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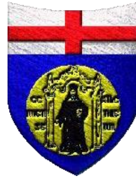

FACOLTA' DI MEDICINA E CHIRURGIA

Department of Experimental Medicine

PhD in Clinical and Experimental Immunology

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Experimental International Thesis

Study of Cytokine-Induced Memory-Like Natural Killer (CIML NK) cells as Possible Tool to Kill Non-Small Cell Lung Cancer (NSCLC) cells and to Contrast their Potential Tumorigenic Properties.

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ABSTRACT

The overall aim of this study is to evaluate the antitumor ability of cytokine-induced memory like (CIML) NK cells in Non-Small Cell Lung Cancer (NSCLC), and ascertain whether their use could provide a functional advantage over conventionally activated NK cells currently employed in clinical trials. A comprehensive phenotypical analysis paired with a functional characterization of CIML NK cells showed that their differentiation was accompanied by an expansion of the $CD56^{bright}$ subset, and that this subset was the main driver of CIML NK effector functions, in terms of both cytotoxic degranulation and cytokine production. Compared to NK cells conventionally activated with IL-2 or IL-15, CIML NK cells displayed higher effector functions and, specifically, higher responsiveness and killing capabilities to NSCLC targets. CIML NK cells were also more effective than conventionally activated NK cells in targeting the $CD133^{+}$ cell subpopulation within *in vitro*-generated NSCLC spheroids. This finding implies that CIML NK cells may significantly affect the tumorigenicity of NSCLC, as the cancer stem cell (CSC) population is generally enclosed in the spheroid-derived CD133+ cells.

Finally, we provide evidence that the ability of CIML NK cells to eliminate $CD133^{+}$ NSCLC cells can be further enhanced by using a trispecific NK cell engager (TriKE) targeting CD133 on CSCs and engaging IL-15 receptor and CD16 on NK cells. In conclusion, we showed that CIML NK cells displayed a superior antitumor activity along with an enhanced capability in controlling tumorigenicity and stemness properties of malignant cells, and, more importantly, we also provide hints for more effective strategies to achieve a better NK cell targeting of tumor cells in NSCLC.

1. INTRODUCTION

1.1. Non-small cell lung cancer

Lung Cancer is the main cause of tumor-related death worldwide, accounting for approximately 1.6 million deaths per year (1). The highest lung cancer mortality rates have been reported in countries such as Turkey, Canada, United States, United Kingdom, and Japan, where the five-year survival rate is only 19% (2,3). The poor prognosis is caused by the late diagnosis of the disease, with most of the patients at an advanced metastatic stage at the time of diagnosis.

Lung tumors are classified as non-small cell lung cancer (NSCLC) or small cell lung cancer (SCLC). Non-Small Cell Lung Cancers (NSCLCs) represent approximately 80-85% of all lung cancers and can be further divided into three subtypes: i) adenocarcinoma, which arises from alveolar cells and accounts for 40% of all NSCLC cases; ii) squamous cell carcinoma, which arises from bronchial epithelium and represents around 30% of all NSCLC diagnoses; and iii) large cell carcinoma, which are pleomorphic malignant epithelial tumors that do not exhibit cytologic, histologic, or immunophenotypic features typical of adenocarcinoma and squamous cell carcinoma (4,5).

The average five-year survival rate for NSCLC varies depending on disease stage and ranges from 63% for Stage I NSCLC, to 7% for Stage IV NSCLC (6). Even though at early stages most tumors are surgically resectable with curative intent, most patients present tumor recurrence. Hence, the inability to prevent tumor recurrence and to effectively treat metastatic disease represent the main causes of the high lethality of this pathology.

The main risk factor for developing lung cancer is tobacco smoking, which is considered responsible for around 80% of cases (3). Continuous cigarette smoking and exposure to secondhand smoke can increase lung cancer risk to up to 50%, and this is particularly associated to the squamous cell carcinoma subtype, whose incidence seems to strongly correlate with cigarette smoke exposure (1). Additional environmental risk factors are pollution and occupational exposure to carcinogens, such as asbestos, silica and diesel fumes (7). Moreover, as reviewed by De Mello et al (3), having a first-degree relative with a history of lung cancer has been reported to increase an individual's risk of

developing the disease by 50%. Besides, about 8% of all cases are currently attributable to genetic predisposition.

