Natural killer (NK) cells are innate immune cells that reside within tissue and circulate in peripheral blood. As such, they interact with a variety of complex microenvironments, yet how NK cells engage with these varied microenvironments is not well documented. The integrin adhesome represents a molecular network of defined and predicted integrin-mediated signaling interactions. Here, we define the integrin adhesome expression profile of NK cells from tonsil, peripheral blood and those derived from hematopoietic precursors through stromal cell coculture systems. We report that the site of cell isolation and NK cell developmental stage dictate differences in expression of adhesome associated genes and proteins. Furthermore, we define differences in cortical actin content associated with differential expression of actin regulating proteins, suggesting that differences in cortical actin homeostasis. Together, these data provide new understanding into the diversity of human NK cell populations and how they engage with their microenvironment.

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Introduction

Human natural killer (NK) cells are commonly defined as CD56+CD3− cytotoxic innate lymphocytes and they play a critical role in identifying and killing virally infected or malignant cells. The importance of NK cells in the control of viral infections is underscored by the clinical course of patients with NK cell deficiencies who experience severe and often life-threatening viral infections (1–3). In addition to circulating NK cells found in peripheral blood, tissue resident NK cell populations are present in organs including liver, lung, spleen, bone marrow, and secondary lymphoid tissue, where they serve unique cytotoxic and regulatory functions (4, 5).

NK cell maturation is marked by the progressive gain of NK cell-associated receptors and functions, and NK cell developmental subsets can be defined as stages 1-6, which are stages of maturation that represent unique cell phenotypes and lineage potentials (6–12). The stage 4 NK cell subset can be further delineated to stages 4A and 4B by expression of NKp80 at stage 4B (13). The predominant NK cell subsets in peripheral blood are stages 4B, 5 and 6, often defined as CD56bright, CD56dim and terminally mature CD56dim respectively, however circulating NK cell and innate lymphoid cell precursors are also found at low frequencies (7–9, 13, 14). CD34+ NK progenitor cells are thought to enter circulation from bone marrow and subsequently seed peripheral sites to continue NK maturation; functionally mature NK cells then re-enter circulation in peripheral blood as stage 4B or stage 5 effectors (7). As such, stage 5 cells predominate in circulation, express perforin and granzymes at baseline, and are considered poised for cytolytic function following recruitment to sites of infection or inflammation. Tissue resident NK cells are generally considered to be stage 4 (CD56bright) NK cells, yet have distinct phenotypes from peripheral blood stage 4 cells and are thought to perform more regulatory functions (4, 10). The differential expression of chemokine receptors and adhesion molecules on NK cells in peripheral blood and tissue have been implicated in the mechanisms that mediate tissue localization and homing of distinct NK cell subsets (4, 5).

