UNIVERSITY OF GENOA

DEPARTMENT OF EXPERIMENTAL MEDICINE

(DIMES)

PhD in Clinical and Experimental Immunology

XXXVII cycle

"Tissue-resident Natural Killer cells in human lung cancer"

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Contents

Abstract

Background: Natural killer (NK) cells, part of the innate lymphoid cell family, represent the third major lymphocytic population in the peripheral blood and are traditionally divided into $CD56^{dim}CD16^{+}$ and $CD56^{bright}CD16^{-}$ subsets. However, recent research has identified tissue-resident(tr) NK cells in different organs, such as lung and liver. These cells are distinguished by the circulating counterpart by expression of markers, such as CD103, CD69, CXCR6, and CD49a responsible for their retention in tissues. Our group and others have recently observed the presence of distinct subsets of tr-NK cells among total lymphocytic infiltrate of NSCLC. While the role of tr-NK cells in normal tissue homeostasis has been, at least in part, clarified, their function in tumor microenvironment (TME) remains poorly understood.

Purpose: The main objective of this study was to investigate the function and ontogeny of tr-NK cells in TME of human NSCLC. We mainly focused on $CD103^{+}$ tr-NK cells, because of their potential to interact with epithelial tumor cells through the recognition of E-cadherin.

Results: We found that tumor-infiltrating $CD103^{+}$ NK cell showed dysregulation in IFN-γ, TNF-α production and degranulation toward tumor targets, but maintained expression of chemotactic factors important for recruitment of Dendritic Cells. Interestingly, their functional abilities can be partially restored in the presence of activating cytokines like IL-15. Regarding the potential origin of $CD103^{+}$ NK cells in tumor, by using a 3D spheroid model of NSCLC, we also observed that only circulating $CD56^{bright}$ NK cells, but not the $CD56^{dim}$ counterpart, could efficiently infiltrate tumor masses. Interestingly, $CD56^{bright}$ infiltrating spheroids acquired CD103 and other features of NK cell counterpart present in the TME.

Conclusions: Our data suggests that steady-state tumor-infiltrating $CD103^{+}$ NK cells show limited pro-inflammatory function despite maintaining expression of chemotactic factors able to recruit other immune effectors. However, these limitations might be overcome by stimulating cytokines. Moreover, our data emphasizes a unique capability for $CD56^{bright}$ NK cell in infiltrating tumor mass and convert to $CD103^{+}$ tr-NK cells. Overall, these results

prompt us to investigate further strategies to stimulate endogenous NK cells to boost antitumor immunity.

1. INTRODUCION

1.1. Human Conventional NK Cells: Subsets, Development, and Functions

Human conventional natural killer (cNK) cells are categorized into two main subsets, based on the expression of the adhesion molecule CD56/NCAM: $CD56^{bright}$ and $CD56^{dim}$. The $CD56^{bright}$ NK cells are primarily known for their enhanced ability to produce cytokines and lack of cytotoxic capability whereas $CD56^{dim}$ NK cells are recognized for their cytotoxic functions(1). In the peripheral blood, $CD56^{dim}$ NK cells are more prevalent, whereas $CD56^{bright}$ NK cells are more commonly found in lymphoid tissues, such as lymph nodes, endometrium, and decidua (2, 3). The development of cNK cells occurs in the bone marrow (BM) (4). In the BM, hematopoietic stem cells (HSCs) differentiate into early lymphoid progenitors (ELPs), which are characterized by high expression levels of cKit, Sca1, and Flt3(5). These ELPs further differentiate into common lymphoid progenitors (CLPs), marked by reduced Sca1 expression and increased interleukin (IL)-7 receptor levels(6).

Various cytokines are crucial for the development of cNK cells, with IL-15 being particularly significant as it utilizes the common gamma chain receptor (7). The expression of the IL-2 receptor (CD122) signifies an irreversible commitment to the NK cell lineage (8). Immature cNK cells are identified by the expression of CD117 and the integrin β2 (DX5) (9). These cells progress through several stages marked by CD27+CD11b-, CD27+CD11b+, and ultimately CD27-CD11b+ (10). The majority of cNK cells that migrate from BM to the peripheral tissues are in the CD27-CD11b+ stage (8).

The primary functions of cNK cells include exerting natural cytotoxicity and producing inflammatory cytokines such as TNF-α, IFN-γ, and GM-CSF (11, 12). Additionally, cNK cells secrete various chemokines like CCL1, CCL3, CCL4, CCL5, and CCL22, which

contribute to creating and maintaining an inflammatory environment (13) , In circulation, human cNK cells express integrins such as αLβ2 (LFA-1), αMβ2 (Mac-1), α4β1 (VLA-4), α5β1 (VLA-5), and α6β1 (VLA-6) (14, 15). The expression of β2 integrins primarily facilitates the adhesion of circulating NK cells to endothelial cells in capillaries and the formation of the immune synapse. In contrast, β1 integrins are associated with mediating tissue residency. The differential roles of β1 and β2 integrins are illustrated by the distinct immune phenotypes observed in leukocyte adhesion deficiency (LAD)-I and LAD-III in humans. Leukocyte adhesion deficiency type I (LAD-I) arises due to a deficiency in β2 integrins, which impedes the exit of NK cell precursors and mature NK cells from the bloodstream and affects the formation of immune synapses (16). Conversely, leukocyte adhesion deficiency type III (LAD-III) is caused by mutations in kindlin-3 or Rap1, which are essential activators of both β1 and β2 integrins. This results in a broader clinical presentation that includes bleeding disorders (17). In LAD-III patients, some integrin functions are either conserved or compensated, potentially through talin facilitating α4β1 to sustain adequate adhesion despite the absence of kindlin-3 (18).

1.2. Deciphering Tissue Residency: Integrin and Non-Integrin Signatures in NK Cell Localization

The tissue residency of both NK and T cells can be characterized by a fundamental transcriptional signature, distinct from integrin-coding genes. Notably, in human NK and T cells, reduced expression of S1PR1 (S1P receptor 1), SELL (L-selectin), RGS1 (regulator of g-protein signaling 1), and KLF3 (Kruppel like factor 3) serves as markers (19, 20). Moreover, human NK cells exhibit heightened CXCR6 expression (21). However, these gene signatures, independent of integrins, intersect with integrin profiles. For instance, CD69+ α1 (CD49a)+ αE (CD103)+ tr-NKs in the human lung exhibit unique non-integrin gene signatures compared to bone marrow and lung CD8 TRM cells (21).

Markers such as α1 (CD49a), collagen IV binder, and αE (CD103), E-cadherin binder, signify tissue retention in T cells (22). Furthermore, the "area code" guiding cell trafficking through

integrin ligand expression extends to specific tissue niches within organs, including the human lung. In the human lung, αE presence likely denotes not only tissue specificity but also spatial localization within tissue. Notably, three distinct populations of tissue-resident NK cells exist in the human lung: CD69+α1−αE−, CD69+α1+αE−, and CD69+α1+αE+. These populations may indicate specific regional localization within the lung. In human skin, β1 positive T cells localize to the dermis but seem unable to position within the epidermis.

This discovery holds significant clinical implications for the progression of psoriasis, highlighting the potential for cellular sequestration within tissue environments based on integrin expression (23). Additionally, the presence of CD103+ (αE+) T cells within lung epithelium indicates αE as a potential marker for epithelial localization, aligning with E-cadherin expression by alveolar epithelial cells and the basement membrane (24). While the exact localization of tr-NK cells in the lung remains undefined, it is plausible that distinct populations, particularly those marked by CD103, are spatially segregated within the epithelium, potentially undergoing predetermined fate decisions that anchor them into long-term tissue residency as they mature (21).

Apart from peripheral blood NK cells, human lungs exhibit the highest frequency of highly differentiated NK cells, with a minority displaying a phenotype consistent with tissue-resident NK cells. In contrast, approximately half of liver NK cells express CD69, indicative of a tissue-resident phenotype (25). Despite CD69 expression, co-expression of CD49a or CD103 is infrequent among liver NK cells in most donors (26). However, in donors where a distinct population of $CD56^{bright}$ CD69+CD49a+ cells are identifiable, these cells often demonstrate a mature phenotype reminiscent of CMV-driven clonal-like expansions (26). Conversely, NK cells in the human uterus display a unique differentiation pathway, concurrently exhibiting characteristics of both immature and mature cells (27). The majority of uterine NK cells co-express CD69, CD103, and CD49a, suggesting a predominantly tissue-resident population (28).

The observed heterogeneity is likely influenced by various organ tissue-specific factors, including the local microenvironment containing distinct soluble factors, parenchymal cells,

and other immune cells, as well as tissue-specific infections. Furthermore, diverse microarchitectures across organs likely impact tissue accessibility and the likelihood of immune cells acquiring a tissue-resident phenotype. For instance, the highly vascularized lungs with less parenchymal tissue compared to the liver and uterus may influence immune cell behavior. Nonetheless, much remains to be elucidated regarding the generation and specialization of tissue-resident and circulating NK cells, particularly in terms of cytotoxicity and cytokine production. Unlike conventional NK cells, the developmental stages of tissue-resident NK cells remain poorly defined, and the existence of distinct precursors for tissue-resident NK cells and the timing of commitment to this lineage are yet to be fully understood. Additionally, whether specific cytokines drive NK cell progenitors towards tissue residency remains an open question.

1.3. Exploring Tissue-Resident Natural Killer Cell Development

Human natural killer (NK) cells, akin to other immune cells, undergo a developmental process originating from hematopoietic stem cells. During gestational week 6, mature NK cells emerge in the liver, with subsequent distribution to various fetal tissues such as the bone marrow, spleen, lymph nodes, lungs, and intestine by the middle of the second trimester (29, 30). Hematopoiesis primarily occurs in the fetal liver during the first trimester and transitions gradually to the bone marrow during the second trimester. In adults, the bone marrow is considered the principal site for NK cell development (31).

The proposed developmental pathway of human NK cells begins with CD34+ hematopoietic stem cells (stage 1), progresses through CD34+CD45RA– cells (stage 2), CD34– CD117+ cells (stage 3), to $CD56^{bright}$ NK cells (stage 4), and culminates in mature $CD56^{dim}$ NK cells (stage 5) (32, 33). However, stages 1–3 encompass heterogeneous cell populations with multipotent capacities, rather than marking the earliest stages of NK cell commitment. For instance, stage 2 cells possess the potential to differentiate into NK cells, B cells, and T cells, while stage 3 cells can differentiate into both NK cells and group 3 innate lymphoid cells (34). Recently, a precursor specifically committed to NK cells (NKP) was identified in human cord blood, bone marrow, and tonsils (35). This NKP, characterized as lineage (LIN)–

CD34+CD38+CD123–CD45RA+CD7+CD10+CD127–, exhibited selective differentiation into NK cells in vitro and in vivo, excluding non-NK innate lymphoid cells, T cells, B cells, or myeloid cells (35). Positioned downstream from a common lymphoid progenitor-like population, this NKP demonstrated the ability to generate T cells, B cells, NK cells, and non-NK innate lymphoid cells at the single-cell level (35).

The existence of distinct NK cell precursors (NKPs) responsible for the development of tissue-resident NK cells remains uncertain in humans, although recent murine studies suggest the presence of specific requirements. For instance, tissue-resident CD49a+ NK cells in the liver, skin, and uterus of mice critically rely on the transcription factor T-bet, whereas conventional NK cells are less influenced by T-bet genetic deletion (36, 37). Moreover, murine NK cells in salivary glands were found to develop independently of the transcription factor NFIL3, unlike conventional NK cells, which depend on NFIL3 (38, 39). Conversely, thymic NK cells in mice depend on the transcription factor GATA3, whereas liver-resident and conventional NK cells do not (40, 41).

Whether similar prerequisites govern the development of human tissue-resident NK cells remains unclear due to the absence of direct methods like genetic barcoding or in vivo fate mapping for tracking NK cell origins in humans. It is plausible that distinct subsets of NK cells originate from hematopoietic precursors within tissues rather than bone marrow. This notion of "peripheral tissue ontogeny" is supported by the identification of CD34+ cells with NK cell potential in various human peripheral tissues. Notably, populations of stage 2 and stage 3 NKPs capable of maturing into NK cells have been observed in the adult human liver (42). Similarly, the human decidua harbors LIN– CD34+CD45+ progenitors capable of differentiating into NK cells upon interaction with stromal cells (43).

The precise identity of these NK cell precursor populations, particularly stage 3 cells, and whether they represent innate lymphoid cells (ILCs) or cells with the potential to differentiate into ILCs, remains uncertain. Furthermore, it remains to be investigated whether the NKPs identified thus far are distinct from those found in bone marrow, cord blood, and secondary

lymphoid tissues (SLTs), and if so, whether they possess an exclusive capacity to give rise to tissue-resident NK cells. According to current knowledge, it is conceivable that NK cell populations in peripheral organs such as the uterus and liver originate, at least partially, from local precursors. The tissue microenvironment in these peripheral organs likely plays a role in shaping the distinctive phenotypic and functional characteristics of these cells.

1.4. Understanding the Role of Diverse Immune Cells in Tumor Microenvironment

The tumor microenvironment (TME) encompasses stromal and immune cells, crucial in shaping cancer progression and influencing responses to therapy (44). Immune cell populations within tumors consist of lymphocytes, including T cells, B cells, and natural killer (NK) cells, as well as various myeloid cells such as granulocytes, monocytes, macrophages, and dendritic cells (DCs). These myeloid cells exhibit diverse and sometimes opposing functions within TME. Generally, intra-tumoral monocytes and M2-polarized macrophages are associated with promoting tumor growth, angiogenesis, and metastasis, contributing to an immunosuppressive milieu and poor clinical outcomes (45).

