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Understanding natural killer cell biology from a single cell perspective

Nikita Subedi [a,b], Liesbeth Petronella Verhagen [a,b], Esmée Michelle Bosman [a], Ilse van Roessel [a], Jurjen Tel [a,b,*]

[a] Laboratory of Immunoengineering, Dept. Biomedical Engineering, Eindhoven University of Technology, Eindhoven, Netherlands
[b] Institute for Complex Molecular Systems, Eindhoven University of Technology, Eindhoven, Netherlands

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ABSTRACT

During the last decade, advances in single cell technologies have ignited increased understanding of natural killer cells (NK cells), which turned out to be far more complex than originally thought. Ample studies have established tissue-specific phenotypic variation within this cell population; however, the functional implication of this vast variation is still unclear. At single-cell level, the function of a NK cell is tightly regulated by several checkpoints however upon proper recognition the cell can deliver a lytic hit as early as 10 min or could take hours before they can kill their target cells. Moreover, only a fraction of NK cells appears to kill target cells while the larger portion of NK cells appear to be non-cytotoxic. All these studies showed that the NK cell compartment is composed of cells with different functional strengths and efficacies, thereby highlighting the necessity of analytical platforms that allow the study of these important innate immune cells at single-cell level.

In this review, we discuss and provide an overview on phenotypical and functional heterogeneity within the NK cell population and subsequently provide information regarding emerging technologies that highlight the importance of single-cell studies to understand the biology of these cells.

1. Introduction

1.1. NK cell biology

In the early seventies, a subset of innate immune lymphocytes capable of inducing spontaneous yet selective cytotoxicity against cancer cells without pre-exposure was recognized and coined as Natural Killer (NK) cells [1,2]. These cells play an important role in anti-viral immunity and immune surveillance by identifying and eliminating transformed or infected cells through distinct recognition mechanisms [3]. NK cells are large granular lymphocytes derived from $CD34^{+}$ hematopoietic progenitor cells that originate in bone marrow, constitute around 10–15% of the total peripheral blood lymphocyte population and have been traditionally defined as $CD3^{-}$ and $CD56^{+}$cells [4].

According to the developmental model proposed by Freud and Caligiuri, the maturation of NK cells involves six distinct stages, starting with the Lin−CD34+CD133+CD244+ hematopoietic stem cells that differentiate into towards CD45RA+ lymphoid-primed multipotential progenitors in Stage 1, and into common lymphoid progenitors. Common lymphoid progenitors then form NK progenitor cells characterized by the loss of CD34 and expression of the surface marker LFA-1 leading to NK cell lineage commitment. Later these cells mature into the CD56bright NK cell subpopulation that subsequently differentiate into the CD56dim NK cell subpopulation. At this stage there is the expression of CD16 and KIRs and finally NK cells differentiate into adaptive NK cells.

Even though the linear model of development gives fundamental information regarding the development of NK cells, several lines of evidence also suggest a more branched model whereby different precursor populations may independently develop into distinct subsets of mature NK cells Fig. 1. One of the examples of such illustration is the experiment that showed that NK cells derived from CMPs and granulocytic monocytic precursors (GMPs) isolated from cord blood could efficiently differentiate into NK cells when cultured in presence of supporting cytokines and stroma. This finding challenged the belief that all NK cells are exclusively derived from CLPs [5]. In addition, in healthy adults, PB CD56- NK cells have been identified, which is an intermediate cell type progressing to CD56dim NK cells, supporting the idea that different NK cell development pathways exist. Also, NK cells, which phenotypically resemble CD56dim cells, have been identified however they lack inhibitory receptors such CD94/NKG2A and KIRs. It is currently unknown how these NK cells can originate from CD56bright NK cells [6].

* Corresponding author at: P.O. Box 513, 5600 MB Eindhoven, The Netherlands.
E-mail addresses: n.subedi@tue.nl (N. Subedi), e.p.varion.verhagen@tue.nl (L.P. Verhagen), e.m.bosman@student.tue.nl (E.M. Bosman), j.tel@tue.nl (J. Tel).

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Existence of these different conflicting theories suggests that there is a need for a more definitive understanding in the field [5,7,8].

Resolving these issues requires an in-depth investigation of NK cell distribution, maturation, function, and transcriptional profiles across different anatomical sites and compartments. Advancing single-cell technologies like RNA sequencing has revealed several tissue-specific patterns related to the development and maturation of multiple lineages. Based on the evidence gathered from the study, Dogra et al. have proposed a model for anatomic control of NK cell development and function that shows site-specific segregation of NK cell maintenance and differentiation [9]. Several other single cell-based studies investigating NK cell development have been discussed in later sections.

1.1.1. NK cell surface receptors and regulation

NK cells have the intrinsic ability to identify "self" to distinguish healthy from target cells (e.g., virus-infected cells or tumor cells). The concept of "dynamic equilibrium" suggests that the stimulation of NK cells and their effector function depends on integrating signals from both activating and inhibitory receptors [10–12]. Virtually all cells express MHC-I molecules (HLA molecules), which function as ligands for inhibitory receptors on the NK cell surface, contributing to self-tolerance. NK inhibitory receptors broadly fall into two subcategories— C-type lectin-like inhibitory receptors (e.g. NKG2A) and killer-cell immunoglobulin-like receptors (KIRs) [13,14]. Apart from MHC class I molecules, other different "self-signals" detected by NK cells, as other inhibitory receptors (e.g., sialic acid binding Siglec receptors) can also regulate cellular activation [3].

In contrast to healthy cells, virus-infected cells or tumor cells lack or have reduced MHC class I expression to avoid antigen-dependent recognition and therefore also have reduced inhibitory signals for NK cells. Simultaneously, activating receptor ligands, such as the Fas death receptor and MHC-I chain-related proteins, are upregulated upon diseased conditions [15]. This causes NK cell activation, leading to target cell elimination through cytotoxicity or secretion of pro-inflammatory cytokines [16]. Different Activation NK cell receptors include KIR family receptors, type II C-type lectin-like molecule (NKG2D), NKG2C heterodimeric receptors, the nectin/nectin-like binding receptors DNAM-1/CD226 and CD2-like receptor- activating cytotoxic cell and Nkp46, Nkp30, and Nkp44 are the superfamily of natural cytotoxicity receptors (NCRs) [17]. These receptors act together with different adaptor proteins to initiate NK cell activation however, combined signaling via inhibitory and activation receptors determine the final functional output [17].

Along with the above-mentioned receptors, NK cells also display surface expression of different cytokine receptors that play an integral role in NK cell regulation. NK cell maturation is dependent on the cytokine microenvironment generated by bidirectional crosstalk with other immune cells such as T cells, dendritic cells (DCs) and macrophages [3,18,19]. An array of different activation, regulatory as well as inhibitory receptors expressed on the NK cell surface and their involvement in regulating NK cells activity has been schematically presented in the Fig. 2.

1.2. Heterogeneity within the NK cell compartment

The phenotypical variation within the NK cell population is marked by the combination and frequency of expressed surface receptors. Traditionally, the NK cell population is classified into two distinct subsets based on their level of CD56 expression: $CD56^{bright}$, and $CD56^{dim}$ NK cells. The $CD56^{bright}$ population is more immunomodulatory and known to be the precursor of the more cytotoxic $CD56^{dim}$ population. The $CD56^{dim}$ population constitutes the largest fraction of peripheral blood conventional NK (cNK) cells while the $CD56^{bright}$ population is more dominantly present in tissues.

Even though this dichotomy is widely accepted, it fails to represent the complexity of NK cells at different maturation stages and cells residing at different anatomical locations. The recently discovered adaptive role of NK cells, along with tissue specific heterogeneity further enhances the complexity [20,21]. All these aspects do not fit the dichotomy dogma, and therefore studying NK cell diversity demands a fresh perspective (Fig. 3).

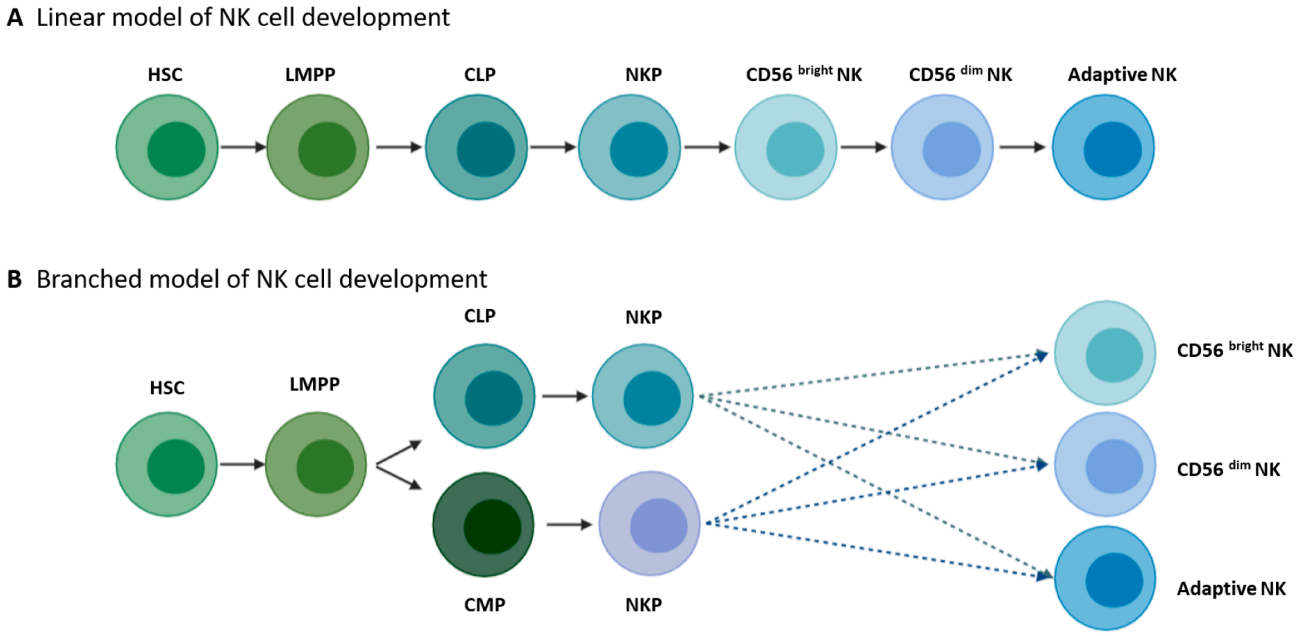

Fig. 1. The linear and branched model of NK cell development and maturation: (A) Linear model explains a direct route of NK cell development from hematopoietic stem cells to the NK cell progenitor sequentially developing into CD56 bright, dim and into adaptive NK cell. **(B)** Branched model suggest that the hematopoietic stem cells differentiate into lymphoid-primed multipotent progenitors (LMPP), which then differentiate toward common lymphoid (CLP) or myeloid progenitors (CMP). These progenitors give rise to individual NK cell progenitors (NKP) into specific NK cell subsets. Figure adapted from Cichocki et al. Front. Immunol. [5]. Figure created using BioRender (https://biorender.com/).