Integrins act as bidirectional signaling hubs between cellular machinery, namely the actin cytoskeleton, and the extracellular microenvironment. As such integrins play particularly critical roles in lymphocyte activation, immune synapse formation and B, T, and NK cell development (15–20). Integrin function is finely tuned and can be regulated by changes in expression, localization and affinity, and their specificity for ligand is dictated in part by the pairing of 18 alpha subunits and 8 beta subunits to form at least 24 unique non-covalently linked obligate heterodimers. NK cells express leukocyte-specific β2 integrins that mediate cell-cell interactions, as well as those that are more broadly expressed and facilitate cell-matrix interactions, including β1 and β7.
technical replicates and performed bulk RNA-Seq. Principal component analysis (PCA) using the 18,475 genes detected by RNA-Seq revealed that both tissue residency and developmental stage are determinants of unique gene expression profiles (Fig. 1A). This analysis revealed that the overall gene expression is more significantly affected by tissue specificity (PC1: 57% of variance) than the developmental stages (PC2: 12% of variance). As such, when phenotypically equivalent stage 4B and 5 cells from peripheral blood or tissue were compared, the two subsets from the same tissue clustered more closely than the two subsets of the same developmental stage (Fig. 1A, Supp. Fig. 2). To further understand the genes contributing to the separation observed between PB and tonsil NK cells, we plotted the 18,475 genes used for PCA by their PC1 loading weights (Fig. 1A). Genes associated with the generally immature phenotype of tonsil NK cells, such as GZMK, KLRC1, and IL7R, were primarily represented by negative weights in PC1, consistent with the clustering of tonsil subsets with negative PC1 values (Fig. 1B). In contrast, peripheral blood NK cells were marked by positive PC1 weights of genes related to NK cytotoxicity, activation, and KIR receptors (B3GAT1, KLRB1, KIR3DL2, KIR2DL1, and IFNGR1) (Fig. 1B). This distribution of gene sets across PC1 suggested that the differences between the transcriptome of tonsil and peripheral blood NK cell subsets also reflected differences in the predominant stages of NK cell maturation found at these sites. In addition, we found cytoskeleton-associated proteins were highly contributing to PC1, suggesting that these are among the genes significantly driving the differences between PB and tonsil NK cell subsets. Specifically, we noted integrins including ITGAD, ITGAE, and ITGA1 within the genes with the lowest PC1 weights (<0.01), while other integrins and actin regulator proteins, such as ITGB2, PXN and ARF1, were within the genes with the highest PC1 weights (>0.01, Fig. 1B). To better understand how integrins were differentially expressed between tissue sites and developmental subsets, we identified the distribution of 229 consensus adhesome genes that have been described previously (30–32) (Supp. Table 1) across PC1 weights (Fig. 1B). Adhesive genes were significantly enriched in the negative end of PC1 (22 adhesive genes in the lowest 5%; Fig. 1B). Further, GO pathway analysis identified pathways associated with cell migration, including regulation of T cell migration (p-value=8.86E-04), dendritic cell migration and positive regulation of cell migration, that were associated with genes with low PC loading weights (Supp. Table 2). These observations suggest that genes which are related to integrin-mediated adhesion and cytoskeletal remodeling are in part driving transcriptional differences between tonsil and PB NK cell subsets. The integrin adhesome network has been described primarily in non-lymphocyte cells (30–32) yet our observations of PC1 weights indicated that distinct patterns of adhesome gene expression are also important in NK cell subsets. To further understand how adhesome genes specifically drive NK cell heterogeneity, we performed PCA only with the 229 consensus adhesome genes (30). Similar to the whole transcriptome PCA (Fig. 1A), PC1 separated tonsil NK cells from PB NK cells, whereas PC2 revealed a tonsil specific progression of adhesome gene expression, suggest-
Distinct patterns of adhesome gene expression. To further define developmental and tissue residency signatures of tonsil and PB NK cell adhesome genes, we identified 5 clusters (clusters A-E) of adhesome genes with K mean clustering (Fig. 2A, B). Cluster A contains genes that are highly expressed in PB NK cells compared to tonsil NK cells. Cluster A includes β1 integrin partners ITGA4 (CD49d), ITGA5 (CD49e), the actin regulator RAC1, and PALLD, a component of actin microfilaments which functions as an actin stabilizer (Fig. 2B, C). Similar to cluster A, cluster B includes genes that are highly expressed in PB NK cells, but an important distinction is that cluster B genes are upregulated with maturation. Leukocyte-specific integrins including ITGAL, ITGAM, ITGB2, and calpain 2 (CAPN2), which functions in focal adhesion disassembly (34), are in this cluster. In addition, cluster B reveals that mature PB stage 5 and 6 NK cells upregulate actin and cytoskeletal regulatory pro-
teins like ABI3, ARF1, ARPC2, PXN and PI3KCA (Fig. 2B, C). Cluster C is comprised of genes that are transiently expressed between tonsil stages 4B and 5, and PB stage 5. This includes genes related to the regulation of the cytoskeleton such as PTK2, GAB1, CORO1A, and ITGAX. Together, genes in clusters A-C show that PB NK cells are characterized by upregulation of actin regulatory proteins and leukocyte associated integrins relative to their tonsil counterparts. Tonsil NK cell subsets were predominantly defined by their preferential expression of adhesome genes belonging to clusters D and E (Fig 2A, B). Cluster D distinguishes tonsil stage 3 and 4A NK cells from the more mature tonsil stage 4B and 5 as well as PB stages 4B-6 (Fig. 2A, B). The genes that constitute cluster D include ITGB7, signaling proteins PRKCA, PAK1, and ADAM12, a disintegrin and metallopeptidase involved in cell migration, proliferation and invasion, and LR1P1, which is predicted to regulate cell migration through Rho GTPases (35, 36) (Fig. 2B, C). Cluster E also reveals that tonsil NK cells preferentially express migration-associated signaling proteins RASAI, SRC, TIAM1, HSPB1 and VIM relative to PB NK cells (Fig. 2B, C). Finally, layilin (LAYN), which binds to talin and localizes to membrane ruffles, and neuropilin (NRP1), a transmembrane glycoprotein that has not been previously described to play a role in NK cell migration (37, 38) are also found in cluster E and are upregulated by tonsil NK cells relative to PB NK cells (Fig. 2B). Taken together, K means clustering of adhesome genes reveals the unique gene expression signatures in tonsil and PB NK cell subsets that are associated with both tissue residency and developmental stage.