Conversely, M1-polarized macrophages and DCs support anti-tumor immunity and are linked with favorable prognoses (46). The involvement of conventional DCs (cDCs) in anti-tumor immunity is attributed to their capacity for antigen presentation and cytokine secretion, crucial for regulating T cell survival and function (47). cDCs comprise at least two subsets: conventional type 1 dendritic cells (cDC1) and conventional type 2 dendritic cells (cDC2). cDC1, reliant on the Batf3 transcription factor, are identifiable by selective expression of DNGR-1 and XCR1, and, in non-lymphoid organs and tumors, additional expression of CD103 with low CD11b expression. These cells excel in engulfing dead tumor cells and transporting tumor antigens to lymph nodes, where they play a pivotal role in priming anti-tumor CD8+ T cells (48).

Furthermore, cDC1 exhibits pivotal functions within tumors themselves. Intertumoral cDC1 play a role in attracting T cells (49), reactivating and amplifying tumor-specific CD8+ T cells (50), and facilitating T cell effector function through the secretion of interleukin (IL)-

12 (51). Human cDC1 is notably scarce within the TME and are often excluded during the early stages of tumor development, potentially impeding anti-tumor immunity and fostering cancer progression. While intra-tumoral cDC1 has not been as extensively investigated in humans as in murine models, their abundance in human melanoma is correlated with T cell infiltration. Moreover, the ratio of cDC1-selective transcripts to macrophage-restricted transcripts can serve as a prognostic indicator for cancer patient survival (49, 50).

Hence, therapeutic interventions targeting the augmentation of cDC1 abundance within tumors or enhancing their activation hold promises for enhancing anti-tumor immunity and potentially improving the efficacy of immunotherapy in cancer patients (49, 50, 52). Nevertheless, the mechanisms governing the abundance of cDC1 at tumor sites remain poorly understood, including whether cDC1s are actively recruited into the TME and whether this process necessitates the involvement of other cell types.

1.4.1. Chemokines in Tumor Microenvironments: Impact on NK Cell Recruitment

Natural killer (NK) cells are a prevalent presence within human tumors, albeit at lower frequencies compared to myeloid and lymphoid cells (2). Augmented NK cell infiltration in the tumor microenvironment (TME) has been correlated with enhanced overall survival among patients afflicted with squamous cell lung cancer (53) and non-small cell lung cancer (NSCLC) (54). Notably, NK cells have recently been implicated in patient responsiveness to anti-PD-1 immunotherapy in metastatic melanoma (55). The recruitment of NK cells to tumors is principally governed by three factors: chemoattractant/receptors, immunomodulation of chemokine axes, and physical barriers. The two primary NK cell subsets, $CD56^{bright}$ and $CD56^{dim}$, exhibit distinct repertoires of chemoattractant receptors, contributing to varied recruitment patterns across tissues. $CD56^{bright}$ NK cells in peripheral blood typically express and respond to ligands for CCR2, CCR5, CCR7, CXCR3, CXCR4, and CD62L, whereas $CD56^{dim}$ NK cells express and respond to ligands for CXCR1, CXCR2, CXCR4, CX3CR1, S1P5, and ChemR23 (2, 56, 57).

In parallel to human NK cell subsets, mouse CD11b SP and CD27 SP NK cells are functionally analogous. Recent single-cell RNA sequencing of metastatic melanoma samples has unveiled transcriptional diversity among NK cells in the TME (58). Nevertheless, despite this transcriptional heterogeneity, $CD56^{bright}$ NK cells have emerged as the predominant subset in the TME across various cancers, including NSCLC and breast cancer (2, 59). The heightened abundance of $CD56^{bright}$ NK cells in the TME of NSCLC and breast cancer has been associated with the downregulation of the chemokine CXCL2, which signals through CXCR2, and the concurrent upregulation of chemokines CXCL9, CXCL10, and CCL19, which signal through CCR7 or CXCR3, within the TME (2).

Likewise, within preclinical mouse lymphoma models, the expression of CXCL9 and CXCL10 by tumor cells, which activate the chemokine receptor CXCR3 on NK cells, plays a crucial role in facilitating NK cell infiltration into the TME (60). CCL5, serving as the ligand for CCR5, uniquely expressed on human $CD56^{bright}$ and mouse CD27 SP NK cells, has also been implicated in the recruitment of NK cells to the TME. Additionally, alternative pathways likely modulate NK cell recruitment, potentially involving the CCL5 axis. In a specific mouse model, tumor-derived progranulin functions to inhibit CCL5 production in an autocrine manner, leading to diminished NK cell infiltration into the TME, compromised tumor control, and heightened metastasis (61).

Concurrently supporting the role of CCL5 in orchestrating NK cell recruitment to the TME, an experimental model of melanoma lung metastasis revealed that IL-33 within the lung TME prompts CCL5 production by CD8+ T cells and eosinophils, subsequently augmenting NK cell recruitment and significantly reducing lung metastases (62). Other investigations demonstrated that exogenous administration or overexpression of IL-33 in transplantable melanoma models engenders heightened recruitment and activation of NK cells within the TME, plausibly mediated through a mechanism involving CCL5 (62). Another unconventional pathway implicated in regulating NK cell recruitment to the TME is governed by the cytokine IL-17D and the chemokine CCL2. In this context, tumor-derived

IL-17D signals to endothelial cells, stimulating the production of CCL2, thereby fostering NK cell recruitment to the TME (63).

Furthermore, CCL27, which activates CCR10 signaling, emerges as another chemokine associated with modulating NK cell recruitment to the TME. Intratumoral administration of adenovirus encoding CCL27 has been shown to enhance NK cell recruitment to the TME in murine models, consequently restraining tumor growth (64). However, conflicting findings from a study suggest that NK cells may not be indispensable for controlling tumor growth, thus clouding the significance of CCL27-mediated recruitment of NK cells to the TME. (65). An intriguing observation is the scarcity of NK cells within the tumor microenvironment (TME) of endometrial cancer, which aligns with diminished production of CCL27, CXCL12, and CCL21 compared to adjacent normal tissue (66). The involvement of CCL27 in cancer is complex due to the potential tumor-cell-intrinsic functions of CCR10, which might promote the proliferation and metastasis of melanoma and breast cancer cells (67). Evidently, further investigations are warranted to comprehensively elucidate the role of CCL27 in orchestrating protective immune responses and influencing cancer progression.

CX3CL1, also known as fractalkine, acts as the ligand for CX3CR1, a chemokine receptor uniquely expressed on cytotoxic $CD56^{dim}$/CD11b SP NK cells. Elevated expression of CX3CL1 serves as a favorable prognostic indicator for patient outcomes and facilitates NK cell infiltration in lung adenocarcinoma (68). Moreover, in human breast cancer tissue, CX3CL1 is downregulated compared to adjacent normal tissue, consistent with the prevalence of $CD56^{bright}$ NK cells within the breast cancer TME (2). Additionally, tumor-mediated production of TGF-β has been found to subvert the CX3CL1–CX3CR1 axis (69). Subsequent studies have demonstrated that TGF-β1 signaling in NK cells induces the expression of microRNA miR-27a-5p, resulting in the downregulation of CX3CR1 expression (70). Notably, the CX3CL1–CX3CR1 signaling axis also plays a role in controlling hepatocellular carcinoma metastasis to the lung, where tumor cells upregulate miR-561-5p, inhibiting CX3CL1 production and consequently reducing NK cell recruitment to the tumor (71).

Collectively, these findings underscore the pivotal role of chemokine signaling in modulating NK cell recruitment to the TME and advocate for targeting these pathways to enhance NK cell presence within tumors. Disruption or modulation of chemokine signaling by two immunomodulatory molecules, HLA-G and CD47, has been associated with alterations in NK cell recruitment to the TME. HLA-G, a member of the nonclassical HLA-class I B genes, exerts potent immune-inhibitory functions. HLA-G expression is confined to the tumor microenvironment (TME) and is absent in surrounding normal tissue. Studies have established a positive correlation between higher HLA-G expression levels and advanced cancer stage or poorer patient outcomes. HLA-G exerts inhibitory effects on NK cells by suppressing their activation, cytokine production, and cytotoxicity through STAT3 downregulation (72, 73). Soluble HLA-G has been shown to decrease the expression of chemokine receptors, including CCR2, CXCR3, and CX3CR1, in human NK cells, indicating a potential impairment of NK cell recruitment to the TME mediated by soluble HLA-G found in the serum of cancer patients (57).

The precise mechanisms by which CD47 regulate NK cell recruitment to the TME remain less elucidated. While CD47 plays a crucial role in inhibiting phagocytosis of cancer cells within the TME, its expression on NK cells enables binding to its ligand thrombospondin-1 (TSP-1), leading to inhibition of NK cell activation and proliferation (60). Blocking TSP-1 binding to CD47 using an anti-CD47 antibody reversed TSP-1–CD47-mediated inhibition, inhibited tumor growth in melanoma-bearing mice, augmented NK cell recruitment to the TME, and enhanced granzyme B and IFN-γ expression in NK cells (74). Further investigations are warranted to delineate the mechanisms underlying NK cell recruitment to the TME following anti-CD47 antibody treatment, yet these findings underscore CD47 as a critical NK cell checkpoint and highlight its potential as a therapeutic target to modulate NK cell numbers in the TME.

Moreover, stromal barriers may influence NK cell recruitment to tumors. Regions of tumors with high levels of extracellular matrix proteins like collagen type IV and laminin were associated with a lack of NK cell infiltration, suggesting that these structures surrounding the

tumor could impede NK cell invasion (75). Consistent with this observation, NK cells are predominantly found in stromal regions rather than in direct contact with tumor cells in human non-small cell lung cancer (NSCLC) tissue (59). Additionally, it has been proposed that even in tumors with elevated expression of NK cell-attracting chemokines, there may not always be a concurrent recruitment of NK cells. These findings underscore the necessity for further research into the physical barriers limiting intratumoral NK cells and emphasize the importance of studying NK cell localization and its impact on a favorable immune response.

1.4.2. Insights into NK Cell Activation in Tumor Microenvironments.

In contrast to T and B lymphocytes, natural killer (NK) cells rely on a repertoire of germline-encoded activating and inhibitory receptors to discern foreign, stressed, infected, or cancerous cells and subsequently induce target cell destruction upon complete activation. Consequently, the integration of complex signals stemming from multiple ligand–receptor interactions underpin NK cell recognition and activation. A pivotal signal for NK cells in identifying potential target cells is the absence or aberrant expression of class I major histocompatibility complex (MHC-I) molecules, a phenomenon termed "missing self-recognition." NK cells constitutively express various Ly49-type inhibitory receptors in mice and killer immunoglobulin-like receptors (KIRs) in humans, alongside the CD94–NKG2A heterodimer, which are critical for NK cell education during development (76, 77).

Inhibitory KIR and Ly49 receptors play crucial roles in NK cell education by recognizing classical polymorphic self-MHC-I molecules, thereby enabling NK cells to discriminate between healthy self-tissue and stressed, infected, foreign, or transformed cells (78). However, alongside the activity of inhibitory receptors, NK cells require activating signals to exert their effector function. Equipped with an array of activating receptors, NK cells are postulated to detect stress-induced ligands on cancer cells. Natural cytotoxicity receptors (NCRs), including NKp46 (NCR1/CD335), NKp44 (NCR2/CD336), and NKp30 (NCR3/CD337), belong to the immunoglobulin (Ig) superfamily and associate with various immunoreceptor tyrosine-based activation motif (ITAM)-containing adaptor proteins to

recruit and activate downstream kinases (e.g., Lck, Fyn, Syk, and ZAP-70) to fully activate NK cells. The identification of cancer cell ligands for NCRs remains an active area of research. Although some ligands for NKp30, such as B7-H6, have been identified, the ligands for NKp46 remain unknown (79). NKp80, characterized by its activating properties in NK cells, engages with activation-induced C-type lectin (AICL, encoded by CLEC2B), which undergoes upregulation upon Toll-like receptor stimulation on myeloid cells (80). Recent investigations have identified platelet-derived growth factor (PDGF)-DD as a ligand for NKp44 through a secretome library screen (81). Activation of NK cells with PDGF-DD results in the secretion of interferon-gamma (IFN-γ) and tumor necrosis factor (TNF), inducing cell cycle arrest in melanoma, ovarian, and breast cancer cells in vitro. Notably, elevated PDGF-DD gene expression correlates with NCR2 and effector cytokine expression and is associated with favorable survival outcomes in glioblastoma patients (82). Consistent with the notion that NCRs can sense soluble mediators, Nidogen-1, an extracellular matrix protein, has been recently identified as a ligand for NKp44 (83). These findings collectively suggest that NK cells can be modulated by secreted molecules interacting with NCRs, presenting a novel avenue for research with potential therapeutic implications.

In addition to NCRs, the lectin-like type 2 transmembrane receptor NKG2D plays a pivotal role in NK cell-mediated tumor cell cytotoxicity. NKG2D, expressed on the majority of NK cells in humans and mice, recognizes a variety of MHC-related ligands that exhibit low expression in healthy tissues but are markedly upregulated in cancer cells (84). Adhesion molecules also contribute to NK cell activation. Lymphocyte function-associated antigen-1 (LFA-1), expressed on NK cells, interacts with intercellular adhesion molecules (ICAMs) on target cells. This interaction enhances NK cell-mediated cytotoxicity by facilitating the polarization of the cytoskeleton machinery, crucial for effective delivery of cytotoxic granules (85). DNAX accessory molecule-1 (CD226/DNAM-1) further facilitates NK cell adhesion, migration, and function (86). Upon binding to its ligands CD155 or CD112, commonly expressed on cancer cells, CD226 promotes NK cell activation and cytotoxicity (87).

