
can support NK cell differentiation from hematopoietic precursor cells, and I further define the migratory and adhesive behaviors of NK developmental intermediates on EL08.1D2 and EL08.1D2-derived cell free matrix. Particularly, NK cell motility is acquired through development and NK cells undergo similar motility on both EL08.1D2 stroma and cell-derived matrix. The information presented in this work defines the extent to which developmentally supportive stroma are required for NK cell development in vitro and provides an avenue into research for alternative substrates and conditions for NK cell development.
I would like to thank my major advisor, Dr. Emily Mace, for her guidance with my work. Additionally, I want to thank everyone in the Orange and Mace lab for providing a supportive environment for me to complete my thesis research. I would not have been able to work nearly as effectively without help from everyone in the group.
Introducing NK cells and their function

1.1. Introduction to NK cells and their function

1.2. NK cell development

1.2.1. Developmental stages

1.2.2. NK cell generation from hematopoietic stem cells

1.3. NK cell migration and adhesion

1.3.1. Role of integrins

1.3.2. Changes in migration and adhesion throughout lymphocyte development

1.4. NK-microenvironment interactions

1.4.1. In vivo development

1.4.2. Interactions with EL08.1D2 stroma

1.4.3. Interactions with ECM

1.5. References

NK cell migration and development on EL08.1D2 stroma

2.1. Introduction

2.1.1. Migration and development

2.2. Materials and methods

2.2.1. Cell culture

2.2.2. CD34+ precursor isolation and flow cytometry

2.2.3. Live-cell imaging and tracking

2.2.4. Analysis

2.2.5. Statistics

2.3. Results
2.3.1. NK cell types exhibit distinct migratory behavior on stromal cells ................. 33
2.3.2. NK cell precursor motility and phenotype changes throughout maturation .. 36
2.3.3. NK cell developmental intermediates in later stages of development exhibit more directed migration............................................................................................ 41
2.3.4. Mode of migration depends on NK cell developmental stage....................... 44
2.3.5. NK cell maturation is accompanied by increased heterogeneity in NK cell migratory behavior. ................................................................................................... 48
2.4. Discussion and conclusions .................................................................................... 51
2.5. References.............................................................................................................. 55

NK cell migration and development on alternate substrates ............................... 58

3.1. Introduction............................................................................................................ 58
3.1.1. Description of cell-derived matrices................................................................ 59
3.1.1.1. CDM composition ...................................................................................... 62
3.1.1.2. Cell adhesion to CDMs .............................................................................. 63
3.1.2. NK cell migration and adhesion through development .................................. 65
3.2. Materials and methods ........................................................................................ 65
3.2.1. Cell culture ....................................................................................................... 65
3.2.2. CDM preparation ............................................................................................. 66
3.2.3. In vitro differentiation from hematopoietic precursor cells ........................... 67
3.2.4. Western Blotting .............................................................................................. 68
3.2.5. CD34+ precursor isolation and flow cytometry ................................................ 68
3.2.6. Acquisition of microscopy images ................................................................... 69
3.2.7. Image analysis and cell tracking ...................................................................... 70
3.2.8. Statistics ........................................................................................................... 71
3.3. Results .................................................................................................................... 71
3.3.1. Cell-derived matrices from EL08.1D2 express common ECM components and support NK cell adhesion ............................................................ 71
3.3.2. CDMs support adhesion and migration of primary NK cells and NK precursors ................................................................................................................. 75
3.3.3. CDMs support NK cell development from hematopoietic precursor cells .... 79
3.3.4. NKDIs have comparable migration on CDM and EL08.1D2 ......................... 82
3.4. Discussion and conclusions .................................................................................... 85
List of Figures

Figure 2.1 – Continuous live cell imaging of human NK cells on stromal cells. 35
Figure 2.2 – Acquisition of intrinsic NK cell migration with differentiation..... 40
Figure 2.3 – NK cell differentiation leads to increasingly directed migration. 43
Figure 2.4 – NK cell differentiation is associated with distinct modes of migration. ................................................................................................................................. 47
Figure 2.5 – NK cell maturation is correlated with increased heterogeneity of migratory phenotype........................................................................................................... 50
Figure 3.1 – Outline of workflow. .................................................................................................61
Figure 3.2 – EL08.1D2 cell-derived matrices contain ECM components and support NK cell adhesion. ...................................................................................................................................................... 74
Figure 3.3 – Primary NK cells and NKDIs adhere to and migrate on CDMs. ...... 78
Figure 3.4 – Cell-derived matrices partially support NK cell development. .....81
Figure 3.5 – NKDIs have comparable migration on CDM and EL08.1D2.........84
Figure 4.1 – EL08.1D2 support for NK cell development. ......................................................... 98
List of Tables

Table 1.1 – NK cell developmental stages........................................................................ 5
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDM</td>
<td>Cell-derived matrix</td>
</tr>
<tr>
<td>DS</td>
<td>Developmental synapse</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>ENK</td>
<td>Ex vivo NK cell</td>
</tr>
<tr>
<td>HSC</td>
<td>Hematopoietic stem cell</td>
</tr>
<tr>
<td>HPC</td>
<td>Hematopoietic precursor cell</td>
</tr>
<tr>
<td>MSD</td>
<td>Mean square displacement</td>
</tr>
<tr>
<td>NKDI</td>
<td>NK cell developmental intermediate</td>
</tr>
</tbody>
</table>
1.1. Introduction to NK cells and their function

Natural killer (NK) cells are a subset of human lymphocytes that play an important role in the innate immune system. As opposed to T and B cell lymphocytes, which are more heavily involved in adaptive immune responses, NK cells provide the first line of defense against infection by targeting diseased cells for killing without the need for prior conditioning. In addition, they release signaling molecules called cytokines which modulate the responses of other immune cells. Humans with a smaller or absent population of NK cells within peripheral blood are more susceptible to viral infection due to reduced ability to kill infected target cells (Biron et al., 1989). NK cells in peripheral blood are defined by their expression of surface protein CD56 as either CD56\textsuperscript{bright} NK cells or CD56\textsuperscript{dim} NK cells, which mark functionally and phenotypically distinct subsets. CD56\textsuperscript{dim} NK cells are specialized
killer cells with high cytolytic activity, expressing high levels of Fcγ receptor III (CD16), which regulates antibody-dependent cell cytotoxicity, in which CD16 binds to antigen-antibody complexes on target cells, resulting in NK cell activation and target cell lysis (Lanier et al., 1988). On the other hand, CD56bright NK cells are CD16+/- with weaker natural cytotoxicity but produce large quantities of immunoregulatory cytokines (Cooper et al., 2001a). Notably, NK deficient patients lacking only the CD56bright NK cell subset still suffer from viral susceptibility, indicating the importance of these cytokine producers for coordinating immune responses (Mace et al., 2013).

For cell killing to take place, NK cells must first migrate to the location of the target cell. Appropriate targets might be found anywhere within the human body; as such, mature NK cells, which typically reside within peripheral blood (PB), must first adhere to vessel walls and transmigrate through tissue to the target site. Upon recognition of a potential target cell, the NK cell must additionally determine if this is indeed an unhealthy cell that needs to be lysed. NK cells display a repertoire of activating and inhibitory surface receptors which they use to assess potential targets. Unhealthy cells downregulate their expression of major histocompatibility complex (MHC) class I proteins, which are recognized by NK cell receptors and thus prime the diseased cell for killing (Karre et al., 1986). Inhibitory or activatory receptors on the NK cell surface, including killer-cell immunoglobulin-like receptors as well as CD94/NKG2A-type receptors, interact with MHC class I molecules on target cells to detect unhealthy cells, which have abnormal MHC class I expression (Braud et al., 1998). Additionally, target cells can be bound by antibodies which
trigger NK cell recognition through the Fcγ receptor CD16 (Lanier et al., 1988). Upon recognition of a target cell, an immunological synapse is formed between the NK and the target cell, through which cytotoxic molecules such as perforin and granzyme pass through into the target, leading to cell death. NK cells are highly efficient; a single NK cell is capable of killing multiple target cells in a process termed “serial killing” (Choi and Mitchison, 2013).

1.2. NK cell development

Lymphocytes originally derive from pluripotent stem cells during embryonic development. Initially, pluripotent stem cells form specialized endothelial cells within the medial region of the embryo which can undergo endothelial-to-hematopoietic transition in order to become hematopoietic stem cells (HSC), which are multipotent cells capable of differentiating into any type of blood cell (Bertrand et al., 2010). These HSCs then bud out of the dorsal aorta, enter circulation, and seed the stem cell niche (Kissa and Herbomel, 2010). For HSCs, this niche is predominantly the bone marrow, where they are subject to developmental signals from surrounding vascular endothelial cells and mesenchymal stroma that may ultimately determine cell fate (Tikhonova et al., 2019). Eventually, NK precursors exit the bone marrow and travel through peripheral blood to secondary lymphoid tissues, which are the site of further NK cell maturation.
1.2.1. Developmental stages

NKDIs can be classified into specific stages of development by assessing expression of key NK cell receptors (Table 1.1). Hematopoietic stem cells, defined by expression of CD34 receptor, gain expression of CD10 to become stage 1 NKDI (Galy et al., 1995). The acquisition of mast/stem cell growth factor receptor, or CD117, demarcates stage 2a of development. Stage 2 is also marked by acquisition of CD122, the common IL-2/IL-15 receptor beta chain, which results in increased responsiveness to these cytokines (Freud et al., 2006). These stage 2a NKDIs gain expression of interleukin-1 receptor and migrate into secondary lymphoid tissues, where they develop into stage 2b developmental intermediates. Importantly, stage 2b NKDI are absent from peripheral blood and bone marrow, suggesting that this developmental transition is restricted to secondary lymphoid tissue (Scoville et al., 2016). In comparison, stage 1 NKDI are present to some degree in peripheral blood, bone marrow, and secondary lymphoid tissue, whereas stage 2a NKDI are enriched in secondary lymphoid tissue and peripheral blood. Indeed, it has been suggested that stage 1 and stage 2a cells derive from bone marrow and enter circulation to traffic to secondary lymphoid tissue where they undergo further maturation (Scoville et al., 2016). Stage 3 is characterized by the loss of the pluripotency marker CD34, followed by the expression of CD94 and NKP80, regulators of NK cell cytotoxicity, which define the transition to stages 4a and 4b respectively (Freud et al., 2016; Freud et al., 2006). Stage 4b, also called CD56<sup>bright</sup> NK cells, eventually upregulate expression of Fcγ receptor CD16 and become stage 5 CD56<sup>dim</sup> NK cells. At this stage, NKDIs exit the secondary lymphoid tissue to primarily circulate within
PB, with CD56<sup>dim</sup> NK cells making up approximately 90% of PB NK cells (Cooper et al., 2001b; Romagnani et al., 2007). Stage 5 of development also marks full acquisition of NK cell cytotoxic function with CD56<sup>dim</sup> NK cells expressing large amounts of perforin and granzyme, cytolytic proteins that potentiate lytic activity. Finally, expression of CD57 marks terminally differentiated stage 6 NK cells, with cells gaining even higher cytotoxic capacity, greater CD16 sensitivity, decreased cytokine responsiveness and decreased proliferation (Lopez-Verges et al., 2010).

<table>
<thead>
<tr>
<th>Stage 1</th>
<th>Stage 2</th>
<th>Stage 3</th>
<th>Stage 4</th>
<th>Stage 5</th>
<th>Stage 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD34&lt;sup&gt;+&lt;/sup&gt;</td>
<td>CD34&lt;sup&gt;+&lt;/sup&gt;</td>
<td>CD34&lt;sup&gt;-&lt;/sup&gt;</td>
<td>CD34&lt;sup&gt;-&lt;/sup&gt;</td>
<td>CD34&lt;sup&gt;-&lt;/sup&gt;</td>
<td>CD34&lt;sup&gt;-&lt;/sup&gt;</td>
</tr>
<tr>
<td>CD117&lt;sup&gt;-&lt;/sup&gt;</td>
<td>CD117&lt;sup&gt;-&lt;/sup&gt;</td>
<td>CD117&lt;sup&gt;-&lt;/sup&gt;</td>
<td>CD117&lt;sup&gt;-&lt;/sup&gt;</td>
<td>CD117&lt;sup&gt;-&lt;/sup&gt;</td>
<td>CD117&lt;sup&gt;-&lt;/sup&gt;</td>
</tr>
<tr>
<td>CD94&lt;sup&gt;-&lt;/sup&gt;</td>
<td>CD94&lt;sup&gt;-&lt;/sup&gt;</td>
<td>CD94&lt;sup&gt;-&lt;/sup&gt;</td>
<td>CD94&lt;sup&gt;+&lt;/sup&gt;</td>
<td>CD94&lt;sup&gt;+&lt;/sup&gt;</td>
<td>CD94&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
<td>CD16&lt;sup&gt;-&lt;/sup&gt;</td>
<td>CD16&lt;sup&gt;-&lt;/sup&gt;</td>
<td>CD16&lt;sup&gt;-&lt;/sup&gt;</td>
<td>CD16&lt;sup&gt;-&lt;/sup&gt;</td>
<td>CD16&lt;sup&gt;-&lt;/sup&gt;</td>
<td>CD16&lt;sup&gt;-&lt;/sup&gt;</td>
</tr>
<tr>
<td>CD57&lt;sup&gt;-&lt;/sup&gt;</td>
<td>CD57&lt;sup&gt;-&lt;/sup&gt;</td>
<td>CD57&lt;sup&gt;-&lt;/sup&gt;</td>
<td>CD57&lt;sup&gt;-&lt;/sup&gt;</td>
<td>CD57&lt;sup&gt;-&lt;/sup&gt;</td>
<td>CD57&lt;sup&gt;-&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Table 1.1 – NK cell developmental stages.