Integrin protein expression and activation reflects tissue specificity of NK cell developmental subsets. Our transcriptomic data suggested that there were significant differences in the expression of integrins between NK developmental subsets and sites of isolation. To define the cell surface expression of CD11a/CD18 [LFA-1], CD11b/CD18 [Mac-1], CD49d/CD29 [VLA-4] and CD49e/CD29 [VLA-5] integrins that were differentially expressed between these populations, we performed flow cytometric analyses of single cell suspensions from tonsil and PB donors (see Supp. Fig. 4 for representative gating strategy and Supp. Tables 3, 4 for antibodies and subsets). Our analysis of leukocyte specific integrins revealed that PB stage 3-6 and tonsil stage 3-5 NK cells from all donors were found to have cell surface expression of all 52 integrins tested (Fig. 3 histograms), with varying degrees of relative expression detectable by measuring mean fluorescence intensity (MFI). Expression of CD11a (integrin αL) was found to be significantly higher in NK cell stages 4B and 5 relative to stage 3 in tonsil, and PB stages 4-6 had significantly higher CD11a MFI relative to stage 3 NK cells (Fig. 3A). Interestingly, our transcriptomic data suggests that ITGAL (integrin αL) is more highly expressed in
PB stage 5 but not stage 4 relative to tonsil (Fig. 2C), yet flow cytometric analysis revealed that CD11a was more highly expressed on the surface of both PB stage 4 and 5 NK cells relative to their counterparts in tonsil (Fig. 3A). Dissimilar to transcriptomic data, which shows increasing levels of integrin αM through PB NK cell maturation, analysis of PB NK cell surface expression of CD11b revealed that expression did not significantly vary between PB NK cell developmental subsets (Fig. 3B). Unlike in PB NK cell subsets, when we analyzed CD11b expression in tonsil NK cell subsets, we saw that tonsil NK cell subsets had a significant increase in the expression of CD11b between stage 5 and stage 3 NK cells (Fig. 3B). Expression of CD11c (integrin αX) did not significantly differ between tonsil NK cell stages (Fig. 3C), yet we observed a significant increase in CD11c between PB stages 3-4B (Fig. 3C), followed by a significant decrease in stage 5. Furthermore, CD103 (integrin αE) expression was consistent between developmental subsets in tonsil, while in PB, although this was not noted by transcriptomic analysis, CD103 was significantly upregulated between stages 3 and 6 as well as stages 4A and 6 (Fig. 3D). Lastly, we analyzed the expression of CD18 (integrin β2) and found that, in agreement with transcriptomic data, CD18 was significantly upregulated in more mature NK cell subsets stage 4B and 5 in tonsil and stage 5 and 6 in PB relative to stage 3 (Fig. 3E). Next, we sought to measure the cell surface expression of CD29 (integrin β1) and integrin β7 associated integrin subunits on phenotypically equivalent tonsil and PB NK cell subsets (Fig. 4). As suggested by our transcriptional data (Fig. 2C), PB NK cells do not significantly upregulate CD49a (integrin α1) (Fig. 4A). On the other hand, tonsil NK cell stages 4A-5 preferentially upregulated CD49a, which is associated with tissue residency, relative to tonsil stage 3 and PB counterparts (Fig. 4A). Both PB and tonsil NK cells did not variably express CD49d (integrin α4) through maturation, yet we found that PB NK cells stages 4 and 5 had increased expression of CD49d relative to their phenotypically equivalent counterpart in tonsil (Fig. 4B). CD49e (integrin α5), a β1 associated integrin receptor for fibronectin domains, is preferentially expressed by more immature stage 3 NK cells and is significantly downregulated through NK cell development in tonsil but to a lesser extent in PB (Fig. 4C). Moreover, both PB and tonsil NK cells downregulated CD29 as they matured from stage 3, albeit the downregulation of CD29 was greater in PB than in tonsil NK cells (Fig. 4D). These data, in parallel to our transcriptomic data, demonstrate that PB and tonsil NK cells differentially regulate cell surface expression of integrins both in a developmental and tissue residency dependent manner.
Integrin conformation on NK cell developmental subsets. Based on the differential expression of integrins between NK cell subsets from peripheral blood and tonsil, we wanted to understand how NK cells from these respective tissues regulate integrin activation, which has implicit effects on cell behavior, shape, and state (30–32). We included mAb24 and HUTS-4 antibodies for detection of open/extended conformation, or activated, CD11a-c/CD18 (LFA-1, MAC-1, integrin αX/β2), and CD49a-f/CD29 (VLA-1-6) respectively (39) (Fig. 5A, C). We found that tonsil NK cells had a greater frequency and MFI of cells with detectable open conformation of CD29 heterodimers when compared to analogous peripheral blood subsets, yet in PB we observe a significant increase in CD29 activation in stage 6 relative to more immature PB NK cells (Fig. 5A, B). We also observed an increased frequency of tonsil stage 4B NK cells with activated CD18 heterodimer compared to peripheral blood stage 4B NK cells (Fig. 5C, D). Taken together, our data suggest that NK cell subsets have differential expression and conformation of integrins on their cell surface, with unique integrin profiles that also reflect their tissue residency.

Expression of integrins on in vitro derived NK cells resembles in vivo patterns. In vitro differentiation of NK cells from CD34+ precursors has been well described as a method of studying human NK cell development (40, 41). While innate lymphocytes undergoing maturation in such systems are thought to progress through stages of differentiation similarly to those in situ (9, 12, 42, 43), the use of xenogenic stromal cells and exogenous cytokines in such systems make it difficult to define the role of the microenvironment in this process. Given the differences between analogous subsets of NK cells isolated from different tissues, we sought to define the expression of integrins on NK cells generated from in vitro differentiation. Primary human CD34+ cells from peripheral blood were isolated (Supp. Fig. 5) and cultured with EL08.1D2 or OP9 stromal cell lines in the presence of cytokines [FLT3L, SCF, IL-3, IL-7, IL-15] (40–43). Following 4 weeks of in vitro NK differentiation, stage 4 and 5 NK cells represented a significant proportion of the CD45+ population (Supp. Fig. 6, 7). We performed flow cytometry with our integrin panel described above after 4 weeks of differentiation on EL08.1D2 or OP9 stromal cells. At this timepoint the frequency of stage 1 and 2 NK cells significantly decreases relative to mature stages 3-5 which represent over 50% of the culture, thus only stage 3-5 NK cells were analyzed (Supp. Fig. 7). As with NK cells from tonsil and PB, CD18 (integrin β2) expression increased with maturation at stages 4 and 5, while CD29 (integrin β1) expression decreased (Fig. 6A). In addition, the MFIs of activated conformations followed a similar trend, showing an increase in open conformation CD18 heterodimers and a decrease in open conformation CD29 heterodimers through development (Fig. 6B, C). Notably, we did not observe a significant difference in integrin expression or conformational activation between in vitro derived NK cells that were generated by culture with OP9 or EL08.1D2 feeder cells. Finally, given the changes in integrin adhesome expression between developmental subsets, we sought to define the density of cellular actin in NK cells generated in vitro. We found that as NK cells matured, they exhibited a significant increase in density of F-actin content following cell permeabilization as measured by intracellular detection of phalloidin by flow cytometry (Fig. 6D). Together our results indicate that in vitro derived NK cells have conserved patterns of integrin expression when compared to ex vivo NK cells and exhibit differential F-actin content in a stage-specific manner.