Another crucial axis of NK cell activation within the tumor microenvironment (TME) is orchestrated by proinflammatory cytokines and danger-associated molecular patterns (DAMPs). Cytokines, upon binding to their respective receptors, augment the activation, survival, proliferation, and maturation of NK cells. Notable cytokines with stimulatory effects on NK cells include IL-2, IL-12, IL-15, IL-18, and IL-21. While IL-2 and IL-15, either individually or in combination with other cytokines, support NK cell survival and proliferation, IL-12 and IL-18 primarily stimulate IFN-γ production and cytotoxicity in NK cells. IL-21 has been shown to enhance NK cell-mediated cytotoxicity by upregulating granzymes and perforin, and the synergistic effect of IL-21 in combination with IL-2 further potentiates NK cell activation (88). Soluble ligands also exert a profound impact on NK cell activation. One such example is soluble HLA-G, capable of activating human NK cells via KIR2DL4, leading to the production of cytokines and chemokines (89). Soluble NKG2D ligands have been observed to inhibit NK cell function by downregulating NKG2D (90). However, conflicting reports exist regarding the role of soluble NKG2D ligands in NK cell activation, as some studies have indicated that soluble forms of high-affinity NKG2D ligands can indeed induce NK cell activation (91). Consequently, the precise role of soluble NKG2D ligands in NK cell activation remains ambiguous.

1.4.3. NK Cell-Mediated Tumor Cell Death

NK cells exhibit the capability to eliminate both local and disseminated tumor cells. Moreover, findings from an eleven-year follow-up study have established a correlation between reduced NK cell killing capacity in peripheral blood and tumor development (92). This observation has sparked an inquiry into the essential role of this rare cell population in protecting against cancer. While the protective nature of NK cell killing is evident, NK cells possess multiple functions, including cytokine and chemokine production, which contribute to shaping the immune response to cancer, thereby potentially amplifying their significance. Consequently, in addition to the direct antitumor activity through cytotoxicity, NK cell-mediated eradication of cancer cells also influences antigen availability for presentation. This holds true not only in the context of normal immune responses but also in clinical scenarios

where monoclonal antibodies are employed to induce antibody-dependent cellular cytotoxicity (ADCC).

The induction of tumor cell death by NK cells can lead to increased release of tumor antigens. Subsequently, this augmented pool of tumor antigens may enhance the presentation of antigens to T cells, thereby potentially augmenting T cell responses against cancer. Consequently, NK cell-mediated killing of tumor cells can occur at the primary tumor site or in disseminated tumors, resulting in the release of tumor antigens that prime an adaptive immune response. However, it is unlikely that this mechanism alone can elucidate the entirety of protection conferred by the presence of NK cells in solid tumors.

1.4.4. NK Cells and Dendritic Cells: Collaborators in Antitumor Immune Responses

The primary mechanisms through which NK cells influence the adaptive immune response to cancer involve cytokine production and modulation of dendritic cell (DC) responses. Upon activation, NK cells produce a diverse array of cytokines, including IFN-γ, GM-CSF, G-CSF, M-CSF, TNF, IL-5, IL-10, IL-13, and others (93). IFN-γ holds significant importance in the context of antitumor immunity and plays a central role in regulating both positive and negative responses to tumors. While the intricate mechanisms underlying IFN-γ-mediated immune responses have been extensively discussed, our focus will be on elucidating the link between NK cell-produced IFN-γ and alterations in the adaptive immune response to cancer. IFN-γ exerts its effects directly on various immune cell populations, including macrophages, DCs, B cells, T cells, and even NK cells themselves. Signaling via IFN-γ in macrophages activates these cells, leading to heightened production of inflammatory cytokines, enhanced phagocytic activity, antigen presentation, and augmented nonspecific cytocidal activity against microbial pathogens and tumor (94).

Furthermore, IFN-γ induces maturation of DCs, resulting in upregulated expression of MHC-I and II molecules, costimulatory molecules, and components necessary for antigen processing and presentation to T cells (95). This heightened antigen presentation capability is complemented by the induction of IL-12 and IL-15 expression in DCs upon IFN-γ

stimulation, both of which play pivotal roles in initiating antitumor responses mediated by CD4+ TH1 and cytotoxic CD8+ T cells (96-98). Direct effects of IFN-γ signaling on T cell function are also noteworthy, as it promotes the development of an antitumor TH1 phenotype in CD4+ T cells and upregulates expression of granzyme and IL-2 receptor on CD8+ T cells, thereby enhancing their cytotoxic potential. Moreover, IFN-γ directly enhances antigen presentation in tumor cells, thereby augmenting tumor immunogenicity (99, 100).

It is crucial to acknowledge that, despite its role as a potent driver of antitumor immunity, IFN-γ also contributes to immune evasion by promoting increased expression of immune suppressive molecules such as programmed death-ligand 1 (PD-L1) on both tumor and myeloid cells within the tumor microenvironment (TME). Consequently, IFN-γ can act as a double-edged sword, and its function may be contingent upon its spatial distribution. Further investigation is warranted to fully elucidate the role of IFN-γ in tumor progression and antitumor immunity. Nevertheless, there exists compelling evidence that IFN-γ production by NK cells serves as a major determinant allowing NK cells to play pivotal roles in shaping adaptive immune responses to cancer and disease (101). The interaction between NK cells and dendritic cells (DCs) underscores the profound impact of innate immune cell crosstalk on adaptive immune responses to disease and cancer. DCs, as key orchestrators of T cell immune responses and pivotal antigen-presenting cells (APCs), bridge the innate and adaptive immune systems, rendering them indispensable partners in NK cell-mediated regulation of adaptive immune responses. Based on developmental, phenotypical, and functional criteria, DCs are broadly categorized into conventional type 1 DCs (cDC1s) and conventional type 2 DCs (cDC2s) in both humans and mice.

Traditionally, cDC1s are recognized as pivotal mediators of cellular immunity against intracellular pathogens and cancer due to their specialization in cross-presenting antigens to CD8+ T cells, whereas cDC2s, exhibiting greater heterogeneity, are presumed to be more efficient in inducing CD4+ T cell responses in cancer (102). Extensive literature underscores the intricate relationship between NK cells and DCs. Early studies suggested that NK cells likely play a significant role in shaping DC responses by modulating these cells, either

through direct killing of immature DCs or by inducing their maturation (103, 104). Thus, akin to common themes in immunology, the interaction between NK cells and DCs may elicit appropriate and complete DC function, yet in certain contexts, it may also exert negative regulation on adaptive immune responses. Nonetheless, cumulative evidence provides unequivocal support for a functional linkage between NK cell activation and antitumor adaptive immune responses.

In addition to their established role in regulating dendritic cell (DC) maturation, recent investigations have unveiled a pivotal upstream role for natural killer (NK) cells in orchestrating DC recruitment, retention, and/or survival within the tumor microenvironment (TME) (105, 106). Studies conducted in a transplantable BRAFV600E mouse model of melanoma have demonstrated that NK cells play a central role in producing the chemokines CCL5 and XCL1/2, which serve to recruit conventional type 1 DCs (cDC1s) into the tumor milieu (105). Notably, the NK cell-mediated recruitment of cDC1s to the tumor is observed only in the absence of tumor-derived prostaglandin E2 (PGE2; Ptgs1–/–/Ptgs2–/–), underscoring the acute sensitivity of NK cell production of CCL5 and XCL1/2 and subsequent cDC1 recruitment to immune-suppressive PGE2.

Moreover, NK cells within the TME have been shown to produce the cytokine FLT3LG, crucial for the generation of cDC1s, and levels of NK cells and FLT3LG expression in the tumor correlate positively with increased cDC1 levels in the TME (106). Collectively, these findings underscore the integral role of NK cells in not only regulating DC maturation and subsequent priming of T cells but also in recruiting and sustaining cDC1 levels within the TME. This multifaceted function of NK cells is of paramount importance, given the critical role TME-resident cDC1s play in bolstering protective immune responses against cancer (102, 106, 107). Taken together, these findings underscore the indispensable role of NK cells in coordinating and initiating the adaptive immune response to cancer.

1.5. Role of Tissue-Resident NK Cells in Human Lung Immunity

The human lung mucosal surfaces are frequently exposed to a myriad of microbes, environmental particles, and various antigens. Consequently, the lung is susceptible to both severe acute and chronic inflammatory conditions and malignancies, including allergies, asthma, microbial infections, and cancer, which collectively contribute significantly to morbidity and mortality rates. Tissue-resident lymphocytes positioned proximal to the airways in the lung encounter many of these potentially pathogenic agents and malignant cells. Depending on their functional responses, these lymphocytes are presumed to play pivotal roles in the pathogenesis of numerous diseases mentioned above. Human lung tissue-resident memory CD8+ T (TRM) cells exhibit a distinctive transcriptional profile (19). Moreover, studies have revealed a positive correlation between the frequency of TRM cells in tumors and clinical outcomes in lung cancer (108). Importantly, the potential exploitation of TRM cells is currently being explored as a promising avenue in human cancer immunotherapy (109).

In contrast to tissue-resident memory CD8+ T (TRM) cells, our understanding of tissue-resident NK (Tr-NK) cells in the human lung remains relatively limited. Most of our knowledge about human NK cells is derived from ex vivo studies of NK cells isolated from peripheral blood. These cells have been observed to respond to virus-infected target cells (110), suggesting that Tr-NK cells in the lung could hold clinical relevance in pathological conditions. Notably, approximately 10% of $CD56^{dim}$CD16+ NK cells and 75% of $CD56^{bright}$CD16− NK cells in the human lung express CD69 (111). CD69, known to inhibit the egress of T cells from tissues by interfering with the sphingosine-1-phosphate receptor (S1P1), is considered a hallmark marker of tissue-resident lymphocytes (112). Additionally, the integrins CD49a (α1 integrin) and CD103 (αE integrin) play crucial roles in retaining lymphocytes in tissues, thereby identifying Tr-NK cells (40).

CD49a binds to collagen IV, a primary component of the basement membrane of the blood-gas barrier in the lung but largely absent in the lung parenchyma. CD103, on the other hand, binds to E-cadherin, expressed by epithelial cells lining the alveolar spaces and bronchial

epithelium of the lung, as well as on lung tumors of epithelial origin (113). Distinct tissue-specific expression patterns of CD49a and CD103 have been observed on NK cells in human liver, tonsil, and endometrium, indicating the presence of discrete subsets of Tr-NK cells in each of these organs (26, 114, 115). Recent investigations of CD49a+ NK cells in the human liver have unveiled subsets of Eomes−T-bet+ and Eomes+ T-bet ^{low} NK cells, suggesting potentially distinct lineages of CD49a+ Tr-NK cells within this organ (26, 116). In contrast, our understanding of the nature of human Tr-NK cells in many other tissues, including the lung, remains comparatively limited.

To date, no published studies have specifically documented the distribution or function of tissue-resident NK cells in human lungs. Given the highly vascularized nature of the lungs, one possibility is that a substantial proportion of NK cells isolated from lung tissue are situated within blood vessels rather than in parenchyma. However, NK cells have been successfully isolated from bronchoalveolar lavage (117), and they have been detected in the parenchyma surrounding lung tumors (59), suggesting that they are present, at least to some extent, outside blood vessels. Nonetheless, whether the enrichment of CD69+CD56^{bright} NK cells in lung tumors reflects a preferential recruitment of these cells from the lung parenchyma or a local expansion of immature NK cells within the tumor remains to be determined. Further research is warranted to elucidate the precise localization and functional significance of tissue-resident NK cells in human lungs.

1.5.1. Integrin Dynamics in Lung Resident NK Cells: Implications for Function and Retention

Considering the differential expression of α1 and αE integrins in the lung, we can speculate on their relative roles in NK cells. Unlike T cells in the lung, tissue-resident NK (tr-NK) cells exhibit poor lytic function when challenged with tumor target cells ex vivo but retain their ability to kill virally infected cells (21). α1+αE+ positive cells demonstrate lower cytotoxicity compared to α1+αE− cells, yet both subsets produce similar quantities of TNF (21). This suggests that αE may influence cells away from a cytotoxic phenotype, possibly through its interaction with E-cadherin, although the functional role of CD103 (αE integrin) is less clearly defined compared to CD49a (α1 integrin) (24).

The expression of α1 integrin indicates plasticity, as its expression can be induced by IL-15 (118). Moreover, α1+ lung tr-NK cells exhibit higher levels of perforin, granzyme B, Ki67, and CCL5, markers indicative of enhanced functional capacity, compared to α1-negative cells (21). While blocking CD49a abolishes cell adhesion and migration on collagen IV in vitro, cells fail to migrate on E-cadherin, and CD103 does not contribute to collagen binding or cell migration (24).

These observations raise questions regarding the relative contributions of α1 and αE integrins and how they are regulated. Is α1 integrin internalized but maintained in readily accessible pools for rapid utilization? Does it remain on the cell surface and alter its function in the presence of αE integrin as part of inter-integrin inside-out signaling? Additionally, α1+αE+ TR-NK cells in the lung exhibit reduced expression of L-selectin compared to α1 or αE negative cells. Furthermore, the expression of S1PR5, a receptor for S1P known for inducing egress from lymphoid tissues, is low in these cells. CD69 antagonizes S1PR1 by causing its removal from the cell membrane and internalization, suggesting that the expression of CD69 and absence of S1PR5 may contribute to the retention of these cells in the tissue (119).

2. AIMS

In the context of cancer, the involvement of NK cells exhibits notable variability among distinct cancer types, revealing significant disparities in the composition of tumor-infiltrating NK cell populations, even if, in general, there is a trend toward accumulation of non-cytotoxic $CD56^{bright}$ NK cells.

As such, the recruitment of non-cytotoxic NK cells may represent a critical mechanism of tumor escape from immune invasion. Our group, recently, described the relationship between the distinctive distribution of non-cytotoxic NK cells in tumor tissues and up-regulation of chemotactic factors specific for this subset in tumor tissues, when compared to adjacent non-tumor counterpart. However, limited information is available regarding the possibility that the relative abundance of non-cytotoxic NK cells could also be dependent on an increased overall number of tr-NK cells.

The function of Tr-NK cells in the setting of cancer remains poorly defined. In addition, another important aspect is that, in contrast to the PB-NK cells, the ontogeny of tr-NK cells is poorly defined in cancer. Whether they derive from mature circulating cells or precursors present in the tissues, is yet to be elucidated. Regarding the presence of tr-NK cells in cancer, preliminary observation by our Lab has shown that discrete subsets of tr-NK cells (expressing CD103, CD69, and CXCR6) accumulate in human solid tumors. In NSCLC, we particularly focused on $CD103^{+}$ NK cells, because of their putative specificity toward binding E-cadherin on epithelial tumor cells. Interestingly, $CD103^{+}$ NK cells showed substantial accumulation in cancer tissues, when compared to matched adjacent uninvolved lung tissues and peripheral blood.