1.2.2. NK cell generation from hematopoietic stem cells

For the purposes of studying NK cell development <i>in vitro</i>, there are several methods available for generating mature NK cells from primary hematopoietic stem cells. NKDIs cultured in the presence of the appropriate cytokines pass through each successive stage of NK development until they become mature NK cells (Dezell et al., 2012; Grzywacz et al., 2006). Additionally, NKDIs can be co-cultured in the presence of a feeder cell line such as EL08.1D2 for greater cell yields and improved
commitment to NK cell lineage (Dezell et al., 2012; Freud et al., 2006; Grzywacz et al., 2006). Presumably, this is because it mimics the adhesive cell-cell contacts occurring within the tissue niche as NKDIs undergo development. In fact, a CD56-containing “developmental synapse” forms between NKDIs and EL08.1D2 stroma \textit{in vitro} that is enriched in actin and L-selectin (Mace et al., 2016). \textit{In vitro} derived NK cells have cytotoxic function and exhibit many of the same surface markers observed on mature NK cells isolated derived from patient blood (Eissens et al., 2012; Grzywacz et al., 2006; Miller and McCullar, 2001; Sivori et al., 2002).

A wide variety of feeder cell types have been shown to have developmentally supportive properties: the murine fetal liver stromal cell line AFT024, the mouse embryonic liver cell line EL08.1D2, as well as many others (Grzywacz et al., 2006; McCullar et al., 2008). However, the AFT024 cell line was inferior to EL08.1D2 in terms of overall NKDI expansion and mostly generated immature CD56- precursors (McCullar et al., 2008). Particularly exciting is the use of human bone marrow stroma to support development, as this is more physiological than the use of murine cell lines (Frias et al., 2008). Notably, this method resulted in generation of both mature NK cells and dendritic cells. Differentiation systems without feeder cell support have also been demonstrated. Dezell et al. used a feeder-free heparin-based method to generate HSC-derived NK cells, resulting in mature NK cells phenotypically similar to those generated by EL08.1D2 co-culture. However, this method resulted in worse NK cell purity and fold expansion (Dezell et al., 2012). Mature NK cells can also be derived directly from human embryonic stem cells (hESCs) or induced pluripotent stem cells (iPSCs), which differentiate into
hematopoietic cells following culture in a spin-aggregated colony (Knorr et al., 2013). Interestingly, in this system a subset of cells naturally differentiates into feeder-like cells expressing markers for endothelial or mesenchymal cells (Knorr et al., 2013). However, the specific roles that feeder cells play in driving NK development are still unclear.

1.3. NK cell migration and adhesion

The ability of NK cells to migrate effectively is crucial to their function as rapid killers of unhealthy cells. A migrating NK cell displays mesenchymal-type migration, a highly conserved behavior between cell types characterized by a high degree of polarization, with a distinct lamellipod structure at the front of the cell and uropod at the back. Rapid actin polymerization at the leading edge results in micro-protrusions which drive the cell forward, but this is counterbalanced by actin retrograde flow, in which actin filaments move toward the uropod (Giannone et al., 2004). This results in a fairly ordered network of parallel actin bundles, as compared to the branched meshwork of actin at the leading edge. Myosin-directed sliding of actin fibers drives the cell forward and retracts the cell rear (Jay et al., 1995). Furthermore, actin retrograde flow drives cell migration through integrins, which tether the lymphocyte to its substrate and provide the necessary friction to drive the cell forward (Hons et al., 2018).

It is important to mention that there is a body of research suggesting that migration in three dimensions is significantly different from the more well-studied
2-d migration. Whereas 2-dimensional migration relies on integrin adhesion to generate forward motion, 3-dimensional migration has been shown to be integrin independent in certain contexts. Instead, cells adopt an amoeboid squeezing mode of migration where actin network polymerization promotes protrusion of the leading edge and myosin II-dependent processes contract the uropod through confined spaces in the 3-d microenvironment (Lammermann et al., 2008). Additionally, cells adapt their migration to the size of the clearance that they need to squeeze through, altering their velocities and persistence lengths (Lautscham et al., 2015). This integrin-independent squeezing is likely to be important for leukocyte migration in tightly confined 3-d spaces, such as during tumor invasion. However, leukocytes can also degrade their local matrix with metalloproteinases and migrate through integrin-dependent mesenchymal-type migration, as observed on 2-dimensional substrates. Indeed, NK cells expressing different levels of matrix metalloproteinases have different migratory properties in Matrigel (Edsparr et al., 2009). Additionally, human umbilical vein endothelial cells display mesenchymal-type migration through Matrigel, and amoeboid-type migration in collagen I gels (Kick et al., 2016). Interestingly, inhibiting MMP activity with batimastat leads to decreased chemotaxis and directedness of migration in Matrigel but not in collagen gels.

### 1.3.1. Role of integrins

Integrins play a key role in NK cell migration by allowing cells to adhere to the substrate. NK cells are known to express a range of integrin molecules, including
integrins LFA-1, VLA-4, VLA-5, and LPAM. LFA-1 is a heterodimer of its alpha subunit CD11a (integrin αL) and beta subunit CD18 (integrin β2) (Hildreth 1983). Similarly, VLA-4 is composed of subunits CD29 (integrin β1) and CD49d (integrin α4) and is known to be involved in NK cell binding to fibronectin in the ECM (Macías et al., 2000). VLA-5 is composed of subunits CD29 (integrin β1) and CD49e (integrin α5) (Gismondi et al., 1991). LPAM is composed of CD49d (integrin α4) and integrin β7 and is involved in mediating cell-cell interactions (Perez-Villar et al., 1996). Mucosal vascular addressin molecule MAdCAM-1, which is expressed on endothelial cells, is responsible for binding to LPAM, but does not bind to either β1 or β2 integrins (Berlin et al., 1993; Perez-Villar et al., 1996). Conjugation of integrins to their ligands induces intracellular signaling pathways that result in actin polymerization and the conversion of intermediate-affinity integrin to high-affinity integrin through conformational changes (Evans et al., 2011; Kim et al., 2003). In this way, NK cells can modulate their level of adhesion when migrating on a substrate.

Ligation of NK cell adhesion molecules has been shown to activate tyrosine-kinase-involved signaling pathways to control cytoskeletal organization within the cell. Integrin binding causes Rap1-GTP-interacting adaptor molecule (RIAM) to recruit talin to the cytosolic portion of the integrin, also forming a complex with Rap1 GTPase (Han et al., 2006). Talin then recruits other adapter proteins such as vinculin, resulting in actin accumulation via the actin-related proteins 2 and 3 (Arp2/3) (Bass et al., 1999; Mace et al., 2010). Talin is additionally connected to the actin machinery via dedicator of cytokinesis 8 molecule (DOCK8), which is a guanine
exchange factor for the Rho GTPase CDC42, and Wiskott-Aldrich syndrome protein (WASp) (Janssen et al., 2016). On NK cells, integrins have also been shown to activate proline-rich tyrosine kinase 2 (PYK-2), a member of the focal adhesion kinase family, to regulate cell polarization and cytotoxicity (Sancho et al., 2000).

Lymphocyte migration is closely related to cell polarization and spreading through cytoskeletal remodeling. Motility is mediated by actin network remodeling within the cell, which is regulated by coordinated signals between integrins, such as LFA-1 and VLA-4, and cytoskeletal components such as actin and tubulin (Shulman et al., 2009; Smith et al., 2005). On NK cells, binding of LFA-1 induces asymmetrical NK cell spreading and increased migration, whereas ligation of NKG2D, an activating receptor that mediates NK cell cytotoxicity, induces symmetrical spreading and reduced migration (Culley et al., 2009). Interestingly, a migrating lymphocyte has high levels of intermediate-affinity LFA-1 at the lamellipodium, whereas high-affinity LFA-1 clusters at a mid-cell region known as the focal zone (Smith et al., 2005). It is suggested that intermediate-affinity LFA-1 is well-suited to rapidly forming and breaking adhesions at the leading edge, while remaining firmly attached at the focal zone.

1.3.2. Changes in migration and adhesion throughout lymphocyte development

As lymphocytes progress through development, they undergo changes in migration and adhesion that reflects variations in the expression of key adhesive and signaling receptors. Broadly, 2-dimensional cell migration can be classified into
three categories depending on the speed and directionality of migration: constrained, random, and directed (Khorshidi et al., 2011). Random migration is the baseline Brownian-type diffusion expected of a cell-sized particle in a medium of given viscosity. Constrained migration is generally slower than random migration and is characterized by small diffusion coefficients suggestive of cell arrest or tethering. Directed migration is defined by a large mean-square displacement, reflective of tracks with above-average speed and a high degree of directionality. Hematopoietic precursor cells isolated from peripheral blood and cultured on EL08.1D2 stroma initially exhibit predominantly constrained migration, but exhibit more frequent directed migration as they progress through NK cell development (Lee and Mace, 2017). A similar pattern has been shown in vivo for T cells using two-photon microscopy, with CD4+ and CD8+ single-positive T cells in human thymic slices exhibiting greater motility than the more immature double-negative and double-positive thymocytes (Halkias et al., 2013). It seems that migratory capacity in lymphocytes is associated with cytotoxic potential, as the highly cytotoxic and more mature CD56dim NK cells exhibit greater mean speeds than CD56bright NK cells (Mace et al., 2016). Additionally, educated NKG2A+ NK cells have greater motility and serial killing capacity than non-educated NKG2A- cells (Forslund et al., 2015).
1.4. NK-microenvironment interactions

1.4.1. In vivo development

Within the human body, NK developmental intermediates undergo constant interactions and signaling with other cell types. In adults, HSCs are maintained within the bone marrow, where they interact with vascular endothelial cells and mesenchymal stromal cells (Kiel et al., 2005). Vascular cells within the bone marrow express pro-haemotopoietic factors such as angiogenin, Notch ligands Dll1 and Dll4, and selectin E, in order to regulate stem cell fate (Tikhonova et al., 2019). Eventually, HSCs exit the bone marrow and migrate through blood vessels to secondary lymphoid tissue, recruited via multi-step adhesion cascades characterized by selectin-mediated tethering, chemotactic signaling, and integrin-mediated firm arrest and diapedesis (Scimone et al., 2006).

While the specific cell-cell interactions that NK developmental intermediates experience in the human body are still largely unknown, studies on other lymphocyte populations or lymphocytes within other species can give insights on this subject. Two-photon microscopy studies of human thymic slices demonstrate that thymocytes acquire increased motility as they mature from double-positive and double-negative cells into mature single-positive T cells (Halkias et al., 2013). Also, Bajenoff et al. showed that thymocytes within the paracortex of the mouse lymph node migrate along a network of fibroblastic reticular cells while B cells rely on follicular dendritic cells (Bajénoff et al., 2006). These fibroblastic reticular cells are responsible for production of the basement membrane in the mouse lymph node,
and also provide structural support for dendritic cells within the lymph node 
(Katakai et al., 2004). Lymph nodes are highly compartmentalized tissues present 
throughout the body where lymphocytes organize and coordinate to fight infection.
Immunohistochemical analysis has revealed that NK cells within mouse lymph node 
reside mostly in the inner medulla and central paracortex and interact closely with 
dendritic cells, possibly because dendritic cells express the NK cell survival signal 
IL-15 (Bajenoff et al., 2006; Ferlazzo et al., 2004; Mattei et al., 2001). Also, during T 
cell development, thymocytes that encounter cells displaying cognate antigen 
undergo cell arrest, calcium flux, and adopt a constrained mode of migration 
(Dzhagalov et al., 2013; Le Borgne et al., 2009). This behavior is likely similar for the 
cognate interactions between NK cells and DCs that establish adaptive immunity. 
Indeed, NK cells within mouse lymph nodes form long and stable contacts with 
dendritic cells (Bajenoff et al., 2006). Given that T cells migrate fairly rapidly, it was 
surprising to see that the first intravital microscopy studies of NK cells within 
mouse lymph node suggested that these NK cells were slowly motile in comparison 
(Bajenoff et al., 2006). However, this has since been shown to be an artifact of cell 
isolation procedures utilizing a CD49b (integrin α2) cross-linking antibody and that 
in fact, NK cells are highly motile within the lymph node, displaying migration 
speeds comparable to B cells (Garrod et al., 2007).

1.4.2. Interactions with EL08.1D2 stroma

It has been shown that NKDI seeded on EL08.1D2 stroma form an interface 
termed the “developmental synapse” characterized by enrichment of CD56, L-
selectin, and F-actin (Mace et al., 2016). Additionally, this site seems to be derived from the uropod of the NK cell based on the expression of uropod markers such as CD43 and moesin. The DS is also the site of rich tyrosine phosphorylation, suggesting that signaling pathways are stimulated through local receptor activation. Indeed, CD56 activation has been shown to induce calcium signaling in neural cells (Doherty et al., 1991). Considered together with the localized enrichment of CD62L that is observed, it seems likely that the DS mediates cell-cell adhesion and may upregulate additional adhesive receptors such as integrins, which are known to activate tyrosine kinases (Sancho et al., 2000). Signaling through the DS may then be responsible for promoting differentiation and survival.