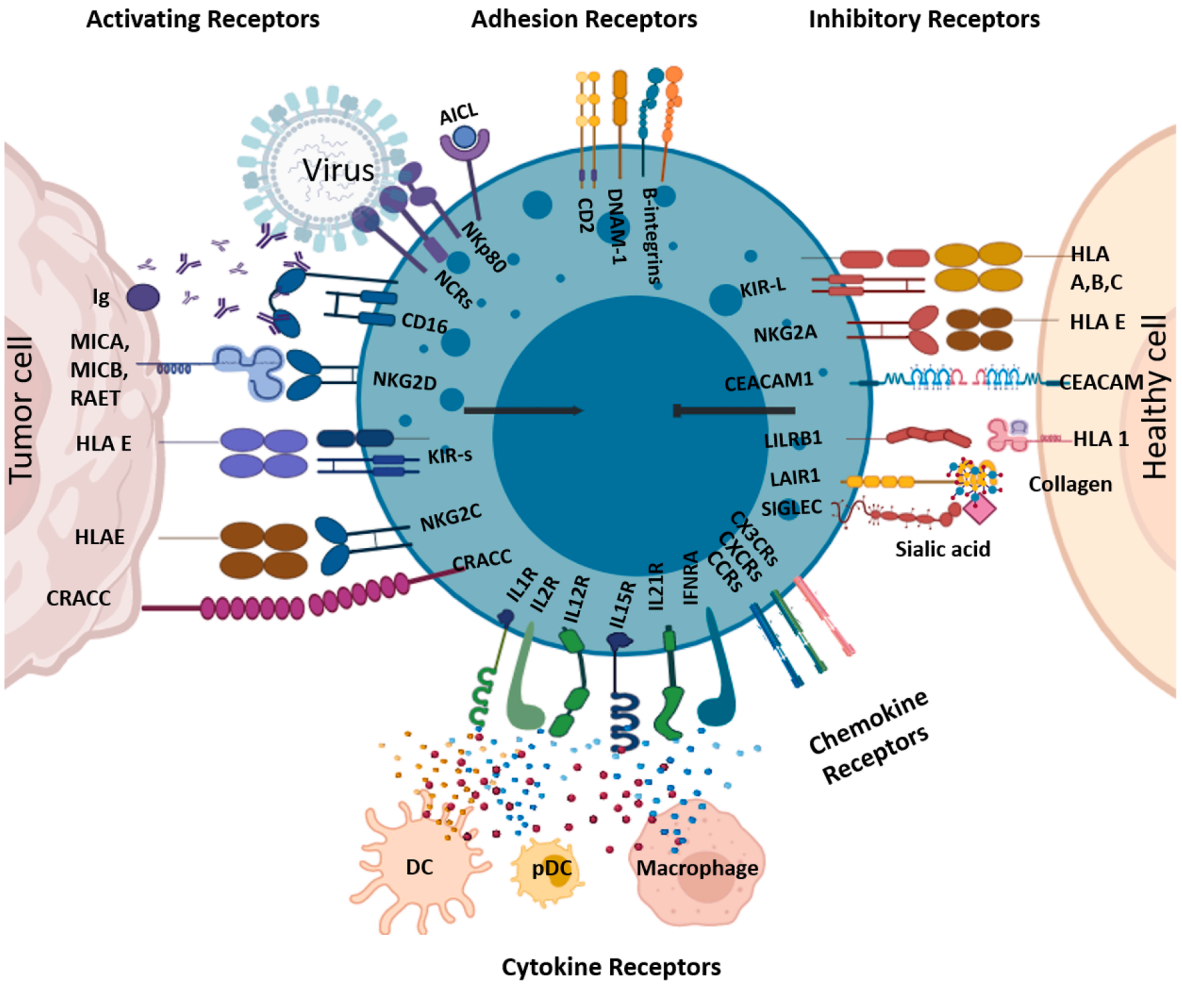

Fig. 2. Regulation of NK cell via its surface receptors: Functional regulation of NK cells involves synergistic signaling via combination of different receptors expressed within their cell surface. Different cell types provide ligands for these receptors that define whether the NK cell gets activated or not. Different activating and inhibitory receptors in Human NK cells and their corresponding ligands along with other chemokines, adhesion receptors and cytokine receptors which are altogether show in the figure. Figure created using BioRender (https://biorender.com/).

1.2.1. Tissue specific NK cell diversity

The ability of lymphocytes to migrate in interstitial tissues is essential for both innate and adaptive immune responses. NK cells play an important role in immune surveillance and instead of remaining confined to peripheral blood, they are widely distributed throughout the body. NK cells are found in healthy skin, gut, liver, lungs, and the uterus during pregnancy [22] along with secondary lymphoid organs. Additionally, human NK cells were also identified in other tissues such as the kidney [23], joints [24], and breast [25] under pathophysiological conditions. Depending on the site and stage of the maturation, NK cells can display different surface markers and functions. cNK cells are mainly found in peripheral blood, migrate to a specific location to exert their effects, and can be further classified as either $CD56^{bright}CD16^{-}$ or $CD56^{dim}CD16^{+}$ cell types.

Specialized tissue resident NK (trNK) cells are identified in various tissues in the human body and contain a higher fraction of $CD56^{bright}$ NK cells. Additionally, they express adhesion molecules, such as CD69, and chemokine receptors, including CCR5 and CXCR6 that preserve trNK cells in tissues and prevent them from entering the circulation. TrNK cells have several features comparable to $CD56^{bright}$ cNK cells and are mainly involved in indirect cytotoxicity. The trNK cells in the bone marrow, secondary lymphoid tissues and liver have an increased expression of the inhibitory receptor CD94/NKG2A and the receptor NKp46, but lower expression of CD94/NKG2C, KIRs and CD16 [26]. Several functional and phenotypic similarities between trNK cells and $CD56^{bright}$ cells suggest that trNK cells could be an immature cell type in transition to become cNK cells [27]. The topological organization of trNK cells within the microenvironment of different organs modulates their functional adaptations. The NK cells that localize in the blood, blood-rich sites such as BM, spleen, and lungs are largely CD56dimCD16+ cells with cytotoxic capacity while NK cells are present only at low frequencies in LNs, tonsils, and throughout the GI tract where they are predominantly CD56brightCD16 cells and show more regulatory functions. TrNK cells at those locations are mostly responsible for interaction with other cells types to maintain homeostasis and thus show more of regulatory function [27]. For example, NK cells at LN are responsible for T cell polarization, while uterine NK cells are mostly responsible for placental vascular remodelling [9]. Crinier et al. profiled the transcriptome of human spleen NK cells and compared them with peripheral blood NK cells. They identified four subsets of human spleen trNK cells of which two subsets lack any blood specific signatures and were therefore unique to spleen [28]. Moreover, three different NK cell subpopulations were discovered in human bone marrow. By pseudotime analysis, a subset of trNK cells (not found in blood) was found to be the precursor of both $CD56^{dim}$-like NK cells and $CD56^{bright}$-like NK cells [29]. These findings support the theory that NK cells enter the blood stream from the bone marrow and subsequently circulate to infiltrate multiple organs. In contrast, evidence also suggests that some trNK cells belong to a distinct lineage of peripheral blood cNK cells. Research showed that those trNK cells can survive in situ for a considerable

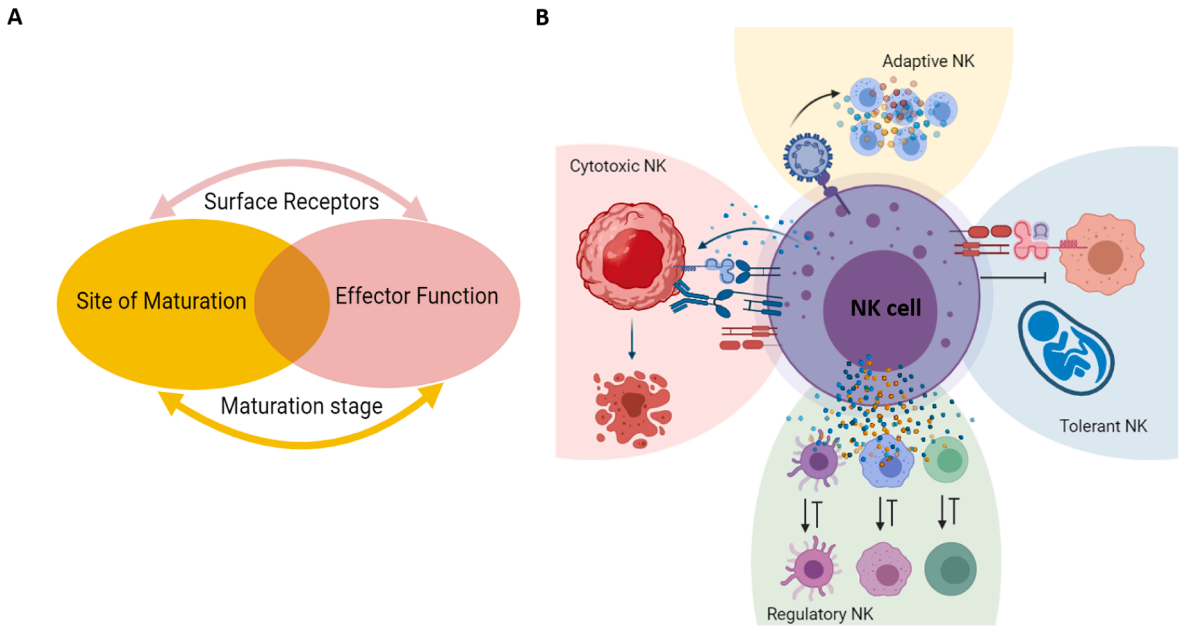

Fig. 3. The NK cell heterogeneity beyond the dichotomy: (A) The NK cell diversity can be categorically explained based on site of maturation and their functional ability. Both the groups can further be correlated via the surface receptors they express and the stage of maturation they are at. **(B)** Based on different functions NK cell display, they can be cytotoxic, regulatory, tolerant or adaptive in nature. Cytotoxic NK cell ($CD56^{dim}$ $CD16^{bright}$ phenotype) display cell lysis as the major function while regulatory sub population ($CD56^{bright}$ phenotypes) are more adept in secreting cytokines such as IFNγ and TNFα. Regulatory NK cells functions in modulating the functions of other immune cells such as DCs, macrophages and T cells. These cells help in quality assessment of DCs and T cells by lysing less mature cells. Tolerant NK cells are passive form of regulatory subsets that plays more important role in maintain the tolerance to the self. Tolerant NK cells are also known for their ability to induce vascular remodeling to provide physiological support to the fetal development. Adaptive NK cell displays a different phenotypical as well as functional characteristic to other NK cells. These cells are found more in chronic viral infections and upon vaccination. Adaptive NK cells are responsible for generating memory NK cells however they show less cytotoxic and secretory ability. Figure created using BioRender (https://biorender.com/).

amount of time, do not translocate to the blood, require different transcription factors (such as Hobit and Tbet) for their development and are presumably terminally differentiated [26]. For instance, some NK cells have different functional roles compared to what has been shown earlier. Profiling tonsil NK cells demonstrated that the majority of tonsil NKs are composed of a $CD56^{+}CD16^{-}$ population that displays contrastingly higher NK activating and cytotoxic transcriptome [30]. These contrasting functional properties shown by the $CD56^{bright}$ population suggest that trNKs possess an independent character that could have their own functional range.

Given their functional similarity and site of residence, ILC1s found in organs traditionally have been understood as trNK cells subtypes [31]. Despite sharing overlapping transcriptomic regions and a dependency towards IL15 for proliferation and stability, the developmental pathways for both the cell types are rather different [32]. NK cell develops strictly from NK cell progenitors that are restricted to generate NK cells but none of the other ILC subsets. Apart from CD200R, no other reliable markers have been identified that distinguish murine NK cells with ILCs [33]. A recent study allowing multi-tissue single-cell analysis deconstructed the complex programs of mouse natural killer and type 1 innate lymphoid cells in tissues to identify unique transcriptional programs underlying both the cell types [34]. The study classified ILC1 as the $Eomes^{-}$ tissue specific NK cells that expresses Zfp683 [34]. Single cell-based applications as such could serve as the molecular guide in resolving the debate also in human immune cells.

1.2.2. Functional NK cell diversity

The NK cell phenotype and maturation state has been integrally conflated with effector function and used as the basis for classifying NK cells into distinct subsets (Fig. 3B). The cells that have superior lytic abilities are known to be cytotoxic NK cells. They are phenotypically defined as $CD56^{dim}$ $CD16^{bright}$ subpopulation with larger expression of lytic granules, and KIRs and are thought to be more mature compared to other NK subsets [35]. The other less mature subsets display more regulatory functions via secretion of different cytokines. These subsets can be further subdivided as the regulatory and the tolerant population [36]. Both subsets are characterized by expression levels of CD56, NKG2A, CD2, CD62L and CCR7 molecules. However, these subsets vary in terms of functions as regulatory NK cells are more responsible for modulating the immune responses from other immune cells and are functionally similar to the trNK cells [36]. The role of the regulatory NK cells has been quite evident in the maturation of dendritic cells, direct activation of adaptive immune cells and killing of immature DCs and over-stimulated macrophages (Fig. 3B). Tolerant NK cells on the other hand show dominant inhibitory characteristics and aid in fetal development during pregnancy as they are mostly found in decidual region [36] (Fig. 3B).

Recently, it was discovered that a few percent of NK cells can develop immunological memory and differentiate into adaptive NK cells [20,37]. Adaptive NK cells were shown to proliferate and differentiate in reaction to a cytomegalovirus (CMV) infection upregulating HLA-E-specific activating receptor CD94/NKG2C [38]. Additionally, antigen-specific NK cell memory was also described in T and B cell deficient mice displaying hapten-specific contact hypersensitivity in skin cells after adoptive transfer of NK cells from a previously sensitized donor [39]. NK cells can undergo differentiation into memory-like effectors once exposed to various cytokines such as IL-12, IL-15, and IL-18 [40]. In the peripheral blood of CMV^{+} individuals, adaptive NK cells were identified and described as $CD56^{dim}CD16^{bright}$, have reduced levels of the surface marker NKp30 and NKp46, and displaying a highly differentiated surface signature, namely, $self^{-}KIR^{+}NKG2A^{-}LILRB1^{+}CD57^{+}Siglec7^{-}$ [41,42]. Unlike cNK cells, Adaptive NK cells are poor responders to IL-12 and IL-18 and show reduced expression of the NCRs. However, upon activation via CD16 and NKG2C, they show rapid proliferation, immense secretion of IFN-γ and enhanced ADCC [43,44]. Furthermore, these specialized subsets have reduced expression of promyelocytic

leukemia zinc finger while DNA methylenation similar to cytotoxic T cells [41,45]. Adaptive NK cells upon continuous activation or chronic viral infection increase expression of check point inhibitors like LAG-3, PD1 thus rendering them into hypofunctional state [46]. All these features make Adaptive NK cells phenotypically as well as functionally different than cNK cells [26].

