From stem cell to immune effector: how adhesion, migration, and polarity shape T-cell and natural killer cell lymphocyte development in vitro and in vivo

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ABSTRACT **Lymphocyte development is a complex and coordinated pathway originating from pluripotent stem cells during embryogenesis and continuing even as matured lymphocytes are primed and educated in adult tissue. Hematopoietic stem cells develop in a specialized niche that includes extracellular matrix and supporting stromal and endothelial cells that both maintain stem cell pluripotency and enable the generation of differentiated cells. Cues for lymphocyte development include changes in integrin-dependent cell motility and adhesion which ultimately help to determine cell fate. The capacity of lymphocytes to adhere and migrate is important for modulating these developmental signals both by regulating the cues that the cell receives from the local microenvironment as well as facilitating the localization of precursors to tissue niches throughout the body. Here we consider how changing migratory and adhesive phenotypes contribute to human natural killer (NK)- and T-cell development as they undergo development from precursors to mature, circulating cells and how our understanding of this process is informed by in vitro models of T- and NK cell generation.**

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INTRODUCTION

Immune cell progenitors are constantly forming contacts with the cells around them as they migrate within the human body both during their development and subsequently as mature functional cells. Diverse immune and nonimmune cell types provide molecular signals, such as secreted cytokines and growth factors, as well as direct surface receptor contacts and a mechanical environment that collectively shape hematopoietic stem cell (HSC) homeostasis, hematopoiesis, and immune cell generation and function. Adhesion and cell migration play critical roles in these processes, including in the generation of immune lineages from $CD34^+$ HSCs. Importantly, the unique adhesive and migratory profile of progenitors is dynamic and thus represents an inherent phenotypic parameter that both defines and shapes discrete stages of differentiation. In this Perspective, we discuss recent developments in our understanding of the role that changing phenotypes of cell migration and adhesion plays in lymphocyte differentiation, with a particular focus on human lymphocytes of the T- and natural killer (NK)-cell lineages. While migration and adhesion are important throughout the life cycle of these lymphocytes, including for their activation and effector functions, here we focus specifically on the importance of these processes on T and NK cell development. Specifically, we discuss what in vitro and in vivo models can tell us about the adhesive and migratory properties of T and NK cell precursors within the environments that support their generation and challenges to understanding the role of polarization, adhesion, and migration in complex microenvironments.

The role of adhesion and migration in adult T-cell and NK cell precursor trafficking in vivo

Lymphocytes originate from HSCs that are first derived by endothelial-to-hematopoietic transition as they bud in response to both extrinsic and intrinsic cues from hemogenic endothelial cells in the

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Abbreviations used: ACD, asymmetric cell division; APC, antigen-presenting cell; DL1, delta-like 1; DL4, delta-like 4; DN3, double-negative 3; HSC, Hematopoietic stem cell; ILC, innate lymphoid cell; LAD, leukocyte adhesion deficiency; NK, natural killer; S1P, sphingosine 1-phosphate.

aortic endothelium of the developing embryo (Medvinsky and Dzierzak, 1996; Bertrand *et al.*, 2010; Boisset *et al.*, 2010; Kissa and Herbomel, 2010; Chen *et al.*, 2011). Generation of these early HSCs is followed by their colonization of the fetal liver, then their expansion and subsequent migration to fetal bone marrow. HSC transit between fetal niches is orchestrated by chemokines and cytokines, including CXCR4 and c-Kit, as well as cadherins, integrins, and selectins (Kinashi and Springer, 1994; Kovach *et al.*, 1995; Levesque *et al.*, 1995; Hirsch *et al.*, 1996; Fraser *et al.*, 2002; Mazo *et al.*, 2002; Emambokus and Frampton, 2003; Ferkowicz *et al.*, 2003; Taoudi *et al.*, 2005; Massberg *et al.*, 2007). In particular, integrins play a key role in guiding embryonic HSC colonization through cell–cell contact within niche environments. Fetal stem cells from β1 integrin-deficient mice fail to populate fetal liver and bone marrow but undergo differentiation in fetal liver cultures, pointing to a specific role for β1 integrin in migration and homing (Hirsch *et al.*, 1996; Potocnik *et al.*, 2000; Scott *et al.*, 2003). In contrast, surface β2 integrins are not present on murine long-term HSCs but play important roles in the generation of multipotent progenitors (Bose *et al.*, 2014; Fathman *et al.*, 2014; Inlay *et al.*, 2014; Leon-Rico *et al.*, 2014). Genetic models in mice reflect the underlying theme that β1 integrins in particular play a nonredundant role in embryonic and adult hematopoiesis, as their deletion largely leads to embryonic lethality, whereas β2 integrins are considered most relevant for effector function and trafficking to sites of inflammation (Bouvard *et al.*, 2001; Papayannopoulou *et al.*, 2001). However, in human fetal liver HSCs, expression of GPI-80, a glycophosphatidylinositol-linked surface protein that mediates adhesion and colocalizes with αMβ2 integrin, marks a self-renewing HSC population and suggests that there is a functional requirement for GPI-80-αMβ2 interactions in mediating adhesion or migration in the niche. Knockdown of either protein led to loss of HSC self-renewal capacity. However, the way in which GPI-80 and αMβ2 function together has not been directly tested (Prashad *et al.*, 2015). Finally, there is also a role for modulation of the extracellular matrix within fetal niches, and matrix metalloproteases likely play a role in the release of embryonic HSCs into circulation (Klein *et al.*, 2015).