Not much is known so far about how the developmental synapse is formed, although it may involve a similar process as the more well-described immunological synapse (IS), the interface formed between NK cells and target cells during NK cell-mediated cytotoxicity. Both synapses are the site of molecular polarization and induce intracellular signaling (Grakoui et al., 1999; Mace et al., 2016). IS formation involves polarization of the microtubule organizing center (MTOC) and lytic granules to the synapse prior to target cell lysis (Hsu et al., 2016). The immunological synapse is initiated by ligation of LFA-1, which then induces accumulation of F-actin and other NK cell receptors at the IS (Grakoui et al., 1999). The role of LFA-1 towards DS formation is currently unknown, although F-actin, CD56, and CD62L accumulation at the DS has been observed (Mace et al., 2016). Furthermore, LFA-1 can be activated by ligands on the target cell to modulate synapse strength (Culley et al., 2009). For example, 2B4 binding to CD48 increases
the binding affinity of LFA-1 by engaging Src tyrosine kinase in an actin-dependent manner (Hoffmann et al., 2011). Overall, NK cell adhesion has been well studied in the context of IS formation but much less so for DS formation.

1.4.3. Interactions with ECM

In order to fully understand NK cell migration in vivo, it is vital to also consider the extracellular matrix, which makes up the non-cellular portion of tissues. The ECM is a complex network of interlinked molecules, including cell adhesion proteins such as collagen, fibronectin, and laminin, to which cells can adhere by utilizing integrin binding. To study NK cell adhesion and migration on ECM, researchers frequently use synthetically created 3D matrices, such as collagen gels. NK cells are capable of migrating through these artificial matrices, and the properties of this migration can be tuned by varying the geometry and chemistry of the matrix (Olofsson et al., 2019; Wolf et al., 2009). Indeed, Edsparr et al. showed that NK cell lines migrate through 3D Matrigels in a manner dependent on matrix metalloproteinases to degrade the local matrix (Edsparr et al., 2009).

The other approach to study cell-ECM interactions is to use more physiologically relevant mimics for tissue ECM; rather than using a chemically generated hydrogel, cell-derived matrices can be generated directly from either cells or tissues. Certain cell types such as fibroblasts or stromal cells secrete ECM components in order to build up the ECM in tissue, and can be used to generate naturally occurring ECM in vitro. These cell-derived matrices contain ECM proteins such as collagen and fibronectin and are frequently used as a more physiologically
suitable mimic for studying cell adhesion and migration to ECM (Kaukonen et al., 2017). An alternate approach is to generate ECM-derived hydrogels by decellularizing and delipidizing human tissues (Sackett et al., 2018). Studies using cell-derived matrices have revealed that integrin $\beta 1$ is critical for cell adhesion to CDM and that CDMs support cell migration (Hakkinen et al., 2011; Scherzer et al., 2015). However, a comprehensive study of NK cell migration and adhesion on cell-derived matrices has not yet been done. Here, I will define NK cell migration throughout development on ECM derived from developmentally supportive stroma. The results presented in this dissertation will help define the minimum requirement for NK cell development and will further the current understanding of NKDI migration and adhesion.
17

1.5. References


NK cell migration and development on EL08.1D2 stroma

2.1. Introduction

As previously mentioned, the most efficient method for in vitro differentiation of human NK cells from CD34+ progenitor cells is through co-culture with an irradiated feeder layer of stromal cells, in particular the EL08.1D2 cell line (Dezell et al., 2012; Grzywacz et al., 2006). NK cells generated in this manner have cytotoxic function and exhibit many of the same surface markers observed on mature NK cells isolated derived from patient blood (Eissens et al., 2012; Miller and McCullar, 2001). Despite the requirement for direct stromal cell contact in this system, the intercellular interactions between the CD34+ progenitors and the EL08.1D2 cells driving this developmental pathway are as of yet unclear and deserve further study. Incubation of CD56bright or CD56dim NK cells on EL08.1D2...
stroma leads to significant non-directed migration on stromal cells. Furthermore, isolation of NK cell developmental intermediates from tonsillar tissue or in vitro differentiation identifies distinct migratory behavior based upon developmental stage (Mace et al., 2016). Progression through differentiation is associated with increased migratory speeds and decreased time spent in arrest. These migratory properties are conserved in both NKDI isolated from tonsillar tissue and NK cells generated in vitro from CD34+ precursors (Mace et al., 2016).

2.1.1. Migration and development

Recent research on NK cells has made it clear that developmental stage is closely tied to cell motility. Incubation of CD56bright or CD56dim NK cells on EL08.1D2 stroma leads to significant NK cell migration, with CD56dim NK cells exhibiting greater mean velocities (Mace et al., 2016). Furthermore, NK cell developmental intermediates isolated from tonsillar tissue or via in vitro differentiation demonstrate distinct migratory behavior based upon developmental stage (Mace et al., 2016). A potential explanation for this phenomenon is that expression of key integrins, such as LFA-1, increases progressively with each stage of development, as shown by RNA-sequencing of progenitor subsets in both tonsils and peripheral blood (Chen et al., 2018).

A similar increase in velocity has been similarly described in developing human T cells, as relatively immature (CD4/CD8 double-negative or double-positive) thymocytes have substantially slower track speeds than their more mature single-positive counterparts (Ehrlich et al., 2009; Halkias et al., 2013). In CD8+ T
cells, an asymmetric distribution of LFA-1 following contact with APCs is maintained through cell division and leads to daughter cells with unequal LFA-1 expression. LFA-1\textsuperscript{high} daughter cells have greater motility and different developmental fates compared to LFA-1\textsuperscript{low} daughter cells (Capece et al., 2017). These results suggest that lymphocytes gain cytotoxic ability throughout development, since greater motility is correlated with more effective cell-mediated cytotoxicity (Choi and Mitchison, 2013; Forslund et al., 2015; Zhou et al., 2017).

Here, I analyze the migratory behavior of NKDI at various stages in development. Taking advantage of modern microscopy systems allowing for long-term live-cell imaging, CD34\textsuperscript{+} hematopoetic precursor cells were seeded on EL08.1D2 stroma and imaged continuously as they matured into CD56\textsuperscript{+} NK cells. By tracking these cell movements, I show that NK cells acquire increased motility progressively through maturation. I further quantify the heterogeneity in the developing NK cell migratory phenotype by classifying single-cell tracks in terms of speed and mode of migration. Together, this defines the acquisition of human NK cell migratory capacity throughout development.

### 2.2. Materials and methods

#### 2.2.1. Cell culture

EL08.1D2 cells stromal cells (a gift from Dr. J. Miller, University of Minnesota) were maintained on gelatinized culture flasks at 32° C in 40.5\% α-MEM (Life Technologies), 50\% Myelocult M5300 (STEMCELL Technologies), 7.5\% heat-
inactivated fetal calf serum (Atlanta Biologicals) with \( \beta \)-mercaptoethanol (10\(^{-5}\) M), Glutamax (Life Technologies, 2 mM), penicillin/streptomycin (Life Technologies, 100 U ml\(^{-1}\)), and hydrocortisone (Sigma, 10\(^{-6}\) M). Culture media was supplemented with 20% conditioned supernatant from previous EL08.1D2 cultures.

For \textit{in vitro} CD34\(^+\) differentiation, 96-well plates were treated with 0.1% gelatin in ultrapure water to promote cell adherence. 50 \( \mu \)L of 0.1% gelatin was deposited on 96-well plates and incubated at room temperature for 30 minutes. Following incubation, wells were washed with DPBS and left for an additional 60 minutes in a sterile culture hood to dry. Gelatinized 96-well plates were pre-coated with a confluent layer of EL08.1D2 cells at a density of 5-10 \( \times \) 10\(^3\) cells per well and then mitotically inactivated by irradiation at 300 rad. Purified CD34\(^+\) hematopoietic precursor cells were cultured at a density of 2-20 \( \times \) 10\(^3\) cells per well on these EL08.1D2 coated plates in Ham F12 media plus DMEM (1:2) with 20% human AB-serum, ethanolamine (50 \( \mu \)M), ascorbic acid (20 mg l\(^{-1}\)), sodium selenite (5 \( \mu \)g l\(^{-1}\)), \( \beta \)-mercaptoethanol (24 \( \mu \)M) and penicillin/streptomycin (100 U ml\(^{-1}\)) in the presence of IL-15 (5 ng ml\(^{-1}\)), IL-3 (5 ng ml\(^{-1}\)), IL-7 (20 ng ml\(^{-1}\)), Stem Cell Factor (20 ng ml\(^{-1}\)), and Flt3L (10 ng ml\(^{-1}\)) (all cytokines from Peprotech). Half-media changes were performed every 7 days, excluding IL-3 after the first week.

NK92 cells (ATCC) were maintained in 90% Myelocult H5100 (STEMCELL Technologies) (Atlanta Biologicals) with IL-2 (200 U ml\(^{-1}\)). YTS cells (a gift from Dr. J. Strominger, Harvard University) were maintained in 85% RPMI 1640 (Life Technologies), 10% fetal calf serum (Atlanta Biologicals), HEPES (Life Technologies,
10 μM), penicillin/streptomycin (Life Technologies, 100 U ml⁻¹), MEM Non-Essential Amino Acids Solution (Thermo Fisher, 1 mM), sodium pyruvate (1 mM), and L-glutamine (2 mM). All cell lines were authenticated by flow cytometry and confirmed monthly to be mycoplasma free.

2.2.2. CD34⁺ precursor isolation and flow cytometry

T and B cell lineage depletion was performed using RosetteSep™ Human Hematopoietic Progenitor Cell Enrichment Cocktail (STEMCELL Technologies) and Ficoll-Paque density gradient centrifugation from routine red cell exchange apheresis performed at Texas Children’s Hospital. Following pre-incubation with RosetteSep, apheresis product was layered on Ficoll-Paque for density centrifugation at 900 g (2,000 r.p.m.) for 20 min (no brake). Cells were harvested from the interface and washed with PBS by centrifugation at 500 g (1,500 r.p.m.) for 5 min then resuspended in fetal calf serum for cell sorting. T- and B- cell depleted cultures were incubated with antibodies for CD34 (clone 561, PE conjugate, BioLegend, 1:100) prior to sorting. Purity of CD34⁺ HPC was roughly 1% at this point. FACS sorting was performed using a BD Aria II cytometer with an 85 μm nozzle at 45 p.s.i. Purity after sorting was >80%. Primary NK cells for short-term imaging were isolated with NK cell RosetteSep.

For FACS analysis of CD34⁺ intermediates, a 6-colour flow cytometry panel was performed on a BD Fortessa using antibodies for CD56 (Clone HCD56, Brilliant Violet 605, BioLegend, 1:200), CD3 (Clone UCHT1, Brilliant Violet 711, BioLegend, 1:200), CD16 (Clone 3G8, PE-CF594 conjugate, BD, 1:300), CD94 (Clone
DX22, APC conjugate, BioLegend, 1:100), CD117 (Clone 104D2, PE/Cy7 conjugate, BioLegend, 1:10), and CD34 (clone 561, PE conjugate, BioLegend, 1:100). Flow cytometry data analysis was performed with FlowJo X (TreeStar Inc.).

2.2.3. Live-cell imaging and tracking

Cells were imaged in 96-well ImageLock plates (Essen Bioscience) on the IncuCyte ZOOM Live-Cell Analysis System (Essen Bioscience) at 37°C every 2 minutes in the phase-contrast mode (10X objective). Images were acquired continuously for a 21-day period. NK cell lines and primary NK cells were labeled using CellTracker Violet (Thermo Fisher) at a working concentration of 15 μM and washed with complete media by centrifugation at 500 g (1,500 r.p.m.) for 5 minutes. Fluorescent images were imported into Imaris (Bitplane) and tracked using the Spot tracking method with the ‘Autoregressive Motion Expert’ setting and removing tracks below an automatic threshold on ‘Track Duration’.

For tracking of NK developmental intermediates, cells were seeded at 2 x 10^3 cells per well on a 96-well ImageLock plate with confluent irradiated EL08.1D2 cells, then imaged at 2 minute intervals for a 24-hour period. Phase-contrast images were segmented using ilastik software by using the ‘2-stage Autocontext’ method for pixel classification to distinguish NKDI from the background (Sommer, Straehle, Köthe, & Hamprecht, 2011; Tu & Bai, 2010). An array of cell positions over time was generated from exported binary segmentation images and imported into Imaris (Bitplane) for tracking analysis. The image set was divided into 24 hour periods for tracking. Tracking was done using the Spot tracking method on Imaris with the
‘Autoregressive Motion’ setting and removing tracks below an automatic threshold on ‘Track Duration’. Individual tracks were then verified by eye and corrected manually where necessary by using Imaris to fix broken or overlapping tracks.

### 2.2.4. Analysis

Track statistics were calculated using Imaris and exported as comma-separated value files which were graphed using GraphPad Prism. Mean speed was defined as the per-track average of all instantaneous speeds calculated at each frame. Displacement was defined as the absolute distance between cell position at the beginning and the end of the 24 hour period of tracking. The straightness parameter was calculated by dividing the displacement by the total path length for each track, so that straightness values close to 1.0 represent highly directed tracks. Arrest coefficient was defined as the percentage of time that the cell stays in arrest based on a threshold on instantaneous speed of 2 μm/min, or approximately one cell body length per image interval. The standard deviation in speed for each track was calculated as the standard deviation in instantaneous velocities observed for a given track. Rose plots were generated by selecting 30 representative tracks per time series and plotting them in Imaris.