Based on this preliminary observation and current knowledge of Tr-NK, my study has been focused on answering the following questions:

1. What are the functional features of tr-NK cells in the setting of NSCLC?
2. What is the origin of tr-NK cells found in human NSCLC?

3. MATERIALS AND METHODS

3.1. Processing of lung tumor tissue specimens and peripheral blood

Samples (neoplastic tissue and adjacent normal lung tissue) were collected from 25 untreated NSCLC patients, who underwent surgical resection of primary lesions and had no other concurrent lung diseases. Tumor stages ranged from stage IA to stage IIIB lung cancer Samples were provided by the Biological Resource Center at IRCCS AOU San Martino-IST-National Cancer Research Institute. The study received approval from the Institutional Ethics Committee of the same institution, and relevant Institutional Review Boards also granted approval. All patients gave written informed consent in accordance with the Declaration of Helsinki.

Samples were collected immediately after surgical procedures. The tumor tissues were thoroughly washed with PBS to eliminate cell debris and red blood cell clusters before their weight was measured. To process, biopsies were minced using scissors, then digested in a digestion solution containing 1mg/mL Collagenase IV (Worthington) and 20 μg/mL DNAse I (Roche) at 37° C with constant shaking. After 90 minutes, digested tissues were then pipetted and cell suspensions filtered through a 70 μm cell strainer to remove large pieces of tissue. The suspensions were then separated by centrifugation on a Ficoll-Hypaque density gradient to isolate mononuclear cells (MNCs). Cells were immediately used for experiments and/or cryopreserved for future usage.

PBMCs were isolated from the buffy coats of 25 healthy donors. The buffy coats were obtained from whole blood donations provided by healthy volunteer donors, generously supplied by the transfusion section of San Martino Hospital in Genoa, Italy. PBMCs were isolated by a density gradient with Ficoll-Hypaque. Depending on purpose, NK cells from tumor and blood were enriched using the MACS untouched NK cell isolation kit (Miltenyi Biotec) or RosetteSep™ Immunodensity Cell Separation (STEMCELL Technologie),according to manufacturers' instructions.

3.2. Flow cytometry.

Antibodies and clones specific for the following proteins were used: CD45 APC-AF750 (clone: J33), CD56 APC (clone: N901) and CD103 FITC (clone: 2G5) all from Beckman Coulter, CD107a eFluor660 (clone: eBioH4A3) and TNF.α eFluor 450 (clone: Mab11) from eBioscience , CD3 APC-R700 (clone: UCHT1), CD69 PE-CF594 (clone: FN50), GM-CSF PE (clone: BVD2-21C11), IFN.γ BB700 (clone: B27), CXCR6 BV421 (clone: 13B1E5), DNAM-1 AF647 (clone: DX11), TIGIT BB700 (clone: 741182), CD49e PE (clone: IIA1) all from BD Biosciences. For indirect immunofluorescence assay: XCL1/2 (clone: E9D3H, Cell signaling), FLT3L (clone: EP1140Y, abcam), anti-CCR4 (clone:24006, R&D System) were used. After incubation with the relevant mAbs, cells were washed, and AF647-conjugated isotype specific goat anti-mouse/rabbit mABs were added and incubated for 30 min at 4°C. Cells were then washed and analyzed by flow cytometry. Samples were then run by Gallios (BeckmanCoulter) cytometers and analyzed by FlowJo 10 software.

3.3. Expansion of tumor-derived CD103+ and CD103- NK cells

NK cells enriched from MNCs derived from neoplastic tissues were sorted with a BD FACSAria III sorter to isolate $CD103^{+}$ and CD103neg NK cells. Part of them were immediately used for analysis, while part of them were seeded in a 96-well round-bottom plate in the presence of IL-15(20 ng/mL) for 10 days. On days 3,6 and 9, half of the culture medium was replenished with fresh medium.

3.4. Degranulation and cytokine expression assay with resting and cultured NK cells

Both resting and cultured sorted CD103+ and CD103neg NK cells, as well as NK cells from blood (PB-NK) were used as effectors in a CD107a assay; the K562 cell line was used as target. To detect spontaneous degranulation, a control sample without target cells was included and an E:T ratio of 2:1. Anti-CD107a mAb was added in each well before co-culture. Effectors and targets were then co-incubated at 37°C for 6 h. After the first hour, Monensin and Brefeldin A (BD bioscience) were added to inhibit cell secretion. At the end

of incubation, cells were first stained for relevant surface markers followed by fixation and permeabilization with Cytofix/Cytoperm (BD Pharmingen) for intracellular detection of IFN-γ, TNF-α, GM-CSF, CCL4, CCL5, XCL1/2 and FLT3L expression by specific mAbs. In addition, to measure maximal functional capability, resting and cultured sorted CD103+ and CD103negNK cells were stimulated with PMA (10ng/ml) and Ionomycin (500ng/ml).

3.5. Soluble factors detection assay

In order to measure the release of factors previously detected by flow cytometry, supernatants derived from tumor-infiltrating NK cells and blood NK cells were analyzed by multiplex magnetic assay (MAGPIX instrument, Luminex). Briefly, sorted resting CD103+ and CD103- tumor-infiltrating NK cells, along with sorted resting $CD56^{bright}$ and $CD56^{dim}$ NK cells from blood were used as effectors in soluble factors release assay. The cells were stimulated overnight with the K562 cell line at an E ratio of 1:1, or for 6 hours with PMA (10 ng/ml) and Ionomycin (500 ng/ml). A control sample without target cells or PMA/ionomycin was also included to measure overnight spontaneous release. Collectively, supernatants derived from the following culture conditions: 1) NK cells only; 2) NK cells and K562; 3) NK cells and PMAi were analyzed, in duplicates, using a custom, commercially available multiplex magnetic assay kit per manufacturer instructions (Milliplex®; MilliporeSigma). Factors of interest included IFN- γ, TNF-α, GM-CSF, CCL4, CCL5, XCL1/2, FLT3L, IL-16 and VEGF. Plates were analyzed on the MAGPIX instrument (Luminex) following manufacturer instructions. Minimum detectable concentrations for each cytokine were: IFN-γ: 0.86 pg/mL; TNF-α: 5.39 pg/mL, GM-CSF: 1.55 pg/mL, CCL4: 0.37 pg/mL, CCL5: 1.58 pg/mL, FLT3L: 0.84 pg/mL and VEGF: 0.98 pg/mL. Concentrations were derived from standard curves set to a 5-parameter (log scale) curve fit.

3.6. Lung Spheroid infiltration assay

In order to explore the differential infiltration abilities of blood-derived NK cell subsets, we established a co-culture of NK cells with three-dimensional multicellular tumor “spheroids” derived from NSCLC cell lines (SW900 cell line). SW900 spheroids were generated in

flatbottom 6-well plates (Ultra-Low attachment multiwell plates, Corning®Costar®, NY, USA) with DMEM-F12 (BioWhittaker®Reagents, Lonza) in serum-free medium (SFM), supplemented with EGF (Peprotech Europe, London UK) at 10ng/ ml final concentration. Spheroids were monitored over time for dimension (perimeter, area and volume) measured using ^{bright} field inverted microscope. SW900 cells were labeled with PKH-67 (green) dye prior to the start of the spheroid generation. At day 7 of spheroids formation, blood NK cells were sorted into $CD56^{bright}$ and $CD56^{dim}$ cells, then labeled with PKH-26 (red) dye and added to the spheroids in stem cell medium supplemented with IL-15 (1ng), at an E:T ratio of 4:1 (4 NK cells: 1 tumor cell) in ultra-low attachment 24-well plates and cultured for up to 5 days. Fluorescence imaging was conducted using Two-photon excitation microscopy. z-stack images were captured from the surface up to the center of spheroids using a two-photon microscope with acousto-optic deflector technology (Femtonics 3d Atlas) and 950 nm or 750 nm as excitation wavelengths (coherent chameleon ultra II laser) and a Nikon 16X IR water immersion objective.

Similar co-cultures were also established to investigate the phenotype of NK cell subsets after infiltration in spheroids to this purpose, after co-culture, tumor spheroids co-cultured with sorted NK cell subsets were harvested and separated from the supernatant containing the surrounding NK cells and residual target cells by a 100µm strainer followed by gravity sedimentation for 5 min in room temperature. Following two washing steps in a volume of 15 ml PBS/2% FCS, at 1500rpm for 1 min, tumor spheroids were dissociated by Gentle Cell Dissociation Reagent for 5 min. Cell suspension was then washed and finally stained for FACS analysis with the following mAbs.

3.7. Statistical analysis

A Tukey's multiple comparison test on a one-way ANOVA was performed to analyze data from difference between tr-NK cells and PB-NK. For the statistical analyses of cytokines protein expression data and NK cell subsets in lung tumor tissues, we performed a paired t test analysis using the GraphPad Prism V10.1.0 (GraphPad Software). A p-value of < 0.05 was considered statistically significant.

4. RESULTS

4.1. CD103+ NK cells represent a distinct subset of tumor-infiltrating NK cells characterized by tissue-resident features

The understanding of natural killer (NK) cells within human peripheral tissues is rapidly advancing. However, a full and detailed understanding of tissue-resident NK cells is still in its early stages, and several key questions and future challenges remain unresolved. Similar to the comprehensive characterization of tissue-resident T cells, there is a need for precise and systematic mapping of NK cells across peripheral tissues. This should focus on the phenotypic variability among NK cells in different tissues and whether markers such as CD103 can be used to reliably identify tissue-resident NK cells.

In addition to NK cells found in peripheral blood, the human lungs appear to contain the highest proportion of highly differentiated NK cells, though a smaller subset exhibits characteristics consistent with tissue-resident NK cells. However, it is already described that NK cells from neoplastic tissues differ from their normal counterpart by being enriched in non-cytotoxic NK cells. Whether this accumulation is associated with higher level of tr-NK cells is not completely understood. In order to analyses the potential heterogeneity of NK cell compartment in NSCLC, and the presence of CD103+ cells, we set out a 10-color flow cytometry panel able to identify distinct subsets of NK cells. NK cells were identified by gating on CD56+ cells among viable CD45 +CD3- CD19- lymphocytes (Figure 1A). Unlike peripheral blood NK (PB-NK) cells, most of NK cells in human lung tumors did not express CD16, which aligns with a non-cytotoxic phenotype (Figure 1B).

To further investigate the phenotypic traits of non-cytotoxic NK cells infiltrating tumors, we examined a panel of markers able to distinguish circulating from tissue-resident NK cells. In particular, we focused on chemokine receptors (CCR7 and CXCR6), integrins (CD103 and CD49e), and NK cell activating receptors (CD69). CXCR6, CD103 and CD69 are considered hallmark markers of tissue resident lymphocytes while CCR7 and CD49e represent a circulating phenotype. The Integrin alpha 5 subunit, also called CD49e, associates with the Integrin beta 1 subunit (CD29) to form the VLA-5 complex. It functions as a receptor for

fibronectin. CCR7 binds to CCL19 and CCL 21 and is a well-known chemotactic receptor that directs adaptive and innate immune cells to secondary lymphoid tissue. CXCR6 is a receptor for the chemokine CXCL16, which exists as a membrane or soluble form. CXCR6 is a marker for resident memory T (TRM) cells that plays a role in immunosurveillance through their interaction with epithelial cells

Most CD16− NK cells in human lung tumors expressed CD69. CD69 has been demonstrated to inhibit the egress of T cells from tissues by interfering with the sphingosine-1-phosphate receptor (S1P1) and is therefore considered as a tissue residency marker. To identify additional markers of tissue residency on lung CD69+ NK cells, we analyzed the expression of chemokine receptors (CCR7 and CXCR6) and integrins (CD103 and CD49e). These markers were significantly expressed on CD16−CD69+ NK cells but absent from CD16+ NK cells (Figure 1B). Interestingly, CD103, CXCR6, and CD69 were frequently co-expressed, although a smaller subset of CD103−CD49e+CCR7+CD69+ NK cells was also observed (Figure 1B).

The heterogeneity observed among these NK cells likely results from organ-specific factors, including the local microenvironment, which feature unique soluble factors, parenchymal cells, and other immune cell populations. The structural diversity of the lung, with its complex micro-architecture, may also play a role in shaping tissue accessibility and the development of a tissue-resident NK cell phenotype. As CD103+CD8+ tissue-resident memory (TRM) cells are often found near or within the lung epithelium where CD103 ligands (such as E-cadherin) are expressed, it is plausible that CD103+ NK cells reside in an intraepithelial location. Collectively, we have observed that a significant subset of primarily CD16− NK cells exhibit the CD103+CD69+CXCR6+ phenotype, potentially representing a specific population of tissue-resident NK cells within human lung tumors.