1.2. Therapeutic strategies

Surgical resection is the most effective treatment in stage I-IIIA NSCLC (5). Patients can also undergo a combination of radiation therapy, chemotherapy, and/or surgery, depending on clinical factors such as tumor size, location, lymph node involvement, underlying co-morbidities, and tolerance to treatment (6).

Targeted therapies and immunotherapy have been recently introduced as first line treatment or adjuvant therapy for a subset of patients. In advanced disease, where targeted therapies or immunotherapies are not applicable, platinum-based chemotherapy like cisplatin often represents the primary option (6,8).

Chemotherapy, and particularly platinum-based drugs, has been the backbone of NSCLC treatment for several years, significantly improving the median overall survival (OS) of patients over best supportive care (15% vs. 5% OS rate in 1 year, respectively) (8,9). Chemotherapy is still prescribed in up-to 80% of patients, as adjuvant therapy, in patients with compromised lymph nodes, neoadjuvant therapy, to reduce the tumor mass before surgery in advanced stages, and as part of palliative systemic care in patients with metastatic disease, or in patients who are not eligible for surgery (10–12).

The frequent adverse effects, with problems of tolerability, and the onset of drug-induced resistance limit the actual effectiveness of these therapies (10).

The development and clinical implementation of targeted therapies has had a huge impact on the prognosis of NSCLC patients. This has been reflected in the increase of 5-year survival rates of patients, which raised from 10.7% in the early 1970s to 19.8% in the 2010s (13). Targeted therapies arose through the identification of molecules that are over-expressed or expressed mainly in tumor cells, as a result of driver mutations affecting proliferation and cell cycle, thus conferring a survival advantage to cancer cells. Molecular tests have now been routinely introduced in clinical practice and have enabled the stratification of patients into different treatment groups based on the presence of specific mutations. Up to 30% of NSCLC patients present mutations in the *EGFR* gene.

These mutations are more common among female patients (mutation rates are 19.7% higher than in males), Asians (rates are 52% higher than in North American populations), non-smokers (rates are 27.8% higher than in past or current smokers), and adenocarcinoma histology (rates are 26.3% higher than in other types). EGFR mutations observed in the histopathology of NSCLC patients consist of either deletions (E19del or E21del) or somatic mutations (L858R) (3,14,15). Anti-EGFR therapy involving the use of tyrosine kinase inhibitors (TKIs), such as erlotinib, gefitinib, afatinib, osimertinib, and dacomitinib, have been approved as either adjuvant therapy in stages IB-IIIA NSCLC or as first-line treatment in patients with metastatic, non-squamous NSCLC whose tumors harbor an EGFR exon 19 deletion or exon 21 L858R mutation (14). Anaplastic lymphoma kinase (ALK) is an additional therapeutic target that has been recently examined. Genetic alterations in ALK are present in around 5% of all NSCLC patients, and they are prevalent in adenocarcinomas and in mild or non-smokers (16). Different types of alterations have been described within the ALK gene including rearrangement or fusion with echinoderm microtubule-associated protein-like 4 (EML4) to form an oncogenic EML4-ALK fusion gene (17). For advanced NSCLC patients whose tumors are positive for an ALK rearrangement, Crizotinib, an ALK inhibitor, is the standard first-line treatment, with patients showing a longer progression-free survival than those receiving standard chemotherapy (16,18).

Despite the promises of targeted therapies in NSCLC patients, only a restricted portion of patients can benefit from these therapies. Moreover, most patients, even those that initially respond to the treatment, will develop resistance in a median of 9 to 13 months after TKI therapy (3).