Primary NK cell subsets have distinct cortical actin densities. Given that flow cytometric analysis of in vitro derived NK cells unexpectedly revealed increased intensity of actin in mature NK cells, we sought to investigate the nature of cortical actin in ex vivo NK cells. Components of the actin cortex include actin, myosin, and regulators of actin polymerization and turnover, including the Arp2/3 complex and formins. Pools of actin monomers provide substrate for the generation and maintenance of the filamentous (F-) actin cortex, as well as the leading edge and immune synapse. Non-muscle mammalian cells contain two structurally similar isoforms of actin monomers, g-actin and b-actin, encoded by the ACTG1 and ACTB genes respectively (44, 45). Given the ubiquitous nature of actin within all cells, it was unsurprising that we found that while there was a trend towards increased ACTB expression in more terminally mature NK cell subsets, there was no significant difference in expression of ACTB or ACTG1 between the isolated subsets of human NK cells in our bulk RNA-Seq dataset (Fig. 7A). To more deeply characterize the nature of filamentous actin in peripheral blood...
and tonsil NK cells performed intracellular detection of phalloidin by flow cytometry as we had previously done for in vitro-derived NK cells (Fig. 6). Strikingly, we found that there was a clear demarcation in actin content, with stage 5 NK cells having higher intensity of phalloidin detection than stage 4 cells (Fig. 7B). More extensive analysis of both tonsil and peripheral blood NK cell subsets showed that actin content was higher in NK cells from stages 5 and 6 in peripheral blood, whereas tonsil NK cell subsets had higher density of phalloidin staining in stage 6 NK cells (Fig. 7C). Given the differences we found by flow cytometry we sought to directly visualize and measure the cortical actin network in freshly isolated peripheral blood NK cells. We performed super-resolution structured illumination microscopy of actin by phalloidin detection in freshly isolated stage 4 and stage 5 cells from peripheral blood. Imaging confirmed our flow cytometry results and we observed a significantly greater density of cortical actin in more mature cells (Fig. 7D). As predicted, CD56 intensity measured by integrated density (MFI*area) was significantly greater in stage 4 CD56*bright NK cells than stage 5 CD56*dim NK cells (Fig. 7E). Similar measurements of actin further validated our observations as we found that integrated density of actin was significantly greater in stage 5 NK cells (Fig. 7F). While such measurements could be reflective of differences in cell size, we found that CD56*dim NK cells did not have a significantly greater volume than CD56*bright cells (Fig. 7G). Therefore, stage 5 NK cells have greater actin density relative to stage 4 cells and this is independent of cell size or activation. Finally, we sought underlying differences in gene expression that could drive the differences in actin density that we observed between NK cell developmental subsets. Further analysis of our RNA-Seq datasets identified key actin nucleation promoting factors and cytoskeletal regulators that were differentially expressed, including ARPC2 (the ARPC2 subunit of the Arp2/3 complex) and PFN1 (profilin) (Fig. 7H). Therefore, while there are a number of adhesome related genes that function in adhesion and migration that have different patterns of differential expression between tissue and peripheral blood, actin nucleating pathways seem to be specifically upregulated in terminally mature NK cells in peripheral blood. This is reflected by a greater density of cortical actin that is detectable in the absence of cellular activation and not linked to expression of actin monomers ACTB and ACTG1.

**Discussion**

Our data suggest that NK cells receive both intrinsic and extrinsic cues which dictate integrin adhesome expression. Tonsil NK cells are representative of secondary lymphoid tissue resident NK cells (8, 10), suggesting that the expression of adhesome components involved in migration and ECM interactions, such as collagen-specific integrins, are preferentially expressed in these tissues relative to peripheral blood. Conversely, peripheral blood NK cells upregulate integrin adhesome components that promote cellular integrity in an environment with constant shear flow and enable cells to bind endothelial cells lining blood vessels during extravasation (39). Previous studies have found that ITGAI, ITGA6, and ITGAD, which are all upregulated in tonsil NK cell populations relative to peripheral blood NK cells, are associated with lymphocyte tissue residency and migration (5, 21, 46, 47). ITGAE (CD103) is upregulated in tissue resident memory CD8^+^ T cells from lung and spleen, as well as in CD56*brigh^CD16^- NK cells from tissue sites such as lung, endometrium, nasal mucosa and intestine, thus providing support for our observations that the adhesome expression of tonsil NK cells includes that of previously characterized tissue resident lymphocytes (5, 23, 46, 47). While less well characterized than integrin αE, integrin αD is expressed on human NK cells and is associated with increased adhesion to ECM components, inside-out signaling-dependent cytokine secretion, and homing to inflammatory sites (48). Our ability to distinguish stage 4A and 4B NK cells and define the up-regulation of β2 integrins, such as ITGAL (CD11a), ITGAM (CD11b), and ITGB2 (CD18), as stage 4A NK cells mature into stage 4B NK cells in tonsil allowed us to further define differences in adhesome profiles that occur at this stage of maturation associated with commitment to NK cell maturation (13). Based on these observations, and the higher expression of cytoskeletal signaling and regulator genes including focal adhesion kinase (PTK2), calpain 2 (CAPN2), and TIAM1, we show that tonsil NK cells have a unique adhesome profile that is defined by higher expression of both integrins and the cytoskeletal machinery that mediates their interactions with tissue.

Our data demonstrate that the largest changes in adhesome gene expression in both peripheral blood and tonsil occur early in NK cell development, and we found many adhesome genes differentially expressed between peripheral blood stages 4B and 5, while none were differentially ex-
pressed between tonsil stages 4B and 5. This suggests that there are still unknown differences between representative stage 4B and 5 NK subsets in tonsil and peripheral blood that go beyond the differences in expression of NK markers commonly used to discriminate developmental subsets between tissues. Still, our observations are in line with previous studies showing that tonsil- and lymph node-resident stage 5 NK cells resemble a more immature-like stage 4B NK cell, with increased expression of CD56 and decreased expression of cytotoxic function-related machinery, whereas stage 4B and stage 5 NK cell subsets in peripheral blood are unique both transcriptionally and phenotypically (4, 5).