In adults, HSCs are maintained within the bone marrow, where they receive signals from surrounding cells in the stem cell niche that regulate self-renewal, expansion, and maturation through local cytokine signaling, cell–cell contacts, and extracellular matrix interactions (Calvi *et al.*, 2003; Zhang *et al.*, 2003; Mendez-Ferrer *et al.*, 2010; Morrison and Scadden, 2014; Wei and Frenette, 2018). The cell adhesion molecule receptors best characterized as being important for adult stem cell niche interactions include selectins, cadherins, integrins (especially β1 integrins), sialomucins, the Ig superfamily, and CD44 (reviewed in De Grandis *et al.*, 2016). While it is beyond the scope of this Perspective to describe these in detail, it is important to note that, as with the transit of cells between niches during development, the maintenance of cells within the niche is orchestrated by a combination of chemokine signals, ligand expression, adhesion receptor expression, and activation state of adhesion molecules. Together, these signals, sometimes referred to as an "area code," act to provide the correct address for stem cells as they are directed to sites that instruct their respective fates in self-renewal or differentiation. These complex mechanisms, particularly when combined with the effects of biomechanical forces, including substrate stiffness (Lee-Thedieck *et al.*, 2012; Choi and Harley, 2017), demonstrate that lineage differentiation is regulated by both stochastic and deterministic cues.

In the adult, common lymphoid progenitors that give rise to T and NK cells exit the bone marrow and migrate through peripheral blood to the thymus or other peripheral tissues, where they are recruited by multistep adhesion cascades characterized by selectin-mediated tethering, chemotactic signaling, and integrin-mediated firm arrest and diapedesis. In the case of homing of thymus-seeding progenitors, the area code that mediates this process is primarily mediated by P-selectin-PSGL1 interactions; chemokine signaling by CCL19, CCL21, and CCL25 through chemokine receptors CCR7 and CCR9; and α4β1 and αLβ2 integrins (Uehara *et al.*, 2002; Rossi *et al.*, 2005; Scimone *et al.*, 2006; Krueger *et al.*, 2010; Zlotoff *et al.*, 2010). Briefly, this process of extravasation is consistent with other contexts in which circulating leukocytes are recruited to tissue. Following egress from bone marrow, circulating thymus-seeding precursors move freely within blood, being carried by circulation (Figure 1). Initial cell surface tethering to thymic endothelium is mediated by interactions between P-selectin on endothelial cells with PSGL-1 on precursors (Rossi *et al.*, 2005; Scimone *et al.*, 2006; Gossens *et al.*, 2009). Following this initial tethering, rolling is mediated by PSGL-1 and CD44, which rapidly form and release bonds with their ligands and activate integrins for the next phase of arrest (Wu *et al.*, 1993; Rossi *et al.*, 2005; Scimone *et al.*, 2006; Graham *et al.*, 2007; Gossens *et al.*, 2009). Arrest is mediated by clustering and affinity regulation of integrins LFA-1 and VLA-4 binding to their ligands ICAM1 and VCAM-1, respectively (Scimone *et al.*, 2006). This firm arrest is followed by crawling toward endothelial junctions and subsequent entry to tissue. Remarkably, it has been estimated that as few as 10 thymic precursors a day enter the thymus, and the signals that recruit these cells are partly dictated by cyclic expression of P-selectin on thymic endothelium that can be modulated in response to decreasing numbers of peripheral T-cells (Zlotoff *et al.*, 2008; Gossens *et al.*, 2009).

While genetic models have suggested that there may be functional redundancy between integrins during seeding of thymic precursors from peripheral blood (Georges-Labouesse *et al.*, 1996; Schmits *et al.*, 1996; Wagner *et al.*, 1996; Scharffetter-Kochanek *et al.*, 1998; Bungartz *et al.*, 2006), the use of a *Fermt3*$^{-/-}$ mouse model, which globally abrogates the affinity-mediated activation of integrins through loss of kindlin activation, demonstrates that postnatal thymic homing is reliant on integrins for extravasation in mice (Moretti *et al.*, 2018). In humans, despite studies demonstrating that kindlin-3 is required for T-cell arrest and spreading, the thymocyte phenotype in individuals with kindlin-3 deficiency (leukocyte adhesion deficiency [LAD] III, LAD-III) is less severe than that of *Fermt3*$^{-/-}$ mice and VLA-4 retains some capacity to support tethering and adhesion and subsequent T-cell precursor entry to the thymus (Manevich-Mendelson *et al.*, 2009). Molecular studies of lymphocytes from individuals with LAD-III, characterized by bleeding disorders, immunodeficiency, and recurrent bacterial infections, demonstrate that initial integrin activation can occur in the absence of kindlin-3, but that integrin bond strengthening and subsequently cell spreading mediated by LFA-1 is impaired (Manevich-Mendelson *et al.*, 2009; Robert *et al.*, 2011). The residual functions found in kindlin–3-deficient cells from LAD-III patients may reflect compensation by other regulators of integrin activation, such as talin, enabling VLA-4 to maintain sufficient adhesiveness in the absence of kindlin-3 to allow for thymic-seeding precursor recruitment (Manevich-Mendelson *et al.*, 2009).