1.2.3. How does heterogeneity arise

As outlined in the aforementioned sections, NK cells display a high level of diversity at single-cell level because of variegated expression of activating and inhibitory receptors, yet the developmental roots and functional consequences of this diversity remains unclear. The developmental model of NK cell origin explains that the diversity in the NK cell repertoire is the functional consequence of developmental stages during its maturation and is maintained by the expression of different surface molecules. A given phenotype for a NK cell subset is the combination of these receptors expressed stochastically within the cells which might further determine its functional capabilities. Studying different developmental phases, it appears as if a group of NK cells starts from a common progenitor cell, however during different developmental stages, they drift from one another due to several intrinsic (genetic) or extrinsic factors (environmental) they are exposed to [26].

1.2.3.1. Intrinsic factors that govern NK cell heterogeneity. Host genetics and epigenetics have one of the important roles in determining the fate of the NK cell diversity and are composed together as one of the "intrinsic factors" in this review. In 1997, the study by Parham et al. showed that each individual expresses a different subset of KIR and suggested that the polymorphic allelic distribution could be one of the important factors regulating the NK cell diversity [47]. More recently, the variation in expression pattern KIRs was correlated with the host genetics [48]. By performing a multiparameter mass cytometry of 28 NK cell panels to phenotype peripheral blood NK cells from 5 sets of monozygotic twins and 12 unrelated adults they showed the existence of more than 100,000 possible NK cell phenotypes and no single phenotype accounted for more than 7% of total NK cells [48]. Receptor patterns and expression levels in identical twins, showed less variability compared to non-related individuals, marking genetics to be one of the determining factors for the observed heterogeneity. Furthermore, the genetic differences within an interracial and interethnic population also greatly influenced the stochastic expression of different cell surface receptors. Different functionally evolved NK cell subsets such as activated NK cells with adaptive memory-like functions are highly linked to epigenetic reprogramming including alterations in DNA methylation [41,49]. Wiencke et al. has provided a model for adaptive NK cell diversification whereby engagement of DAP12-coupled activating receptors results in epigenetic imprinting of the ZBTB16 locus, resulting in loss of promyelocytic leukemia zinc finger protein expression [49]. Promyelocytic leukemia zinc finger protein expression deficient NK cells also display stochastic hypermethylation of promoters encoding signaling proteins SYK, EAT-2, DAB2, and FcεRγ, resulting in the diversification of the adaptive NK cell repertoire.

In addition to host genetics and epigenetics, another crucial intrinsic factor that governs cellular diversity is the involvement of different transcription factors [50–52]. Closer analysis of different organ-specific NK cell clusters showed discrete developmental stages at different points of differentiation. This study supported the notion that the developmental progression of NK cells goes from CD56bright to CD56dim NK cells and showed the existence of a "transitional" population with intermediate expression of CD56 or CD57 surface molecules that links the two phenotypes [53]. Similar developmental subsets consisting of mature, transitional, and terminal NK cell subsets were identified in murine bone marrow NK cells. Later it was identified that different developmental stages were regulated by different transcription factors thus leading to heterogeneity [54,55]. It is widely appreciated that cNK cells differ from trNK by a different set of transcription factors during differentiation. For example, the development of liver-resident NK cells is dependent on the transcription factors Hobit and *T*-bet, whereas differentiation of cNK cells requires expression of the transcription factors Eomes and only moderately *T*-bet [56,57].

All these studies show how host intrinsic factors play an important role in modulating variation within NK cells. Even though host genetics determines the expression pattern of different KIRs together with selective transcription translation and post-translational modifications, external factors such as cytokine signaling, cell–cell interactions, epigenetic modifications and immunological experience further affects the gene expression at multiple levels generating multiple layers of heterogeneity within NK cell compartment.

1.2.3.2. Extrinsic factors that govern NK cell heterogeneity. NK cell development is equally affected by cues from the environment wherein they reside. Examples of such extrinsic factors include, NK cell education, the expression level of MHC-I molecules and, furthermore, different environmental cues such as cytokines, growth factors and pathogenic interactions, that shape the NK cell development process.

The expression pattern and the affinity of different KIRs identify self-molecules during maturation play an important role in inducing the functional variation within the NK cell compartment. By the process of "education", these cells can tune the functional ability of cells in an MHC-dependent or independent way [58,59]. Thus, generated educated population can identify self from foreign and have acquired more adept effector function while some with non-reactive or lacking expression of inhibitory receptors remain hypofunctional [60]. Around 10–20% less educated or uneducated cells, that might be considered potentially harmful co-exist together with functionally superior NK cells [61,62]. These uneducated NK cells have a high threshold for activation, thus maintaining the self-tolerance in the body [61,63]. They are also thought to play an important role during inflammatory microenvironments, like viral and bacterial infections and certain tumor invasions [64,65]. The state of education could be reversed by the cytokines, thus providing the possibility of changing the functional fate of the NK cells [66]. Thus educated, uneducated, non-binding and weakly-binding NK cells play their part in maintaining host immunity [65]. Apart from the host's genetics, which can affect the stochastic expression of different inhibitory receptors on their surface, NK cell education also depends on the expression of HLA molecules which varies among the population. Thus, in the process of developing functionally competent NK cells, a new level of diversity is generated within and between individuals.

NK cells can sense different chemokines that direct them towards the site of infection or adapt a certain functionality in response to cytokines being secreted. The role of cytokines, such as IL-15, in inducing intralineage plasticity to maintain phenotypical and functional diversity within NK cell repertoires has been recently identified [67]. IL15 helps in controlling the cellular metabolism via activating the mammalian target of rapamycin (mTOR) and is essential for NK cell survival, development and proliferation [68]. The role of IL15 has also been studied in maintaining the functional plasticity therefore providing the opportunity to tune different functional state within NK cell repertoire. Either way, the physiological as well as the pathological environment shapes the phenotype of NK cells in such a way that they are capable to serve a particular function. NK cells that have attained full maturation and education, leave the site of generation, and take up residence at the peripheral tissues. At the tissue level, these cells are exposed to different tissue specific cytokines, growth factors and pathogens. This difference in exposure can generate different phenotypical variants that are opted to fulfill specific tasks. For example, decidual NK cells found in the uterus have a distinct phenotype compared to peripheral blood NK cells. These cells share common features to $CD56^{bright}$ cNK cells and secrete several chemokines like XCL1, CCL1 and angiogenic factors such as, vascular endothelial growth factor (VEGF), placental growth factor

(PLGF), Ang1, and Ang2 that are involved in vascular remodeling and therefore promote the fundamental physiological process of pregnancy [69,70].

NK cell diversity expands with immune experience, which means that the more cells are exposed to environmental stimuli, the more there is diversification. At birth, NK cells have very low diversity but have high capacity for a range of responses. This variation enhances with age and over the course of a lifetime exposure, the NK repertoire diversifies, hence, becoming functionally specialized and unique to an individual. Even though the NK cell repertoire stabilizes over time, their adaptability to respond against novel antigens decreases [71]. The high NK cell diversity was identified to be significantly associated with 2.5 folds higher risk of HIV-1 acquisition [71].

The tumor microenvironment (TME) can steer and affect NK cell heterogeneity. This variation impairs the effectiveness of NK cells by limiting their capacity to infiltrate tumors, thus dampening the cytolytic function of tumor-infiltrating NK cells. Additionally, the TME induces several immunosuppressive soluble factors such as TGFβ, prostaglandin E2 (PGE2), adenine, indoleamine 2,3-dioxygenase (IDO) and a range of ligands that inhibit their function, including KIRs, CD94/NKG2A, PD1, CTLA4, TIM3, TIGIT, CD96, KLRG-1, LAG3, and, recently discovered, IL-1R8 [72,73]. Another aspect contributing to the impaired NK cells function in the TME is the poor availability of nutrients, such as glucose and glutamine. Research showed that $CD56^{bright}$ NK cells require glycolysis to produce IFN-γ and promote their survival, while the lung TME favors more gluconeogenesis due to up-regulation of the fructose diphosphates enzyme [74]. This leads to inhibition of glycolysis and eventually leading to impaired NK cell viability.

Taken together, several studies have shown that heterogeneity is the function of intrinsic as well as extrinsic factors eventually shaping different stages within the development, thereby generating wide variations within cell types and resulting in the diverse NK cell populations (Fig. 4).

1.3. Probing heterogenous NK cell effector functions in the single cell era

The wide diversity displayed by NK cells accommodates its versatility, given that these cells do not undergo receptor recombination and rearrangement like other lymphocytes. Although efforts were made to attain an improved understanding of cellular heterogeneity and defining the high degree of phenotypic diversity among human NK cells, it remains largely understudied. Also, very little is known to what extent the vast phenotypical variation is correlated with the functional diversity. The field has long been hampered by the sole use of conventional population-based technologies, which cannot dissect this complex variation since it can only estimate the average distribution of responses [75]. For long, flow cytometry and microscopy-based techniques have been the gold standard for the analysis of cellular behavior. These tools have the ability to measure with single cell resolution, however, lack single cell manipulation and often involve activation of cells in bulk-assays, which ultimately leads to paracrine and juxtacrine signaling among cells [76]. Recent breakthroughs in single-technologies such as mass cytometry, single-cell RNA sequencing (scRNA-seq), and a combination of different microfluidics and micro systems-based studies, integrated with flow cytometry and microscopy, have allowed ventures to study, identify, and correlate the phenotypical characteristics with functional diversity. In the following section, we will discuss how single-cell technologies with their specific features (Table 1) have facilitated studying different NK cell functions to understand NK cell biology with a new perspective.

1.3.1. Single NK cell-mediated cytotoxicity

An efficient NK cell-mediated cytotoxic response is a function of balanced engagement of activation and inhibitory receptors on the cell surface and lytic pathway evoked by NK cells to induce target cell death. The largest fraction (~80%) of cNK cells is composed of cytotoxic ($CD56^{dim}$ $CD16^{bright}$)NK cells, however, studies showed that for healthy individuals, only a few percentages of the target cells are lysed at a 1:1 effector: target ratio [77–81]. Earlier studies using conventional methodologies had already established the variation in the killing ability within NK cells. However, these variations were thought to be projected because of nonuniform timings of interaction and stimulation, insufficient duration of interactions, or a different number of interacting partners [82–86]. Since the conventional assays relied on net lysis of target cells and expression of degranulation markers such as CD107a, they were not able to dissect the NK/target cell interaction besides providing the quantitative measure of the target cell death [82]. Therefore, several details of NK/Target cell interaction such as the lytic

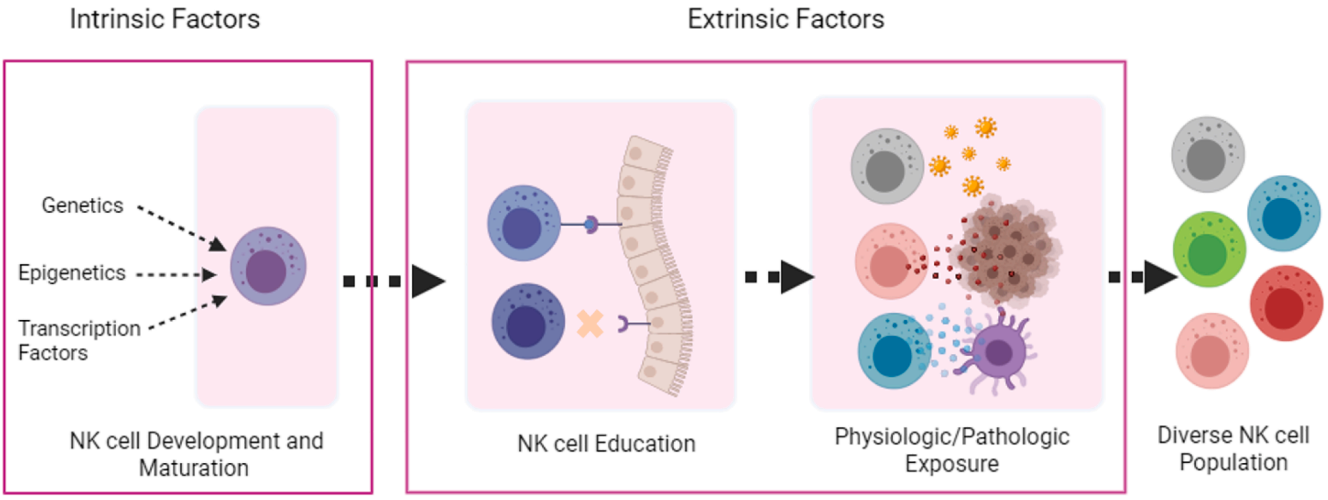

Fig. 4. Origin of Heterogeneity: The NK cell diversity is acquired at different levels during the overall life cycle of NK cells. These variations are already acquired during the developmental phase of NK cells where different intrinsic factors such as genetics, epigenetics and transcription factors play an important role. Furthermore, upon maturation, NK cells go through an education process that prevents the cells from self-harm. However, there are also the population that do not match the HLA proteins upregulated by "self" cells thus making them more responsive towards acute viral infections. The third level of variation are induced due to different environmental exposure such as tissue specific cytokines, cross talk with other immune cells, tumor infiltration and encounter with infected cell types. Upon all these exposures, a repertoire of NK cells with different functional capabilities and preferences are generated thus resulting to unexpectedly wide diversity within the cell population. Figure created using BioRender (https://biorender.com/).