When considering these differences between NK cells isolated from different sites, we sought to better define how in vitro derived cells align with primary cells. In vitro studies have demonstrated a functional requirement for integrins, specifically VLA-4 (α4β1), in facilitating T cell precursor interactions with OP9-DL1 stroma and priming double negative T cells to receive Notch signaling (49). When we consider integrin β1 and integrin β2 expression, we observed that, similar to primary human NK cells, in vitro-derived NK cells have changes in integrin expression between stages 3
and 4 of development. Specifically, NK cells transition from having relatively high integrin β1 in early stages of in vitro NK cell development to having low integrin β1 and high integrin β2 expression during stages 4-5. The transition between high expression of integrin β1 to integrin β2 suggests that the upregulation of integrin β2 is a key feature of NK maturation in vitro and occurs in concert with the acquisition of CD94 and downregulation of CD117 (c-kit). The similarities in the patterns of changes of integrin β1 and integrin β2 density that we observe between in situ- and in vitro-derived NK cells suggest that relative density of integrin expression may be an intrinsically programmed feature of NK cell maturation. In contrast to the expression of total integrin subunits, we found that the relative frequencies of cells with activated conformation of CD18 and CD29 heterodimers differed between in vitro and in situ-derived cells. Measuring the amount of activated integrin on the surface of NK cells allowed us to understand differences in the regulation of these subunits between tissue sites and demonstrated that integrin activation is dependent on both the microenvironment and developmental stage of NK cells. Factors that could contribute to the observed differences in integrin activation between in vitro and in situ-derived cells include differences in ECM components and the exogenous use of cytokines, particularly IL-15 (50).

Our finding that NK cells at later stages of development, namely stage 5 in peripheral blood and stages 4 and 5 in vitro, had increased density of cortical actin was surprising. While we did see a slight increase in the expression of the actin monomer ACTB, the difference in cortical actin density as measured by both flow cytometry and super-resolution microscopy was orders of magnitude greater in stage 5 (CD56dim) NK cells than stage 4 cells (CD56bright). Further, it should be noted that we did not activate the cells with integrin or activating receptor ligation. As such, the differences we observed were that of the cortical actin meshwork, not activation-induced actin remodeling. In contrast with other cell surface receptors such as L-selectin and CD94 that appear to have intermediate expression as cells progress from stage 4 to stage 5 (11, 51), we found a sharp demarcation in phalloidin intensity between CD56bright and CD56dim cells. Shear flow, such as that found in circulation, induces rapid lymphocyte morphological changes prior to tissue extravasation, suggesting that the CD56dim NK cells found primarily in circulating peripheral blood may have increased cortical density in response to shear stresses or actin polymerization induced by tethering or transendothelial migration. However, given that the CD56bright (stage 4) population isolated from peripheral blood had uniformly lower phalloidin staining, it seems unlikely that this is the case unless CD56dim NK cells have uniquely undergone transendothelial migration. The increased expression of actin nucleating proteins, including actin-related protein 2/3 complex subunit 2 and profilin, in stage 5 peripheral blood cells suggests that the higher expression of these correlates with this higher actin density, and ARPC2 is a known regulator of cortical actin thickness (52). More detailed investigations into actin dynamics and architecture in primary NK cells will be necessary to link the phenotypic differences that we observed with functional outcomes.

While here we have been guided by the consensus integrin adhesome, what remains to be defined is the spatial information regarding the formation of integrin adhesion complexes and signaling islands. Studies of the integrin adhesome are generated by proteomic data and further probed by high- and super-resolution microscopy that provides critical spatial information about how such complexes are formed and function (28–30, 32, 53, 54). An additional caveat of our approach is that both flow cytometry and gene expression data consider only discrete integrin subunits, yet their function and ligand specificity must be considered in the context of their obligate heterodimeric structure. However, by defining the expression of adhesome components at the gene expression level, and validating some of these by protein expression, we lay the foundation for future studies that will better define the role of integrins through multi-scale approaches.

**Methods**

**Primary NK cell isolation from peripheral blood and tonsil samples.** All human tissues used in the RNA-seq experiments were collected under a protocol approved by The Ohio State University Institutional Review Board. Human pediatric tonsils were obtained fresh through the Cooperative Human Tissue Network (CHTN) from Nationwide Children’s Hospital (Columbus, OH), and peripheral blood was obtained through the American Red Cross as previously described (13, 33). Single cell suspensions were enriched for NK lineage cells using a bivalent antibody RosetteSep (StemCell Technologies)-based method (55), and then the resultant enriched NK cell fractions were labeled with antibodies (see Supp. Table 3 for a complete list of flow antibodies) and finally sorted to purity using a BD FACS Aria II cell sorter. Purities were validated post-sort and all samples had purity greater than 99%. Human tonsil NK cell subsets were defined and sorted as follows: stage 3 (Lin−CD117+CD94−NKp80−CD16−), stage 4a (Lin−CD94−NKp80−CD16+), stage 4b (Lin−CD94+NKp80+CD16−), stage 5 (Lin−NKp80+CD16+); human blood NK cell subsets were defined and sorted as follows: stage 4b (Lin−NKp80+CD16−CD57−), stage 5 (Lin−NKp80+CD16+CD57−), stage 6 (Lin−NKp80+CD16+CD57+). For these sorting experiments, Lin = CD3, CD14, CD19, CD20, CD34.