In contrast to defined requirements for the recruitment of thymic-seeding precursors, the requirements for recruitment and seeding of precursors that give rise to NK cells within tissue in the adult have not been described. The generation of mature NK cells is dependent on bone marrow precursors, as bone marrow ablation abrogates development and bone marrow transplantation can restore

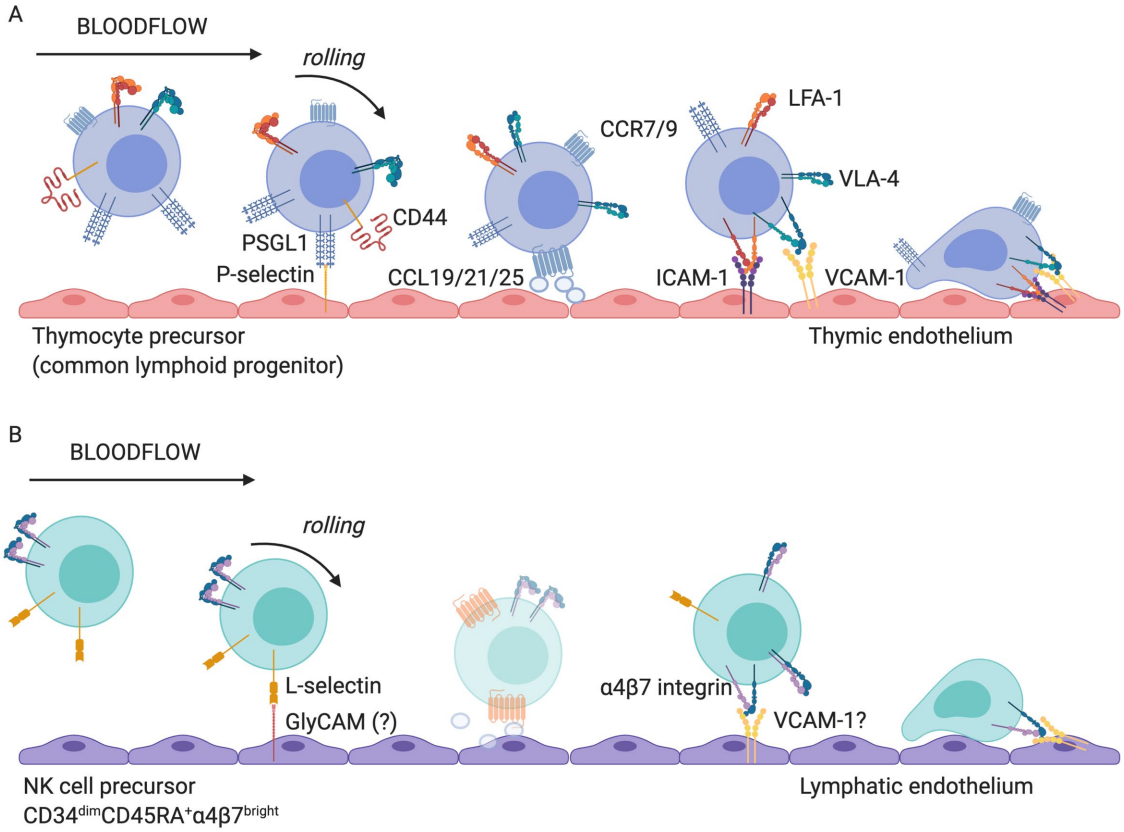

FIGURE 1: Recruitment of postnatal early T- and NK cell precursors from circulation to sites of development. (A) Following egress from the bone marrow, common lymphoid progenitors can be detected in circulation prior to seeding peripheral organs for further development to T- and NK cell lineages. Recruitment of thymus seeding precursors (top) is initially mediated by binding of PSGL1 on circulating precursors to P-selectin on thymic endothelium and hyaluronic acid-independent binding of CD44. This is followed by signaling through chemokine receptors CCR7 and CCR9 binding to their ligands CCL19, CCL21, and CCL25, which leads to increased affinity of integrins for their ligands. Arrest and firm adhesion are mediated by β1 and β2 integrins binding to their ligands VCAM-1 and ICAM-1, respectively, which also subsequently regulate crawling into tissue. (B) Progenitors that seed secondary lymphoid tissue and generate human NK cells are $CD34^{dim}CD45RA^{+}\alpha4\beta7^{bright}$. While less well understood than T-cell precursor homing, their recruitment to secondary lymphoid tissue is proposed to be initiated by L-selectin on precursors binding to its ligands on lymphatic endothelium. The chemokine signal that may be acting in this context is unknown, and chemokine receptor expression on this subset of cells has not been defined (depicted by opacity at chemokine engagement step). The high expression of integrin α4β7 on NK cell precursors suggests that it may be acting to mediate firm arrest and subsequent extravasation, most likely mediated by binding to VCAM-1 on lymphatic endothelium, although this hasn't been demonstrated directly. Diagram is not to scale and is simplified for depiction of key cell surface receptors.