The introduction of immunotherapy based on the use of immune checkpoint inhibitors (ICI) has shown great responsiveness and has significantly improved the survival rate of patients with advanced stage NSCLC (19–21). ICIs targeting the PD-1/PD-L1 pathway showed particularly interesting results in NSCLC (16). A wide variety of anti-PD-1 monoclonal antibodies (mAbs) such as nivolumab, pembrolizumab and cemiplimab, and anti-PD-L1 mAbs like atezolizumab and durvalumab have been successfully tested in preclinical and clinical settings (19,21). All these ICIs, binding either to the ligand or to

the receptor, can prevent the induction of inhibitory signals generated consequently to the ligand/receptor interaction. ICIs are currently offered as a first-line therapy for NSCLC or in combination with other immunotherapies or chemotherapy (19,22). Notwithstanding the unprecedented impact of ICIs, this class of treatments do not fully address the therapeutic requirements of all NSCLC patients, considering also that only a fraction of tumors express PD-L1. In fact, just 10–15% of patients demonstrate sustained long-term benefits in response to ICIs, while up to 30%, after an initial response, acquire resistance and show a rapid disease progression (23).

Despite the recent advances and the advent of new treatment options, the prognosis of NSCLC patients with advanced-stage or high-risk cancer remains poor. Additionally, even if NSCLC patients are diagnosed at an early-stage, it has been estimated that more than 50% will progress and develop distant-organ metastasis, most commonly to the bone (40%), brain (35%), liver (22 %) and adrenal glands (10%) (24).

Due to the profound phenotypic, biochemical, and metabolic alterations that accompany and characterize metastasis, therapeutic interventions directed against proliferating cancer cells, including targeted therapies centered on oncogenic drivers and conventional chemotherapies, have proven effectiveness only for primary neoplasms but have failed to demonstrate clinical efficacy against metastases (25).

In addition, chemotherapeutics commonly used in the treatment of NSCLC may have divergent effects on tumor spreading. Indeed, these treatments can both promote host´s ineffective inflammatory responses, consequent to cytotoxic damage, and apply a selective pressure leading to the expansion of drug-resistant $CD133^+$ cancer stem cells (CSCs), which are endowed with a high ability to disseminate and initiate distant metastasis (26,27).

1.3. Mechanisms underlying metastatic progression.

The process of metastatic dissemination to distant organs is complex and takes place through a series of events referred to as metastatic cascade, involving five major steps (28):

1) Local proliferation of tumor cells forming a primary lesion that will acquire

additional genetic alterations.

2) Migration of tumor cells toward newly formed or pre-existing capillary vessels or sentinel lymph nodes to reach circulation.
3) Survival of circulating tumor cells (CTCs) migrating either as single tumor cells or tumor clusters in the peripheral blood, by evading immune responses until they encounter those conditions that are permissive for extravasation.
4) Tumor cell engraftment of distant tissues upon extravasation and establishment of a premetastatic niche.
5) Persistence of tumor cells in a silent state at the metastatic site until proliferation is resumed to establish clinically detectable metastatic tumors (macrometastases).

Accumulating evidence suggests that metastatic spreading occurs not only at advanced stages, but also in the early phases of the disease. Indeed, CTCs can persist for a prolonged period in the blood stream prior to the formation of macrometastases, especially when CTCs are organized into circulating tumor cell clusters, which are more prone to immune evasion (28,29).

Tumor clusters of CTCs are composed of 2 to over 50 tumor cells and originate either from intravasation of cancer cell clumps, arising from the primary tumor via vessels and lymphatic ducts, or from collective migration of tumor cells aggregating directly in circulation (29–32). However, the latter hypothesis has been confuted, at least in the context of breast cancer, by Aceto et al. (30).

CTC clusters display a particularly high metastatic potential by maintaining cell clonal proliferation and by protecting the innermost cells from the stresses of circulation and from immune surveillance by shielding them from mechanical forces and host immune cells (33). Experimental evidence has shown that clustered CTCs do not only possess a structural advantage over single CTCs but also display an altered phenotype that facilitates metastasis, featured by increased expression of antiapoptotic proteins, such as Bcl-2 and by the entry into an inactive dormancy state in which the expression of proliferation markers such as Ki67, is suppressed (34). Additionally, it has been found

that these tumor clusters express higher levels of stem cell and therapeutic resistance markers (34,35). The clinical relevance of these findings has been highlighted in recent studies revealing that, by forming these structures, tumor cells metastasize at 20-100 times greater efficiency and, as such, they have been defied as a negative prognostic factor in cancer patients in the context of different tumors (34,36,37).