For flow cytometry, whole blood was obtained by venipuncture from healthy donors or as discarded apheresis product from patients undergoing routine red blood cell exchange at Columbia University Medical Center. Alternatively, we acquired RBC-low Leukocyte-enriched Buffy Coats from the New York Blood Bank as an alternative source of primary NK cells for flow cytometric analysis. Primary NK cell subsets from blood were enriched with NK cell RosetteSep (StemCell Technologies). Blood was layered onto Ficoll-Paque density gradient followed by centrifugation at 2,000 rpm for 20 mins (no brake). NK cells were collected from the density...
gradient interface and washed with PBS by centrifugation at 1,200 rpm for 7 mins. NK cells were resuspended in PBS 10% FCS and counted, then either resuspended in PBS for flow cytometry or cryopreserved in fetal calf serum with 10% DMSO at a concentration of 1-2.5 x 10^6 cells per ml. Tonsil samples for dissociation and flow cytometry analysis were acquired from routine tonsillectomies performed on pediatric patients at Columbia University Irving Medical Center. Tissue samples were placed in a sterile dish with PBS and manually dissociated by mincing into a cell suspension. The cell suspension was then passed through a 40 µm filter to obtain a single cell suspension and washed with PBS by centrifugation at 1,200 rpm for 7 mins. Cells were either resuspended in PBS for flow cytometry or FCS 10% DMSO freezing media at a concentration of 5-10 x 10^6 cells and cryopreserved prior to use. CD34+ precursors for in vitro experiments were isolated from whole blood obtained by venipuncture from two healthy donors. Mononuclear blood cells were isolated from donors were incubated with anti-CD34 antibody (Supp. Table 3) prior to cell sorting. CD34+ cells were isolated by FACS sorting on a BD Aria II cytometer with an 85 µm nozzle at 45 p.s.i. Sorted cells were confirmed to be >90% CD34+ and were cultured directly after isolation on previously irradiated EL08.1D2 or OP9 cells as described below. Primary cells, apheresis and tonsils were obtained in accordance with the Declaration of Helsinki with the written and informed consent of all participants under the guidance of the Institutional Review Boards of Ohio State University and Columbia University.

Peripheral blood and tonsil NK cell bulk RNA sequencing and analysis. Freshly sorted blood and tonsil NK cells were pelleted, and total RNA was isolated using the Qiagen RNeasy Mini Kit (Qiagen, Active Motif). Directional poly-A RNA sequencing libraries were prepared and sequenced as 42-bp paired-end reads on an Illumina NextSeq 500 instrument (Illumina) to a depth of 33.2 – 48.0 x 10^6 read pairs (Active Motif). Alignment to human genome (hg19 build) was done using TopHat. Transcriptome assembly and analysis was performed using Cufflinks and expression was reported as FPKM. Fragments per kilobase per transcript per million mapped read (FPKM) gene expression data were obtained from pre-filtered and normalized bulk RNA sequencing raw data and imported to iDEP 0.9 (56). FPKM bulk RNA seq data (Supp. Table 5) was processed using the source code available on iDEP 0.9 (56) following the recommended parameters. Pathway analysis was performed on iDEP 0.9 transformed RNA seq data using PGSEA ranking and KEGG pathways (56, 57), filtering on pathways with 15-2000 genes and FDR cutoff of 0.2. Prism 8.0 (GraphPad Software) was used to visualize data.

In vitro NK cell differentiation. EL08.1D2 cells were a gift from Dr. Jeffrey Miller (University of Minnesota) and were cultured as previously described (58) in culture flasks pre-treated with 0.1% gelatin. Cells were maintained at 32°C in 40.5 z-MEM (Life Technologies), 50% Myelocult M5300 (StemCell Technologies), 7.5 heat-inactivated fetal calf serum (Atlanta Biologicals) with β-mercaptoethanol (10⁻⁵ M), Glutamax (Life Technologies, 2 mM), penicillin/streptomycin (Life Technologies, 100 U ml⁻¹), and hydrocortisone (Sigma, 10⁻⁶ M), supplemented with 20% conditioned media. OP9 cells (ATCC) were cultured in nongelatinized culture flasks at 37°C in alpha minimal essential media with 20% heat inactivated FBS and 1% Penicillin/Streptomycin (Life Technologies, 100 U ml⁻¹). Prior to in vitro NK cell differentiation, 10⁴ EL08.1D2 cells were seeded into 96-well flat bottom plates pre-coated with 0.1% gelatin, while OP9 cells were similarly seeded into nongelatinized 96-well flat bottom plates. Cells were grown to confluence then subjected to mitotic inactivation by irradiation at 30 Gy. Following FACS sorting, CD34+ cells were cultured in NK cell differentiation media containing Ham F12 media plus DMEM (1:2) with 20% human AB serum, ethanalamine (50 µM), ascorbic acid (20 mg ml⁻¹), sodium selenite (5 µg ml⁻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). CD34+ cells were seeded onto irradiated EL08.1D2 or OP9 at a density of 2 x 10^5 cells per well and incubated at 37°C with weekly half media exchanges.

Primary and in vitro derived NK cell flow cytometric analysis. Flow cytometry to quantify integrin expression was performed using antibodies as described in Supplemental Table 3 and Fig. 5. Cryopreserved primary NK cells were thawed and resuspended in RPMI 10% FCS then immunostained at the concentrations indicated. For intracellular phallolidin staining cells were first incubated with antibodies for surface receptors, fixed and permeabilized using CytoFix/Cytoperm (BD Biosciences), then incubated with directly conjugated phallolidin. Data were acquired on a Bio-Rad ZE5 Cell Analyzer then exported to FlowJo 10 (BD Biosciences) for analysis. Integrin subunit MFI of primary tonsil and peripheral blood NK cell subsets were used to directly compare integrin expression of populations of cells collected on the same day. For flow cytometry of in vitro derived NK cells, cells were isolated at weekly time points and immunostained (Supplemental Table 3). NK developmental subsets were identified and analyzed using the gating strategy described in supplemental figures 4, 5, 6 and 7. Data were plotted and statistical analysis was performed using Prism 8.0 (GraphPad Software).