populations of lytic NK cells (Lotzova *et al.*, 1993; Miller *et al.*, 1994; Kim *et al.*, 2002; Fathman *et al.*, 2011). Bone marrow is also likely a site of generation of mature NK cells in both mice and humans, as a spectrum of NK cell developmental intermediates can be found there (Kim *et al.*, 2002; Eissens *et al.*, 2012). However, similar spectra of human NK cell developmental intermediates are found in peripheral tissue, particularly secondary lymphoid tissue, with later intermediate stages also found in spleen (Freud *et al.*, 2006; Eissens *et al.*, 2012). As such, an outstanding question in human NK cell biology is the relative contribution to circulating human NK cells that are contributed by these respective environments and the identity of the early precursors that transit from bone marrow to peripheral sites to undergo further maturation (Eissens *et al.*, 2012; Freud *et al.*, 2014).

Relative to the mechanisms that dictate thymic settling by common lymphoid progenitors, the signaling that directs precursors to seed tissue microenvironments that can give rise to mature NK cells is poorly understood. The earliest hematopoietic progenitors found within human secondary lymphoid tissue that preferentially give rise to mature NK cells are $CD34^{dim}CD45RA^{+}\alpha4\beta7^{bright}$ (Freud *et al.*, 2005). These cells are also present in bone marrow and circulation, and peripheral blood but not lymph node cells with this phenotype highly expresses L-selectin (Freud *et al.*, 2005). The expression of L-selectin and integrin β7 suggests that they may be directing these cells to lymphoid tissue prior to their subsequent differentiation. While selectin-deficient mice do not have gross deficiencies in NK cell development, $L\text{-selectin}^{-/-}$ mice have reduced frequencies of NK cells in secondary lymphoid tissue, supporting a requirement for trafficking of immature or mature NK cells to these tissues (Sobolev *et al.*, 2009). More recently, circulating innate lymphoid cell (ILC) precursors that can give rise to all ILC and NK cell lineages have been described in peripheral blood; however, whether these represent the dominant NK cell precursor and, if so, how these are trafficked into peripheral tissues remains unknown; adhesion and migration receptors on these cells have not been characterized (Montaldo *et al.*, 2014; Scoville *et al.*, 2016; Lim *et al.*, 2017).

T-cell development occurs in the thymus, and progression from a thymic-seeding precursor to mature T-cell is marked by the coordinated migration of cells through the cortex and the medulla where they undergo positive and then negative selection (reviewed in Ladi *et al.*, 2006; Hu *et al.*, 2015). Differentiation and spatial localization of thymic progenitors is again orchestrated by chemokines, cytokines, stromal cells, and the extracellular matrix, as well as other biochemical signals such as Notch signaling (Petrie and Zuniga-Pflucker, 2007). As such, spatially localized chemokine function includes the specification of integrin and adhesion receptor expression to enable selective binding to cortical or medullary thymic stromal elements (Ehrlich *et al.*, 2009). On entry to the thymus at the cortico-medullary junction and commitment to the T-cell lineage, thymocytes expressing the chemokine CXCR4 undergo chemotaxis toward CXCL12 in the cortex, mediated in part by α4β1 on thymocytes binding to VCAM-1 on cortical thymic epithelial cells (Salomon *et al.*, 1997; Kim *et al.*, 1998; Campbell *et al.*, 1999; Bleul and Boehm, 2000; Prockop *et al.*, 2002; Ara *et al.*, 2003; Plotkin *et al.*, 2003; Ki *et al.*, 2014). Once in the cortex, cells undergo T-cell receptor β rearrangement, and double-negative 3 (DN3) cells localize to the outer capsule for expansion following β-selection (Porritt *et al.*, 2003). The chemokine signals that drive localization to the subcapsule are incompletely understood, although CCR9 expression, which occurs first at the DN3 stage, may contribute to this stage (Campbell *et al.*, 1999; Bleul and Boehm, 2000; Wurbel *et al.*, 2006; Hu *et al.*, 2015). Following positive selection, thymocytes undergo rapid migration inward to the medulla, where CCR7 and CCR4 play critical roles in guiding thymocyte localization (Ueno *et al.*, 2004; Witt *et al.*, 2005; Choi *et al.*, 2008; Ehrlich *et al.*, 2009; Le Borgne *et al.*, 2009; Choi *et al.*, 2013; Cowan *et al.*, 2014). Once in the medulla, central tolerance is enforced by the interaction of single-positive thymocytes with antigen-presenting cells (APCs), including dendritic cells and medullary thymic epithelial cells, displaying self-antigens from peripheral tissues. The relatively brief amount of time that thymocytes spend in the medulla undergoing negative selection means that migration at this stage is rapid and optimized to facilitate efficient scanning of APCs. This rapid migration is promoted by CCR7, which binds CCL21, and requires Rap1 and Mst1 (also known as Stk4), which activates integrins to bind ICAM-1 and localizes LFA-1 to the leading edge (Amsen *et al.*, 2000; Ehrlich *et al.*, 2009; Hyun *et al.*, 2012; Ueda *et al.*, 2012). As such, negative selection is dependent on expression of integrins LFA-1, VLA-4, and VLA-5 (Utsumi *et al.*, 1991; Salomon *et al.*, 1994; Paessens *et al.*, 2008; Linhares-Lacerda *et al.*, 2010; Bose *et al.*, 2014; Gottrand *et al.*, 2015). Mature thymocytes then exit the thymus in response to sphingosine 1-phosphate (S1P) gradient signaling which is accompanied by down-regulation of VLA-5 (Allende *et al.*, 2004; Cotta-de-Almeida *et al.*, 2004; Matloubian *et al.*, 2004). Thymocyte development also includes selective constitutive activation of α4β1 integrin on double-positive T-cells which facilitates their binding to fibronectin and VCAM-1 and demonstrates that activation, in addition to expression, can generate specificity of integrin binding (Salomon *et al.*, 1994). While most of these findings have been reported from mouse models, the importance of MST1, which is the mammalian homologue of Drosophila Hippo, has also been demonstrated by the identification of patients with *MST1* deficiency. The clinical phenotype of these patients includes autoimmunity, presumably as a result of impaired negative selection, and patient lymphocytes have impaired binding to ICAM-1 under shear flow and deficient chemotaxis (Abdollahpour *et al.*, 2012; Crequer *et al.*, 2012; Nehme *et al.*, 2012; Halacli *et al.*, 2015; Dang *et al.*, 2016).