The negative prognostic value of clustered CTCs has been recently validated in NSCLCs, in which their presence in the pulmonary and peripheral circulation of patients at early disease stages undergoing tumor surgical resection was predictive of poor prognosis and disease progression; thus emphasizing that therapeutic strategies aimed at eliminating clustered CTCs may hold promise in preventing and controlling NSCLC metastatic disease (34).

In recent years, renewed attention has been given to the key role of NK cell-mediated immunosurveillance in controlling and preventing metastatic disease, and on the innate ability possessed by these cells to target and eradicate tumor cells, including cancer stem cells (38,39). Clinical evidence has supported the key role of NK cells in controlling metastasis. An inverse correlation between the levels of circulating or tumor-infiltrating NK cells and the onset and severity of metastases at clinical presentation has been reported in patients with gastric, colorectal, renal and prostate carcinomas, suggesting that NK cells may be a promising therapeutic target for the prevention and treatment of metastasis (40–43).

1.4. Human NK cell biology

Natural killer cells (NK cells) are a critical component of the innate immune system and are characterized by their rapid response to and strong cytotoxic activity against virus-infected and malignant cells without requiring prior antigen exposure. Beyond their innate effector properties, NK cells also exert regulatory functions in adaptive immunity through the secretion of an array of proinflammatory cytokines/chemokines, such as IFN-γ, TNF-α, CCL3, CCL4 and CCL5, which results in a shift of the Th1/Th2 balance towards a Th1 immune response (44–46). They are derived from Cluster of Differentiation (CD)34^{+} hematopoietic progenitors in the bone marrow, and their differentiation is thought to be

completed in the secondary lymphoid tissues, including tonsils, spleen, and lymph nodes (45).

NK cells constitute approximately 5-15% of all peripheral blood lymphocytes, and have an average life span of 2 weeks, thus necessitating constant replenishment from bone marrow precursors (46,47). They are as well present in lymphoid organs, like the spleen, and in non-lymphoid peripheral tissues as tissue-resident NK cells (48).

Phenotypically, human NK cells are defined by the lack of CD3–T-cell receptor (TCR) complex, characteristic of T cells, and by the expression of CD56 (neural cell adhesion molecule – NCAM), an immunoglobulin (Ig) superfamily glycoprotein that is thought to take part in the interaction between NK cells and target cells, and to have a role in NK cell motility (49). NK cells can be divided into two major subsets based on the expression levels of the two main NK cell surface markers, CD56 and CD16 (FcγRIII). This latter is a transmembrane glycoprotein of the Ig superfamily known to induce antibody-dependent cellular cytotoxicity (ADCC) by binding with low affinity the Fc portion of target-opsonizing IgGs (50).

Based on the surface abundance of CD56 and CD16, NK cells are conventionally classified as $CD56^{bright}CD16^{\pm}$ and $CD56^{dim}CD16^{high}$ (44). $CD56^{dim}CD16^{high}$ NK cells are considered a terminally differentiated and mature subset and represent around 90% of circulating NK cells. This subset is particularly relevant for its involvement in natural cytotoxicity and ADCC, as they are endowed with a large content of cytolytic granules containing perforin and granzyme B (51). On the other hand, $CD56^{bright}CD16^{\pm}$ NK cells are defined as an immature subset and constitute about 10% of all circulating NK cells. They exhibit low levels of perforin and consequently sustain only limited cytotoxic responses, but undergo robust proliferation upon cytokine stimulation, and are an important source of cytokines, including Interferon (IFN)-γ, Tumor-Necrosis Factor (TNF)-α, and Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF), which are involved in immune regulation (52). $CD56^{bright}CD16^{\pm}$ NK cells relying on the expression of L-selectin (CD62L) and CC-chemokine receptor 7 (CCR7) can extravasate from circulation into tissues and lymph nodes, where they constitute the main NK cell subset (53,54).

Despite this classification, it is still under debate whether these cell types represent functionally distinct NK cell subsets or different stages of maturation. In addition, the relative proportion of $CD56^{bright}$ and $CD56^{dim}$ NK cells in tissues can be very different from that observed in the peripheral blood. Indeed, as previously introduced, in lymph nodes, spleen, tonsils and liver, most tissue-resident NK cells are $CD56^{bright}$ NK cells, and they have now been shown to be phenotypically and functionally distinct from conventional peripheral blood NK cells (48,55).