For mean squared displacement (MSD) analysis and classification of migration modes, I developed custom MATLAB scripts based on a previously described method for transient migration behavior analysis (Khorshidi et al., 2011). Calculation of MSD was done using @msdanalyzer, a publicly available MATLAB class for MSD analysis of particle trajectories (Tarantino et al., 2014). To implement
the method for transient migratory period analysis, each track was analyzed using a sliding window approach and calculating the MSD corresponding to each window. The MSD data was fit to a curve to estimate the degree of curvature by doing a linear fit against the log-log of the MSD and finding the slope. The diffusion coefficient of a cell in two dimensions is proportional to the slope of the MSD, so to calculate this I did a simple linear fit against the MSD. For fitting purposes, I only considered the first 6 points of the MSD, as previously described. Track segments were then classified as directed, constrained, or random migration depending on thresholds on these values which were set to be the same as those previously described (Khorshidi et al., 2011). The threshold for diffusion coefficient was set at 4.2 μm²min⁻¹, calculated based on the typical diameter of an NK cell, with all track segments having a smaller diffusion coefficient classified as constrained migration. On the other hand, all segments with an MSD curvature α above 1.5 were defined as directed migration. All remaining segments after these thresholds were applied were classified as diffusive migration.

2.2.5. Statistics

Statistical analysis was calculated using Prism 6.0 (GraphPad). Ordinary one-way ANOVA was used to compare track statistics. Outliers were removed from exported track statistics using the ROUT method in Prism with Q = 1%. For all tests P<0.05 was considered significant.
2.3. Results

2.3.1. NK cell types exhibit distinct migratory behavior on stromal cells

NK cell lines and ex vivo NK cells undergo migration on EL08.1D2 stromal cells when assayed over relatively short periods of imaging (Mace et al., 2016). To compare their migratory behavior and validate the sensitivity of our long-term imaging system, NK92 and YTS cell lines, and ex vivo NK cells (eNK) were labeled with CellTracker Violet dye, and co-cultured them with EL08.1D2 cells for 24 hours. Along with CD34+ NK cell progenitors, these cells were continuously imaged at 2 minute intervals, and following acquisition cell migration was tracked (Figure 2.1A). eNK and NK cell lines had distinct migratory properties classified by mean speed, straightness, and arrest coefficient. eNK cells had more directed motion overall, characterized by significantly higher track straightness and lower arrest coefficient (frequency of time cells were in arrest) than both cell lines (Figure 2.1B, C). There were significant differences in motility between the two NK cell lines, with NK92 cells having higher overall speeds than YTS cells (1.163±0.33 µm/min vs. 0.7785±0.14 µm/min), although straightness was not significantly different (Figure 2.1B, C). Compared to CD34+ NK cell progenitors, both NK92 cells and mature ex vivo NK exhibited greater mean speed and spent less time in arrest (Figure 2.1B). This experiment validated that cell movements could be accurately tracked and revealed interesting variations between NK cell types in terms of motility.
YTS or NK92 NK cell lines or ex vivo human NK cells (eNK) were co-cultured with an EL08.1D2 monolayer for 24 hours in a 96-well plate. Images were acquired continuously every 2 minutes and cells were tracked using a fluorescent label. A) Representative phase-contrast images of each cell type with randomly selected sample tracks overlaid. Insets show zoomed-in views of single-cell tracks. Scale bar=300 μm. B) Mean track speed (top), straightness (center), and arrest coefficient (bottom) of NK cells were calculated. Error bars indicate s.d. Means with significant differences were determined by ordinary one-way ANOVA with Tukey’s multiple comparison test **p<0.01, ****p< 0.0001). Data is representative of three independent experiments. C) Rose plots of representative tracks from the different cell lines. n=30 per graph. Figure adapted from Lee, BJ, “Acquisition of cell migration defines NK cell differentiation from hematopoietic stem cell precursors,” Molecular Biology of the Cell, 2018.
2.3.2. NK cell precursor motility and phenotype changes throughout maturation

As eNK cells show significant motility on stromal cells, and acquisition of migratory behavior is associated with NK cell development in vitro and in vivo I sought to define the migratory behavior of NK cell precursors throughout development (Mace et al., 2016). Specifically, I aimed to measure cell migratory properties with minimally invasive imaging in a large field of view in order to not bias analyses with cells that may leave the imaging field. Using our long-term imaging system described above, human NK cells were generated in vitro from purified CD34+ hematopoietic precursor cells co-cultured on a developmentally supportive monolayer of EL08.1D2 stromal cells (Grzywacz et al., 2006; Mace et al., 2016). Cells were imaged continuously every 2 minutes throughout the 21-day period required for maturation into NK cells. Individual cells were segmented and tracked for parameters including track speed, length and displacement to measure how their migratory behaviors evolved throughout differentiation (Figure 2.2A). As expected, NK cell progenitors at the 0-day time point had significantly slower speeds than enriched NK cells (Figure 2.2B, 2.1B). All tracking was done on phase-contrast images because the fluorescent cellular dyes did not persist throughout the entire four-week period. Using this method enabled the tracking of single cells for extended periods of imaging and visual inspection confirmed that average cell track length increased significantly throughout the time of differentiation (Figure 2.2B).
To quantify the migratory behavior of developing NK cells early in differentiation, tracks were extracted from continuous 24 hour periods at days 0, 7, 14 and 21. Mean speed and displacement increased significantly at each progressive time point (Figure 2.2B). Initially, CD34\(^+\) cells had minimal speed (0.85±0.20 \(\mu\)m/min), track length (273.6±107.9 \(\mu\)m), and displacement (19.68±11.98 \(\mu\)m) over the initial 24 hour period. However, at day 21, mean speed was 1.48±0.48 \(\mu\)m/min, consistent with previously reported migration speeds of mature NK cells (Khorshidi et al., 2011; Mace et al., 2016). Path length increased over time as well but was only significant between the 7-day and 14-day time points (Figure 2.2B). Given the significant differences between days 0 and 14, an additional experimental replicate was performed where cells were tracked for consecutive 24-hour movies over the first 14 days of imaging. Significant variations in mean speed, displacement, and path length were observed between daily time points, which cumulatively accounted for the overall trends previously described at the weekly time points (Figure 2.2C). Based on these results, I concluded that the acquisition of track speed and length in developing NK cells occurs progressively throughout the initial stages of differentiation.

Progression of NK cell development was additionally monitored by flow cytometry analysis of NK cell developmental markers at days 0, 7, 14, and 21. I analyzed cells for expression of CD117 and CD94, which, when considered with CD34, identify developmental stages 1-4, and CD16, a marker of NK cell terminal maturation (Eissens et al., 2012; Freud et al., 2006). NK cells undergo differentiation through four linear stages: stage 1 cells are CD34\(^+\)CD117\(^-\)CD94\(^-\), stage 2 cells are
CD34\(^+\)CD117\(^+\)CD94\(^-\), stage 3 cells are CD34\(^-\)CD117\(^+\)CD94\(^-\), and stage 4 cells are CD34\(^-\)CD117\(^+/-\)CD94\(^+\). Gating based on FSC and SSC for the NKDI population, I observed that increasing frequencies of cells underwent differentiation to stage 4 (27% at Day 21), which was accompanied by a decreasing frequency of immature cells at later time points (Figure 2.2D).
Figure 2.2 – Acquisition of intrinsic NK cell migration with differentiation.

CD34+ HPCs were seeded on a monolayer of EL08.1D2 cells. Cells were imaged continuously in phase-contrast mode for 21 d. Images were acquired every 2 min, and tracking was done over a 24-h period for each time point. FACS analysis was performed weekly to monitor expression of developmental markers. (A) Representative images of NK cell intermediates with randomly selected tracks 0 (i), 7 (ii), and 21 (iii) d after the start of the experiment. Scale bar = 50 μm. (B) Mean speed, displacement, and path length of NK cell developmental intermediates were measured at 7-d intervals as indicated. Error bars indicate SD. ****p < 0.0001 by ordinary one-way ANOVA with Tukey’s multiple comparisons test. n = 932 (0D), 803 (7D), 134 (14D), and 148 (21D). (C) Mean speed, displacement, and path length of cells from continuous tracking from the first 14 d are shown as 24 h segments. Error bars indicate SD. Means with significant differences as analyzed by ordinary one-way ANOVA with Tukey’s multiple comparison test are shown (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001). (D) FACS analysis of NK cell maturation markers. Predicted NK cell developmental stage based on phenotype as described in the text is shown in Roman numerals. All data shown are representative of three independent experiments. Figure adapted from Lee, BJ, “Acquisition of cell migration defines NK cell differentiation from hematopoietic stem cell precursors,” Molecular Biology of the Cell, 2018.
2.3.3. NK cell developmental intermediates in later stages of development exhibit more directed migration.

Given the increasing speed and track length associated with migration of developing NK cells, I sought to further quantify the degree of directed migration in NK cell tracks by calculating the straightness and arrest coefficients. This was performed first on tracks extracted from 7-day intervals (Figure 2.3A). An increase in straightness and decrease in arrest coefficient over time was observed for the weekly time points (Figure 2.3A). While CD34+ HPC (0D) had a track straightness of 0.074±0.05, at the 21D time point the mean straightness index was 0.149±0.10. Similarly, arrest coefficient decreased from 0.993±0.01 to 0.884±0.1 between the 0 and 21-day time points. This was in agreement with the increase in mean speed observed for the later time points. As demonstrated for the significant changes in track speed between days 14 and 21, straightness and arrest coefficient similarly had the greatest changes in this time interval (Figure 2.3A). Although significant differences from day to day were detected, the overall change in these statistics is relatively small compared to those observed between days 0 and 21 (Figure 2.3B).
Figure 2.3 – NK cell differentiation leads to increasingly directed migration.

(A) Straightness and arrest coefficient for NK cell tracks at weekly time points. Error bars indicate SD. Means with significant differences as analyzed by ordinary one-way ANOVA with Tukey's multiple comparison test are shown (**p < 0.01, ***p < 0.001, ****p < 0.0001). n = 932 (0D), 803 (7D), 134 (14D), and 148 (21D). (B) Straightness and arrest coefficient for NK cell tracks at daily time points. Error bars indicate SD. Means with significant differences are determined by ordinary one-way ANOVA with Tukey's multiple comparison test (*p < 0.05, ***p < 0.001, ****p < 0.0001). All data shown are representative of three independent experiments. Figure adapted from Lee, BJ, “Acquisition of cell migration defines NK cell differentiation from hematopoietic stem cell precursors,” Molecular Biology of the Cell, 2018.
2.3.4. Mode of migration depends on NK cell developmental stage.

Migrating cells can either exhibit directed motion, constrained motion, or random diffusion. Random diffusion represents the type of movement expected of a cell of a certain size due to Brownian motion (Khorshidi et al., 2011). In this case, the mean square displacement (MSD) will be linear with time, whereas directed or constrained tracks will deviate above or below the linear trend, respectively. To characterize the diffusivity of our NK cell precursors, I calculated the MSD of NK cell tracks at the weekly time points (Figure 2.4A). In agreement with our previous measurements of track length and displacement, MSD progressively increased over time, starting at 546.8±549.6 μm$^2$ at day 0 for $t=450$ minutes and increasing to 6405.0±10447.7 μm$^2$ at day 21 for the same $t$ value.

While these values reflected a population-based measurement, many cells exhibited complex behaviors with multiple modes contained within a single track. To classify cell tracks by their transient properties, I implemented a previously described method for analyzing NK cell migration (Khorshidi et al., 2011). Using this method for each track, both the diffusion coefficient $D$ and the diffusion exponent $\alpha$ were calculated. These define the slope and curvature of the MSD curve, respectively. Tracks were classified by mode of migration by thresholding on these values (Figure 2.4B). Applying this analysis to all tracks for a given time point gave the fraction of time cells spent in either constrained versus directed migration. At day 0, 99.2% of cells exhibit purely constrained motion and 0% of cells exhibit purely directed motion (Figure 2.4C). This had changed significantly by day 21, with
39.2% of cells exhibiting purely constrained tracks and 3.4% of cells exhibiting purely directed motion (Figure 2.4D). These results suggest that the increase in mean speed over developmental stage that was described previously is due to a greater propensity for more mature cells to undergo directed migration. By analyzing the mean squared displacement of cell tracks, significantly more directed walks were observed at later stages of NK cell differentiation. This could reflect a migration strategy adopted by more functional NK cell intermediates to maximize target cell killing.
Figure 2.4 – NK cell differentiation is associated with distinct modes of migration.