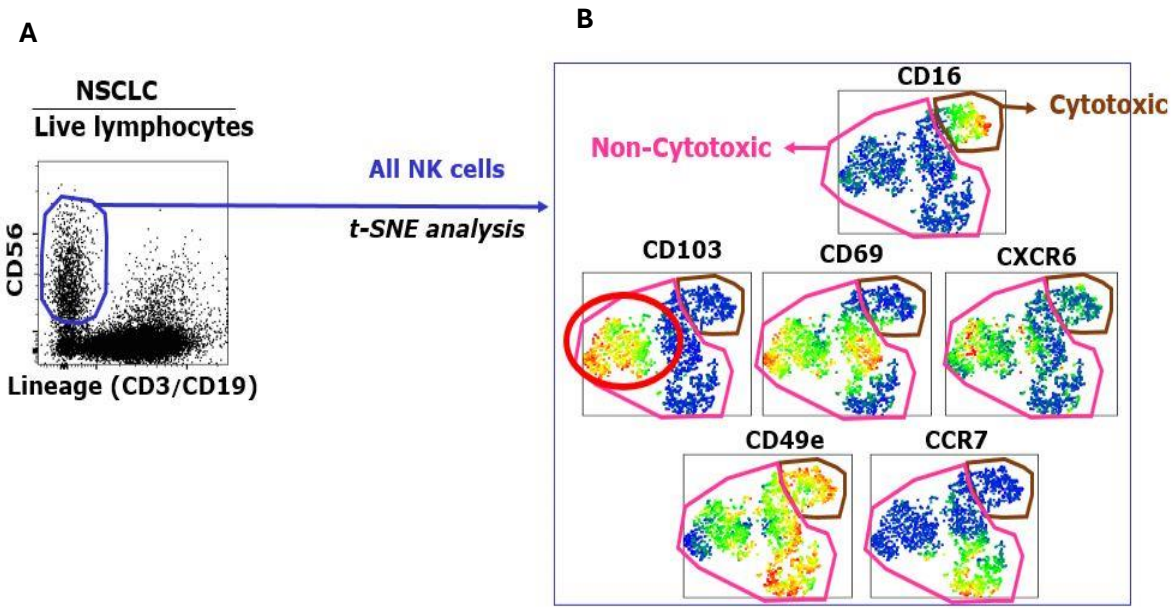


Figure 1. CD103 as a Key Marker for Identifying Tissue-Resident NK Cells in Human Lung Tumors. (A) Gating strategy to identify NK cells in a single-cell suspension from lung tumor tissue. (B) t-SNE plots showing the expression of tissue residency and circulating markers on CD16+ or— and CD16^{-} tumor infiltrating NK cells

4.2. CD103+ Tumor infiltrating NK (TINK) cells exhibit diminished responsiveness toward target cells.

Given the distinctive accumulation of CD103+ NK cells in tumor tissues compared to matched normal lung tissues, we endeavored to conduct a thorough analysis of their functional capabilities. Our focus was on evaluating the function of unstimulated CD103+ tr-NK cells isolated from the TME through in vitro experiments utilizing tumor cells as targets. We found that CD103+ NK cells displayed restricted responsiveness towards K562 cells, a prototypic tumor target cell line lacking HLA class I expression, as indicated by the CD107a-degranulation assay. Similar findings were observed for CD103neg NK cells. In contrast, peripheral blood-derived NK cells exhibited higher degranulation when exposed to the same target (Figure 2A).

Moreover, under identical stimulation, resting CD103+ NK cells showed minimal production of IFN-γ, TNF-α, and GM-CSF, consistent with the trend observed in resting CD103neg NK cells.

Conversely, despite being impaired in the secretion of pro-inflammatory cytokines, they exhibited consistent secretion levels of CCL4 and XCL1/2 in response to tumor cells (Figure 2A). As expected, peripheral blood-derived NK cells displayed higher levels of CCL4 compared to TINK cells, even if among the two TINK subsets, CD103+ had a trend toward higher production than the CD103- counterpart. (Figure 2B). With regard to XCL1/2, no significant differences were observed between blood NK cells and TINK subsets.

These results suggest that resting TINK, particularly CD103+ NK cells isolated from tumor tissues, exhibit limited pro-inflammatory responsiveness against tumor cells, but still maintaining capacity to release chemiotactic factors for other leukocytes, such as macrophages and DCs.

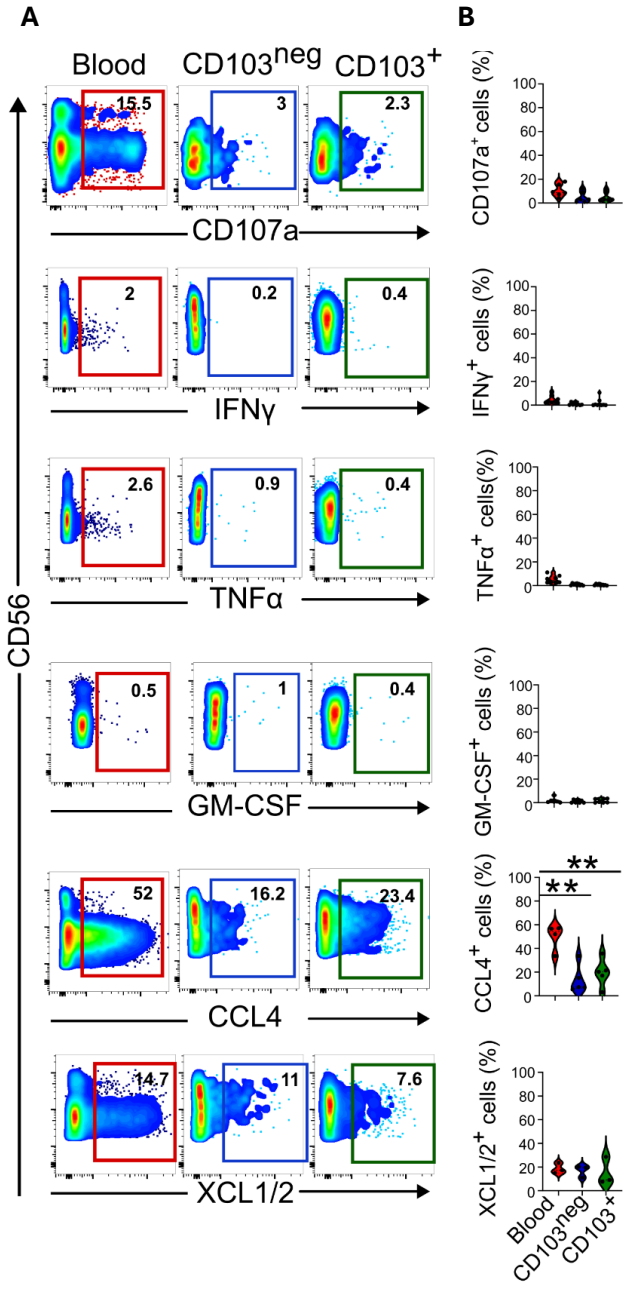


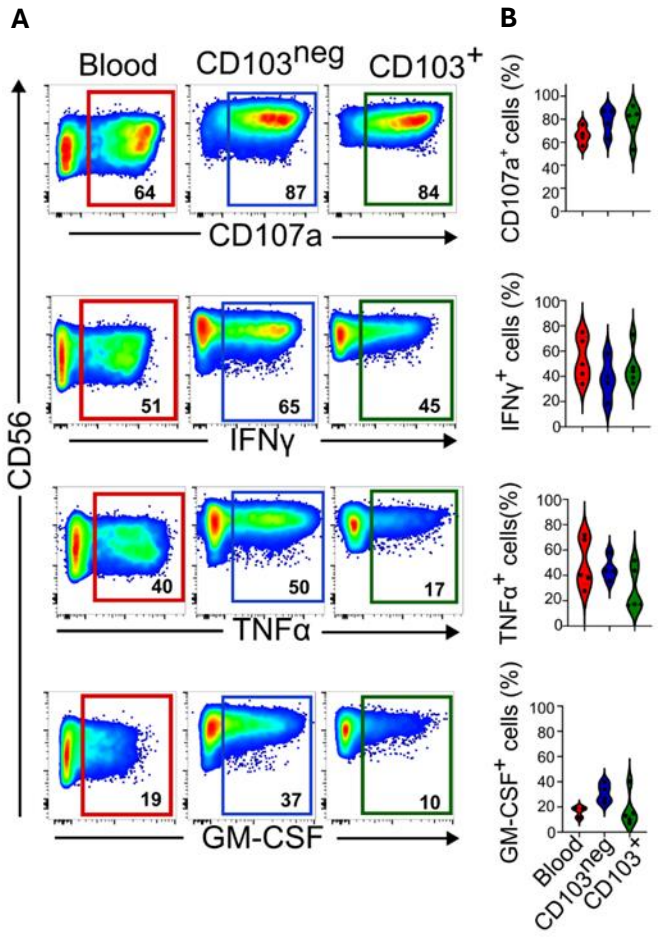
Figure 2. NK cells derived from human lung tumors are hypofunctional, even after exposure to tumor cells. (A) Representative pseudocolor plots of degranulation and cytokines/chemokines production by NK cells freshly isolated from PBMCs and lung tumor tissue. The latter further sorted in CD103+ and CD103- cells Samples were stimulated with K562 cells. (B) Summary of data showing degranulation, IFN-γ, TNF-α, GM-CSF, CCL4 and XCL1/2 production in PB-NK cells and TINK cells in the presence of tumor cells. *P < .05 and **P < .01.

4.3. CD103+ TINK cells can be rescued by IL-15 treatment.

IL-15 is a cytokine crucial for development, maintenance and function of NK cells and TRM cells. It is produced by dendritic cells, epithelial cells, and alveolar macrophages following inflammation in the lung. Given the importance of IL-15 for NK cell and TRM cell function, as well as the potential importance of tr-NK cells in inflammatory responses in the lung, we also analyzed different subsets of NK cells in human lung with respect to responses to IL-15.

To investigate whether IL-15 stimulation could rescue the functional capabilities of CD103+ NK cells we also performed a stimulation of PB-NK cells and sorted CD103+ and CD103 – lung tumor NK cell subsets with IL-15 for 10 days and then employing them in co-culture experiments against K562. This stimulation resulted in an increased degranulation and higher production of IFN -γ, TNF-α, and GM-CSF by CD103+ NK cells compared to their resting counterparts. However, CD103+ NK cells still exhibited the same level of IFN-γ, TNF-α, and GM-CSF compared to $CD103^{neg}$ NK cells and PB-NK cells (Figure 3A and B). Taken together, the present analysis of CD103+ NK cells reveals that they are intrinsically functional and furthermore are able to upregulate molecules associated with effector functions upon stimulation with IL-15.

Figure 3. Hyporesponsive TINK cells could be rescued by IL-15 stimulation. (A) Representative pseudocolor plots of degranulation and cytokines production by NK cells after 10 days incubation in medium containing IL-15 (20ng/ml). (B) Summary of data showing degranulation, IFN-γ, TNF-α, and GM-CSF production after IL-15 treatment.



4.4. Tumor infiltrating CD103+ NK cells can produce Dendritic cells chemoattractant.

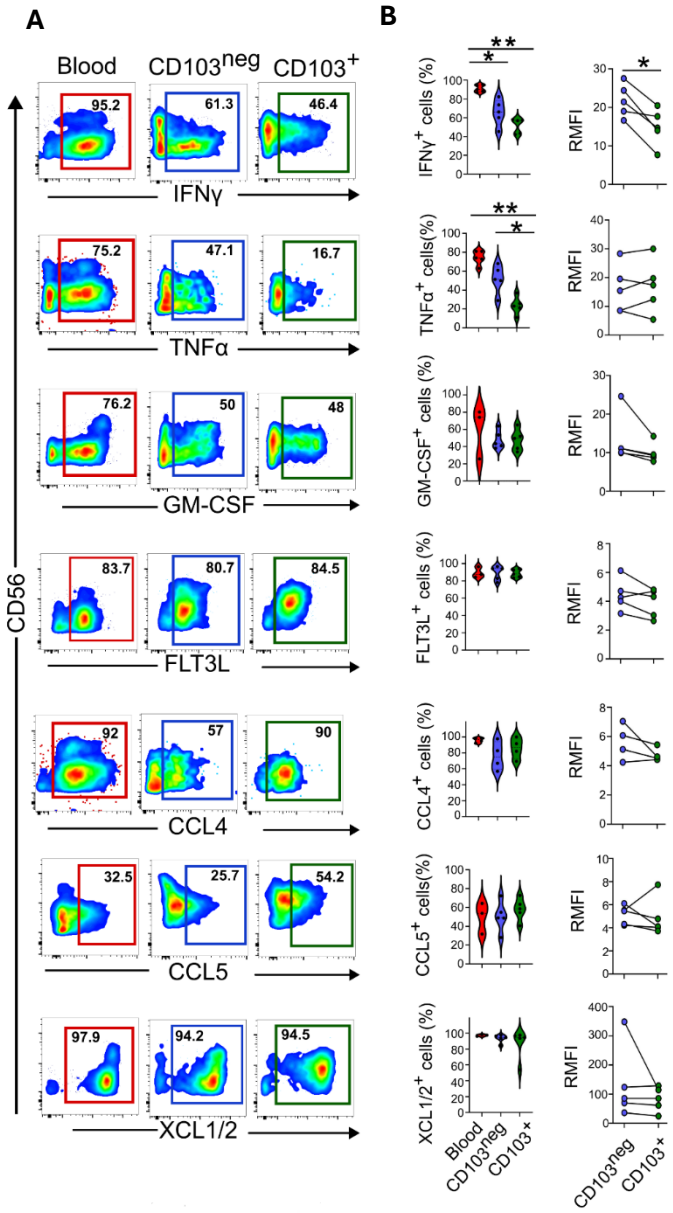
Given the distinctive enrichment of CD103+ NK cells within TME, we sought to explore their functional characteristics further. We have shown that human lung NK cells are hypofunctional in response to physiological stimulation, i.e. tumor cells. However, we do not know whether this is due to an impaired capability of recognizing tumor cells, thus we here set out to extend this functional analysis to analyze the maximal potential of CD103+ tissue-resident NK (tr-NK) cells within the tumor microenvironment (TME). Therefore, we stimulated intertumoral NK cells with phorbol 12-myristate 13-acetate (PMA) and ionomycin and analyzed intracytoplasmic expression of IFN-γ, TNF-α, GM-CSF, CCL5, CCL4, FLT3L and CXCL1/2 in tr-NK cells and PB-NK cells using flowcytometry staining.