Table 1
Table summarizing different single-cell analysis tools discussed in this review in terms of their specific features that allowed studying different aspects of NK cell biology [1–21,23–93,95–124].

NK cell study	Single cell tool	Features	References
NK cell development and maturation	Single RNA Sequencing	• Overal transcriptional profile that can identify the developemental and maturation clusters • Identifies roles of different transcritption factors responsible for variation	[9,27,28,29,30,34,107]
Migration and homing	Single RNA Sequencing	• Identify differnt organ specific signature that induces homing • Identifies different chemokine receptors upregulated by NK cells that are responsible to tissue specific trafficing	[53]
	Microwell based microsystem with microscopy	• Can mimic invivo microenvironment including ECM and collagen • 3D migratory profiles can be monitored of individual cells	[79,91,111,112,113]
	Microfluidic system	• Provision of stable chemical gradients to induce migration of NK cells	[106,110]
Cytotoxicity	Microwells with microscopy	• Facilitate Cell pairing • Real time monitoring of cells • Possibilities for cell retrieval	[78,79,91,92,87]
	Droplet based Microfluidicswith microscopy	• Facilitates cell compartmentalization • High throughput cell visualization in real time • Highly tunable cell pairing	[77,88,94,96,99,101]
	Hydrodynamic traps	• Facilitates cell pairing • Realtime monitoring	[82]
Immune-regulation	Microengraving in microwells with microscopy	• Real time monitoring of secretion and cell interaction • Washing steps can be included	[100]
	Droplet based Mircofluidics with FACS and microscopy	• Real time as well as end point measurement • High sensitivity due to low droplet volume • High throughput visualization	[101,103]
	Microfluidic system	• Facilitates cell pairing • Washing and multiple stimulation is possible	[80]
Memory	Single RNA Sequencing	• Identifies the trait for adaptive cells	[29,114,116]

potential of a single cell, independent participation of each NK cell, and the spatiotemporal parameters of interaction dynamics were missed, thus masking the true intrinsic cellular heterogeneity underlying the population [87]. Several groups have developed cytotoxicity assays at a single-cell level that allowed investigating these different aspects of cell-mediated cytotoxicity [76,88,89]. One of the important challenges for developing such platforms remains efficient cell pairing to facilitate NK cell interactions with their target cells in high throughput while continuously monitoring their dynamics in real-time.

Microfluidic platforms such as micro/nano wells, oil–water droplets and hydrodynamic traps facilitate the efficient cell pairing and enable studying difference in cytolytic behavior of these cells in the single cell resolution [77,89,90]. Microwell-based platforms provide a restricted space for NK cells for studying cellular interactions and thus monitor their contact dynamics with the target cells (Fig. 5A). Longer and frequent contacts ensured higher cytotoxicity in activated NK cells compared to resting ones [91]. Along with the contact duration, the variation was also observed in the speed with which NK cells lyse its target cell death with having a group of cells exerting effect within 10 min of interaction while the other group taking more than hours to lyse. This variation has been coupled to the quantity of lytic molecules delivered by individual NK cell, however, the mechanism involved governing the variation in the lytic content of each NK cells is still poorly understood [77,79].

Interestingly, around 6% of highly potent NK cells also known as serial killers could deliver multiple hits and are responsible for the majority of lysis within short span of time [79]. Studying the cytolytic behavior of a serial killer requires tracking an individual cell with multiple target cells, which is impractical with conventional techniques. Serial killing involves sequential activation of NK cells, where the individual cell dissociates with the lysed target cell and moves to another cell. Sequential activation of individual NK cells has been studied using microwells to associate the serial killing ability with the surface markers and granular content of each NK cell upon encountering a new target cell [92]. By studying these cells at single cell level, the role of CD16 in modulation of perforin secretion has become clear. Shedding of CD16 proved important for the detachment from opsonized targets and thus for enhancing NK cell survival and serial engagement of target cells.

Apart from the intrinsic cytotoxic potential, several studies focused on understanding the dynamics of NK cell interactions with target cells. Since tumor cells themselves can have a variegated expression of surface molecules, the response of NK cells towards an individual cancer cell can vary extremely [93]. Exploiting droplet-based microfluidics to pair NK cells with different patient tumor cells, Sarkar et al. showed that the tumor cells from two diffuse large B-cell lymphoma patients had a similar contact durations with NK cells while primary Burkitt lymphoma cells made longer contacts and were lysed at later times [88]. Studies as such could help in identifying the specificity of a single NK cell towards different types of target cells can thus have a great impact in tailoring the strategies for NK cell-based immunotherapy(Fig. 5B, C). Most of the droplet-based cytotoxicity studies that facilitate cellular interactions unfortunately lack in sufficient data points with desired cell pairing [88,94]. Since immune cells can be highly heterogeneous in their effector functions, throughput is vital to discover small and distinct subsets. To address this complex heterogeneity, we recently developed a cytotoxicity platform that allows the longitudinal visualization of around 20,000 cell containing droplets with different E:T ratios [77]. We showed that roughly 20% of single NK cells displayed cytotoxic behavior thus implying that the NK cells constitute cells with different strengths in executing effector functions and that roughly (Fig. 5B).

NK cells use various molecules to initiate a cytotoxic effect against infected or malignant cells. By utilizing CD16 receptors that opsonize antibody bound target cells, NK cell can induce cytotoxicity via antibody dependent cellular cytotoxicity (ADCC). Analysis of this effector function has vital clinical relevance in evaluating the efficacy of antibody-mediated immunotherapy. Although a few studies have emerged that allowed measuring ADCC at a single cell level, they are still limited to technical development while information regarding the functional aspect still awaits [95,96].

In summary, studying NK cells at single cell level can become beneficial in identification of subpopulation with distinct killing abilities and can be used for determining the specificity of NK cells against different target cells.

1.3.2. Regulatory function of a single NK cell

During an infection, the NK cells interact with different other immune cells, such as DCs, macrophages that secret IL12, and other NK cell-stimulating cytokines. The activated NK cells secrete different pro-

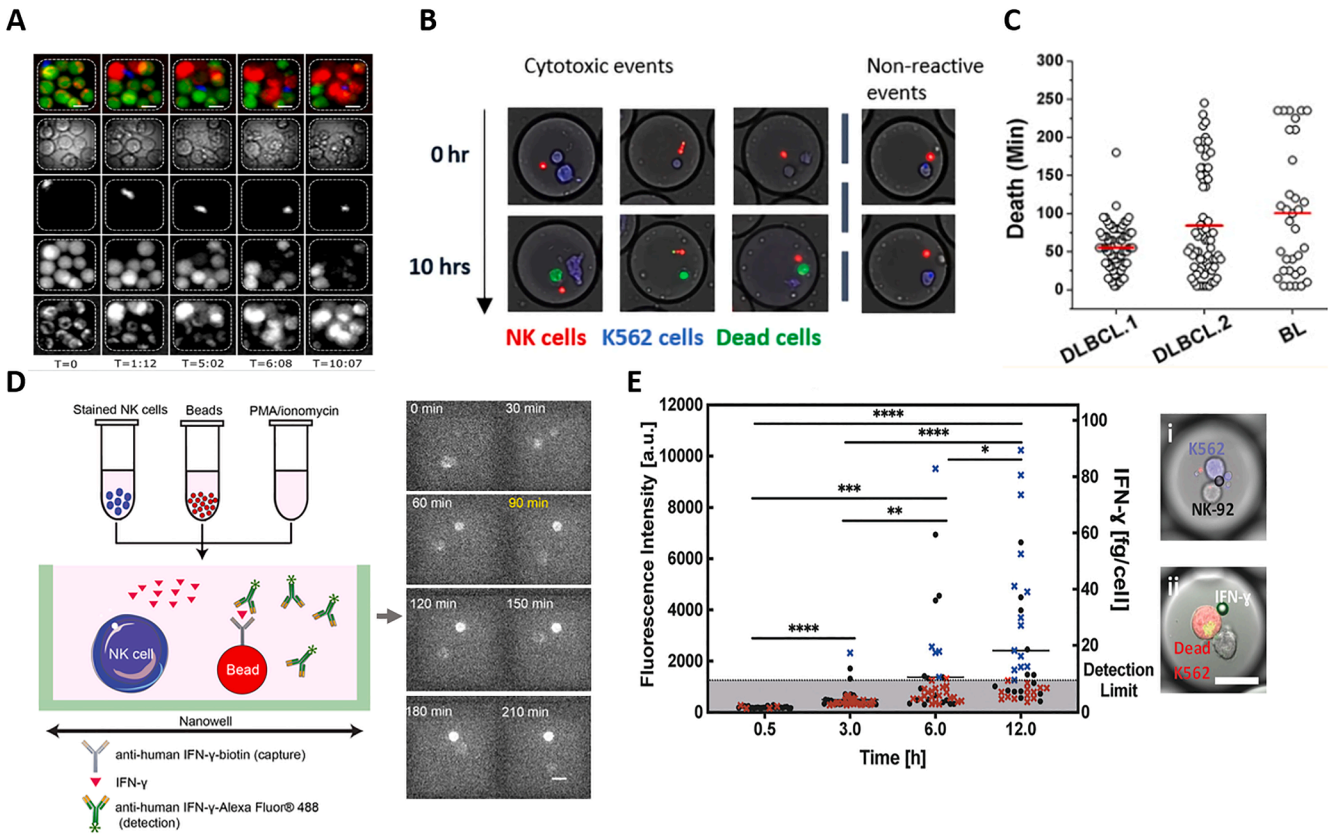

Fig. 5. Single cell-based approach to study NK cell effector functions: (A) The microchip platform allows interaction between NK cells and target cells within the silicon-glass microwell arrays that fit into a microscopic platform. A single platform contains 32,400 individual wells (≈50 μm × 50 μm) arrayed to facilitate screening with a 10× objective. Time-lapse imaging showing a serial killer NK cell (blue) lysing 8 target cells (alive: green; dead: red) within a 12-hour time frame. Adapted from Guldevall et al., Front. Immunol., 2016. **(B)** Water-oil emulsions of around 70pL allow compartmentalization of NK-Target cell pair, thus preventing external paracrine interaction. The figure adapted from Subedi et al., SR., 2021 shows a real-time observation of droplets with NK cells (red) and K562 (blue) and positive cytotoxic events (green). **(C)** Single cell-based approaches have provided the ability to screen the lytic dynamics of NK cells with different target cells. The graph represents the variation in the lytic duration of NK cells with different patient-derived tumor cells: Diffuse large B cell lymphoma (DLBCL1 and DLBCL2) and Burkitt lymphoma (BL), in the droplets-based cytotoxicity assay. Adapted from Sarkar et al., Front Immunol. 2017. **(D)** Schematic of sandwich immunoassay in the nano-well-based platform for detecting cytokine secretion from single NK cells that allowed dynamic tracking of the IFN-γ secretory activity. Scale bar = 10 μm. Adapted from An et al., PLOS, 2017. **(E)** Droplet-based combinatorial assay to monitor cytotoxicity together with IFN-γ secretion simultaneously with sensitivity ranging at femtogram level. Adapted from Antona et al., Adfm. 2020.

inflammatory cytokines, such as IFN-γ, TNF-α, granulocyte–macrophage colony-stimulating factors but with systemic inflammation, a high amount of IL12 is released that induces NK cells to secrete IL10 together with other cytokines activation [97–99]. IL10 dampens the activation of macrophages and DCs but it has been shown to enhance the effector function of NK cells [99]. Thus, through cytokines secretion and intracellular interaction, NK cells induce immunity while maintaining the homeostasis within the system. Quantification of cytokines can be easily achieved at the population level using conventional immunoassays, however, no information about the identity, numbers or activity of the individual secreting cells can be extracted from such studies. Technological advances allow for zooming in on individual producers and the direct measurement of cellular functionality (Fig. 5D). Bead-based molecular sensors in a nano-well array enabled to dynamically profile the secretion of IFN-γ from single NK cells [100]. The cytotoxic ($CD56^{dim}CD16^{+}$) NK cells were found to secrete IFN-γ in less than 3 h upon interaction with target cells, however, the secretion rate and amount varied depending on the donor. Another study using similar approach showed the association of IFN-γ secretion with the motility of NK cells during target cell interaction while no association with its cytolytic abilities had been established. Later a combinatorial assessment of cytotoxicity with secretion of a single NK cell using droplet based platform found around 30% cytolytic NK cells being positive for secretion of IFN-γ [101]. Small volume of droplets ranging from few picolitres easily allowed this high sensitivity (fg cell-1) required for the detection of secretion from an individual cell which was not possible to achieve in a well based assay [101,102] (Fig. 5E). Thus, integrated analysis of cytolytic activity and secretory activity of single NK cells has revealed a variegated association within two functions which would not be possible to study in population level without tracking individual cells. The application of functionalized nanoparticles together with droplet-based microfluidics further allowed the simultaneous activation and measurement of IFN-γ secreted by NK cells [103]. In this way different single-cell platforms contributed to studying the immunoregulatory role of NK cells by elucidating the correlation between secretion and cytolysis. Apart from IFN-γ secretion, several groups have focused on understanding the role of NK cells in modulating the function of other immune cells, such as DCs and T cells [104–106]. scRNA-seq of the nasopharyngeal carcinoma TME showed the high prevalence of cytotoxic NK cells that secreted high amounts of XCL1 and XCL2 [107]. Both are ligands for XCR1 receptors that were over-expressed in DCs in the TME, indicative for a role in recruiting DCs to the TME by NK cells. It has provided new insights in how NK cells modulate the adaptive and innate immune systems to maintain immune homeostasis.