NK cell development from lymphocyte precursors can likely occur in multiple tissue microenvironments, including bone marrow, but the best defined of these is secondary lymphoid tissue (Freud *et al.*, 2005, 2006, 2017). While the continuum of NK cell development in tissue has been reviewed elsewhere (Di Vito *et al.*, 2019), human NK cell developmental intermediates can broadly be defined as stages 1–6, of which stages 1–4 predominantly found in tissue and stages 5–6 ($CD56^{dim}$ NK cells) represent the majority of cells in circulating peripheral blood. As $CD56^{dim}$ NK cells have been detected within efferent lymph vessels, it is suggested that NK precursors exit secondary lymphoid tissue to primarily circulate within peripheral blood at this stage of development (Romagnani *et al.*, 2007). This hypothesis is supported by the decreased expression of L-selectin on mature NK cells, which correlates with their decreased efficiency of adhesion to ligands on lymph node high endothelial venules relative to less mature NK cells (Frey *et al.*, 1998).

While the spatial localization of T-cells within environments that support their development has been well described, it is unclear how migration within a supportive tissue microenvironment supports NK cell development, and the spatial localization of NK cell developmental intermediates within human lymphoid tissue has not been well mapped. Of relevance to human NK cell development, peripheral blood NK cells express α4β7, α4β1 (VLA-4), and α5β1 (VLA-5), which mediate their adhesion to fibronectin and VCAM-1 (Gismondi *et al.*, 1991; Perez-Villar *et al.*, 1996). They additionally express α6β1 (VLA-6), which binds laminin and may be playing a role in interactions in developmentally supportive microenvironments (Santoni *et al.*, 1991). β2 integrins, including αLβ2 (LFA-1) and αMβ2 (Mac-1), are highly important for NK cell function through their mediation of adhesion to target cells and contribution to NK cell activation (Bryceson *et al.*, 2009; Urlaub *et al.*, 2017). However, the seemingly normal numbers and subsets of NK cells in β2 integrin-deficient LAD-1 patients in the presence of highly deregulated target cell killing demonstrate that β2 integrins are less important for NK cell development than they are for function (Kohl *et al.*, 1984; Kuijpers *et al.*, 1997; Castriconi *et al.*, 2007). Finally, other nonintegrin cell adhesion molecules likely play critical roles in migration and NK cell development, including the glycoprotein CD44, as its deletion in mice leads to impaired NK cell terminal maturation (Delfino *et al.*, 1994). As with T-cells, there are most certainly as yet undefined changes in expression of adhesion and migration-related receptors that dictate NK cell development. This likely includes discrete changes in the expression of integrins and adhesion receptors and/or their baseline affinity that accompanies the transition from tissue residency to a circulating, shear flow environment following NK cell terminal maturation, although this has not been defined with high resolution. These likely also reflect a response to microenvironment, and despite linear models of NK cell development being widely accepted, recent studies highlighting the plasticity of ILC lineages have highlighted the responsiveness of these cells to environmental cues.

The role of adhesion and migration during T and NK cell development in vitro

Given the demonstrated importance of complex signaling from the microenvironment in the maintenance, differentiation, and homing of both HSCs and lymphoid precursors, how can these signals be recapitulated in vitro to guide stem cell fate and lymphocyte development? Coculture systems that support the maintenance and retention of long-term reconstitution potential of HSCs on stromal cells of human, primate, and mouse bone marrow or mouse fetal liver origin have been well-described (Paul *et al.*, 1991;

Moore *et al.*, 1997; Punzel *et al.*, 1999; Nolta *et al.*, 2002; Punzel *et al.*, 2002; Oostendorp *et al.*, 2005; Hutton *et al.*, 2006; Weisel *et al.*, 2006; Kokkaliaris *et al.*, 2016). Similar systems are also used to selectively promote the generation of NK cells or T-cells from $CD34^+$ precursors by coculture with developmentally supportive stromal cells in concert with cytokine treatment (Miller *et al.*, 1994, 1999; Briard *et al.*, 2002; Schmitt and Zuniga-Pflucker, 2002; De Smedt *et al.*, 2004; Schmitt *et al.*, 2004; La Motte-Mohs *et al.*, 2005; Woll *et al.*, 2005; de Pooter *et al.*, 2006; Grzywacz *et al.*, 2006; McCullar *et al.*, 2008; Beck *et al.*, 2009; Herrera *et al.*, 2017; Zhao *et al.*, 2018).