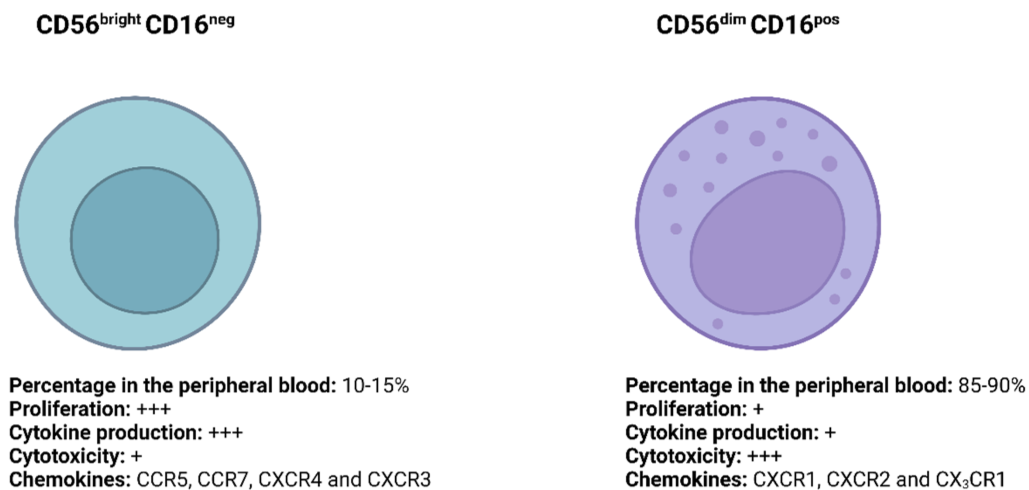

Figure 1. Main NK cell subsets in peripheral blood. $CD56^{bright}$ and $CD56^{dim}$ NK cell subsets exhibit different phenotype, and functional and proliferative activity.

1.4.1. The NK cell receptor repertoire

NK cell activity is regulated by a balance of activating and inhibitory signals transmitted by a large array of germline DNA-encoded surface receptors that, unlike T and B cell receptors, do not undergo gene rearrangement or affinity maturation. Upon ligand engagement, these receptors transmit either inhibitory or activating signals that control NK cell activation. The balance of the activating and inhibitory signals, generated in response to the ligand/receptor interactions, dictates the activation status of NK cells (56). The NK cell receptor repertoire essentially comprises three major inhibitory and activating receptor families: Killer immunoglobulin-like receptors (KIRs), natural cytotoxicity receptors (NCRs) and C-type lectin-like receptors, which are described in detail in the next

sections. Through this repertoire of receptors, NK cells exhibit a clear capacity to recognize (and be activated by) infected or transformed cells, in which the expression of activating ligands is commonly increased and inhibitory ligands are decreased or even lost (57,58). By exploiting the same receptor repertoire, NK cells avoid attacking healthy cells, which maintain low expression levels of NK cell activating ligands and high levels of human leukocyte antigens class I (HLA-I), which are the ligands of the most effective inhibitory NK receptors (KIRs and CD94:NKG2A – see below) (57).

Functional response of NK cells is generally determined by the integration of signals coming from the variable engagement of different activating and inhibitory receptors (59). Only when the balance between activating and inhibitory signals reaches the activation threshold, NK cell functions including cytokine and chemokine secretion, and cellular cytotoxicity are induced. The requirement for activating receptor combinations helps prevent unrestrained activation of NK cells and provides flexibility in sensing and responding to environmental stimuli. CD16 is the only receptor that can activate NK cells on its own, without any additional activation through other receptors (59).

Importantly, simultaneous engagement of activating receptors and high affinity inhibitory receptors results in a dominant inhibitory transduction signal that abrogate the signals initiated via the activating pathways (60).

Mature and functional NK cells express at least one inhibitory receptor specific for self-HLA class I molecules. Indeed, during the cell differentiation process, only NK cells expressing inhibitory receptors recognizing self-HLA class I molecules undergo a process called "licensing or education", consisting of the acquisition of functional competencies in terms of cytotoxic ability and cytokine secretion (61). This process ensures, on one hand, self-tolerance towards healthy cells and on the other hand, an efficient response against transformed cells, which usually lack or down-regulate HLA class I expression and acquire or up-regulate the expression of ligands for the non-HLA specific activating NK receptors (62).