1.3.3. Single NK cell migration

The tissue-specific homing is guided by different organ-specific elements, such as chemokine and non-chemokine molecules during

physiological and pathological conditions [108]. It is well known that the two major peripheral blood NK cell subsets express substantially different chemokine receptors that only partially overlap [22,109]. $CD56^{dim}$ NK cells express CXCR1, CXCR2, CXCR3 and to some extent CCR5 whereas $CD56^{brigt}$ NK cells only expresses CXCR1, CXCR3 and high CCR5. More recently, scRNA-seq showed the variegated expression of different chemokine receptors for different phenotypes that led to functional trafficking of NK cells [53]. The active NK cells at a steady-state down-regulated CXCR4 (a bone marrow homing chemokine) as they become functionally mature [53].

NK cell migration in vivo encounters several challenges posed by the tissue microenvironment. Populations-based in vitro migration assays (e.g., the commonly used transwell assay) are not able to include these features and thus do not provide a thorough assessment of migratory conditions for NK cells. To overcome this limitation, several microfluidic-based systems that mimic the in vivo microenvironment such as stable chemical gradient for chemotaxis, and inclusion of extracellular matrix to support cell motility, have been developed [106,110]. To monitor the migration dynamics upon NK cell activation, a collagen-based microwell system has been developed that allowed long-term imaging of NK–target cell interactions within a confined 3D volume [111]. Even though these studies represent NK cells cultures in bulk, tracking the movement of single NK cells enabled the identification of heterogeneous migratory behavior. NK cells exhibited a range of different migratory behaviors, like (i) alternate between periods of random movement, (ii) directed migration, and (iii) migration arrest. Similar transient variations in the NK cell migration profile were identified and NK cells commonly displayed a stop and go behavior while the factors determining such behavior are poorly understood [112]. IL2 activated NK cells displayed a more directed migratory phenotype and were more likely to employ “motile scanning” of the target-cell surface during conjugation compared to the non-activated cells [91].

These single cell-based studies reveal the variation in migratory behavior of NK cells and the effect of microenvironment on their migration pattern. These findings would not be possible without tracking individual cells which further enhances the importance of single cell study in migration studies of NK cells.

1.3.4. Single NK cell and memory

The memory like adaptive function of NK cells has been described a decade ago during CMV infection [20,113]. Recently, scRNA-seq studies revealed an increased frequency of adaptive NK cells in $CMV^{positive}$ donors while a small proportion of adaptive NK cells were still found in $CMV^{negative}$ individuals [114]. Furthermore, a small percentage of these adaptive NK cells were also found in bone marrow and spleen [29]. These studies showed that adaptive NK cells persist long after the induction of the anti-CMV immune response and have augmented effector functions upon reinfection. Additionally, the ability of NK cells to develop adaptive functions was also identified in case of vaccination [115]. Antigen-specific recall responses by human NK cells were observed in viral antigen-challenged skin of adult volunteers who had chickenpox as children, thus, suggesting towards the possible contribution of human NK memory response after immunization. Furthermore, during active tuberculosis infection, the cytotoxic fraction of NK cells was found to be depleted, however, adaptive NK cells still persisted longer in circulation [116]. Several single cell-based studies provided insight into the newly identified memory-like role of NK cells in terms of their phenotypical features, tissue-specific distribution, overall function, and longevity.

1.4. Conclusion and outlook

Decades of research have established that NK cells are a highly heterogeneous cell type. Initially, this heterogeneity is genetically determined, primarily due to variation in HLA and KIR gene families. Immunological experiences, physiological and pathological exposure also compiles into the variation later, thus generating unique NK cell phenotypes. Single-cell technologies have provided means to unravel this diversity and have taken the NK cell research into an additional dimension. The earlier mentioned studies have provided in-depth knowledge of the structural, molecular, and functional capabilities of these cell types. Even though each study provides important information in a certain aspect, connective research describing the complete NK cell biology is still missing. For example, identification of serial killers via microfluidic tools is limited to qualitative study while not much information about these cells at the molecular level is available. Similarly, the use of RNA-seq technologies has elucidated tissue-specific NK cell diversity based on their phenotypical variation, however, the downstream analysis of the functional aspect is lacking.

Understanding cellular heterogeneity can potentially aid in developing superior cell-based therapies to treat infection, inflammation, and cancer [117]. Applications originating from single-cell-based studies can lead to the identification of new pathways and cellular interactions, which can enhance immunotherapy [118–120]. Single cell-based platforms could also be used to study the efficacy of immunomodulating drugs in enhancing the activity of NK cells [121]. The relevance of comparing functional heterogeneity between different trNK cells and cNK cells must be emphasized to reveal the role of these NK cells in human physiology and diseases [118,122]. Numerous information generated from the single cell-based studies that have been used for immunotherapeutic approaches [92,116,123,124].

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author contributions

NS, LVV, JT conceptualized the manuscript. NS, EMB, IvR wrote the manuscript. JT wrote and verified the manuscript.

References

[1] R. Kiessling, E. Klein, H. Wigzell, “Natural” killer cells in the mouse. I. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Specificity and distribution according to genotype, Eur. J. Immunol. 5 (2) (1975) 112–117, https://doi.org/10.1002/eji.1830050208.
[2] A. Moretta, C. Bottino, M.C. Mingari, R. Biassoni, L. Moretta, What is a natural killer cell? Nat. Immunol. 3 (1) (2002) 6–8, https://doi.org/10.1038/ni0102-6.
[3] E. Vivier, E. Tomasello, M. Baratin, T. Walzer, S. Ugolini, Functions of natural killer cells, Nat. Immunol. 9 (5) (2008) 503–510, https://doi.org/10.1038/ni1582.
[4] M.A. Caligiuri, Human natural killer cells, Blood 112 (3) (2008) 461–469, https://doi.org/10.1182/blood-2007-09-077438.
[5] F. Cichocki, B. Grzywacz, J.S. Miller, Human NK cell development: One road or many? Front. Immunol. (2019) https://doi.org/10.3389/fimmu.2019.02078.
[6] B. Grzywacz, J.S. Miller, M.R. Verneris, Developmental stages and pathways of NK cell maturation, Nat. Kill. Cells (2010), https://doi.org/10.1016/B978-0-12-370454-2.00001-6.
[7] Q. Chen, W. Ye, W. Jian Tan, K.S. Mei Yong, M. Liu, S. Qi Tan, E. Loh, K. TE Chang, T. Chye Tan, P.R. Preiser, J. Chen, Delineation of Natural Killer Cell Differentiation from Myeloid Progenitors in Human, Sci. Rep. 5 (1) (2015), https://doi.org/10.1038/srep15118.
[8] C. Wu, B. Li, R. Lu, S. Koelle, Y. Yang, A. Jares, A. Krouse, M. Metzger, F. Liang, K. Loré, C. Wu, R. Donahue, I.Y. Chen, I. Weissman, C. Dunbar, Clonal tracking of rhesus macaque hematopoiesis highlights a distinct lineage origin for natural killer cells, Cell Stem Cell. 14 (4) (2014) 486–499, https://doi.org/10.1016/j.stem.2014.01.020.