The generation of mature T or NK cells is most efficient when cells have direct contact with stroma, although the contact-dependent signals between human immune precursors and stromal cells, which are frequently of murine fetal liver or bone marrow origin, have not been well defined. Certain conserved ligands are key determinants of lineage differentiation, and expression of Notch ligands delta-like 1 (DL1) or delta-like 4 (DL4) on OP9 stromal cells facilitates the generation of mature NK cells or T-cells from $CD34^+$ precursors, whereas OP9 stroma in the absence of these ligands is less supportive of NK cell development and not permissive of T-cell development (Kodama *et al.*, 1994; Schmitt and Zuniga-Pflucker, 2002; La Motte-Mohs *et al.*, 2005; Freud *et al.*, 2006; Bachanova *et al.*, 2009; Beck *et al.*, 2009; Haraguchi *et al.*, 2009; Felices *et al.*, 2014). Whether integrin-mediated adhesion to stromal cells contributes to the supportive contribution of stromal cells is not known; however, the presence of cell matrix components on stromal cell lines suggests these interactions play a role in promoting differentiation. There is strong evidence that suggests that interplay between adhesion molecules and other signals is required for the optimal generation of mature cells in these systems. Expression of the β1 integrin ligand VCAM-1 on OP9-DL4 cells or its presentation via adsorption to a solid surface significantly enhances cell migration and Notch pathway activation and synergistically increases production of T-cell progenitors in vitro when compared with Notch ligands alone (Shukla *et al.*, 2017). While β1 and β7 integrins on T-cell precursors can bind an array of ligands, including fibronectin, binding to VCAM-1 is specifically sufficient when combined with DL4 to generate T-cell developmental intermediates at a frequency comparable to OP9-DL4 stroma. Collectively these data indicate that synergy between integrin and Notch ligands, which also leads to enhanced cell migration, is a key driver of in vitro differentiation of T-cells and defines the importance of adhesion and integrin-mediated signaling in amplifying Notch signaling in this process. While it would not be predicted that selectins are relevant in a two-dimensional static in vitro culture, L-selectin is induced on NK cell developmental intermediates generated in vitro, and this induction is dependent on contact with stroma (Bachanova *et al.*, 2009; Mace *et al.*, 2016). This may be a result of Notch signaling, as incubation on OP9-DL1 but not OP9 induces up-regulation of L-selectin on naïve T-cells (Kondo *et al.*, 2017). Surprisingly, however, neutralizing antibody to L-selectin reduces spontaneous migration of NK cells on stroma, suggesting that there may be a role for selectins in mediating migration, even in the absence of shear flow in a two-dimensional coculture system (Mace *et al.*, 2016).

Finally, the generation of mature NK cells from embryonic stem cells and induced pluripotent stem cells represents an additional model to study the requirements for stroma and adhesion in differentiation. Human pluripotent stem cell systems have traditionally been a two-step approach in which pluripotent stem cells are differentiated first into $CD34^+$ hematopoietic precursors and then mature NK cells following secondary culture on murine-derived stroma (Woll *et al.*, 2005). This system has since been modified such that embryoid body formation includes the generation of $CD34^+$ hematopoietic precursors and $CD34^+CD31^+$ endothelial cells and $CD34^+CD73^+$ mesenchymal stromal cells that support NK cell development in a single continuous culture (Knorr *et al.*, 2013). In addition to CD31 and CD73, these autologous stroma express MHC class I molecules, and as such this is an exciting model to test the role that stromal cells play in NK cell differentiation and the role that cell migration plays in the spatial localization of both stroma and precursors in this system.

Migratory phenotypes throughout T-cell and NK cell development

Lymphocyte migration within the tissue microenvironment is amoeboid and rapid (>5 μm/min) and includes seemingly integrin-independent migration in confined three-dimensional environments (Friedl *et al.*, 1998; Lammermann *et al.*, 2008; Krummel *et al.*, 2014). Within lymphatic organs the linear model of chemokines activating integrins and inducing adhesion, as is found under shear flow in circulation, is not maintained and instead chemokine and integrin signaling act independently (Hons *et al.*, 2018). While many of the chemokine receptors that mediate rolling and extravasation from circulation are also functional within tissue, such as CCR7, in lymphoid organs CCR7 ligation triggers cortical actin flow, and integrins couple this actin flow to substrate through friction (Hons *et al.*, 2018). As such, even the same players that regulate migration can function very differently in different environments, namely, confined three-dimensional environs and under high shear flow in circulation.