(A) MSD of tracks acquired at weekly time points. Graph is truncated at 450 min because few cell tracks persist for longer. Error bars indicate SD. (B) Representative NK cell track after 21 d of development shown with segments corresponding to each migration mode labeled. (C) Fraction of time spent in either constrained, random, or directed motion for each cell after 0 d of development. n = 932. (D) Fraction of time spent in either constrained, random, or directed motion for each cell after 21 d of development. n = 148. All data shown are representative of three independent experiments. Figure adapted from Lee, BJ, “Acquisition of cell migration defines NK cell differentiation from hematopoietic stem cell precursors,” Molecular Biology of the Cell, 2018.
2.3.5. **NK cell maturation is accompanied by increased heterogeneity in NK cell migratory behavior.**

While a global increase in cell motility was observed over time, I also observed increasing heterogeneity of cell migratory behaviors with progressive NK cell maturation. Plotting the distribution of mean speeds for the 7-day time intervals, I observed that although mean speeds progressively increased over time, later time points still retained a few cells exhibiting very low speeds (Figure 2.5A). Individual analysis of single cell tracks showed that this increased variation in speed is accompanied by more diversity in migration behaviors (Figure 2.5A). Increased standard deviation of track velocities at later time points was seen when track statistics from both the weekly and consecutive daily datasets were measured (Figure 2.5B). Mean standard deviation increased from 0.39±0.11 μm/min at day 0 to 1.07±0.51 μm/min by day 21. This heterogeneity was due in part to the shift towards complex behaviors; at the beginning of tracking, cells exhibited primarily low-speed constrained tracks, whereas at later times there was a greater frequency of behaviors such as tracks resembling Lévy-type super-diffusive walks, characterized by a series of short, constrained motions interspersed with periods of highly directed motion (Harris et al., 2012). Additionally, a small subset of mature cells exhibited purely ballistic migration, travelling in essentially a straight line for the duration of migration, particularly at later time points (14 and 21 days).
Figure 2.5 – NK cell maturation is correlated with increased heterogeneity of migratory phenotype.

(A) Number of alternations between migration modes for cell tracks observed at the weekly time points. n = 932 (0D), 803 (7D), 134 (14D), 148 (21D). Insets: Rose plots of representative tracks. n = 20 per graph. (B) SD of the instantaneous speeds observed for each track for daily time points and weekly time points. Error bars indicate SD. Significance between means determined by ordinary one-way ANOVA with Tukey’s multiple comparisons test: **p < 0.01, ***p < 0.001, ****p < 0.0001. n = 932 (0D), 803 (7D), 134 (14D), and 148 (21D). All data shown are representative of three independent experiments. Figure adapted from Lee, BJ, “Acquisition of cell migration defines NK cell differentiation from hematopoietic stem cell precursors,” Molecular Biology of the Cell, 2018.
2.4. Discussion and conclusions

Here, I observed that NK cell progenitors demonstrated significantly increased cell motility as they matured into functional NK cells. Continuous tracking of NKDI throughout development reveals that cells progressively acquire greater migratory capacity as well as more heterogeneous migration behaviors. A similar increase in velocity has been similarly described in developing human T cells, as relatively immature (double-negative and double-positive) thymocytes have substantially slower track speeds than their more mature single-positive counterparts (Ehrlich et al., 2009; Halkias et al., 2013). It seems clear that increased migration is acquired throughout lymphocyte development as a means for enhancing cytolytic effectiveness. Additionally, changes in NK cell receptor phenotype through development likely drives this increased migratory ability. For example, integrins αL and β2, the components of LFA-1, are upregulated through development whereas integrins β1 and β7 are downregulated (Chen et al., 2018). Also, expression of IL-2/IL-15 receptor beta chain CD122 is gained at stage 2 of development, which might promote directed NK cell migration through increased responsiveness to IL-15 (Allavena et al., 1997; Freud et al., 2006). On average, observed progenitor cell velocities at all stages were similar to typical velocities seen for immortalized NK cell lines as well as those seen in other studies looking at lymphocyte motility, both in vitro (Khorshidi et al., 2011; Zhou et al., 2017) and in vivo (Ehrlich et al., 2009; Miller et al., 2002).
By analyzing the mean squared displacement of cell tracks, I observed significantly more directed walks at later stages of NK cell differentiation. This could reflect a migration strategy adopted by more functional NK cell intermediates to maximize target cell killing. Many cells measured at later time points exhibited seemingly Lévy-type walks, characterized by periods of extended cell arrest interspersed with short, highly directional movements. CD8+ T cells similarly utilize Lévy walks, with computational modeling suggesting that this behavior increases the efficiency of locating target cells compared to a purely random walk (Harris et al. 2012). Inhibiting T cell turning, such as through deletion of the non-muscle myosin motor myosin 1g, leads to decreased detection of rare antigens and supports the idea that an inability to effectively perform Lévy-type walks results in a less efficient search strategy (Gerard et al., 2014). In NK cells, similar transient periods of arrest have been previously described to correspond to the formation of conjugates with target cells and cell-mediated killing, although they can also occur spontaneously (Khorshidi et al., 2011). Interestingly, IL-2-activated NK cells, which have greater cytolytic activity than unstimulated cells, spend much less time in arrest while also forming twice as many cell contacts compared to resting NK cells, suggesting that regulation of this behavior correlates with relevant functional differences (Olofsson et al., 2014). Therefore, the acquisition of this specific mode of migration may represent a previously unappreciated component of functional maturation. Alternatively, the seeking behavior that develops may enable the location of key developmental cues that are required for NKDI to proceed through
differentiation. It will be of interest to determine whether this behavior is a requirement for, or a product of, NK cell maturation.

At least initially, migration of NK cell developmental intermediates involves interactions between NK cell progenitors and EL08.1D2 stromal cells. NK cell intermediates may initially preferentially adhere to viable stromal cells through integrin ligands or other cell adhesion molecules, causing a bias towards more constrained migration. However, after 10-14 days of in vitro culture, stromal cells often underwent apoptosis, possibly due to NKDI-mediated cytotoxicity. This loss of confluent stromal cells may lead to the increased frequency of directed walks I measured as there are fewer stromal cells to provide a constraining effect. Surprisingly, I observed that NKDI continued to exhibit transient periods of arrest at the later time points even when fewer stromal cells remained adherent to the plate. As EL08.1D2 stroma are capable of secreting ECM components, it is possible that the NKDI continue to migrate on these even after live stroma have exited the system.

Intravital microscopy demonstrates that lymphocytes closely interact with the stromal microanatomy in lymph nodes and form frequent cell-cell contacts, suggesting that direct interactions with stroma drives NK cell migration (Bajénoff et al., 2006; Beuneu et al., 2009; Miller et al., 2002). NK cells also interact with EL08.1D2 stromal cells through a uropod-derived structure termed the developmental synapse, at which CD56 and CD62L are enriched (Mace et al., 2016). Given the requirement for EL08.1D2 stroma in the efficient generation of mature NK cells in this system, yet the cross-species nature of these interactions, it is likely that
conserved molecules such as ECM components are key contributors to the
generation of mature NK cells. As NKDi continue to migrate and exhibit significant
adhesion even following loss of EL08.1D2 confluence, it is possible that the
secretion of ECM components by the stromal cell line continues to act as a platform
for NKDi migration and development. Alternatively, developmentally supportive
stromal cells may secrete chemokines such as SDF-1 that contribute to NK cell
migration (Aiuti et al., 1997). Given the requirement for direct stromal cell contact
with progenitors for the generation of mature NK cells, however, it is likely that at
least part of the stromal cell contribution to NK cell development is as providing a
supportive substrate.

In summary, I have shown that NK cells acquire motility throughout
development, progressing from a mostly constrained migratory phenotype to
complex walks and more directed migration. The transition to this mode of
migration strategy may enable more efficient target cell killing by increasing the
rate at which NK cells can conjugate with targets. Additionally, the increased
heterogeneity in cell migration at later stages may correspond to NK cells with
different functional capabilities, with cells exhibiting greater motility being more
effective at cell-mediated cytotoxicity. Continuing to study NK cell dynamics at the
single-cell level will likely lead to greater understanding of their development and
function.
2.5. References


Chapter 3

NK cell migration and development on alternate substrates

3.1. Introduction

Having determined that NKDI develop on EL08.1D2 stroma and also acquire increased migration through development, I was interested in further defining the requirement of cell migration in NK cell development. To study this, I used cell-derived matrices created directly from EL08.1D2 stroma. As a substrate in which structural support was retained but cell-cell effects were removed, CDMs were used as a model system to study the requirement for and migration in NKDI development. Given that HPCs treated with CD56 blocking antibodies to inhibit migration display impaired development, we hypothesized that NKDI would still be able to undergo some degree on development on ECM alone (Mace et al., 2016).
3.1.1. Description of cell-derived matrices

Cell-derived matrices are becoming increasingly popular as a model of tissue due to their physiological similarity. Within tissue, the non-cellular portion is termed the extracellular matrix and consists of a variety of proteins, enzymes, and macromolecules that provide scaffolding and biochemical support to surrounding cells. Large structural proteins such as collagen, fibronectin, and laminin make up a large portion of the ECM and can serve as a scaffolding for cells *in vivo* through interactions with integrins on the cells' surfaces. The mature ECM is the result of ECM proteins being secreted by cells throughout tissue development. The generation of cell-derived matrices is a mimic of this natural process, as CDMs are generated entirely from cells such as stroma or fibroblasts *in vitro* (Figure 3.1). First, cell culture plates are coated with gelatin to promote cell adhesion. As gelatin is soluble in aqueous solution, plates are then treated with glutaraldehyde to crosslink the gelatin and enhance its stability in solution (Yang and Ou, 2005). Next, cells are added to the plates and allowed to adhere overnight. After a confluent monolayer is formed, ascorbic acid is cycled into the culture media for a period of 7 days in order to promote CDM formation. Following this, the cellular and nuclear debris is removed, leaving a completed cell-derived matrix that can then be used for further experiments.
Add EL08.1D2 stroma

- Gelatin coating
- Gelatin crosslinking

- Cells adhered to plate
- Cells cultured in ascorbic-acid containing media for 7 days
- Cells deposit matrix

- Lyse cells with chemical treatment
- Remove cellular and nuclear debris
- CDM ready for use

CDM validation

CDM characterization

Microscopy

WB

Cell migration
Cell culture plates are coated with gelatin and seeded with EL08.1D2 stromal cells. Cells are allowed to adhere overnight and grown until confluent. Once confluent, ascorbic acid is cycled into the media for 7 days to promote matrix formation. During matrix extraction, cells are lysed with a chemical treatment and nuclear debris is removed with DNAse. CDMs can then be validated by microscopy and western blotting, or used for functional studies such as NK cell migration.
3.1.1.1. CDM composition

CDMs have been successfully produced from cell types such as fibroblasts or stromal cells and from a wide variety of species, including human, mouse, or monkey (Erdoğan et al., 2017; Hellewell et al., 2017). Despite this variance, there is striking similarity in the composition of the resulting CDMs. Mass spectrometry analysis of CDMs from RCS cells, a rat chondrosarcoma cell line, showed that cell-derived matrices are mostly made up of ECM proteins such as fibronectin, collagen, matrilin (Hellewell et al., 2017). In addition, a large percentage consists of thrombospondin-family proteins, which are capable of binding to fibronectin or collagen to mediate cell-ECM interactions. Many cytoskeletal proteins such as tubulin or actin, which are ubiquitous within cells, are absent from cell-derived matrix; this fact can be used to assess CDM purity through methods such as western blotting, as these proteins will only be detected in direct cell lysates. Unsurprisingly, ECM derived directly from tissue through decellularization and delipidization has a very similar proteomic phenotype as cell-derived matrix, also consisting mostly of collagen, fibrillin, and laminin (Sackett et al., 2018). Indeed, human pancreatic tissue can be decellularized to produce acellular ECM and ECM hydrogels suitable for in vitro studies of cell function (Sackett et al., 2018). In addition, atomic force microscopy on cell-derived matrices has confirmed that they have a similar stiffness to in vivo ECM (Soucy et al., 2011). These studies justify the use of cell-derived matrix as a model of cell activity within tissue and as a preferable alternative to synthetic hydrogels such as Matrigel or collagen gels. Here, I use ECM derived directly from EL08.1D2 as a model system where cell-cell effects are absent but
adhesive and migratory support is kept, and determine the effect on cell adhesion, migration, and development.

### 3.1.1.2. Cell adhesion to CDMs

Cell adhesion to CDMs is dependent on integrin binding to extracellular components. Integrin β1 regulates binding to collagen and fibronectin, and these interactions can be further modulated by the thrombospondin family of proteins within ECM (Macías et al., 2000; Murphy-Ullrich and Hook, 1989). Integrin α5 and αv are also involved in regulating motility on CDMs, as migration speed and directionality is affected when integrin activity is blocked (Erdogan et al., 2017). Integrin ligation leads to an intracellular signaling cascade characterized by recruitment of proteins such as talin, vinculin, WASp, and leading to actin network polymerization through Arp2/3 (Mace et al., 2010; Ye et al., 2010). Indeed, fibroblasts cultured on cell-derived matrix display distinct focal adhesion with enrichment of vinculin, actin, and integrin β1 (Hakkinen et al., 2011). Fibroblasts also migrate faster on CDMs than collagen-coated plates, possibly due to increased adhesive capacity. In addition, it has been suggested that integrins are critical for modulating the positioning and activation of MMPs, which cells use to migrate through ECM (Wolf and Friedl, 2009).

### 3.1.2. NK cell migration and adhesion through development

In the previous section, I showed that NK cell migration correlates with developmental stage. CD56bright NK cells exhibited significantly faster cell migration
and underwent less frequent arrests than less developed NKDI. Also, NKDI derived in vitro from CD34+ precursor cells exhibited progressively greater cell velocity and less frequent cell arrest as they matured (Lee and Mace, 2017). With this knowledge, I hypothesized that cell-cell interactions between NKDI and EL08.1D2 stroma drive cell migration while concurrently provide developmentally supportive signaling to promote an NK cell fate.