Interestingly, not all factors required PMAi for activation. Indeed, after parallel testing with different stimulation conditions (i.e. PMAi, tumor targets, no stimulation), we observed as some factors were even downregulated in the presence of these stimulations. This is the case of CCL5 and FLT3L, which were constitutively present and detected in the cytoplasm at higher levels without stimulation. Notably, CD103+ NK cells expressed higher levels of CCL5 in comparison to CD103- and PB-NK cells (Figure 4A), suggesting a specific role for CD103+ tissue-resident NK (tr-NK) cells in production of CCL5 (RANTES) in the human lung tumor. Regarding pro-inflammatory cytokines, consistent with previous data, even with PMAi, did not allow strong production, as resting CD103+ NK cells produced lower levels of IFN-γ, TNF-α, than CD103-, but similar levels of GM-CSF. Notably, all subsets exhibited maximal production of CCL4, XCL1/2, and FLT3L chemokines (Figure 4A). These findings indicate that resting tumor-infiltrating NK (TINK) cells have selective reduced responsiveness to PMAi compared to circulating NK cells (Figure 4B). Regarding IFN-γ, CD103+ NK cells not only had a lower frequency of positive cells but also produced significantly less cytokine, as measured by the ratio of mean fluorescent intensity (MFI) of IFN-γ^+ to IFN-γ- cells in CD103+ and matched CD103- NK cells (Figure 4C). Overall, TINK subsets produced lower levels of cytokines than PB)-derived NK cells.

C

Figure 4. The functional capacity of human lung tumor infiltrating NK cells (TINKs) is largely compromised, even in response to PMAi stimulation.

(A) Representative pseudocolor plots illustrating factors production by NK cells isolated from both PBMCs and lung tumor tissues. Samples were cultured in medium supplemented with PMAi. (B) Summary of frequencies of cytokines producing PB-NK cells and CD103+ and CD103- TINK cells. (C) Summary data of factors production by CD103+ and CD103- TINK cells. *P < .05, **P < .01



4.5. Functional Heterogeneity in Intratumoral NK Cells: X-shift Reveals Distinct Subsets with Impaired APC Support Potential

To enhance the assessment of dysfunctionality in CD103+ cells and to explore distinct functional states within intratumoral NK cells beyond conventional methodologies, we undertook a reanalysis of data utilizing automated clustering techniques (specifically X-shift). Our focus was on discerning the expression patterns of critical factors for DC recruitment and stimulation, such as GM-CSF, IFN-g, and XCL1/2 within intratumoral NK cells sourced from three different patients, with peripheral blood (PB) NK cells from three HD serving as controls (Figure 5A and B).

The application of X-shift clustering revealed the identification of eight distinct clusters. Notably, seven of these clusters (1, 2, 4, 5, 6, 7, 8) exhibited the expression XCL1/2, aligning with the widespread presence of these factors in NK cells. Cluster 4 notably demonstrated strong expression of all factors. Conversely, cluster 3 emerged as a distinct group devoid of all three factors, exhibiting preferential expression in intratumorally NK cells (Figure 5A and B).

Consistent with prior knowledge regarding PB NK cell subsets, PB-CD56^{bright} NK cells demonstrated enrichment in clusters 4 and 6, exhibiting varying levels of expression for all three factors, whereas PB-CD56^{dim} cells were composed of clusters 7 and 8, expressing XCL1/2 and IFN-g but lacking GM-CSF expression (Figure 5C).

Upon comparison between CD103+ NK cells and PB-CD56^{bright} NK cells, we observed a significant increase in cluster 3 (lacking all factors) alongside increases in clusters 2, 5, and 8, which express XCL1/2 but exhibit negative or low levels of IFN-g and/or GM-CSF. Additionally, CD103+ NK cells displayed a marked decrease in cluster 4. Comparing CD103+ to CD103neg NK cells revealed a substantial increase in cluster 2, couple with the disappearance of cluster 7 and a decrease in cluster 8, indicating impaired IFN-g secretion when intratumorally NK cells adopt CD103+ characteristics (Figure 5C). These findings underscore the influence of the tumor microenvironment on intratumorally CD103+ NK cells, suggesting potential deficits in supporting antigen-presenting cell (APC) maturation.

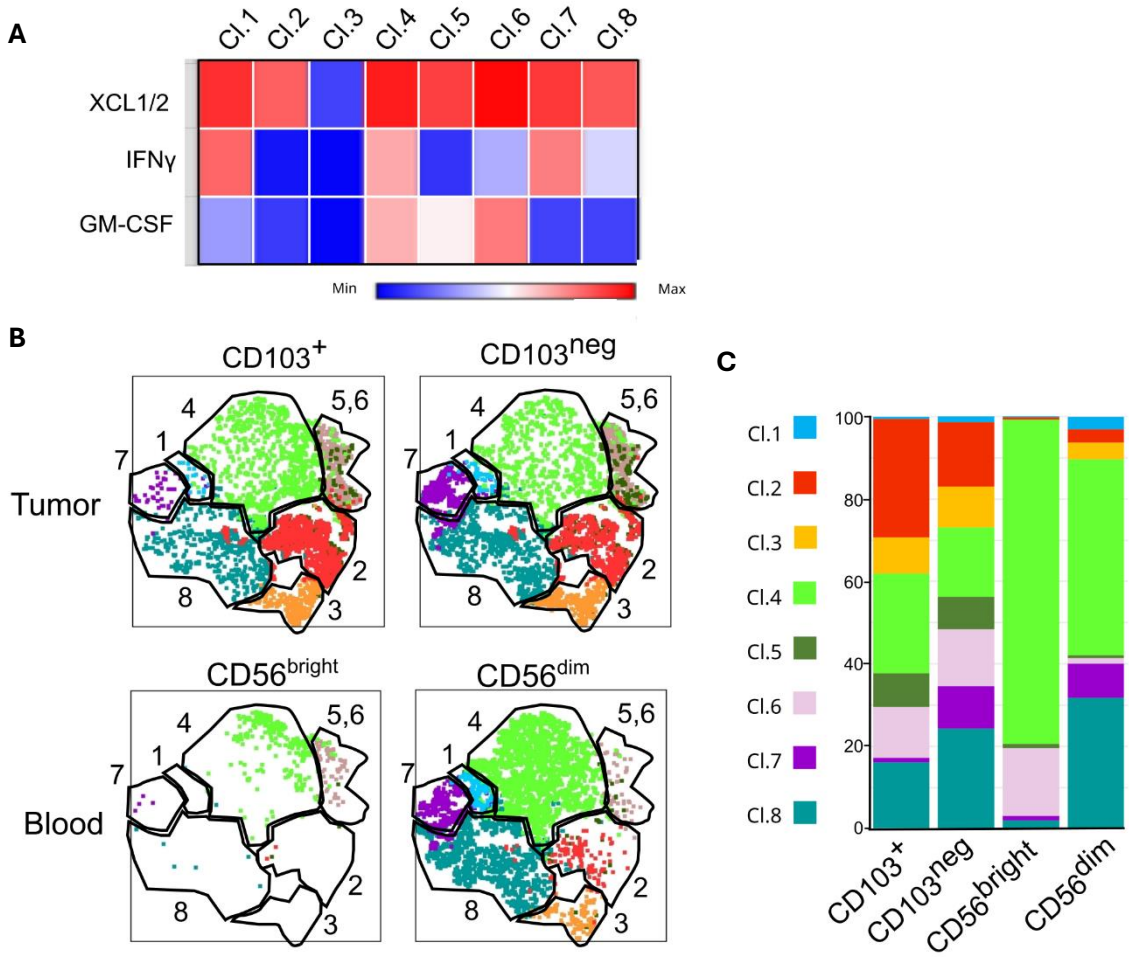


Figure 5. Insights into cytokine co-expression and functional diversity in TME revealed by tSNE analysis. (A) Heat map summary of co-expression of XCL1/2, IFN-γ, and GM-CSF by different clusters. (B) tSNE projections identifying the different TINK/PB-NK clusters and(C) frequency of each cluster in the different cell subsets.

4.6. Tumor-infiltrating NK Cells: soluble factor resource in the early stages of tumor development

Studies conducted in a mouse model of melanoma have demonstrated that NK cells play a central role in producing the chemokines CCL5 and XCL1/2, which serve to recruit conventional type 1 DCs (cDC1s) into the tumor milieu(105). Moreover, NK cells within the TME have been shown in mouse model to produce the cytokine FLT3LG, crucial for the generation of cDC1s, and levels of NK cells and FLT3LG expression in the tumor correlate positively with increased cDC1 levels in the TME(106). Collectively, these findings underscore the integral role of NK cells not only regulating DC maturation and subsequent priming of T cells, but also in recruiting and sustaining cDC1 levels within the TME. So, we hypothesized that human tr-NK cells could be able to release these cytokines toward different stimuli.

As previously shown (Fig. 2 and 3), by flow cytometry we observed expression of some of these factors, and, in particular, it seemed that some of them were poorly released upon target stimulation, but only after PMAi. Conversely, other factors were constitutively expressed and/or released at high level upon interaction with tumor target cells. In order to verify the actual release of relevant factors we analyzed supernatants derived from different culture conditions. Samples were analyzed using a custom, commercially available multiplex magnetic assay kit per manufacturer instructions (Milliplex®; Millipore Sigma). Samples were analyzed on the MAGPIX instrument (Luminex) to quantify IFN-γ, TNF-α, GM-CSF, FLT3L, CCL4 and CCL5. To recapitulate data obtained by flow cytometry, we sorted CD103+ and CD103- tumor-infiltrating NK (TINK) cells, along with $CD56^{bright}$ and $CD56^{dim}$ NK cells from peripheral blood, then we cultured these cells alone, with target cells (at a 1:1 ratio), or stimulated using PMA and ionomycin (PMAi).

Significant increases in the secretion of IFN-γ, TNF-α, GM-CSF, CCL5 and CCL4 were observed when NK cells were cultured with K562 cells or stimulated with PMAi, compared to NK cells cultured alone (Figure 6). Notably, the secretion of IFN-γ, TNF-α, GM-CSF, and CCL4 were highest during stimulation with PMAi, when compared to either co-culture with

K562 cells (Figure 6). Interestingly, FLT3L release did not show any measurable increase in response to either K562 or PMAi stimulation.

In line with our previous FACS analysis, NK cells isolated from lung tumor biopsies (CD103+ and CD103-) demonstrated lower cytokine secretion capacity than peripheral blood NK cells ($CD56^{bright}$ and $CD56^{dim}$), indicating reduced functional activity in the tumor microenvironment.

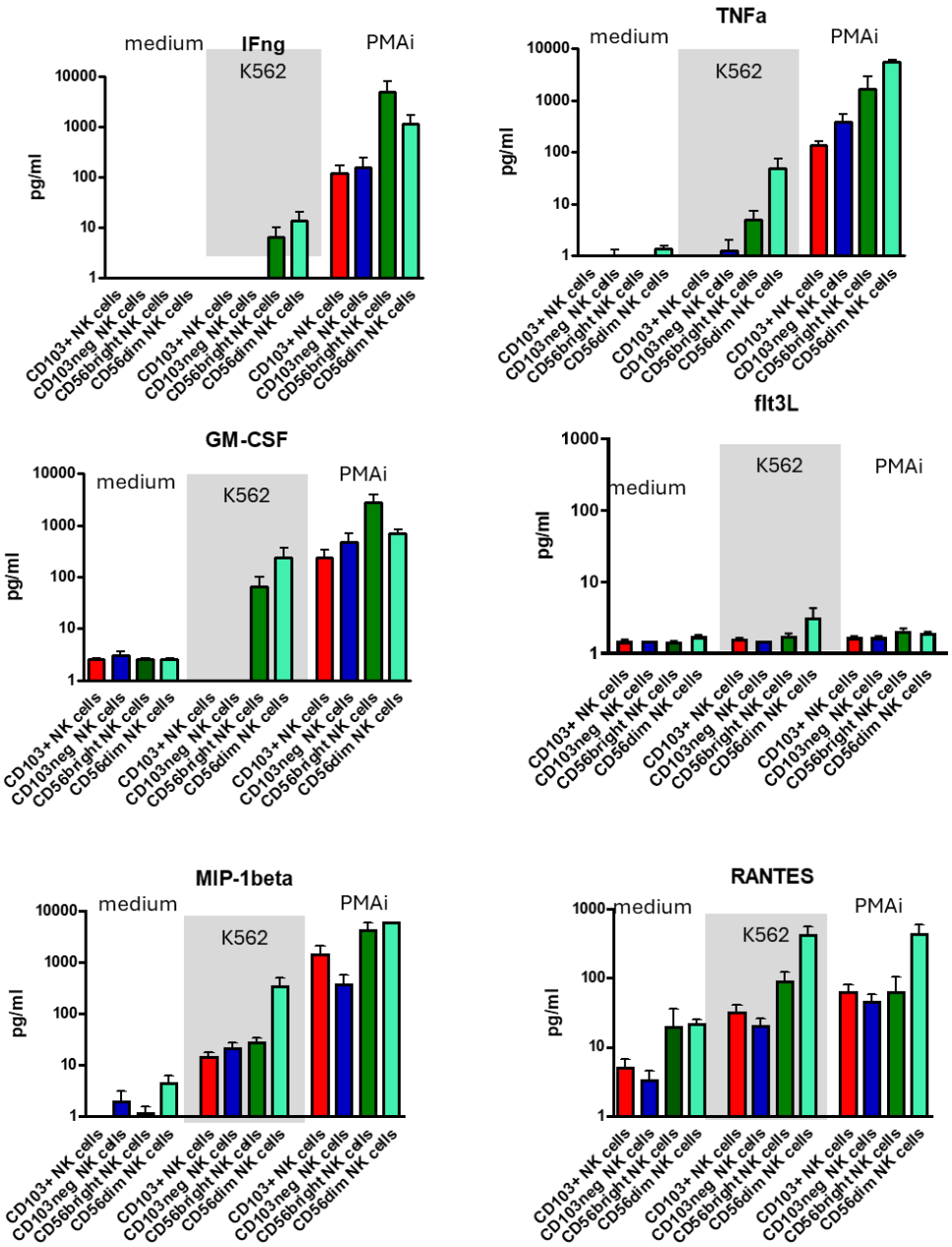


Figure 6. Quantification of soluble factors secretion by NK cells in response to different stimuli. Summary plots of MAGPIX data depicting the concentrations of IFN-γ, TNF-α, GM-CSF, FLT3L, CCL4 and CCL5 in the supernatants of NK cells under different conditions: NK cells cultured alone for 18 hours, NK cells stimulated with PMAi for 6 hours, or NK cells co-cultured with K562 cells for 18 hours.