[9] P. Dogra, C. Rancan, W. Ma, M. Toth, T. Senda, D.J. Carpenter, M. Kubota, R. Matsumoto, P. Thapa, P.A. Szabo, M.M. Li Poon, J. Li, J. Arakawa-Hoyt, Y. Shen, L. Fong, L.L. Lanier, D.L. Farber, Tissue Determinants of Human NK Cell Development, Function, and Residence, Cell 180 (4) (2020) 749–763.e13, https://doi.org/10.1016/j.cell.2020.01.022.
[10] L. Moretta, A. Moretta, Unravelling natural killer cell function: Triggering and inhibitory human NK receptors, EMBO J. 23 (2) (2004) 255–259, https://doi.org/10.1038/sj.emboj.7600019.
[11] E. Vivier, J.A. Nunès, F. Vély, Natural killer cell signaling pathways, Science 306 (5701) (2004) 1517–1519.
[12] M.T. Orr, L.L. Lanier, Natural Killer Cell Education and Tolerance, Cell 142 (6) (2010) 847–856, https://doi.org/10.1016/j.cell.2010.08.031.
[13] W.M. Yokoyama, B.F.M. Plougastel, Immune functions encoded by the natural killer gene complex, Nat. Rev. Immunol. 3 (4) (2003) 304–316, https://doi.org/10.1038/nri1055.
[14] P. Parham, MHC class I molecules and KIRS in human history, health and survival, Nat. Rev. Immunol. 5 (3) (2005) 201–214, https://doi.org/10.1038/nri1570.
[15] D.H. Raulet, N. Guerra, Oncogenic stress sensed by the immune system: Role of natural killer cell receptors, Nat. Rev. Immunol. 9 (8) (2009) 568–580, https://doi.org/10.1038/nri2604.
[16] L. L, Up on the tightrope: natural killer cell activation and inhibition, Nat. Immunol. (2009).
[17] Y.T. Bryceson, M.E. March, H.-G. Ljunggren, E.O. Long, Synergy among receptors on resting NK cells for the activation of natural cytotoxicity and cytokine secretion, Blood 107 (1) (2006) 159–166, https://doi.org/10.1182/blood-2005-04-1351.
[18] J.C. Sun, L.L. Lanier, NK cell development, homeostasis and function: Parallels with CD8 + T cells, Nat. Rev. Immunol. 11 (10) (2011) 645–657, https://doi.org/10.1038/nri3044.
[19] E. Vivier, D.H. Raulet, A. Moretta, M.A. Caligiuri, L. Zitvogel, L.L. Lanier, W. M. Yokoyama, S. Ugolini, Innate or adaptive immunity? The example of natural killer cells, Science (80-.) 331 (6013) (2011) 44–49.
[20] J.C. Sun, J.N. Beilke, L.L. Lanier, Adaptive immune features of natural killer cells, Nature 457 (7229) (2009) 557–561, https://doi.org/10.1038/nature07665.
[21] S. Paust, C.A. Blish, R.K. Reeves, T.C. Pierson, Redefining Memory: Building the Case for Adaptive NK Cells, J. Virol. 91 (20) (2017), https://doi.org/10.1128/JVI.00169-17.
[22] P. Carrega, G. Ferlazzo, Natural killer cell distribution and trafficking in human tissues, Front. Immunol. (2012), https://doi.org/10.3389/fimmu.2012.00347.
[23] J.S. Schleypen, N. Baur, R. Kammerer, P.J. Nelson, K. Rohrmann, E.F. Gröne, M. Hohenfellner, A. Haferkamp, H. Pohla, D.J. Schendel, C.S. Falk, E. Noessner, Cytotoxic markers and frequency predict functional capacity of natural killer cells infiltrating renal cell carcinoma, Clin. Cancer Res. 12 (3) (2006) 718–725, https://doi.org/10.1158/1078-0432.CCR-05-0857.
[24] N. Dalbeth, R. Gundle, R.J.O. Davies, Y.C.G. Lee, A.J. McMichael, M.F.C. Callan, CD56 bright NK Cells Are Enriched at Inflammatory Sites and Can Engage with Monocytes in a Reciprocal Program of Activation, J. Immunol. 173 (10) (2004) 6418–6426, https://doi.org/10.4049/jimmunol.173.10.6418.
[25] J. Faget, C. Biota, T. Bachelot, M. Gobert, C. Dezutter-Dambuyant, I. Treilleux, S. Goddard-Leon, J.Y. Blay, C. Caux, C. Menetrier-Caux, Abstract 1341: Early detection of tumor cells by innate immune cells leads to Treg recruitment through CCL22 production by tumor cells, in (2010), https://doi.org/10.1158/1538-7445.am10-1341.
[26] A.G. Freud, B.L. Mundy-Bosse, J. Yu, M.A. Caligiuri, The Broad Spectrum of Human Natural Killer Cell Diversity, Immunity 47 (5) (2017) 820–833, https://doi.org/10.1016/j.immuni.2017.10.008.
[27] D.K. Sojka, B. Plougastel-Douglas, L. Yang, M.A. Pak-Wittel, M.N. Artyomov, Y. Ivanova, C. Zhong, J.M. Chase, P.B. Rothman, J. Yu, J.K. Riley, J. Zhu, Z. Tian, W.M. Yokoyama, Tissue-resident natural killer (NK) cells are cell lineages distinct from thymic and conventional splenic NK cells, Elife (2014), https://doi.org/10.7554/eLife.01659.
[28] A. Crinier, P. Milpied, B. Escalière, C. Piperoglou, J. Galluso, A. Balsamo, L. Spinelli, I. Cervera-Marzal, M. Ebbo, M. Girard-Madoux, S. Jaeger, E. Bollon, S. Hamed, J. Hardwigsen, S. Ugolini, F. Vély, E. Narni-Mancinelli, E. Vivier, High-Dimensional Single-Cell Analysis Identifies Organ-Specific Signatures and Conserved NK Cell Subsets in Humans and Mice, Immunity 49 (5) (2018) 971–986.e5, https://doi.org/10.1016/j.immuni.2018.09.009.
[29] A. Crinier, P.-Y. Dumas, B. Escalière, C. Piperoglou, L. Gil, A. Villacreces, F. Vély, Z. Ivanovic, P. Milpied, É. Narni-Mancinelli, É. Vivier, Single-cell profiling reveals the trajectories of natural killer cell differentiation in bone marrow and a stress signature induced by acute myeloid leukemia, Cell. Mol. Immunol. 18 (5) (2021) 1290–1304, https://doi.org/10.1038/s41423-020-00574-8.
[30] Å.K. Björklund, M. Forkel, S. Picelli, V. Konya, J. Theorell, D. Friberg, R. Sandberg, J. Mjösberg, The heterogeneity of human CD127+ innate lymphoid cells revealed by single-cell RNA sequencing, Nat. Immunol. 17 (4) (2016) 451–460, https://doi.org/10.1038/ni.3368.
[31] M.L. Robinette, A. Fuchs, V.S. Cortez, J.S. Lee, Y. Wang, S.K. Durum, S. Gilfillan, M. Colonna, Transcriptional programs define molecular characteristics of innate lymphoid cell classes and subsets, Nat. Immunol. 16 (3) (2015) 306–317, https://doi.org/10.1038/ni.3094.
[32] M.G. Constantinides, B.D. McDonald, P.A. Verhoef, A. Bendelac, A committed precursor to innate lymphoid cells, Nature 508 (7496) (2014) 397–401, https://doi.org/10.1038/nature13047.
[33] O.-E. Weizman, N.M. Adams, I.S. Schuster, C. Krishna, Y. Pritykin, C. Lau, M. A. Degli-Esposti, C.S. Leslie, J.C. Sun, T.E. O'Sullivan, ILC1 Confer Early Host Protection at Initial Sites of Viral Infection, Cell 171 (4) (2017) 795–808.e12, https://doi.org/10.1016/j.cell.2017.09.052.
[34] A.P. McFarland, A. Yalin, S.-Y. Wang, V.S. Cortez, T. Landsberger, R. Sudan, V. Peng, H.L. Miller, B. Ricci, E. David, R. Faccio, I. Amit, M. Colonna, Multi-tissue single-cell analysis deconstructs the complex programs of mouse natural killer and type 1 innate lymphoid cells in tissues and circulation, Immunity 54 (6) (2021) 1320–1337.e4, https://doi.org/10.1016/j.immuni.2021.03.024.
[35] L. Moretta, Dissecting CD56dim human NK cells, Blood 116 (19) (2010) 3689–3691, https://doi.org/10.1182/blood-2010-09-303057.
[36] B. Fu, Z. Tian, H. Wei, Subsets of human natural killer cells and their regulatory effects, Immunology 141 (4) (2014) 483–489, https://doi.org/10.1111/imm.12224.
[37] V. Stary, G. Stary, NK Cell-Mediated Recall Responses: Memory-Like, Adaptive, or Antigen-Specific? Front. Cell. Infect. Microbiol. (2020) https://doi.org/10.3389/fcimb.2020.00208.
[38] L.L. Liu, A. Pfefferle, V.O. Yi Sheng, A.T. Björklund, V. Béziat, J.P. Goodridge, K. J. Malmberg, Harnessing adaptive natural killer cells in cancer immunotherapy, Mol. Oncol. (2015), https://doi.org/10.1016/j.molonc.2015.10.001.
[39] J.G. O'Leary, M. Goodarzi, D.L. Drayton, U.H. von Andrian, T cell- and B cell-independent adaptive immunity mediated by natural killer cells, Nat. Immunol. 7 (5) (2006) 507–516, https://doi.org/10.1038/ni1332.
[40] R. Romee, S.E. Schneider, J.W. Leong, J.M. Chase, C.R. Keppel, R.P. Sullivan, M. A. Cooper, T.A. Fehniger, Cytokine activation induces human memory-like NK cells, Blood 120 (24) (2012) 4751–4760, https://doi.org/10.1182/blood-2012-04-419283.
[41] H. Schlums, F. Cichocki, B. Tesi, J. Theorell, V. Beziat, T. Holmes, H. Han, S. C. Chiang, B. Foley, K. Mattsson, S. Larsson, M. Schaffer, K.-J. Malmberg, H.-G. Ljunggren, J. Miller, Y. Bryceson, Cytomegalovirus infection drives adaptive epigenetic diversification of NK cells with altered signaling and effector function, Immunity 42 (3) (2015) 443–456, https://doi.org/10.1016/j.immuni.2015.02.008.
[42] M. Gumá, A. Angulo, C. Vilches, N. Gómez-Lozano, N. Malats, M. López-Botet, Imprint of human cytomegalovirus infection on the NK cell receptor repertoire, Blood 104 (12) (2004) 3664–3671, https://doi.org/10.1182/blood-2004-05-2058.
[43] J. Lee, T. Zhang, I. Hwang, A. Kim, L. Nitschke, MinJae Kim, J. Scott, Y. Kamimura, L. Lanier, S. Kim, Epigenetic Modification and Antibody-Dependent Expansion of Memory-like NK Cells in Human Cytomegalovirus-Infected Individuals, Immunity 42 (3) (2015) 431–442.
[44] L. Quatrini, M. Della Chiesa, S. Sivori, M.C. Mingari, D. Pende, L. Moretta, Human NK cells, their receptors and function, Eur. J. Immunol. 51 (7) (2021) 1566–1579, https://doi.org/10.1002/eji.202049028.
[45] M. Luetke-Eversloh, Q. Hammer, P. Durek, K. Nordström, G. Gasparoni, M. Pink, A. Hamann, J. Walter, H.D. Chang, J. Dong, C. Romagnani, Human Cytomegalovirus Drives Epigenetic Imprinting of the IFNG Locus in NKG2Chi Natural Killer Cells, PLoS Pathog. (2014), https://doi.org/10.1371/journal.ppat.1004441.
[46] A. Merino, B. Zhang, P. Dougherty, X. Luo, J. Wang, B.R. Blazar, J.S. Miller, F. Cichocki, Chronic stimulation drives human NK cell dysfunction and epigenetic reprograming, J. Clin. Invest. 129 (9) (2019) 3770–3785, https://doi.org/10.1172/JCI12591610.1172/JCI125916DS1.
[47] N.M. Valiante, M. Uhrberg, H.G. Shilling, K. Lienert-Weidenbach, K.L. Arnett, A. D'Andrea, J.H. Phillips, L.L. Lanier, P. Parham, N.M. Valiante, M. Uhrberg, H. G. Shilling, K. Lienert-Weidenbach, K.L. Arnett, A. D'Andrea, J.H. Phillips, L. L. Lanier, P. Parham, Functionally and structurally distinct NK cell receptor repertoires in the peripheral blood of two human donors, Immunity (1997).
[48] A. Horowitz, D.M. Strauss-Albee, M. Leipold, J. Kubo, N. Nemat-Gorgani, O. C. Dogan, C.L. Dekker, S. Mackey, H. Maecker, G.E. Swan, M.M. Davis, P. J. Norman, L.A. Guethlein, M. Desai, P. Parham, C.A. Blish, Genetic and environmental determinants of human NK cell diversity revealed by mass cytometry, Sci. Transl. Med. 5 (208) (2013), https://doi.org/10.1126/scitranslmed.3006702.
[49] J.K. Wiencke, R. Butler, G. Hsuang, M. Eliot, S. Kim, M.A. Sepulveda, D. Siegel, E. A. Houseman, K.T. Kelsey, The DNA methylation profile of activated human natural killer cells, Epigenetics 11 (5) (2016) 363–380, https://doi.org/10.1080/15592294.2016.1163454.
[50] T.D. Holmes, R.V. Pandey, E.Y. Helm, H. Schlums, H. Han, T.M. Campbell, T. T. Drashansky, S. Chiang, C.-Y. Wu, C. Tao, M. Shoukier, E. Tolosa, S. Von Hardenberg, M. Sun, C. Klemann, R.A. Marsh, C.M. Lau, Y. Lin, J.C. Sun, R. Månsson, F. Cichocki, D. Avram, Y.T. Bryceson, The transcription factor Bcl11b promotes both canonical and adaptive NK cell differentiation, Sci. Immunol. 6 (57) (2021), https://doi.org/10.1126/sciimmunol.abc9801.
[51] D. Wang, S. Malarkannan, Transcriptional regulation of natural killer cell development and functions, Cancers (Basel) 12 (6) (2020) 1591, https://doi.org/10.3390/cancers12061591.
[52] J. Bi, X. Wang, Molecular Regulation of NK Cell Maturation, Front. Immunol. (2020), https://doi.org/10.3389/fimmu.2020.01945.
[53] C. Yang, J.R. Siebert, R. Burns, Z.J. Gerbec, B. Bonacci, A. Rymaszewski, M. Rau, M.J. Riese, S. Rao, K.-S. Carlson, J.M. Routes, J.W. Verbsky, M.S. Thakar, S. Malarkannan, Heterogeneity of human bone marrow and blood natural killer cells defined by single-cell transcriptome, Nat. Commun. 10 (1) (2019), https://doi.org/10.1038/s41467-019-11947-7.
[54] J. Zhao, S. Zhang, Y. Liu, X. He, M. Qu, G. Xu, H. Wang, M. Huang, J. Pan, Z. Liu, Z. Li, L. Liu, Z. Zhang, Single-cell RNA sequencing reveals the heterogeneity of liver-resident immune cells in human, Cell Discov. 6 (1) (2020), https://doi.org/10.1038/s41421-020-0157-z.