Microscopy and image analysis. For structured illumination microscopy primary NK cells were enriched from peripheral blood of healthy human donors using RosetteSep (Stemcell Technologies). Freshly isolated cells were pre incubated with anti-CD56 Alexa Fluor 647 (clone HCD56, Biolegend, 1:100) for 20 minutes in a conical tube then incubated on #1.5 coverslips that had been pre-coated with poly-L-lysine for an additional 20 minutes at 37°C 5% CO2. Following incubation, cells were fixed on the coverslip with
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Supplemental Figure 1: Frequency of CD57+CD16+CD56<sup>dim</sup> stage 6 NK cells and other developmental subsets in tonsil and PB. A) Representative dot plots of stage 5 and 6 cell counts in PB and tonsil samples, gated on CD45<sup>+</sup>CD3<sup>+</sup>CD19<sup>+</sup>CD14<sup>-</sup>CD117<sup>-</sup>CD94<sup>+</sup>/CD56<sup>dim</sup>/CD16<sup>+</sup>. B) Frequency of NK stage 6 subsets within CD56<sup>dim</sup>/CD16<sup>+</sup> NK cells were calculated from 5 Tonsil and 4 PB donors. Means were compared using Mann-Whitney test P value=0.0159.
Supplemental Figure 2: NK cell gene expression is dependent on developmental stage and tissue residency. A) Hierarchical heatmap of top 2,000 variably expressed genes of peripheral blood and tonsil NK cell subsets, not filtered on adhesome genes. B) Schematic of hierarchical clustering of peripheral blood and tonsil NK cell subsets based on total gene expression. C) Correlation matrix of peripheral blood and tonsil NK cells based on top 2,000 variably expressed genes ranked by standard deviation.
Supplemental Figure 3: Comparison of peripheral blood and tonsil NK cell gene expression reveals that adhesome related genes are variably expressed between tissue sites. A) Differential gene expression analysis of peripheral blood and tonsil NK cells highlighting genes with ≥2-fold cutoff and FDR ≥0.1 (red = up in peripheral blood, blue = down in peripheral blood) B) GO Biological Process Pathway analysis of top differentially expressed genes in peripheral blood relative to tonsil NK cells. Green arrows indicate pathways related to adhesome genes.
Supplemental Figure 4: Representative flow cytometric gating strategy for analyzing peripheral blood and tonsil NK cell subsets. A) peripheral blood NK cells. B) Tonsil NK cell subsets. Numbers indicated NK cell development stages isolated with each gate.
Supplemental Figure 5: Representative flow cytometric gating strategy for sorting CD34 positive cells from NK cell enriched peripheral blood samples. A) Representative unstained enriched NK cell progenitors from peripheral blood mononuclear cells used to set FACS gating for CD34+ cell sorting. B) FACS sorting of CD34+ cells from PE anti-CD34 stained NK enriched peripheral blood mononuclear cells. C-D) Representative flow cytometry of enriched NK cells and FACS sorted CD34+ cells used for in vitro NK cell differentiation experiments.
Supplemental Figure 6: Representative flow cytometric gating strategy for discriminating in vitro NK cell developmental subsets after weeks of culture with either (A) EL08.1D2 or (B) OP9 feeder cells.
Supplemental Figure 7: Representative flow plots depicting maturation of in vitro NK cell populations after one and four weeks of culture. After 4 weeks of culture in vitro NK cells become (A) CD94 and (B) perforin positive.
### Adhesome genes used for bulk RNA Seq analysis