Two-photon imaging of murine T-cells in vivo has illustrated their dependence on stromal architecture and chemokines for migratory behaviors that include diffusive Brownian random walks, super-diffusive Levy walks, subdiffusive pauses, and, in some instances, ballistic migration (reviewed in Krummel *et al.*, 2016). Murine NK cells at steady state in lymph node are highly motile yet more confined than $CD8^+$ T-cells and undergo rapid, transient interactions with local dendritic cells (Beuneu *et al.*, 2009). Despite this dependence on extrinsic factors, however, these migratory phenotypes can also be cell intrinsic, as suggested by the maintenance of T-cell speed and arrest patterns when imaged ex vivo (reviewed in Mrass *et al.*, 2010). Similarly, the acquisition of stage-specific, complex migratory phenotypes by human NK cells generated in vitro suggests that these patterns of motility accompany a program of differentiation that includes regulated acquisition of determinants of migratory capacity (Mace *et al.*, 2016; Lee and Mace, 2017).

Hematopoietic precursors isolated from peripheral blood and cultured on stromal cells exhibit predominantly constrained motion initially but acquire motility throughout development in a two-dimensional EL08.1D2 coculture model of NK cell differentiation (Mace *et al.*, 2016; Lee and Mace, 2017). In particular, T-cell development is reliant on migratory search strategies that optimize activation and differentiation in complex microenvironments. Two-photon microscopy studies in human thymic slices demonstrate that $CD4^+$ and $CD8^+$ single-positive T-cells have greater motility than less mature double-negative and double-positive cells (Halkias *et al.*, 2013). Additionally, thymocytes undergoing negative selection with cognate antigen undergo cell arrest, calcium flux, and demonstrate a highly constrained mode of migration compared with progenitors undergoing positive selection (Melichar *et al.*, 2013). In this way, thymocyte motility supports T-cell development by allowing cells to tune the duration and number of TCR signals that they receive during selection.

During in vitro NK cell development, hematopoietic precursors seeded on developmentally supportive stromal cells attain greater motility and transition from a predominantly constrained migration phenotype to a highly heterogenous phenotype consisting of cells switching among constrained, random, and directed migration as they progress through NK developmental stages (Mace *et al.*, 2016; Lee and Mace, 2017). Similar to thymocytes, early NK progenitors may adopt a confined mode of migration to tune their receptor signaling with stroma in the developmental niche; however, the nature of these signals in the context of NK cell development is not defined. The final stages of NK cell maturation are also marked by changes in migratory phenotype, as highly cytotoxic $CD56^{dim}$ NK cells incubated on stromal cells exhibit greater mean velocities than the weakly cytotoxic $CD56^{bright}$ NK cells, and educated $NKG2A^{+}$ NK cells have greater motility and serial killing capability than their non-educated $NKG2A^{-}$ counterparts (Forslund *et al.*, 2015; Mace *et al.*, 2016).

The role of cell polarity in T-cell and NK cell development in vitro and in vivo

An additional element of lymphocyte differentiation is the establishment and maintenance of cell polarity, which in lymphocytes can be generated by migration or the formation of the immunological synapse. While lymphocytes were once thought to lose cell polarity while undergoing cell division during differentiation, the emergent paradigm of asymmetric cell division (ACD) in T-cell development and activation has defined a role for maintenance of polarity through cell–cell contact during mitosis leading to divergent cell fates (Chang *et al.*, 2007; Russell, 2008; Arsenio *et al.*, 2015). Formation of an immunological synapse between a T-cell and an APC during T-cell activation leads to T-cell polarization and subsequent ACD in which the proximal daughter, on inheriting greater density of T-cell receptors, metabolic components, and transcription factors, becomes the effector cell and the distal daughter becomes the memory cell (Chang *et al.*, 2007; Arsenio *et al.*, 2015). Given the integrin-mediated adhesion at the immunological synapse between T-cells and APCs, ACD in T-cell activation includes an asymmetric distribution of LFA-1 following contact with APCs which is maintained through cell division and leads to daughter cells with unequal LFA-1 expression. $LFA\text{-}1^{high}$ daughter cells have increased retention in lymph nodes, greater stability of T-cell-APC conjugates, and differential effector functions compared with $LFA\text{-}1^{low}$ daughter cells (Capece *et al.*, 2017). This study demonstrated that unequal inheritance of cell adhesion and migration receptors can contribute to the divergent T-cell fates generated by ACD in addition to similar partitioning of signaling, metabolic, and transcriptional regulators.