Cell-derived matrix provide a simplified model in which cell-cell interactions are removed from the system, as these have been shown to be involved in NK cell migration and adhesion. In the previous chapter, I described striking changes in NKDI migration behaviors throughout development; however, the relative contribution of the EL08.1D2 stroma towards NKDI motility was not clear. Considering that CD56 is suggested be involved in homotypic binding at the DS, it is interesting that inhibition of CD56 ligation with a blocking antibody greatly reduces NK cell motility on EL08.1D2 stromal cells (Mace et al., 2016). Indeed, it has been suggested that NCAM regulates integrin-dependent cell motility by acting as a carrier for integrin endocytosis (Diestel et al., 2007). NK cell conjugation to target cells through IS formation often leads to temporary cell arrest (Khorshidi et al., 2011). Additionally, more motile NKG2A+ educated NK cells have increased target cell conjugation and probably of killing compared to their NKG2A- counterparts (Forslund et al., 2015). Whereas mature NK cell motility is affected by IS formation, it seems plausible that NKDI migration is regulated by DS formation and the resulting activation of signaling pathways. In this context, it is useful to look at NKDI migration on cell-derived matrices, given that cell-cell synapse formation and
signaling leading to cell arrest is not possible in this system. Here, I study NKDI development, migration, and adhesion on EL08.1D2 stromal-cell-derived matrices as a model substrate in which cell-cell signaling is removed but the adhesive support is retained. Given that these matrices are derived directly from EL08.1D2 stroma, the adhesions that they support are likely to be very similar to those experienced by NKDI migrating on EL08.1D2 in the \textit{in vitro} differentiation experiment described in Chapter 2. Using this model system, these results will elucidate the minimum requirement for NK cell developmental support, as well as how migration and adhesion are tied to development.

\section*{3.2. Materials and methods}

\subsection*{3.2.1. Cell culture}

EL08.1D2 cells stromal cells (a gift from Dr. J. Miller, University of Minnesota) were maintained on gelatinized culture flasks at 32° C in 40.5% $\alpha$-MEM (Life Technologies), 50% Myelocult M5300 (STEMCELL Technologies), 7.5% heat-inactivated fetal calf serum (Atlanta Biologicals) with $\beta$-mercaptoethanol (10$^{-5}$ M), Glutamax (Life Technologies, 2 mM), penicillin/streptomycin (Life Technologies, 100 U ml$^{-1}$), and hydrocortisone (Sigma, 10$^{-6}$ M). Culture media was supplemented with 20% conditioned supernatant from previous EL08.1D2 cultures.

NK92 cells (ATCC) were maintained in 90% Myelocult H5100 (STEMCELL Technologies) (Atlanta Biologicals) with IL-2 (200 U ml$^{-1}$). All cell lines were authenticated by flow cytometry and confirmed monthly to be mycoplasma free.
3.2.2. **CDM preparation**

First, 96-well flat-bottom cell culture plates were treated with 0.1% gelatin in ultrapure water. 50 µL of 0.1% gelatin was deposited on 96-well plates and incubated at 37°C for 1 hour. Following incubation, wells were washed with sterile PBS. 50 uL of 1% (vol/vol) glutaraldehyde (Sigma-Aldrich) was then added for a 30 minute incubation at room temperature. After, wells were washed with PBS and treated with 1 M glycine (Sigma) for 2 min at room temperature. Wells were washed with PBS and stored overnight at 4°C in EL08.1D2 cell culture media supplemented with 1% pen-strep (Life Technologies). EL08.1D2 cells were trypsinized and added to the gelatinized 96-well plates at a density of 10,000–100,000 cell/cm² in a 150 uL volume. Cells were incubated at 32°C until formation of a confluent monolayer. Once a monolayer formed, culture media was replaced with a solution of culture media supplemented with ascorbic acid (50 µg ml⁻¹; Sigma). Media was replaced every other day with freshly made ascorbic acid supplemented media for 7-10 days, due to the instability of ascorbic acid in solution.

After this treatment, cells were extracted by aspirating off media and washing the cells with PBS. Stromal cells were denuded by adding 50 µL of extraction solution consisting of 2% (vol/vol) NH₄OH (Sigma), 0.5% (vol/vol) Triton X-100 (Fisher) in PBS, which was first pre-warmed to 37°C. After a 3 minute incubation at room temperature, extraction buffer was pipetted off and wells were washed twice with PBS. Next, 50 µL of DNase I (10 µg ml⁻¹; Sigma-Aldrich) was deposited and incubated at 37°C for 30 minutes. Then the DNase solution was
removed, followed by washing twice with PBS. The resulting CDMs were stored at 4°C in PBS supplemented with 1% (vol/vol) penicillin-streptavidin.

3.2.3. *In vitro* differentiation from hematopoietic precursor cells

For *in vitro* CD34+ differentiation on EL08.1D2, 96-well plates were treated with 0.1% gelatin in ultrapure water to promote cell adherence. 50 μL of 0.1% gelatin was deposited on 96-well plates and incubated at room temperature for 30 minutes. Following incubation, wells were washed with DPBS and left for an additional 60 minutes in a sterile culture hood to dry. Gelatinized 96-well plates were pre-coated with a confluent layer of EL08.1D2 cells at a density of 5-10 x 10^3 cells per well and then mitotically inactivated by irradiation at 300 rad. For *in vitro* CD34+ differentiation on CDM, 96-well plates containing cell-derived matrix were prepared as previously described. For *in vitro* differentiation on low adhesion plates, special low adhesion plates (Corning) were used without additional preparation.

Purified CD34+ hematopoietic precursor cells were cultured at a density of 2-20 x 10^3 cells per well on these EL08.1D2 coated plates in Ham F12 media plus DMEM (1:2) with 20% human AB- serum, ethanolamine (50 μM), ascorbic acid (20 mg l-1), sodium selenite (5 μg l-1), β-mercaptoethanol (24 μM) and penicillin/streptomycin (100 U ml-1) in the presence of IL-15 (5 ng ml-1), IL-3 (5 ng ml-1), IL-7 (20 ng ml-1), Stem Cell Factor (20 ng ml-1), and Flt3L (10 ng ml-1) (all cytokines from Peprotech). Half-media changes were performed every 7 days, excluding IL-3 after the first week.
3.2.4. Western Blotting

EL08.1D2 stroma were lysed in RIPA buffer (Thermo Fisher) with 1% vol/vol HALT inhibitor (Thermo Fisher) at a concentration of 10⁷ cell ml⁻¹. Samples were mixed with LDS buffer and DTT, separated on a 4-12% Bis-Tris gel, transferred with a nitrocellulose membrane, then blocked with skim milk. For CDM western blots, matrix was scraped directly into LDS/DTT solution pre-heated at 95°C for 5 minutes. Fibronectin was detected with a polyclonal rabbit anti-fibronectin antibody (1:1000; Abcam) and actin with mouse anti-actin (1:5,000; Sigma) followed by either goat anti-mouse 680 or goat anti-rabbit 800 (1:10,000; LiCor). Proteins were detected on a LiCor Odyssey.

3.2.5. CD34⁺ precursor isolation and flow cytometry

T and B cell lineage depletion was performed using RosetteSep™ Human Hematopoietic Progenitor Cell Enrichment Cocktail (STEMCELL Technologies) and Ficoll-Paque density gradient centrifugation from routine red cell exchange apheresis performed at Texas Children’s Hospital. Following pre-incubation with RosetteSep, apheresis product was layered on Ficoll-Paque for density centrifugation at 900 g (2,000 r.p.m.) for 20 min (no brake). Cells were harvested from the interface and washed with PBS by centrifugation at 500 g (1,500 r.p.m.) for 5 min then resuspended in fetal calf serum for cell sorting. T- and B- cell depleted cultures were incubated with antibodies for CD34 (clone 561, PE conjugate, BioLegend, 1:100) prior to sorting. Purity of CD34⁺ HPC was roughly 1% at this point. FACS sorting was performed using a BD Aria II cytometer with an 85 μm
nozzle at 45 p.s.i. Purity after sorting was >80%. Primary NK cells for short-term imaging were isolated with NK cell RosetteSep.

For FACS analysis of CD34+ intermediates, a 6-colour flow cytometry panel was performed on a Bio-Rad ZE5 Cell Analyzer using antibodies for CD56 (Clone HCD56, Brilliant Violet 605, BioLegend, 1:200), CD3 (Clone SK7, Brilliant Violet 711, BioLegend, 1:100), CD16 (Clone 3G8, PE-CF594 conjugate, BD, 1:300), CD94 (Clone DX22, APC conjugate, BioLegend, 1:100), CD117 (Clone 104D2, PE/Cy7 conjugate, BioLegend, 1:10), and a Zombie Aqua Viability Dye (1:100, BioLegend). Flow cytometry data analysis was performed with FlowJo X (TreeStar Inc.).

### 3.2.6. Acquisition of microscopy images

For tracking of NK developmental intermediates, cells were seeded at 2 x 10^3 cells per well on a 96-well ImageLock plate with confluent irradiated EL08.1D2 cells, then imaged at 2 minute intervals on the IncuCyte ZOOM Live-Cell Analysis System (Essen Bioscience) at 37 °C in the phase-contrast mode (10X objective). Images were acquired for a 1 hour time span each week for a total of 21 days.

For microscopy, EL08.1D2 cells or cell-derived matrices were prepared in on circular microscope coverslips in 24-well cell culture plates. Plates were blocked with heat denatured 2% BSA/PBS for 1 hour at 37 °C. Next, ex vivo NK (ENK) cells from lineage-depleted apheresis or NK92 cells were incubated on samples for 30 minutes. Directly conjugated antibodies to surface antigens CD56 (1:50, Clone HCD56, BioLegend) and CD29 (1:50, Clone TS2/16, BioLegend) were added at this
Samples were fixed with 4% PFA/PBS for 20 minutes at room temperature, then washed three times with PBS and treated with 0.2% Triton X-100/PBS for 10 minutes. Samples were stained with polyclonal rabbit anti-collagen I antibody (1:100, Novus Biologicals), followed by phalloidin (Thermo Fisher). Samples were washed and coverslips were removed and mounted onto microscope slides in ProLong Gold (Life Technologies).

### 3.2.7. Image analysis and cell tracking

For analysis of collagen confluence, microscopy images showing collagen distribution were imported into FIJI, and manually thresholded to remove background (Schindelin et al., 2012). The confluence was then recorded based on the ‘Area Fraction’ measurement. For actin footprint measurement, a freehand selection was drawn around cells and the selection area was then measured.

Cell tracking was performed using the TrackMate plugin in FIJI, by manual tracking. Track statistics were exported from FIJI and the data was imported into MATLAB, which was also used to generate the rose plots. Mean speed, straightness, and arrest coefficient were calculated using custom scripts in MATLAB. Mean speed was defined as the per-track average of all instantaneous speeds calculated at each frame. Displacement was defined as the absolute distance between cell position at the beginning and the end of the 24 hour period of tracking. The straightness parameter was calculated by dividing the displacement by the total path length for each track. Arrest coefficient was defined as the percentage of time that the cell
stays in arrest based on a threshold on instantaneous speed of 2 μm/min, or approximately one cell body length per image interval.

3.2.8. Statistics

Statistical analysis was calculated using Prism 8.0 (GraphPad). Ordinary one-way ANOVA was used to compare track statistics. Mann-Whitney U test was used to compare actin footprint data. For all tests P<0.05 was considered significant.

3.3. Results

3.3.1. Cell-derived matrices from EL08.1D2 express common ECM components and support NK cell adhesion

Cell-derived matrices were generated from EL08.1D2 stroma as described previously (Kaukonen et al., 2017). CDMs were stained with collagen I antibody and imaged by confocal microscopy. Analysis of microscopy images revealed that cell-derived matrices contained a collagen network that was significantly more confluent than that of EL08.1D2 stroma, with a mean confluence of 13.76%±9.99 versus 0.29%±0.50 (Figure 3.2A, B). As expected, control microscopy slides without EL08.1D2 stroma did not display a collagen network, even after treatment for CDM production (ascorbic acid exposure for a week, followed by addition of cell denudation buffers and DNase). Mean confluences were 0.009%±0.016 and 0.467%±0.552 for cell-free controls with and without CDM treatment, respectively. Interestingly, confocal microscopy revealed that EL08.1D2 expressed a minor but
significant level of collagen expression (Figure 3.2D). To determine if CDMs also expressed alternate ECM proteins, I performed western blots on solubilized CDM and EL08.1D2 cell lysates with a fibronectin antibody. Indeed, fibronectin content was detected on solubilized CDM but not on EL08.1D2 lysates (Figure 3.2C). The presence of an actin band on the EL08.1D2 lysates but not solubilized CDM indicated that there was no cellular contamination in the CDM samples. These experiments demonstrated that cell-derived matrices were in fact being produced from EL08.1D2 stroma.