[55] C. Yang, J.R. Siebert, R. Burns, Y. Zheng, A. Mei, B. Bonacci, D. Wang, R. A. Urrutia, M.J. Riese, S. Rao, K.S. Carlson, M.S. Thakar, S. Malarkannan, Single-cell transcriptome reveals the novel role of t-bet in suppressing the immature NK gene signature, Elife (2020), https://doi.org/10.7554/eLife.51339.
[56] S. Gordon, J. Chaix, L. Rupp, J. Wu, S. Madera, J. Sun, T. Lindsten, S. Reiner, The Transcription Factors T-bet and Eomes Control Key Checkpoints of Natural Killer Cell Maturation, Immunity 36 (1) (2012) 55–67, https://doi.org/10.1016/j.immuni.2011.11.016.
[57] S. Lunemann, G. Martrus, H. Goebels, T. Kautz, A. Langeneckert, W. Salzberger, M. Koch, M.J. Bunders, B. Nashan, K.P.J.M. Van Gisbergen, M. Altfeld, Hobit expression by a subset of human liver-resident CD56bright Natural Killer, Sci. Rep. (2017), https://doi.org/10.1038/s41598-017-06011-7.
[58] S. Kim, J. Poursine-Laurent, S.M. Truscott, L. Lybarger, Y.-J. Song, L. Yang, A. R. French, J.B. Sunwoo, S. Lemieux, T.H. Hansen, W.M. Yokoyama, Licensing of natural killer cells by host major histocompatibility complex class I molecules, Nature 436 (7051) (2005) 709–713, https://doi.org/10.1038/nature03847.
[59] K.-M. Lee, M.E. McNerney, S.E. Stepp, P.A. Mathew, J.D. Schatzle, M. Bennett, V. Kumar, 2B4 Acts As a Non-Major Histocompatibility Complex Binding Inhibitory Receptor on Mouse Natural Killer Cells, J. Exp. Med. 199 (9) (2004) 1245–1254, https://doi.org/10.1084/jem.20031989.
[60] Y. He, Z. Tian, NK cell education via nonclassical MHC and non-MHC ligands, Cell. Mol. Immunol. 14 (4) (2017) 321–330, https://doi.org/10.1038/cmi.2016.26.
[61] S. Cooley, F. Xiao, M. Pitt, M. Gleason, V. McCullar, T.L. Bergemann, K. L. McQueen, L.A. Guethlein, P. Parham, J.S. Miller, A subpopulation of human peripheral blood NK cells that lacks inhibitory receptors for self-MHC is developmentally immature, Blood 110 (2) (2007) 578–586, https://doi.org/10.1182/blood-2006-07-036228.
[62] C. Pfeifer, A.J. Highton, S. Peine, J. Sauter, A.H. Schmidt, M.J. Bunders, M. Altfeld, C. Körner, Natural Killer Cell Education Is Associated With a Distinct Glycolytic Profile, Front. Immunol. 9 (2018), https://doi.org/10.3389/fimmu.2018.0302010.3389/fimmu.2018.03020.s001.
[63] N.C. Fernandez, E. Treiner, R.E. Vance, A.M. Jamieson, S. Lemieux, D.H. Raulet, A subset of natural killer cells achieves self-tolerance without expressing inhibitory receptors specific for self-MHC molecules, Blood 105 (11) (2005) 4416–4423, https://doi.org/10.1182/blood-2004-08-3156.
[64] F. Li, H. Wei, H. Wei, Y. Gao, L. Xu, W. Yin, R. Sun, Z. Tian, Blocking the natural killer cell inhibitory receptor NKG2A increases activity of human natural killer cells and clears hepatitis B virus infection in mice, Gastroenterology 144 (2) (2013) 392–401, https://doi.org/10.1053/j.gastro.2012.10.039.
[65] N. Tarek, J.-B. Le Luduec, M.M. Gallagher, J. Zheng, J.M. Venstrom, E. Chamberlain, S. Modak, G. Heller, B.o. Dupont, N.-K. Cheung, K.C. Hsu, Unlicensed NK cells target neuroblastoma following anti-GD2 antibody treatment, J. Clin. Invest. 122 (9) (2012) 3260–3270, https://doi.org/10.1172/JCI6274910.1172/JCI62749DS1.
[66] K. Juelke, M. Killig, A. Thiel, J. Dong, C. Romagnani, Education of hyporesponsive NK cells by cytokines, Eur. J. Immunol. 39 (9) (2009) 2548–2555, https://doi.org/10.1002/eji.200939307.
[67] A. Pfefferle, B. Jacobs, H. Netskar, E.H. Ask, S. Lorenz, T. Clancy, J.P. Goodridge, E. Sohlberg, K.-J. Malmberg, Intra-lineage Plasticity and Functional Reprogramming Maintain Natural Killer Cell Repertoire Diversity, Cell Rep. 29 (8) (2019) 2284–2294.e4, https://doi.org/10.1016/j.celrep.2019.10.058.
[68] A. Marçais, J. Cherfils-Vicini, C. Viant, S. Degouve, S. Viel, A. Fenis, J. Rabilloud, K. Mayol, A. Tavares, J. Bienvenu, Y.-G. Gangloff, E. Gilson, E. Vivier, T. Walzer, The metabolic checkpoint kinase mTOR is essential for IL-15 signaling during the development and activation of NK cells, Nat. Immunol. 15 (8) (2014) 749–757, https://doi.org/10.1038/ni.2936.
[69] L.M. Gaynor, F. Colucci, Uterine natural killer cells: Functional distinctions and influence on pregnancy in humans and mice, Front. Immunol. (2017), https://doi.org/10.3389/fimmu.2017.00467.
[70] J. Hanna, D. Goldman-Wohl, Y. Hamani, I. Avraham, C. Greenfield, S. Natanson-Yaron, D. Prus, L. Cohen-Daniel, T.I. Arnon, I. Manaster, R. Gazit, V. Yutkin, D. Benharroch, A. Porgador, E. Keshet, S. Yagel, O. Mandelboim, Decidual NK cells regulate key developmental processes at the human fetal-maternal interface, Nat. Med. 12 (9) (2006) 1065–1074, https://doi.org/10.1038/nm1452.
[71] D.M. Strauss-Albee, J. Fukuyama, E.C. Liang, Y. Yao, J.A. Jarrell, A.L. Drake, J. Kinuthia, R.R. Montgomery, G. John-Stewart, S. Holmes, C.A. Blish, Human NK cell repertoire diversity reflects immune experience and correlates with viral susceptibility, Sci. Transl. Med. 7 (297) (2015), https://doi.org/10.1126/scitranslmed.aac5722.
[72] S. Platonova, J. Cherfils-Vicini, D. Damotte, L. Crozet, V. Vieillard, P. Validire, P. André, M.-C. Dieu-Nosjean, M. Alifano, J.-F. Régnard, W.-H. Fridman, C. Sautès-Fridman, I. Cremer, Profound coordinated alterations of intratumoral NK cell phenotype and function in lung carcinoma, Cancer Res. 71 (16) (2011) 5412–5422, https://doi.org/10.1158/0008-5472.CAN-10-4179.
[73] N.C. Horton, S.O. Mathew, P.A. Mathew, Novel Interaction between Proliferating Cell Nuclear Antigen and HLA I on the Surface of Tumor Cells Inhibits NK Cell Function through NKp44, PLoS One (2013), https://doi.org/10.1371/journal.pone.0059552.
[74] J. Cong, X. Wang, X. Zheng, D. Wang, B. Fu, R. Sun, Z. Tian, H. Wei, Dysfunction of Natural Killer Cells by FBP1-Induced Inhibition of Glycolysis during Lung Cancer Progression, Cell Metab. 28 (2) (2018) 243–255.e5, https://doi.org/10.1016/j.cmet.2018.06.021.
[75] N. Sinha, N. Subedi, J. Tel, Integrating Immunology and Microfluidics for Single Immune Cell Analysis, Front. Immunol. 9 (2018) 2373, https://doi.org/10.3389/fimmu.2018.02373.
[76] E. Forslund, K. Guldevall, P.E. Olofsson, T. Frisk, A.E. Christakou, M. Wiklund, B. Önfelt, Novel microchip-based tools facilitating live cell imaging and assessment of functional heterogeneity within NK cell populations, Front. Immunol. 3 (2012) 1–7, https://doi.org/10.3389/fimmu.2012.00300.
[77] N. Subedi, L.C. Van Eyndhoven, A.M. Hokke, L. Houben, M.C. Van Turnhout, C.V. C. Bouten, K. Eyer, J. Tel, An automated real-time microfluidic platform to probe single NK cell heterogeneity and cytotoxicity on-chip, Sci. Rep. 11 (2021) 17084, https://doi.org/10.1038/s41598-021-96609-9.
[78] K. Guldevall, L. Brandt, E. Forslund, K. Olofsson, T.W. Frisk, P.E. Olofsson, K. Gustafsson, O. Manneberg, B. Vanherberghen, H. Brismar, K. Kärre, M. Uhlin, B. Önfelt, Microchip screening platform for single cell assessment of NK cell cytotoxicity, Front. Immunol. (2016), https://doi.org/10.3389/fimmu.2016.00119.
[79] B. Vanherberghen, P.E. Olofsson, E. Forslund, M. Sternberg-Simon, M. A. Khorshidi, S. Pacouret, K. Guldevall, M. Enqvist, K.J. Malmberg, R. Mehr, B. Önfelt, Classification of human natural killer cells based on migration behavior and cytotoxic response, Blood. 121 (2013) 1326–1334, https://doi.org/10.1182/blood-2012-06-439851.
[80] A. Manuscript, Nihms582539, 15 (2014) 128–135. Doi: 10.1038/ni.2796.Single-cell.
[81] A.E. Christakou, M. Ohlin, B. Vanherberghen, M.A. Khorshidi, N. Kadri, T. Frisk, M. Wiklund, B. Önfelt, Live cell imaging in a micro-array of acoustic traps facilitates quantification of natural killer cell heterogeneity, Integr. Biol. (United Kingdom) 5 (2013) 712–719, https://doi.org/10.1039/c3ib20253d.
[82] B. Dura, M.M. Servos, R.M. Barry, H.L. Ploegh, S.K. Dougan, J. Voldman, Longitudinal multiparameter assay of lymphocyte interactions from onset by microfluidic cell pairing and culture, Proc. Natl. Acad. Sci. 113 (26) (2016) E3599–E3608, https://doi.org/10.1073/pnas.1515364113.
[83] D.M. Davis, Mechanisms and functions for the duration of intercellular contacts made by lymphocytes, Nat. Rev. Immunol. 9 (8) (2009) 543–555, https://doi.org/10.1038/nri2602.
[84] G. Iezzi, K. Karjalainen, A. Lanzavecchia, The duration of antigenic stimulation determines the fate of naive and effector T cells, Immunity 8 (1) (1998) 89–95, https://doi.org/10.1016/S1074-7613(00)80461-6.
[85] D.M. Underhill, M. Bassetti, A. Rudensky, A. Aderem, Dynamic interactions of macrophages with T cells during antigen presentation, J. Exp. Med. 190 (12) (1999) 1909–1914, https://doi.org/10.1084/jem.190.12.1909.
[86] T. Rothoeft, A. Gonschorek, H. Bartz, O. Anhenn, U. Schauer, Antigen dose, type of antigen-presenting cell and time of differentiation contribute to the T helper 1/T helper 2 polarization of naive T cells, Immunology 110 (4) (2003) 430–439, https://doi.org/10.1111/j.1365-2567.2003.01758.x.
[87] Y.J. Yamanaka, C.T. Berger, M. Sips, P.C. Cheney, G. Alter, J.C. Love, Single-cell analysis of the dynamics and functional outcomes of interactions between human natural killer cells and target cells, Integr. Biol. (United Kingdom) 4 (10) (2012) 1175, https://doi.org/10.1039/c2ib20167d.
[88] S. Sarkar, P. Sabhachandani, D. Ravi, S. Potdar, S. Purvey, A. Beheshti, A. M. Evens, T. Konry, Dynamic analysis of human natural killer cell response at single-cell resolution in B-Cell Non-Hodgkin Lymphoma, Front. Immunol. 8 (2017) 1–13, https://doi.org/10.3389/fimmu.2017.01736.
[89] B. Dura, S.K. Dougan, M. Barisa, M.M. Hoehl, C.T. Lo, H.L. Ploegh, J. Voldman, Profiling lymphocyte interactions at the single-cell level by microfluidic cell pairing, Nat. Commun. 6 (1) (2015), https://doi.org/10.1038/ncomms6940.
[90] A. Yalçın, Y.J. Yamanaka, J.C. Love, Analytical technologies for integrated single-cell analysis of human immune responses, Methods Mol. Biol. 853 (2012) 211–235, https://doi.org/10.1007/978-1-61779-567-1_16.
[91] P.E. Olofsson, E. Forslund, B. Vanherberghen, K. Chechet, O. Mickelin, A.R. Ahlin, T. Everhorn, B. Önfelt, Distinct migration and contact dynamics of resting and IL-2-activated human natural killer cells, Front. Immunol. 5 (2014) 1–10, https://doi.org/10.3389/fimmu.2014.00080.
[92] K. Srpan, A. Ambrose, A. Karampatzakis, M. Saeed, A.N.R. Cartwright, K. Guldevall, G.D.S.C. De Matos, B. Önfelt, D.M. Davis, Shedding of CD16 disassembles the NK cell immune synapse and boosts serial engagement of target cells, J. Cell Biol. 217 (9) (2018) 3267–3283, https://doi.org/10.1083/jcb.201712085.
[93] D.R. Makanga, F. Da Rin de Lorenzo, G. David, C. Willem, L. Dubreuil, N. Legrand, T. Guillaume, P. Peterlin, A. Lebourgeois, M.C. Béné, A. Garnier, P. Chevallier, K. Gendzekhadze, A. Cesbron, K. Gagne, B. Clemenceau, C. Retière, Genetic and molecular basis of heterogeneous nk cell responses against acute leukemia, Cancers (Basel) 12 (7) (2020) 1927, https://doi.org/10.3390/cancers12071927.
[94] S. Antona, I. Platzman, J.P. Spatz, Droplet-Based Cytotoxicity Assay: Implementation of Time-Efficient Screening of Antitumor Activity of Natural Killer Cells, ACS Omega 5 (38) (2020) 24674–24683, https://doi.org/10.1021/acsomega.0c03264.
[95] S.C. Hsiao, H. Liu, T.A. Holstlaw, C. Liu, C.Y. Francis, M.B. Francis, Real Time Assays for Quantifying Cytotoxicity with Single Cell Resolution, PLoS One (2013), https://doi.org/10.1371/journal.pone.0066739.
[96] S. Sarkar, W. Kang, S. Jiang, K. Li, S. Ray, E. Luther, A.R. Ivanov, Y. Fu, T. Konry, Machine learning-aided quantification of antibody-based cancer immunotherapy by natural killer cells in microfluidic droplets, Lab Chip. 20 (13) (2020) 2317–2327.
[97] A.M. Abel, C. Yang, M.S. Thakar, S. Malarkannan, Natural killer cells: Development, maturation, and clinical utilization, Front. Immunol. (2018), https://doi.org/10.3389/fimmu.2018.01869.
[98] D.H. Kaplan, V. Shankaran, A.S. Dighe, E. Stockert, M. Aguet, L.J. Old, R. D. Schreiber, Demonstration of an interferon γ-dependent tumor surveillance