ACD has additionally been demonstrated to play a critical role in T-cell development, as OP9-DL1 stroma, which support T-cell development in vitro, generate asymmetry through polarization of Notch and CXCR4 leading to asymmetric distribution of Notch, Numb, and α-Adaptin in daughter cells (Pham *et al.*, 2015; Charnley *et al.*, 2019). Thymocyte polarity is regulated by the Scribble polarity complex, a conserved regulator of ACD (Yu *et al.*, 2006). The incubation of $Scribble^{-/-}$ thymocytes on OP9-DL1 led to reduced ACD and, notably, reduced cellular expansion and differentiation, particularly in progression through the DN3 stage of development (Pham *et al.*, 2015). Further studies in vitro and in intact thymus demonstrated that establishment of polarity is via the formation of a bona fide immunological synapse with stromal cells and that Notch1 and CXCR4 function to regulate the formation of this synapse, with Notch1 acting as an active driver of polarity through orientation of α-Adaptin and Numb (Allam *et al.*, 2019; Charnley *et al.*, 2019). Interestingly, in the context of the studies showing that the presence of VCAM-1 can enhance Notch signaling (Shukla *et al.*, 2017), functionalization of surfaces with both VCAM-1 and Notch1 reduced the polarization of DN3a cells, although downstream differentiation was not affected (Charnley *et al.*, 2019). Taken together, these findings are impactful as they ascribe conserved mechanisms of regulation of ACD by the Scribble complex to lymphocytes. Importantly, they also suggest a role for regulation of access to the niche, in the form of CXCR4 signaling and stromal cell interactions, as key determinants of downstream thymocyte fate through immune synapse formation.

The elegant use of the OP9-DL1 system to describe T-cell ACD during development provides a compelling model to dissect the orchestration of fate determination. It is interesting in this context to consider how contact with Notch ligand-expressing stromal cells may be differentially affecting commitment to T- and NK cell fates. Both T-cells and NK cells undergo spontaneous migration on stromal cells, which in the case of T-cells is punctuated by arrest, establishment of polarity through an immunological synapse, and ACD leading to fate specification (Pham *et al.*, 2015; Charnley *et al.*, 2019). Despite the demonstrated importance of Notch signaling for the generation of NK cells in vitro (Bachanova *et al.*, 2009; Beck *et al.*, 2009; Haraguchi *et al.*, 2009; Felices *et al.*, 2014), a similar paradigm of ACD for NK cell development has not been found. Similarly, the role of CXCR4 in regulation of NK cell development in vitro has not been described, although it is required for generation of mature NK cells in mice (Noda *et al.*, 2011). While ACD has not been detected in NK cell development or function, NK cells do form a synapse with EL08.1D2 stromal cells that is characterized by enrichment of L-selectin and CD56 and is a site of signaling through calcium flux and tyrosine phosphorylation (Mace *et al.*, 2016). Given its structure and the enrichment of uropod-associated proteins including CD43 and moesin, it seems that this synapse is uropod-derived and can act as a tether to stroma through high-affinity adhesions that promote differentiation and survival. The significance of these observations, made in a two-dimensional culture system, on in vivo NK cell development is not well-defined; however, this structure is highly reminiscent of chemokine-mediated, LFA-1-dependent T-cell tethering that precedes antigen-specific immune synapse formation (Friedman *et al.*, 2006). This distinctive phenotype includes the apparent prevention of migration through high-affinity adhesion at the uropodal tether, which is accompanied by leading edge ruffling and pseudopodial protrusions. In T-cells, this tethering primes the cell for activation by increasing sensitivity to antigen on contact with an APC, on which the uropod is released, and an immunological synapse is formed (Friedman *et al.*, 2006).

Ultimately, whether there are truly differential roles for Notch and CXCR4-driven polarity or ACD in T-cells as opposed to NK cells, or whether a failure to detect ACD is a technical feature of studying NK cells, is unknown. More carefully delineating the contribution of conserved regulators of differentiation to adhesion, migration, polarization, and differentiation will no doubt shed light on important determinants of development in innate and adaptive immunity.

Challenges and future perspectives

An ongoing challenge in the field continues to be understanding the tissue-specific signals that govern transition from hematopoietic precursor to mature immune cell in a complex environment, particularly in humans. Even within model organisms, imaging single-cell dynamics with minimal perturbation at sufficient spatial and temporal resolution has limited our ability to visualize and subsequently define many of the cell biological mechanisms that govern lineage decisions, particularly in innate immune cells. Technological

advances that will likely drive new discoveries include advances in optical microscopy that enable better visualization within tissue, such as selective plane illumination microscopy, tissue clearing, and stochastic optical reconstruction microscopy (Abe *et al.*, 2016; Hu *et al.*, 2016; Li *et al.*, 2017). While imaging mass cytometry, multiplexed ion beam imaging, and histo-cytometry have been available for some time (Gerner *et al.*, 2012; Angelo *et al.*, 2014; Giesen *et al.*, 2014), these advances in spatial single-cell profiling, especially those linked to transcriptional information, are driving new insights into cell identity and interactions in tissue (Moffitt *et al.*, 2016; Xia *et al.*, 2019). These advances will be required to answer key outstanding questions that remain in the field. From an overarching perspective, the greatest challenge remains in linking single-cell behavior and identity with complex microenvironments and understanding how cell dynamics are shaped by these parameters. Other, more specific challenges lie particularly in understanding the role of adhesion and migration in NK cell development. These questions include better defining the nature and phenotype of tissue-seeding NK cell (or ILC) precursors and determining the adhesion and chemokine signals that are required for this seeding. Finally, more comprehensive understanding of the contact-dependent signals that shape T- and NK cell development in vitro will provide important insights into the best way to generate mature cells using in vitro systems, which have important implications for immunotherapy and cell-based therapeutics.

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