To determine if CDMs supported NK cell adhesion, I imaged microscope slides of NK92 cells incubated on CDM and EL08.1D2 stroma. NK92 were seeded on each substrate, allowed to adhere for 30 minutes, and then fixed and stained with antibodies against actin and collagen (Figure 3.2D). Confocal microscopy revealed that NK92 adhered to both CDM and EL08.1D2 stroma, although NK92 plated on CDMs seemed to display greater spreading characterized by long, thin filopodia-like extensions. Quantitative image analysis confirmed that NK92 cells exhibited a significantly larger actin footprint on CDMs compared to EL08.1D2 stroma, with a mean footprint of $165.0 \pm 60.4 \, \mu m^2$ on CDMs compared to $90.2 \pm 16.9 \, \mu m^2$ on stroma (Figure 3.2E). This suggests greater integrin activation of NK cells on CDMs, given that integrin ligation and subsequent outside-in signaling is correlated with cell spreading and adhesion (Culley et al., 2009; Moser et al., 2009).
Figure 3.2 – EL08.1D2 cell-derived matrices contain ECM components and support NK cell adhesion.

A) Fluorescence microscopy images of conditions used to validate CDM production. Only slides containing EL08.1D2 that underwent treatment for CDM production (top left) produced a collagen network. Slides with EL08.1D2 that were untreated (bottom left), slides without EL08.1D2 that underwent treatment for CDM production (top right), and slides without EL08.1D2 that were untreated (bottom right) did not display collagen. B) The confluence of the collagen network was measured for each condition. Error bars represent s.d. Means with significant differences were determined by ordinary one-way ANOVA with Tukey’s multiple comparison test (****p< 0.0001). C) Western blot for fibronectin on EL08.1D2 or CDM lysates. D) Fluorescence microscopy images showing NK92 spreading on CDM versus EL08.1D2. Arrows denote NK92 cells. Scale bar = 10 um. E) Quantification of cell footprint in each condition. Error bars indicate s.d. Means with significant differences were determined by Mann-Whitney U test (****p< 0.0001).
3.3.2. CDMs support adhesion and migration of primary NK cells and NK precursors

Having confirmed that CDMs supported NK92 adhesion, I repeated these experiments with healthy donor-derived ENK cells to determine if primary cells also adhered to CDM. ENK were isolated from human peripheral blood, incubated on CDM or EL08.1D2 stroma, and fixed and stained with antibodies against actin, CD29, and CD56. Confocal microscopy of cells again showed the presence of filopodia-like extensions characterized by CD29 and CD56 enrichment, demonstrating that CD29 is important for adhesion to both stroma and ECM (Figure 3.3A). Z-stack information revealed that CDMs were somewhat 3-dimensional with a depth comparable to that of the EL08.1D2 monolayer, and NK cells were capable of invading into the CDM. Once again, primary NK cells had slightly greater spreading on CDMs, with a mean actin footprint of 40.0±12.3 µm² on CDM compared to 36.2±11.8 µm² on stroma (Figure 3.3B).

As CDMs supported adhesion of both primary NK and NK92 cells, I hypothesized that cell migration of NKDI on CDM would be comparable to that of NKDI on stroma. CD34+ HPCs were isolated from peripheral blood and seeded on either CDM, EL08.1D2, or a specialized low-adhesion plates coated with a hydrophilic, neutrally charged hydrogel to inhibit cell attachment. Immediately after cell addition, live-cell imaging was performed with image acquisition every 2 minutes (Figure 3.3C). As expected, HPCs had comparable migration on CDM and EL08.1D2 (Figure 3.3D, 3.5C). In contrast, HPCs did not migrate on low adhesion
plates, with cell motions being predominantly the result of random drift. Together, these results suggest that CDMs support adhesion and migration of primary NK cells.
Figure 3.3 – Primary NK cells and NKDIs adhere to and migrate on CDMs.

A) ENKs were allowed to adhere to either EL08.1D2 or cell-derived matrix for 30 minutes and then fixed and stained with fluorescent antibodies. Images show maximum intensity projection of z-stacks. Scale bar = 10 um. B) Quantification of cell footprint in each condition. Error bars indicate s.d. Means with significant differences were determined by Mann-Whitney U test (*p< 0.05). C) CD34+ HPCs were isolated from peripheral blood and seeded on various substrates and imaged every 2 minutes. Cell migration was tracked following image acquisition. Scale bar = 50 um. D) Rose plots of HPC tracks in each condition.
3.3.3. CDMs support NK cell development from hematopoietic precursor cells

Having established that CDMs support NK cell adhesion and migration, and given that EL08.1D2 stroma support NK cell development, in order to elucidate the requirement of NK cell migration for proper cell maturation, the logical next step was to assess NK development on CDMs. CD34+ HPCs were isolated from peripheral blood and seeded on either CDM, stroma, or low adhesion plates. 2,000 HPCs were seeded in each well of a 96-well cell culture plate. Flow cytometry analysis for developmental markers was performed weekly. Indeed, at 21 days, NKDI showed similar maturation on EL08.1D2 and CDMs but failed to survive on low adhesion plates (Figure 3.4). Expression of CD56 and CD94 was observed for the first two conditions, denoting that NKDI had successfully reached stage 4 and 5 of NK development. However, receptor expression was slightly decreased in the CDM condition, with 49.1% of NKDIs positive for CD94 compared to 65.9% of NKDIs positive in the EL08.1D2 condition (Figure 3.4B). Similarly, only 63% of NKDIs in the CDM condition were positive for CD56 expression, whereas 80.3% of NKDIs were positive in the EL08.1D2 condition. Additionally, cell counts showed that NK survival and proliferation was decreased in the CDM condition, with 57,500 live NKDI remaining compared to 350,000 live NKDI in the EL08.1D2 condition (Figure 3.4C). Confirming the flow cytometry data, cell counting also demonstrated a complete lack of surviving cells in the low adhesion condition. Together, these results suggest a requirement for adhesion/migration for NK cell development; HPCs on low adhesion plates completely failed to develop into mature NK cells, in
contrast to those plated on CDM or EL08.1D2, which proliferated and matured. However, both cell maturation and survival of NKDI on CDM was inferior to that of the EL08.1D2 plates, suggesting that adhesion is necessary but not sufficient for NK development.
A) CD34+ HPCs were isolated from peripheral blood and seeded on CDM, EL08.1D2, or low adhesion plates. After 21 days, cells were harvested and analyzed by flow cytometry to determine cell maturation. Cells were gated based on FSC/SSC and a live-dead marker for live lymphocytes. B) Bar graphs showing percentage of cells positive for either CD94 or CD56 in each condition. C) Number of live cells present for each condition. Data is representative of three independent experiments.
3.3.4. NKDIs have comparable migration on CDM and EL08.1D2.

Given that NKDI were capable of maturing on cell-derived matrices, I hypothesized that they would have similar migratory behavior on CDM and EL08.1D2. CD34+ hematopoietic precursor cells were isolated from patient blood and seeded on either CDM or EL08.1D2. In conjunction with the weekly flow cytometry for developmental markers, cells were also imaged every 2 minutes and migration was tracked at weekly time points (Figure 3.5). Indeed, tracks were fairly similar between the two conditions at all weekly time points (Figure 3.5B, 3.3D). For example, the mean speed of tracks was 2.14 ± 0.79 μm/min on EL08.1D2 after 0 days, and 1.93 ± 0.98 μm/min on CDMs (Figure 3.5C). After 21 days, this increased to 3.626 ± 0.72 μm/min on EL08.1D2 and 2.936 ± 0.79 μm/min on CDMs; this was consistent with previously reported migration speeds of mature NK cells in similar conditions (Khorshidi et al., 2011; Mace et al., 2016). Similarly, after 0 days, mean straightness was 0.21 ± 0.14 on EL08.1D2 and 0.23 ± 0.11 on CDM, and mean arrest coefficient was 0.60 ± 0.19 on EL08.1D2 and 0.67 ± 0.26 on CDM. In fact, the two conditions were not significantly different at all, except for straightness after 7 days, which was significantly higher on CDMs, with a mean straightness of 0.57 ± 0.13 compared to 0.28 ± 0.15 on EL08.1D2. This is likely due to the greater proliferation in the EL08.1D2 condition causing less space for each individual NKDI to migrate around in.
Figure 3.5 – NKDiS have comparable migration on CDM and EL08.1D2.

CD34+ HPCs were isolated from peripheral blood and seeded on CDM or EL08.1D2 stroma. Cell migration was imaged every 2 minutes and cells tracks were determined at each week. A) Representative tracks showing HPC migration on CDM and EL08.1D2 stroma. Scale bar = 50 um. B) Rose plots of cell tracks on CDM and EL08.1D2 at each week. C) At each week, the mean speeds (left), straightnesses (middle), and arrest coefficient (right) of cell tracks was determined. Error bars indicate s.d. Means with significant differences were determined by ordinary one-way ANOVA with Tukey’s multiple comparison test (* p< 0.05). Data is representative of three independent experiments.
3.4. Discussion and conclusions

In this section, I have described EL08.1D2 cell-derived matrices as an alternative substrate for NK in vitro differentiation that supports NK cell development but with impaired survival. These cell-derived matrices are composed together of ECM macromolecules that are naturally secreted by EL08.1D2 stroma under correct conditions. I confirmed that CDMs contain expected ECM components, including fibronectin and collagen, and that these macromolecules are arranged in a dense matrix similar to tissue ECM.

Interestingly, both NK92 and ENK had greater spreading on CDM compared to EL08.1D2 stroma. This may reflect greater integrin activation in the CDM condition, as LFA-1 ligation on NK cells is known to be involved in cell spreading and migration (Culley et al., 2009). Indeed, integrins are responsible for attaching the NK cell to structural proteins within the ECM, such as collagen and fibronectin. VLA-4, consisting of CD94d (integrin α4) and CD29 (integrin β1) is involved in fibronectin binding to ECM (Macías et al., 2000). Integrin β1 is additionally responsible for facilitating adhesion to collagen (Staatz et al., 1990). The lack of significant difference in cell motility despite the disparity in adhesion may be due to additional activating or inhibitory receptor interactions present between NKDI and EL08.1D2 that are absent in the CDM condition. For example, co-ligation of LFA-1 with an activating receptor leads to symmetrical spreading, but co-ligation with of an inhibitory receptor leads to decreased spreading (Culley et al., 2009). Also, cell spreading on CDMs has been shown to be integrin-specific, as integrin β1
knockdown cells have greater area on ECM while β3 knockdown cells exhibit the same spreading as wild type cells (Costa et al., 2013). While spreading on CDMs was frequently uniform and symmetric, I did notice a greater degree of polarization with NK cells on EL08.1D2 stroma, although this was not statistically significant. This may be related to the capability for the formation of a developmental synapse between NK cells and EL08.1D2 (Mace et al., 2016). The DS is derived from the uropod and is the site of rich signaling as determined by tyrosine activation and calcium flux. While the possibility of a similar synapse forming between CDM and NK cells is unlikely due to the network-like structure of CDM, the integrin activation observed on this substrate suggests that signaling may still be taking place within the NK cell.

Previously, I observed that hematopoietic precursor cells on low adhesion plates fail develop into mature NK cells and do not display migration on this substrate. This suggests a previously undescribed requirement for adhesion and migration in NK cell development. Indeed, it has been shown that integrins are necessary for lymphocyte migration during development (Arroyo et al., 1996). Also, blocking CD56 activity during NK cell development led to impaired motility and development (Mace et al., 2016). While CD56 is not typically described as an adhesion receptor, it has been shown to regulate integrin-dependent cell migration, possibly through modulation of integrin activity (Diestel et al., 2005; Diestel et al., 2007). While integrin activation was observed on both CDM and EL08.1D2, there was not a significant difference in cell motility between these two conditions. Thus, it is possible that the difference in NK cell survival and differentiation is due to the
EL08.1D2 stroma releasing pro-survival signals, either in the form of cytokines or growth factors, or biochemical signaling between EL08.1D2 and NK precursors promoting survival and NK cell fate. For example, expression of VCAM-1, the ligand for integrin VLA-4, on OP9 cells has a synergistic effect with Notch signaling to promote T cell differentiation \textit{in vitro}, and inhibition of the aryl hydrocarbon receptor on hematopoietic progenitor cells promotes cell fate in an OP9-DL1 differentiation model (Angelos et al., 2017; Shukla et al., 2017). Also, stromal cells are secretors of cytokines and growth factors, and in particular the EL08.1D2 cell line supports HPC survival even without direct contact (Leuning et al., 2018; Oostendorp et al., 2005). Specifically, stromal cell-derived factor-1β is released by bone marrow-derived mesenchymal stromal cells and promotes stem cell survival (Herberg et al., 2013). However, it is not impossible that the pro-survival factors are totally absent in ECM derived from EL08.1D2. Given that cytokines are known to be “sticky” and can bind molecules such as heparin, it is possible that some cytokines are retained within CDM (Kishimoto et al., 2009). Through this mechanism, heparin has been used in a cell-free system to support NK cell differentiation from hematopoietic stem cells \textit{in vitro}, as it is suggested that heparin binding inhibits cytokine degradation in solution and presents them in a more physiological manner (Dezell et al., 2012). Interestingly, heparan sulfate, a structurally similar analog of heparin, is found within the extracellular matrix of nearly all mammalian tissue and binds to signaling proteins such as FGF; This binding has a stabilizing effect and promotes the biological effectiveness of the growth factor (Yayon et al., 1991). Heparan sulfate proteoglycans also bind to cytokine IL-7 and present it to murine
lymphocytes in *in vitro* cultures of whole bone marrow cells to promote lymphocyte development (Borghesi et al., 1999). This is especially interesting given that IL-7 is necessary for the *in vitro* NK cell differentiation described in this dissertation and suggests a migration-independent mechanism through which CDMs support NK cell development. Clearly, further work on the specific role of CDMs in supporting NK cell development is necessary.