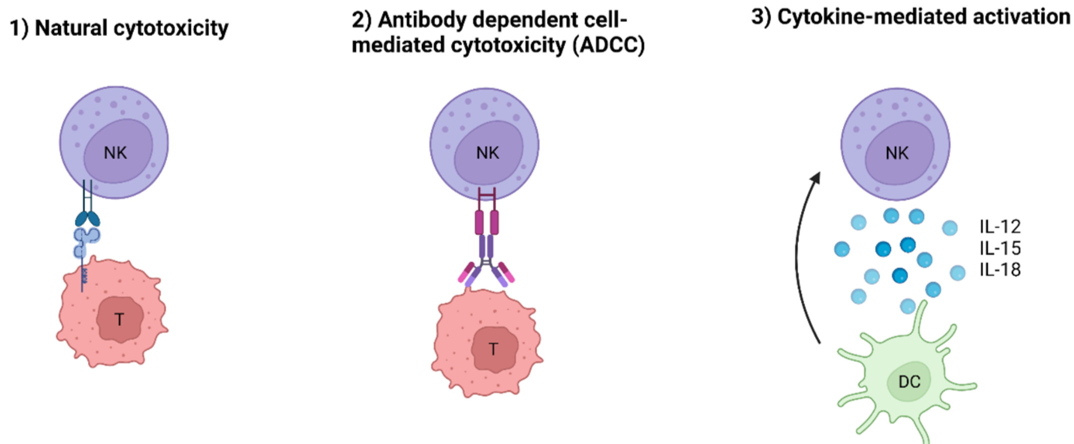

Figure 2. Regulation of NK cell activity. NK cells can respond to different types of stimuli: The engagement of activating receptors (1) triggers their cytotoxic activity and cytokine release. The induction of the same activities is also mediated by ADCC (2). The exposure to different cytokines activates NK cells, enhances their cytotoxic potential, and induces IFN-γ and TNF-α release on certain NK cell subsets (i.e. $CD56^{bright}CD16^{dim}$ NK cells) (3).

1.4.2. Killer immunoglobulin-like receptors (KIRs)

Human KIRs represent a family of innate immune receptors structurally belonging to the Ig superfamily and are encoded within the highly polymorphic leukocyte receptor complex (LCR) located on the chromosome region 19q13.4 (63). The KIR family includes twelve members, with a certain degree of polymorphism, each recognizing different HLA-I allelic variants as ligands. They are type I transmembrane glycoproteins with two (KIR2D) or three (KIR3D) Ig-like extracellular domains with either a short (S) or long (L) cytoplasmic domain (64,65). The length of the cytoplasmic domain dictates the functional property of the KIRs. A long cytoplasmic domain, for instance, mediates an inhibitory signal by the presence of one or two immunoreceptor tyrosine-based inhibitory motifs (ITIMs), which upon receptor ligation, become phosphorylated and recruit tyrosine phosphatases such as the Src homology 2 domain-containing phosphatase (SHP)-1 and SHP-2 (3–5), responsible for the inhibition of various NK cell–mediated effector functions. In contrast, a short cytoplasmic domain mediates an activating signal through the receptor association with adaptor proteins (DAP12)

bearing immunoreceptor tyrosine-based activating motifs (ITAMs) (58,64). KIRs with two and three Ig domains bind to HLA-C/-G and HLA-A/-B molecules, respectively (65). KIR2DL1 (CD158a), KIR2DL2/DL3 (CD158b1/b2) and KIR3DL1/DL2 (CD158e/k) represent the most prominent inhibitory KIRs, whereas KIR2DS4 is the most prevalent activating KIR (65).

KIRs represent the leading group of negative regulators within the signaling pathways controlling NK cell activity (65). These receptors, together with the NKG2A:CD94, ensure the fail-safe mechanisms that prevent the unwanted attack of self, healthy cells. As described above, however, some members of the KIR family actually mediate activating rather than inhibitory signals. The role of these receptors in the biology of NK cells is not completely understood. Such activating KIRs can recognize HLA-I allelic determinants, and there is evidence suggesting that, in the context of the KIR-HLA-I interaction, they could be involved in the defense against viral infections or in the control autoimmune diseases by attacking activated autoreactive T cell clones (66–69).