| Gene1 | Gene2 | Gene3 | Gene4 | Gene5 | Gene6 |
|-------|-------|-------|-------|-------|-------|
| AB11  | DLC1  | ITGB1BP1 | PEA1K | SMPX  |       |
| AB12  | DNM2  | ITGB2  | PFN1  | SORBS1|       |
| AB13  | DOCK1 | ITGB3  | PIK3CA| SORBS2|       |
| ABL1  | ELMO1 | ITGB3BP | PIP5K1C| SORBS3|       |
| ACTB  | ENAH  | ITGB4  | PKD1  | SOS1  |       |
| ACTN1 | ENG   | ITGB5  | PLAUR | SPTLC1|       |
| ADAM12| EZR   | ITGB6  | PLCG1 | SRC   |       |
| AGAP2 | FABP3 | ITGB7  | PLD1  | SSH1  |       |
| AJUBA | FBLM1 | ITGB8  | PLEC  | STARD13|      |
| AKT1  | FERMT1| KCNH2  | PPFA1 | STAT3 |       |
| ANKR28 | FERMT2| KEAP1  | PPML1 | SVIL  |       |
| ARF1  | FERMT3| KTN1   | PPMLM | SYK   |       |
| ARGAP24| FHL2  | LASP1  | PP2CA | SYNM  |       |
| ARGAP26| FLNA  | LAYN   | PRKACA| TENC1 |       |
| ARGAP32| FYN   | LDB3   | PRKCA | TES   |       |
| ARGAP5 | GAB1  | LIMK1  | PRNP  | TESK1 |       |
| ARGH12 | GIT1  | LMS1   | PTEN  | TGFBI1|       |
| ARGH2  | GIT2  | LMS2   | PTK2  | THY1  |       |
| ARGHE6  | GNB2L1| LPP    | PTKB2 | TIA1  |       |
| ARGHE7  | GRB2  | LPXN   | PTPN1 | TLN1  |       |
| ARPC2  | GRB7  | LRPI   | PTPN11| TNS1  |       |
| ASAP2  | HAX1  | LYN    | PTPN12| TRIO  |       |
| ASAP3  | HRAS  | MACF1  | PTPN2 | TRIP6 |       |
| BCAR1  | HSPA2 | MAPK1  | PTPN6 | TRPM7 |       |
| BCARD3 | HSPB1 | MAPK8  | PTPRA | TSPAN1|       |
| CALR   | ILK   | MAPK8P3| PTRF  | TUBA1B|       |
| CAPN1  | ILKAP | MARCKS | PTRPH | VASP  |       |
| CAPN2  | INPP5D| MMP14  | PTTPRO| VAV1  |       |
| CAPS8  | INPPL1| MSN    | PVR   | VAV2  |       |
| CASS4  | INSR  | MYH9   | PXN   | VAV3  |       |
| CAV1   | IRS1  | MYOM1  | RAC1  | VCL   |       |
| CBL1   | ITGA1 | NCAM1  | RAPGEF1| VIM   |       |
| CD151  | ITGA10| NCK2   | RASA1 | ZFYVE21|      |
| CD47   | ITGA11| NDEL1  | RAVER1| ZYX   |       |
| CEACAM1| ITGA2 | NEDD9  | RDX   |       |       |
| CFL1   | ITGA28| NEXN   | RHOA  |       |       |
| CIB1   | ITGA3 | NF2    | RNF185|       |       |
| CIB2   | ITGA4 | NISCH  | RNF5  |       |       |
| CIB2   | ITGA5 | NRP1   | ROC1K |       |       |
| CORO1A | ITGA6 | NRP2   | SDC4  |       |       |
| CORO1B | ITGA7 | NUDT16L1| SDCBP|       |       |
| CORO2A | ITGA9 | OSTF1  | SH2B1 |       |       |
| CRK    | ITGAD | PABPC1 | SH3KBP1|       |       |
| CRKL   | ITGAE | PAK1   | SHARPIN|       |       |
| CSK    | ITGAL | PALLD  | SHC1  |       |       |
| CSR1P  | ITGAM | PARVA  | SIRPA |       |       |
| CTNNBP2NL| ITGAV| PARVB  | SLC16A3|       |       |
| CYTH2  | ITGAX | PDE4DIP| SLC3A2|       |       |
| DEF6   | ITGB1 | PDPK1  | SLC9A1|       |       |

**Supplemental Table 1:** Adhesome gene list for RNA sequencing analysis of peripheral blood and tonsil NK cells. Genes were identified from the consensus integrin adhesome (reference 28).

**Supplemental Table 2:** GO Pathway terms (see Excel sheet)
### Supplemental Table 3: Adhesome antibody panel for flow cytometric analysis and sorting of NK cells.

| Marker | Fluorophore | Identifier | Source             |
|--------|-------------|------------|--------------------|
| CD17   | BV711       | 313230     | Biolegend          |
| CD14   | BV421       | 325628     | Biolegend          |
| CD16   | PB          | 302021     | Biolegend          |
| CD16   | PE-CF594    | 562320     | BD Biosciences     |
| CD19   | BV421       | 302234     | Biolegend          |
| CD3    | BV421       | 344834     | Biolegend          |
| CD34   | AF700       | 343526     | Biolegend          |
| CD45   | BUV737      | 748719     | BD Biosciences     |
| CD56   | BV605       | 318334     | Biolegend          |
| CD57   | BV510       | 393313     | Biolegend          |
| CD94   | BUV395      | 743954     | BD Biosciences     |
| NKP80  | PE-Vio615   | REA845     | Miltenyi Biotec    |
| Activated CD29 | FITC | FCMA1389F (HUTS4) | Millipore          |
| CD11a/CD18 | APC | 363410 (mAb 24) | Biolegend          |
| CD11b  | APC-CY7     | 301342     | Biolegend          |
| CD11c  | BV650       | 301638     | Biolegend          |
| CD14   | Viogreen    | 130-096-875 | Miltenyi Biotec    |
| CD16   | AF700       | 557920     | BD Biosciences     |
| CD20   | Viogreen    | 130-096-904 | Miltenyi Biotec    |
| CD3    | Viogreen    | 130-097-582, 130-096-910 | Miltenyi Biotec |
| CD49d  | BV785       | 304314     | Biolegend          |
| CD49e  | APC         | 328012     | Biolegend          |
| CD56   | BV421       | 562751     | BD Biosciences     |
| CD57   | APC-H7      | 555618     | BD Biosciences     |
| CD94   | PerCP-Cy5.5 | 562361     | BD Biosciences     |
| Human CD45 | BV786 | 563716     | BD Biosciences     |
| KIR2D  | PE          | 130-092-888 | Miltenyi Biotec    |
| KIR3DL1/2 | PE       | 130-095-205 | Miltenyi Biotec    |
| NKp80  | APC         | 130-094-845 | Miltenyi Biotec    |
| Perforin | PE        | 308106     | Biolegend          |
| Phalloidin | AF568   | A12380     | Thermo Fisher      |
| Total CD18 | PE-Cy7 | 302109     | Biolegend          |
| Total CD29 | APC-Cy7 | 303008     | Biolegend          |
| Total CD29 | FITC    | 303016 (TS2/16) | Biolegend         |

### Supplemental Table 4: Gating strategy utilized for discriminating natural killer cell developmental subsets.

### Supplemental Table 5: Complete gene list (see Excel sheet)