### 3.5. References


Chapter 4

Significance and future directions

4.1. Summary

In this work, I have provided a comprehensive study of NK cell development, migration, and adhesion on developmentally supportive EL08.1D2 stroma and ECM derived from these stromal cells. In Chapter 2, I demonstrated that EL08.1D2 stroma support migration of NK92, YTS, and primary ENK cells, and that primary NKDI cells seeded on these stroma develop into mature NK cells over the course of 21 days. Additionally, maturing NKDI acquire increased migratory capacity as they develop, with progressive increases in mean speed and straightness and decreased time spent in arrest. Cell tracks were classified as either constrained, random, or directed migration, revealing that less mature NKDI spent most of their time exhibiting constrained migration with only a few migratory cells, while more
matured cells exhibited much more directed and random migration characterized by greater mean speeds and overall displacement for a given interval. Unsurprisingly, the heterogeneity of the migratory phenotype also increased at later time points, with cells exhibiting much more frequent alternation between migratory modes at 21 days into the experiment. In Chapter 3, I expanded on these previous results by introducing cell-derived matrix as a substrate that would support cell adhesion and migration but without any cell-cell signaling present, and described NKDI adhesion, migration, and differentiation on this CDM. ECM derived from EL08.1D2 expressed expected ECM components, including collagen and fibronectin, and supported adhesion of both NK92 as well as primary ENK cells, with greater cell spreading observed on CDMs as compared to EL08.1D2 stroma. Additionally, NKDI migrated on both CDMs and EL08.1D2 stroma, but not on low adhesion plates which would preclude cell adhesion. Over the course of a 21 day differentiation experiment, cell migration was not significantly different between NKDI on CDM or EL08.1D2. However, NK cell differentiation was impaired in the CDM condition as compared with NKDI plated on EL08.1D2 stroma, with less expression of developmental markers CD56 and CD94. Additionally, less total number of cells were present in the CDM condition, indicating a defect in cell survival.
4.2. Significance

Together, these results suggest that adhesion alone is not sufficient to support normal NK cell development; there must be an additional requirement for cell-cell signaling between NKDI and EL08.1D2. This signaling might occur through the previously described developmental synapse, the uropod-derived interface that forms between NKDI and EL08.1D2 (Mace et al., 2016). It might also occur through longer-range interactions such as through secretion of vesicles, cytokines, or other signaling molecules. However, cell-derived matrix does provide some level of developmental support, as NKDI did exhibit significant albeit impaired development and survival on CDMs. This suggests the possibility of a stroma-cell-free system for in vitro differentiation of NK cells from CD34+ HSCs, which is exciting due to clinical barriers associated with using a murine cell line such as EL08.1D2. Indeed, it might be feasible to generate a developmentally supportive ECM substrate from scratch, with a more comprehensive description of EL08.1D2-derived ECM components that could be gained from methods such as mass spectrometry.

As NKDI migration is not significantly different on EL08.1D2 or CDM, I propose that in this context the changes in migration observed throughout development are predominantly the result of differences in the phenotype of the cell adhesome at each developmental stage. Although chemokines such as SDF-1 are released by stromal cells and have been shown to influence HSC migration by activating integrins, these chemokines must not be relevant to our study as they
cannot possibly be present in the CDM condition (Aiuti et al., 1997; Peled et al., 2000). RNA-seq data has shown that the cell adhesome, particularly the expression level of integrins, undergoes significant changes through NK cell development. Some integrins, such as LFA-1, upregulate their expression at later stages, while others such as integrin β1 and β7 are downregulated (Chen et al., 2018; Freud et al., 2005). Given the increase in directed migration observed for more mature NK cells, NK migration is likely integrin-specific, potentially with LFA-1 as the main driver of cell migration. Indeed, it has been shown that different integrins have unique signaling mechanisms and overall contributions to adhesion and migration (Mostafavi-Pour et al., 2003).

Although EL08.1D2 stroma have previously been described as one of the optimal methods for generating a large, pure population of mature NK cells from CD34+ HSCs, it seems the requirement for these cells may not substantial as previously believed (Grzywacz et al., 2006). Indeed, NKDI can undergo full development on cell-derived matrices, which only provide adhesive and migratory support for the cells. The EL08.1D2 stromal cells do not seem to be releasing chemokines as previously discussed, but they may be releasing signaling molecules such as cytokines to promote cell survival. Stromal cells have been shown to release various cytokines and growth factors such as HGF, VEGF, FGF, IL-6, and IL-8 (Leuning et al., 2018). Particularly, the UG26.1B6 and EL08.1D2 stromal cell lines have been shown to support the maintenance and survival of hematopoietic stem cells, even without direct contact (Oostendorp et al., 2005). Thus, EL08.1D2 might
still play some role in supporting NKDI survival, as I observed significantly
decreased final cell counts for HPCs initially seeded on CDMs.

Another explanation for the difference in efficiency of NK cell development on CDMs is that contact-dependent biochemical signaling between NKDI and EL08.1D2 is critical for proper survival and differentiation. While EL08.1D2 maintenance of hematopoietic stem cells is not contact-dependent, proper NK cell maturation is reduced when cell-cell contact is prevented in a Transwell system (McCullar et al., 2008; Oostendorp et al., 2005). However, the specific biochemical pathways through which EL08.1D2 stroma support NK cell development are unclear. It has been suggested that these interactions are focused at the DS between NKDI and EL08.1D2 stroma, and may take place through homotypic interactions between CD56 on NKDI and stroma (Mace et al., 2016). Studies using OP9 stroma, which also support generation of mature NK or T cells, has shown that Notch ligands DL1 and DL4 on stroma are key lineage determinants (Freud et al., 2006; La Motte-Mohs et al., 2005). Similarly, Wnt proteins, which are secreted signaling factors, have been shown to be involved in T cell development, although a role in NK cell development has not been established. Double knockout mice lacking Wnt1 and Wnt4 demonstrated reduced thymic cell number and impaired T cell differentiation (Staal et al., 2001). Expression of VCAM-1 on OP9 cells or in a cell-free system significantly enhances cell migration and Notch pathway activation and increases production of T cell progenitors when compared to Notch ligands alone (Shukla et al., 2017). In mice, expression of Notch ligand Delta1 promotes development of NK
cells from HSCs (DeHart et al., 2005). Feedback between Notch and integrins is likely bidirectional, as Notch signaling can also activate β1 integrins (Leong et al., 2002). Integrin binding may also drive NK cell maturation by supporting other biochemical signals in a similar fashion.
Given that NKDI can undergo development and migration on cell-derived matrices as well as EL08.1D2 stroma, I suggest that EL08.1D2 may support NK cell development through release of signaling factors promoting survival or cell fate determination. However, contact-dependent biochemical interactions between EL08.1D2 and HSC are also likely critical. At stage 1 of development, NKDI migrate on either substrate through integrin β1 and integrin β7 adhesions. Over the course of development, these integrins are downregulated and LFA-1 is upregulated to become the primary driver of cell migration. Additionally, EL08.1D2 exit the system, possibly through NK cell killing, and mature NK cells gain the capacity to secrete cytokines.
Limitations and future directions

The data presented in this work could be built upon in several ways. In Chapter 2, I provided a comprehensive analysis of NK cell motility through development. However, I chose to focus on population-level NK cell migration dynamics, rather than perform single-cell analysis of motility. For example, high-resolution microscopy of NKDC migration would be useful to determine the specific function of various adhesome receptors, such as integrins, during NK development. Given that the developmental synapse between NKDC and EL08.1D2 stroma has been described to be critical for NK cell development, it would be useful to further define the DS with high-resolution confocal microscopy (Mace et al., 2016). While CD56 and CD62L were observed to localize to the DS, expression of other critical adhesion receptors such as integrins is currently unknown. Receptor engagement at the developmental synapse could be determined by looking at co-localization between integrins and their corresponding ligands on the stroma. For example, CD56 engages in homotypic interactions and is expressed on both NK and EL08.1D2 cells (Mace et al., 2016). NK cells present LFA-1 integrin which binds to ICAM-1, as well as VLA-4 integrin which binds to fibronectin and VCAM-1 (Macías et al., 2000; Marlin and Springer, 1987). Additionally, integrin activation could be measured by determining expression of talin, vinculin, and Pyk-2 at the DS. Given that the DS is uropod-derived, DS formation should correlate with cell polarity. Thus, it would be useful to perform measurements of NKDC circularity or axis ratio on EL08.1D2, in
addition to measuring cell footprint. Further high-resolution live-cell microscopy would allow the analysis of the temporal dynamics of DS formation, such as rate of formation and dissociation, or whether DS formation is correlated with cell arrest.

In Chapter 3, I have presented cell-derived matrices as an alternate substrate that supports development of NKDI but results in poor cell survival. Given that there is no motility defect on CDM, it seems likely that EL08.1D2 stroma have some mechanism for enhancing survival of NKDI. While proteomic analysis of the EL08.1D2 has already been performed, to our knowledge there has not yet been a comprehensive study of the EL08.1D2 secretome (Ledran et al., 2008). Such a study would reveal any pro-survival cytokines or growth factors being released by EL08.1D2 during *in vitro* differentiation. While here I have found no significant difference in NKDI motility on CDM or EL08.1D2 at the population level, migration at the single-cell level might be a different case. High-resolution live-cell microscopy would once again be useful here to define the role of specific integrins. The use of blocking antibodies to target certain integrins would also paint a better picture of integrin-specific NKDI motility. Additionally, the specific receptor-ligand signaling interactions between EL08.1D2 and NKDI are still unclear. Further studies to closely analyze the effects of known regulators of lymphocyte development, such as CD56 and Notch, would be useful to elucidate the role of these interactions (Mace et al., 2016; Shukla et al., 2017).
Furthermore, in Chapter 3 I compared NKDI adhesion on EL08.1D2 and CDM by analyzing changes in cell footprint area. Actin footprint was greater on the CDM condition, suggesting a greater activation of integrin receptors. Cell adhesion could be cross-validated by applying a functional adhesion assay such as the inverted centrifugation assay or even a rolling cell adhesion (Franco et al., 2006; Shetty et al., 2014). Studies of rolling cell adhesion are supposedly more physiologically relevant, since the system mimics cells flowing through blood vessels or within secondary lymphoid tissues. \textit{In vivo}, this seems to occur following the origination in the bone marrow of CD34$^+$ common lymphoid progenitor cells, which then circulate through peripheral blood to secondary lymphoid tissues throughout the body, such as the tonsil or lymph nodes (Freud et al., 2005; Galy et al., 1995). Again, blocking antibodies could be applied against specific adhesome receptors to study receptor-specific cell adhesion.

Here, I have studied NKDI motility in an \textit{in vitro} system, as fairly little is known about NK cell migration within the body through development. Of course, the tissue residency of NKDI at various stages is somewhat defined, as it is known that stage 1 NKDI reside in the bone marrow, and eventually migrate to tonsil and lymph node secondary lymphoid tissue where stage 3 NKDI reside (Scoville et al., 2016). There, they undergo further maturation and exit as CD56$^{\text{bright}}$ NK cells and CD56$^{\text{dim}}$ NK cells which circulate within peripheral blood (Cooper et al., 2001; Romagnani et al., 2007). However, the specific localization of NKDIs within tissue is unknown, as is what other cells are present within the surrounding niche. Insights might be gained
into this subject by using immunohistochemical approaches to study primary patient tissue. Immunohistochemistry would provide a high-resolution description of NKDI localization within tissue, as well as any interactions with surrounding lymphocytes or stromal cells. Another approach would be to use intravital microscopy methods to study NK cell motility directly within primary tissue, although this would clearly be restricted to murine studies. One such study, although performed on T cell lymphocytes, has already revealed that lymphocyte migration within lymph nodes is spatially associated with the stromal cell network (Bajénoff et al., 2006).

Another limitation of this work is that motility has only been studied in the context of NKDI migration on a 2-dimensional substrate. While the Incucyte microscope used in this dissertation allows for accurate tracking of NKDI migration over long time periods, it is unfortunately not designed for 3-dimensional imaging. This is concerning, given that lymphocytes have been shown to be capable of an additional integrin-independent mode of migration in 3-dimension system similar to amoeboid squeezing (Lammermann et al., 2008). However, even within three dimensions, in less confined microenvironments cells can still utilize the integrin-dependent mesenchymal-type migration described in this dissertation (Friedl et al., 1998). While it is not immediately clear how to translate a differentiation system dependent on an EL08.1D2 monolayer into three dimensions, it is a different case altogether for cell-derived matrices. 3D CDM have been well defined in the literature and the thickness of cell-derived matrix can be tuned by adjusting the treatment
used for CDM production (Hakkinen et al., 2011). Indeed, fibroblasts migrating through 2D and 3D CDM have very different motility and morphology. In fact, confocal microscopy has revealed that NKDI are capable of invading into cell-derived matrix, despite the fact that the CDMs used here were similar to EL08.1D2 stroma in their overall thickness. Thus, I present this work here with the hope that it may benefit and inspire such innovative studies in the future.
4.4. References


stromal cells is determined by surface structure of the microenvironment. Sci Rep 8, 7716.


NK cells display longer telomeres and acquire features of CD56dim NK cells upon activation. J Immunol 178, 4947-4955.