4.7. CD49e and CD103 identify putative stages of tr-NK cell development in tumor

As previously discussed, a primary focus of our study is the ontogeny of tissue-resident natural killer (tr-NK) cells. We hypothesize that CD103+ NK cells may originate from CD103- NK cells, which are recruited from peripheral blood under tumor microenvironment (TME) stimuli, with transforming growth factor-beta (TGF-β) playing a critical role in this process. To support this hypothesis, we analyzed the expression of CD49e in tumor-infiltrating NK cells, with a specific focus on the CD103+ NK cell subset, using flow cytometry (Fig. 7A). CD49e serves as a marker predominantly expressed by circulating NK cells, with higher expression in the $CD56^{bright}$ compared to the $CD56^{dim}$ subset, but negative/downregulated in tr-NK cells(120) .

Our analysis revealed that the CD103+ NK cell population is not entirely negative for CD49e expression. Rather, it includes cells with intermediate levels of CD49e expression, as well as a negative subset. In contrast, the majority of the remaining NK cells (~60%) were identified as CD49e+CD103-, suggesting they may represent recently recruited NK cells from the circulation, with a minor contamination from true peripheral blood cells. Based on the observed CD49e and CD103 expression patterns in both intratumoral and circulating NK cells, we propose a model of progressive conversion from circulating to tissue-resident NK cells. Specifically, this trajectory appears to involve a transition from $CD49e^{+}$CD103- NK cells through an intermediate phase, culminating in the appearance of CD103+CD49e- NK cells (Figure 7B).

To further validate this hypothesis, we assessed the expression of additional markers on three NK cell populations: CD103-CD49e+, CD103+$CD49e^{int}$, and CD103+CD49e-. These markers included both tissue-residence markers (CXCR6 and TIGIT) and circulating NK cell markers (CCR7 and DNAM1), providing insight into the potential conversion of circulating NK cells into tissue-resident cells (Figure 7C). Flow cytometric analysis of these markers revealed a pattern consistent with our hypothesis. CD49e+CD103- cells, which are closely related to circulating NK cells, exhibited high CCR7 expression and low CXCR6 expression. As cells transitioned to the CD103+$CD49e^{int}$ population, CCR7 expression decreased while CXCR6 expression progressively increased, reaching its highest levels in the

CD103+CD49e- population. A similar trend was observed for DNAM1 and TIGIT. Specifically, CD49e+CD103- cells displayed high DNAM1 and low TIGIT expression, while the CD103+CD49e^{int} and CD103+CD49e- populations demonstrated a marked decline in DNAM1 and a corresponding increase in TIGIT (Figure 7C). Although our analysis involved a limited number of markers, the results support the notion that tumor-infiltrating NK cells undergo a conversion process. These cells likely originate from circulation, extravasate into the tissue, and subsequently acquire a tissue-resident phenotype.

An additional question regarding the biology of CD103+ NK cells pertain to their potential for expansive growth within the tumor microenvironment (TME). Specifically, we sought to determine whether the CD103+ NK cell population arises from the proliferation of non-tissue resident NK cells at the tumor site. To address this question, we evaluated the expression of Ki67, an intranuclear marker associated with cell proliferation, which is widely utilized in tumor diagnostics to identify proliferating cells. Recent advancements have also enabled its application in laboratory settings, where intranuclear staining can highlight the proliferative component within a cell population.

In this instance, we employed flow cytometry to perform surface marker staining in conjunction with intranuclear staining to identify the Ki67 marker. The data obtained from this analysis are presented in Figure 7D. Relating to the hypothesis of NK cell conversion from circulating to tumor-infiltrating cells, we observed a significant percentage of Ki67+ cells within the CD103+CD49e- population, indicating a high level of proliferation. Additionally, elevated Ki67 levels were also detected in the intermediate CD103+CD49e^{int} population, further supporting the notion of proliferation and derivation from tissue resident cells.

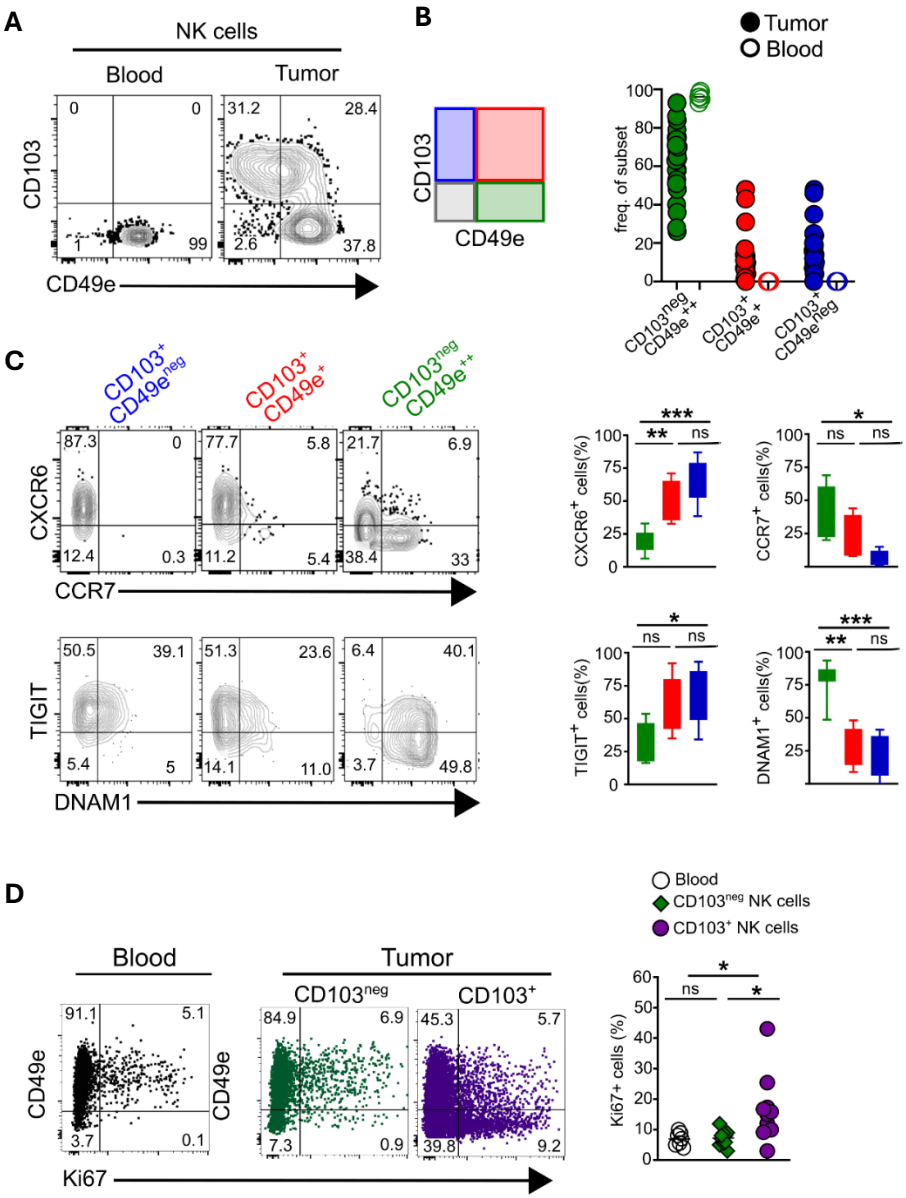


Figure 7. CD103+ NK cell populations in tumor tissue exhibit markers of tissue residency and proliferation. (A) Representative flow plots of CD49e and CD103 expression by NK cells freshly isolated from PBMCs and lung tumor tissue. (B) Subsets: CD49e+CD103- (green), CD49e+CD103+ (red), and CD49e-CD103+ (blue). Frequencies of CD49e+CD103-, CD49e+CD103+ and CD49e-CD103+ cells among NK cells in lung tumor tissue (solid circles) and peripheral blood (open circles) . (C) Representative flow plots and data summary of circulating and tissue residency surface marker expression on NK cells from lung tumor tissue in CD49e+CD103- (green), CD49e+CD103+ (red), and CD49e-CD103+ (blue). (D) Representative dot plot and summary of data of differential expression level of ki67 between fresh total blood and tumor CD103neg or CD103+ NK cells. *P < .05, **P < .01, and ***P < .001.

4.8. Peripheral blood CD56^{bright} NK cells efficiently adhere and infiltrate tumor spheroids.

We hypothesized that the two circulating NK cell subsets (CD56^{bright} and CD56^{dim}) may exhibit distinct capabilities in infiltrating tumor masses. To investigate this aspect, we set out experiments mimicking NK cell infiltration into tumor spheroids. NK cell subsets were co-cultured solid tumor spheroids grown from SW900 cells at effector-to-target (4:1) ratios in the presence of 1 ng/ml of IL-15, for 5 days. Solid tumor spheroids grown in the absence of NK cells served as negative control. Through Two-photon excitation microscopy, we observed that CD56^{bright} NK cells accumulated and infiltrated into the center of the tumor spheroid, as evidenced by representative images captured at 12 different Z planes, indicating their localization within the spheroid (Figure 8). Conversely, CD56^{dim} NK cells adhered poorly and did not infiltrate spheroids, at least day 5 of co-culture.

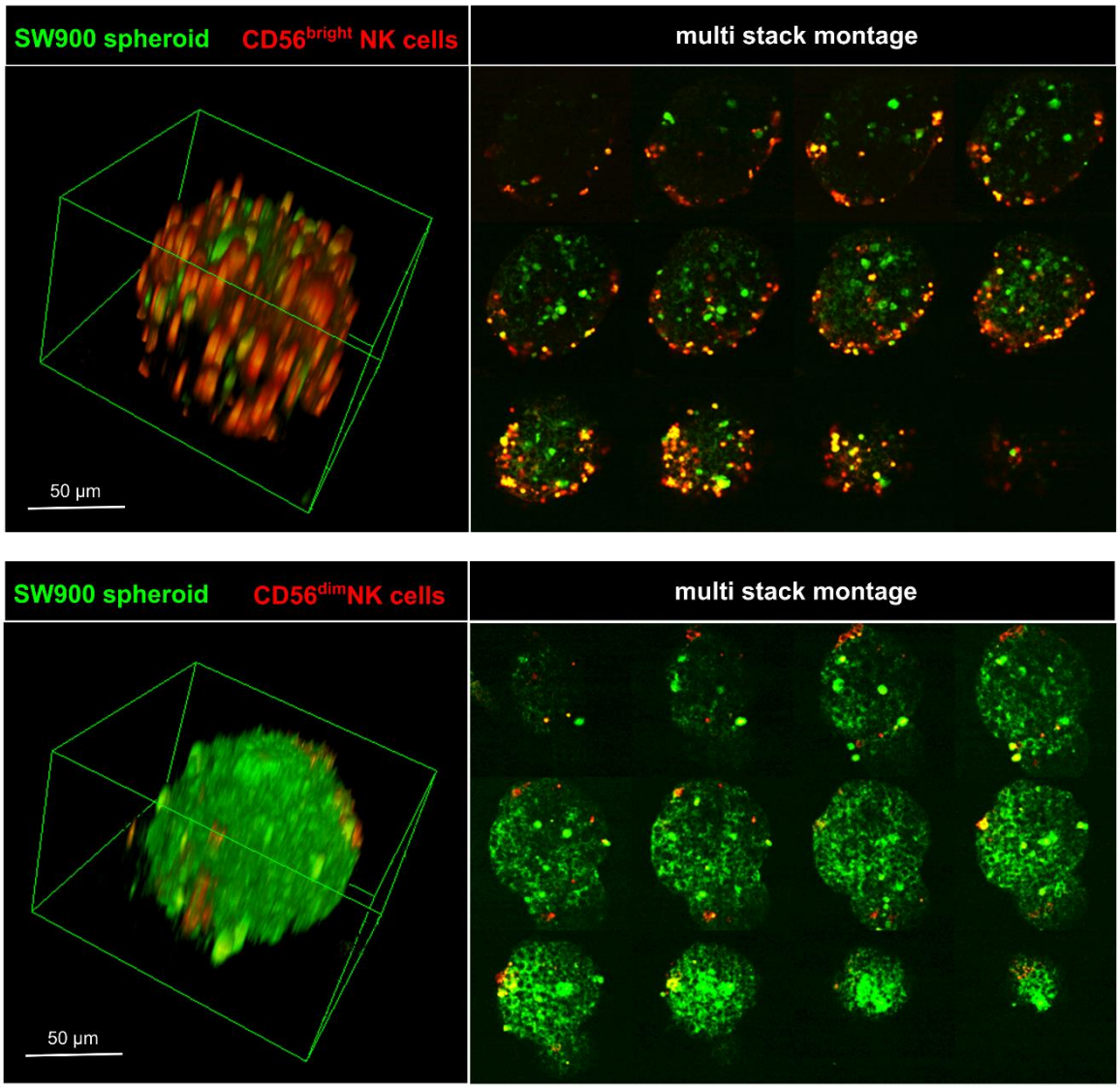


Figure 8. Interaction of CD56^{bright} and CD56^{dim} NK Cell subsets with NSCLC Tumor Spheroids. Representative images obtained through Two-photon excitation microscopy illustrate the distinct infiltration patterns of NK cell subsets into tumor spheroids. CD56^{bright} NK cells (labeled red) are observed to accumulate and infiltrate the central region of the tumor spheroid (labeled green), while CD56^{dim} NK cells predominantly adhere to and accumulate at the periphery.