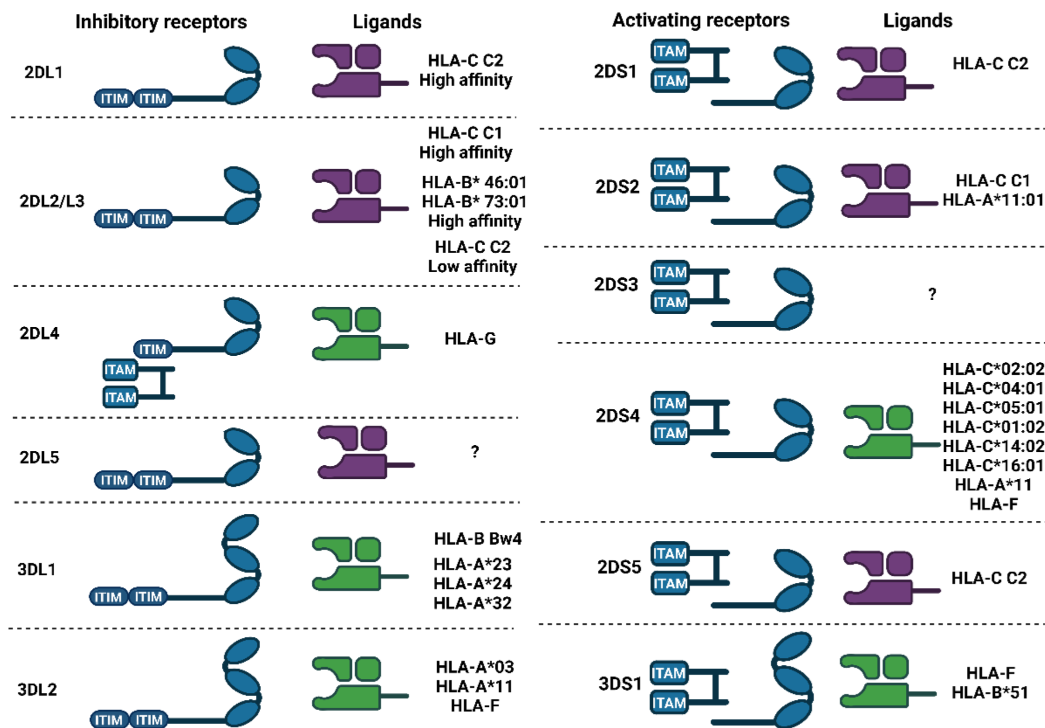

Figure 3. Killer cell immunoglobulin-like receptors (KIR). KIRs have either 2 or 3 Ig extracellular domains (2D or 3D). KIRs can have either long or short cytoplasmic tails (L or

S). The long cytoplasmic tails contain ITIMs, while short tails interact with DAP12 containing ITAMs. KIRs recognize as ligand HLA molecules, such as HLA-A, -B, -C and -G.

1.4.3. Natural cytotoxicity receptors (NCRs)

NCRs are type I transmembrane glycoproteins belonging to the Ig superfamily and utilize extracellular Ig-like domains for ligand binding. Human NK cells express three types of NCRs, NKp30 (NCR3 or CD337), NKp44 (NCR2 or CD336), and NKp46 (NCR1 or CD335). While NKp46 and NKp30 are constitutively expressed on both resting and activated NK cells, NKp44 expression is limited to activated NK cells. NCRs bind to ITAM-bearing adaptor proteins FcεRI-γ, and CD3-ζ (NKp30 and NKp46) and KARAP/DAP12 (NKp44) which are responsible for signal transduction (62,70). Several membrane-bound, intracellular, and soluble extracellular NCR ligands have been identified, including molecules derived from viruses, bacteria, and parasites, molecules associated to tumor tissues and tumor cells, or, finally, induced on activated immune cells, such as DCs. The most known ligands are represented by hemagglutinin (HA) and hemagglutinin-neuraminidase (HN) of different viruses, as ligands for NKp46 and NKp44, B7-H6 and HLA-B-Associated Transcript 3 (BAT3)/ BCL2-Associated Athanogene 6 (BAG6), as ligands for NKp30, Nidogen-1, PDGF-DD, the 21spe isoform of mixed-lineage leukemia protein-5 (MLL-5) 21spe-MLL5, Proliferating Cell Nuclear Antigen (PCNA), and HLA-DP*04:01, as ligands for NKp44, complement factor P (CFP) as ligand for NKp46 (62,71–73). In addition, heparan sulfate proteoglycans have been shown to interact with all the NCRs. Of note, not all of the indicated ligands induce NCR activating signaling. For example, PCNA, and the soluble forms of some of the ligands (sB7-H6 and sNidogen-1) can inhibit NCR-mediated cell triggering (73–75).