system in immunocompetent mice, Proc. Natl. Acad. Sci. U. S. A. (1998), https://doi.org/10.1073/pnas.95.13.7556.
[99] Z. Wang, D.i. Guan, J. Huo, S.K. Biswas, Y. Huang, Y. Yang, S. Xu, K.-P. Lam, IL-10 Enhances Human Natural Killer Cell Effector Functions via Metabolic Reprogramming Regulated by mTORC1 Signaling, Front. Immunol. 12 (2021), https://doi.org/10.3389/fimmu.2021.619195.
[100] X. An, V.G. Sendra, I. Liadi, B. Ramesh, G. Romain, C. Haymaker, M. Martinez-Paniagua, Y. Lu, L.G. Radvanyi, B. Roysam, N. Varadarajan, Single-cell profiling of dynamic cytokine secretion and the phenotype of immune cells, PLoS One (2017), https://doi.org/10.1371/journal.pone.0181904.
[101] S. Antona, T. Abele, K. Jahnke, Y. Dreher, K. Göpfrich, I. Platzman, J.P. Spatz, Droplet-Based Combinatorial Assay for Cell Cytotoxicity and Cytokine Release Evaluation, Adv. Funct. Mater. 30 (46) (2020) 2003479, https://doi.org/10.1002/adfm.202003479.
[102] K. Eyer, R.C.L. Doineau, C.E. Castrillon, L. Briseño-Roa, V. Menrath, G. Mottet, P. England, A. Godina, E. Brient-Litzler, C. Nizak, A. Jensen, A.D. Griffiths, J. Bibette, P. Bruhns, J. Baudry, Single-cell deep phenotyping of IgG-secreting cells for high-resolution immune monitoring, Nat. Biotechnol. 35 (10) (2017) 977–982, https://doi.org/10.1038/nbt.3964.
[103] J. Park, Y. Shin, J.M. Kim, S.H. Kweon, A.Y. Song, Y. Baek, J. Kim, D. Cho, H. S. Kim, J. Doh, Multifunctional Microparticles with Stimulation and Sensing Capabilities for Facile NK Cell Activity Assay, ACS Sens. 6 (3) (2021) 693–697, https://doi.org/10.1021/acssensors.0c02138.
[104] A. Laroni, R. Gandhi, V. Beynon, H.L. Weiner, IL-27 imparts immunoregulatory function to human NK cell subsets, PLoS One (2011), https://doi.org/10.1371/journal.pone.0026173.
[105] T.D. Holmes, E.B. Wilson, E.V.I. Black, A.V. Benest, C. Vaz, B. Tan, V.M. Tanavde, G.P. Cook, Licensed human natural killer cells aid dendritic cell maturation via TNFSF14/LIGHT, Proc. Natl. Acad. Sci. U. S. A. 111 (52) (2014) E5688–E5696, https://doi.org/10.1073/pnas.1411072112.
[106] J. Hipolito, H. Peretz-Soroka, M. Zhang, K.e. Yang, S. Karimi-Abdolrezaee, F. Lin, S. Kung, A new microfluidic platform for studying natural killer cell and dendritic cell interactions, Micromachines 10 (12) (2019) 851, https://doi.org/10.3390/mi10120851.
[107] Y.-P. Chen, J.-H. Yin, W.-F. Li, H.-J. Li, D.-P. Chen, C.-J. Zhang, J.-W. Lv, Y.-Q. Wang, X.-M. Li, J.-Y. Li, P.-P. Zhang, Y.-Q. Li, Q.-M. He, X.-J. Yang, Y. Lei, L.-L. Tang, G.-Q. Zhou, Y.-P. Mao, C. Wei, K.-X. Xiong, H.-B. Zhang, S.-D. Zhu, Y. Hou, Y. Sun, M. Dean, I. Amit, K. Wu, D.-M. Kuang, G.-B. Li, N.a. Liu, J. Ma, Single-cell transcriptomics reveals regulators underlying immune cell diversity and immune subtypes associated with prognosis in nasopharyngeal carcinoma, Cell Res. 30 (11) (2020) 1024–1042, https://doi.org/10.1038/s41422-020-0374-x.
[108] F.-D. Shi, H.-G. Ljunggren, A. La Cava, L. Van Kaer, Organ-specific features of natural killer cells, Nat. Rev. Immunol. 11 (10) (2011) 658–671, https://doi.org/10.1038/nri3065.
[109] P. Carrega, I. Bonaccorsi, E. Di Carlo, B. Morandi, P. Paul, V. Rizzello, G. Cipollone, G. Navarra, M.C. Mingari, L. Moretta, G. Ferlazzo, CD56 bright Perforin low Noncytotoxic Human NK Cells Are Abundant in Both Healthy and Neoplastic Solid Tissues and Recirculate to Secondary Lymphoid Organs via Afferent Lymph, J. Immunol. 192 (8) (2014) 3805–3815, https://doi.org/10.4049/jimmunol.1301889.
[110] S. Mahmood, S. Nandagopal, I. Sow, F. Lin, S.K.P. Kung, Microfluidic-based, live-cell analysis allows assessment of NK-cell migration in response to crosstalk with dendritic cells, Eur. J. Immunol. 44 (9) (2014) 2737–2748, https://doi.org/10.1002/eji.201344244.
[111] P.E. Olofsson, L. Brandt, K.E.G. Magnusson, T. Frisk, J. Jaldén, B. Önfelt, A collagen-based microwell migration assay to study NK-target cell interactions, Sci. Rep. 9 (2019) 1–10, https://doi.org/10.1038/s41598-019-46958-3.
[112] M.A. Khorshidi, B. Vanherberghen, J.M. Kowalewski, K.R. Garrod, S. Lindström, H. Andersson-Svahn, H. Brismar, M.D. Cahalan, B. Önfelt, Analysis of transient migration behavior of natural killer cells imaged in situ and in vitro, Integr. Biol. 3 (7) (2011) 770, https://doi.org/10.1039/c1ib00007a.
[113] S. Paust, H.S. Gill, B.-Z. Wang, M.P. Flynn, E.A. Moseman, B. Senman, M. Szczepanik, A. Telenti, P.W. Askenase, R.W. Compans, U.H. von Andrian, Critical role for the chemokine receptor CXCR6 in NK cell-mediated antigen-specific memory of haptens and viruses, Nat. Immunol. 11 (12) (2010) 1127–1135, https://doi.org/10.1038/ni.1953.
[114] S.L. Smith, P.R. Kennedy, K.B. Stacey, J.D. Worboys, A. Yarwood, S. Seo, E. H. Solloa, B. Mistretta, S.S. Chatterjee, P. Gunaratne, K. Allette, Y.-C. Wang, M. L. Smith, R. Sebra, E.M. Mace, A. Horowitz, W. Thomson, P. Martin, S. Eyre, D. M. Davis, Diversity of peripheral blood human NK cells identified by single-cell RNA sequencing, Blood Adv. 4 (7) (2020) 1388–1406, https://doi.org/10.1182/bloodadvances.2019000699.
[115] R. Nikzad, L.S. Angelo, K. Aviles-Padilla, D.T. Le, V.K. Singh, L. Bimler, M. Vukmanovic-Stejic, E. Vendrame, T. Ranganath, L. Simpson, N.L. Haigwood, C.A. Blish, A.N. Akbar, S. Paust, Human natural killer cells mediate adaptive immunity to viral antigens, Sci. Immunol. 4 (35) (2019), https://doi.org/10.1126/sciimmunol.aat8116.
[116] Y. Cai, Y. Dai, Y. Wang, Q. Yang, J. Guo, C. Wei, W. Chen, H. Huang, J. Zhu, C. Zhang, W. Zheng, Z. Wen, H. Liu, M. Zhang, S. Xing, Q. Jin, C.G. Feng, X. Chen, Single-cell transcriptomics of blood reveals a natural killer cell subset depletion in tuberculosis, EBioMedicine. 53 (2020) 102686, https://doi.org/10.1016/j.ebiom.2020.102686.
[117] V.Y.S. Oei, M. Siernicka, A. Graczyk-Jarzynka, H.J. Hoel, W. Yang, D. Palacios, H. Almåsbak, M. Bajor, D. Clement, L. Brandt, B. Önfelt, J. Goodridge, M. Winiarska, R. Zagozdzon, J. Olweus, J.-A. Kyte, K.-J. Malmberg, Intrinsic functional potential of NK-Cell subsets constrains retargeting driven by chimeric antigen receptors, Cancer Immunol. Res. 6 (4) (2018) 467–480, https://doi.org/10.1158/2326-6066.CIR-17-0207.
[118] L.F. de Andrade, Y. Lu, A. Luoma, Y. Ito, D. Pan, J.W. Pyrdol, C.H. Yoon, G.-C. Yuan, K.W. Wucherpfennig, Discovery of specialized NK cell populations infiltrating human melanoma metastases, JCI Insight 4 (23) (2019), https://doi.org/10.1172/jci.insight.133103.
[119] J. Cursons, F. Souza-Fonseca-Guimaraes, M. Foroutan, A. Anderson, F. Hollande, S. Hediyeh-Zadeh, A. Behren, N.D. Huntington, M.J. Davis, A gene signature predicting natural killer cell infiltration and improved survival in melanoma patients, Cancer Immunol. Res. 7 (7) (2019) 1162–1174, https://doi.org/10.1158/2326-6066.CIR-18-0500.
[120] J. Ni, X.i. Wang, A. Stojanovic, Q. Zhang, M. Wincher, L. Bühler, A. Arnold, M. P. Correia, M. Winkler, P.-S. Koch, V. Sexl, T. Höfer, A. Cerwenka, Single-Cell RNA Sequencing of Tumor-Infiltrating NK Cells Reveals that Inhibition of Transcription Factor HIF-1α Unleashes NK Cell Activity, Immunity 52 (6) (2020) 1075–1087.e8, https://doi.org/10.1016/j.immuni.2020.05.001.
[121] J.M.R. Van der Meer, R.J.A. Maas, K. Guldevall, K. Klarenaar, P.K.J.D. de Jonge, J.S.H. van Evert, A.B. van der Waart, J. Cany, J.T. Safrit, J.H. Lee, E. Wagena, P. Friedl, B. Önfelt, L.F. Massuger, N.P.M. Schaap, J.H. Jansen, W. Hobo, H. Dolstra, IL-15 superagonist N-803 improves IFNγ production and killing of leukemia and ovarian cancer cells by CD34+ progenitor-derived NK cells, Cancer Immunol. Immunother. (2021), https://doi.org/10.1007/s00262-020-02749-8.
[122] D. Brownlie, M. Scharenberg, J.E. Mold, J. Hård, E. Kekäläinen, M. Buggert, S. Nguyen, J.N. Wilson, M. Al-Ameri, H.-G. Ljunggren, N. Marquardt, J. Michaëlsson, Expansions of adaptive-like NK cells with a tissue-resident phenotype in human lung and blood, Proc. Natl. Acad. Sci. U. S. A. 118 (11) (2021), https://doi.org/10.1073/pnas.2016580118.
[123] M.C. Costanzo, D. Kim, M. Creegan, K.G. Lal, J.A. Ake, J.R. Currier, H. Streeck, M. L. Robb, N.L. Michael, D.L. Bolton, N.J. Steers, M.A. Eller, Transcriptomic signatures of NK cells suggest impaired responsiveness in HIV-1 infection and increased activity post-vaccination, Nat. Commun. 9 (1) (2018), https://doi.org/10.1038/s41467-018-03618-w.
[124] A.R. Schuurman, T.D. Reijnders, A. Saris, I. Ramirez Moral, M. Schinkel, J. de Brabander, C. van Linge, L. Vermeulen, B.P. Scicluna, W.J. Wiersinga, F.A. Vieira Braga, T. van der Poll, Integrated single-cell analysis unveils diverging immune features of COVID-19, influenza, and other community-acquired pneumonia, Elife. (2021), https://doi.org/10.7554/elife.69661.