4.9. Circulating CD56^{bright} NK cell transition to tissue-resident phenotype within Tumor Spheroids

Given the pronounced ability of CD56^{bright} NK cells over CD56^{dim} NK cells to adhere and infiltrate spheroids, we speculated on their potential acquisition of tissue-resident (tr)-NK cell features, associated with concomitant downregulation of markers associated with recirculation. To address this question, the same setting in 4.8 was also employed and the phenotype of NK cells associated with spheroid was assessed by flow cytometry.

Notably, CD56^{bright} cells from the spheroid exhibited acquired expression levels of CD103 compared to NK cells cultured alone, as soon as a culture of 5 days(Figure 9A). As hypothesized, CD56^{bright} NK cells, additionally, displayed elevated expression of other tr-markers such as CD69 and CXCR6 (Figure 9B). DNAM-1 mediates cellular adhesion to other cells bearing its ligands, nectin molecule CD112 and nectin-like protein CD155 to promote lymphocyte signaling, lymphokine secretion and cytotoxicity of NK cells. DNAM1 expression was notably downregulated in infiltrating NK cells (Figure 9B). These findings corroborate our earlier observation, suggesting that CD56^{bright} NK cells exhibit heightened capacity to infiltrate tumor masses and acquire phenotypic features similar to NK cells isolated from tumor tissues.

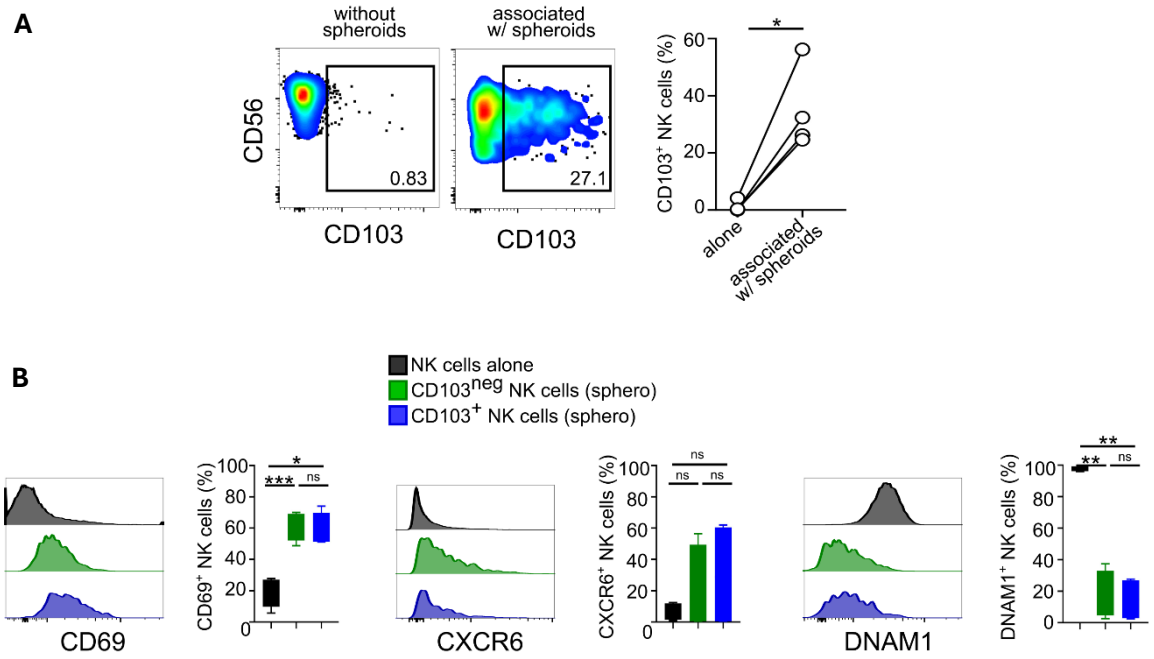


Figure 9. CD56^{bright} NK cells in lung tumor spheroid are characterized by CD103 upregulation. (A) Representative pseudocolor plots showing CD103 expression by CD56^{bright} NK cells incubated in medium alone (unstimulated) or cocultured with SW900 spheroid. (B) Representative overlays (left) and frequency (right) of expression of CD69, CXCR6 and DNAM-1 on CD103^{+} and CD103^{-} spheroid infiltrating cells and NK cells alone. *P < .05, **P < .01, and ***P < .001.

5. DISCUSSION

This study sought to understand whether tissue-resident NK cell could infiltrate and reside within the tumor microenvironment of non-small cell lung cancer. Our preliminary observations have identified distinct putative subsets of tissue-resident NK cells, based on expression of peculiar tr-markers such as CD69, CXCR6 or CD103. We then focused on CD103+ NK because of their potential capability to interact with tumor cells. Based on this hypothesis, following further analyses, we have characterized CD103+ NK cells as a unique subset of tumor-infiltrating NK cells, displaying distinct tissue-residency characteristics, altered functionality, and a potential critical role in modulating the tumor immune landscape. The findings presented in our study collectively highlight their complex interactions within the TME, offering novel insights into their dual functionality as both immune regulators and tumor suppressors. Integrating these results with recent and foundational studies may expand our understanding of their role in tumor immunology and therapeutic opportunities.

Based on our data and current knowledge on tissue-resident NK cells, this study shed light on the growing body of knowledge regarding the biology of tissue-resident NK cells and highlights the opportunities for novel immunotherapies targeting subsets of tumor-resident NK cells.

CD103+ NK cells are characterized by their expression of tissue-residency markers such as CD103, CXCR6, and CD69, which facilitate their retention in epithelial-rich environments. These markers are central to their interaction with epithelial cells and their adaptation to the local microenvironment. For example, CD103 binds to E-cadherin, a ligand expressed by

epithelial cells, thereby anchoring NK cells within tissues. Previous studies provided critical evidence of CD103 as an important marker on NK cells and its role in immune cell localization within normal lung tissues, establishing its importance in maintaining immune surveillance in epithelial tumors(21). Similarly, research has emphasized CXCR6 as a key mediator of tissue residency, further supporting the hypothesis that these markers are indispensable for NK cell positioning within the TME(121).

Distinct from the highly cytotoxic $CD56^{dim}$ NK cells that dominate peripheral blood, CD103+ NK cells are more related with $CD56^{bright}$ NK cells. These cells are known for their cytokine-secreting capabilities and interactions with other immune cells. The differentiation of $CD56^{bright}$ NK cells into tissue-resident CD103+ NK cells is heavily influenced by the TME's environmental signals, including cytokines and chemokines. Previous research has demonstrated that local environmental cues, such as integrins and cytokine gradients, can drive NK cells to adopt tissue-specific phenotypes(122, 123). The transition of NK cells from circulating $CD56^{bright}$ precursors to tissue-resident CD103+ phenotypes underscores their remarkable plasticity and adaptability. Research identified CD103 and CD49a as key integrins mediating tissue retention, highlighting their importance in establishing NK cell residency within the TME(122, 123).

The findings from our study corroborate these observations, highlighting the possibility that circulating $CD56^{bright}$ NK cells could convert to CD103+ NK cells, upon conditioning by tumor microenvironment.

Using 3D tumor spheroid models, we observed that that peripheral blood-derived $CD56^{bright}$ NK cells infiltrate and acquire a tissue-resident phenotype. This transition could be driven by TGF-β1 and IL-15, underscoring the plasticity of NK cells within tumors.

Previous studies described how IL-15 and TGF-b1 might sustain circulating NK cell-conversion into tr-NK cells, providing potential therapeutic targets for enhancing their anti-tumor capabilities(124). Consistent with this, in our setting, we might suspect a synergy between the same factors, even if also a cell-to-cell contact seems necessary, since NK cells infiltrating spheroids expressed consistently higher levels of CD103 than NK cells present in the supernatant of the co-culture, but not infiltrating the tumor mass (data not shown) The findings underscore the dynamic interplay between circulating and tissue-resident NK cells, emphasizing the therapeutic potential of favoring this transition to improve outcomes in NSCLC. One of the most significant findings of this study is the impaired functionality of CD103+ NK cells within the TME. Despite their retention in tumor sites, these cells exhibit reduced capacity to degranulate in the presence of target cells and lower expression of pro-inflammatory factors, as evidenced by diminished production of critical effector cytokines like IFN-γ, GMCSF and TNF-α. These impairments align with observations that TME-derived factors, including TGF-β1 and hypoxia, might suppress NK cell activity and alter their phenotypes(125). Furthermore, research highlighted how TGF-β1 drives the polarization of NK cells toward a regulatory phenotype, further diminishing their ability to mount effective anti-tumor responses(126).

Interestingly, this study found that CD103+ NK cells retain the ability to secrete soluble factor such as CCL5. CCL4, FLT3L and XCL1/2, are essential for recruiting dendritic cells

(and other immune effectors) to the tumor site. This dual role—reduced direct cytotoxicity but preserved immune recruitment capacity—underscores the complexity of CD103+ NK cells in the TME. Previous studies emphasized the NK cell-DC axis in promoting anti-tumor immunity in mouse models, highlighting the importance of NK cell-derived chemokines in recruiting and activating DCs(105, 127), however what subset of tumor-infiltrating NK cells is better equipped for this function, is still not clear. Additionally, research demonstrated that this axis is critical in checkpoint therapy-responsive tumors in mouse models and human, providing further context for the chemokine-secreting functionality observed in CD103+ NK cells(106). Taking together, our observations on functionality of CD103+ NK cells, together with their supposed localization in contact with tumor cells, might be relevant to support a localized immune response at the tumor site.

IL-15 is well-known for its role in promoting NK cell survival, proliferation, and effector functions. Research showed that IL-15 reactivates hypofunctional NK cells in the TME, enabling them to reengage in anti-tumor immunity(128). Further studies highlighted IL-15's role in sustaining NK cell inflammatory profiles, reinforcing its therapeutic potential(129). Interestingly, our data suggests a relevant role of IL-15 in restoring proliferation and the functionality of tumor-infiltrating CD103+ NK cells. This study demonstrated that IL-15 treatment partially rescued their cytotoxic potential, enhancing both cytokine production and degranulation.

The ability of IL-15 to enhance both the cytotoxic and immune-recruitment capacities of CD103+ NK cells might suggest this cytokine as a key component of combination therapies. Advanced delivery systems, such as IL-15-conjugated nanoparticles, liposomes, or fusion

proteins, offer opportunities to enhance its stability and bioavailability in the TME. Additionally, combining IL-15 with immune checkpoint inhibitors, such as anti-PD-1 or anti-CTLA-4 antibodies, could synergize innate and adaptive immune responses, offering a powerful strategy for overcoming TME-induced suppression and improving therapeutic efficacy in NSCLC.

Beyond their direct cytotoxic functions, CD103+ NK cells might play a role in immune recruitment within the TME. This study demonstrated that CD103+ NK cells secrete chemokines such as FLT3L, XCL1/2 and CCL5, which attract cDC1 populations critical for T cell priming and antigen presentation. Previous research emphasized the importance of the NK cell-DC axis in fostering robust anti-tumor immunity(127, 130). The findings of this study corroborate a potential role of CD103+ NK cells in modulating the immune composition and activity of the TME. These functions are not exclusive of tumor-infiltrating $CD103^{+}$ NK cells, as we showed that $CD103^{neg}$ counterpart can secrete pivotal factors. However, if further investigations will confirm the localization of CD103+ NK cells in contact with tumor cells, their ability can represent an advantage in mounting anti-tumor responses.

The heterogeneity of CD103+ NK cells, as revealed by X-shift clustering, further underscores their complexity. This analysis identified functional subsets of CD103+ NK cells with distinct chemokine production profiles, suggesting that different subpopulations may serve specialized roles within the immune microenvironment. Previous studies observed phenotypic diversity among tissue-resident NK cells, which they attributed to local cytokine and stromal interactions(124, 131). Understanding this heterogeneity is essential for

developing targeted therapies that leverage specific subsets of NK cells to maximize therapeutic outcomes.

This study highlights the dual role of CD103+ NK cells as immune recruiters and impaired cytotoxic effectors, presenting unique challenges and opportunities for therapeutic intervention. By targeting the suppressive mechanisms of the TME, such as TGF-β1 and hypoxia, alongside enhancing NK cell activity through cytokines like IL-15, therapies can potentially reinvigorate the anti-tumor immune response. Future approaches may involve combining cytokine-based therapies with novel checkpoint inhibitors or epigenetic modulators to maximize therapeutic efficacy.

Emerging technologies, such as adoptive NK cell therapies, offer exciting prospects for leveraging CD103+ NK cells. Engineering these cells with enhanced resistance to TME-induced suppression or improved chemokine secretion profiles could significantly bolster their efficacy in solid tumors. Furthermore, integrating spatial transcriptomics and advanced imaging techniques could deepen our understanding of the spatial dynamics of CD103+ NK cells and their interactions within the TME, paving the way for precision medicine approaches.

Altogether, this comprehensive investigation into CD103+ NK cells in NSCLC shed light on their phenotypic and functional characteristics, emphasizing their role in immune recruitment, modulation, and therapeutic potential. By integrating experimental findings with recent literature, this study advances our understanding of NK cell biology in the tumor setting and lays the groundwork for innovative immunotherapies targeting this subset, as well

as other subsets of tumor-infiltrating NK cells. Future research should continue to explore the molecular mechanisms driving their heterogeneity and adaptability, fostering the development of precise and effective strategies to harness the full potential of tissue-resident NK cells in cancer treatment.

6. ACKNOWLEDGEMENT

First and foremost, I wish to express my profound gratitude to my tutor, **Professor Guido Ferlazzo**, for his invaluable guidance, steadfast support, and encouragement throughout the course of my doctoral studies. His mentorship has been fundamental to my academic development and the realization of this work.

I am equally grateful to **Professor Paolo Carrega**, whose assistance and expertise have been instrumental to every aspect of this project. His insightful advice, constructive feedback, and unwavering dedication have significantly contributed to the successful completion of this research.

I would also like to extend my sincere appreciation to **Dr. Paola Orecchia**, **Dr. Monica Parodi**, and **Dr. Massimo Vitale** for their indispensable support during the experimental phase of my thesis. Their technical expertise, patience, and commitment greatly facilitated the progress and success of my research.

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