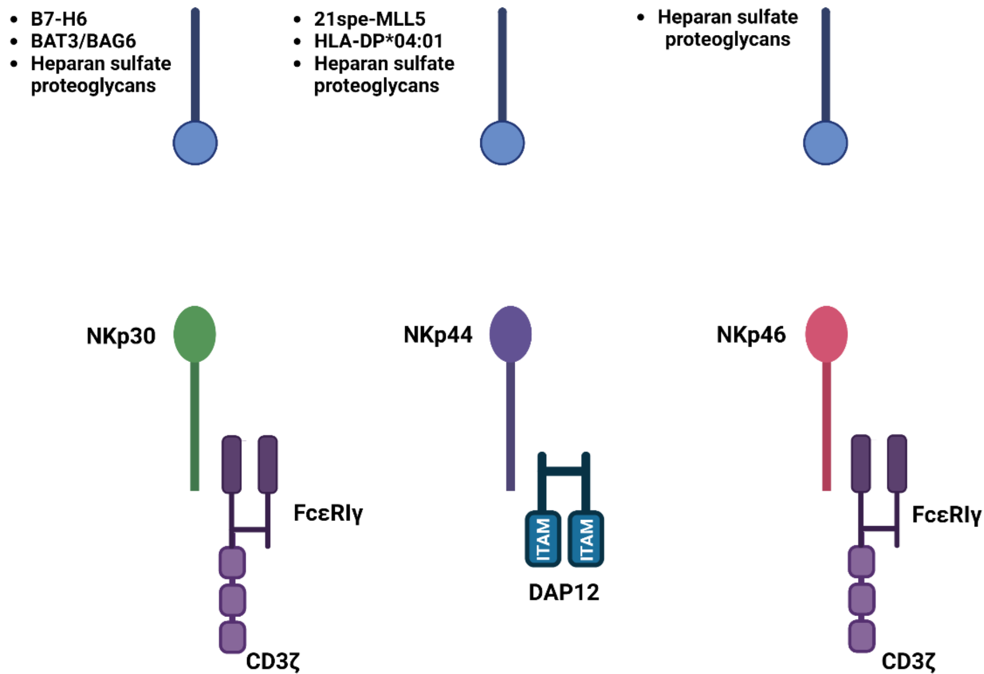

Figure 4. Schematic representation of NCRs NKp30, NKp44 and NKp46 expressed on the surface of NK cells recognizing ligands expressed by tumor cells.

Altogether, the acquisition of NCRs during NK cell maturation correlates with a high cytolytic activity against tumor target cells and, vice versa, downregulation of NKp30, NKp44, and NKp46 correlates with a deficient NK cytolytic response (60).

1.4.4. CD94/NKG2 Receptors

The family of C-type lectin-like receptors comprises the NK group 2 (NKG2) subfamily that, similarly to the KIR family, includes both inhibitory and activating receptors. NKG2 receptors are type II C-type lectin-like membrane glycoproteins, including seven different members, such as NKG2A (CD159), NKG2C (CD159c), NKG2D (CD314), NKG2E, NKG2F and NKG2H. NKG2A, NKG2C, NKG2E, NKG2F and NKG2H form heterodimers with CD94 through disulfide bonds, whereas NKG2D forms homodimers (58,76,77).

The inhibitory receptor CD94:NKG2A contains ITIMs in its cytoplasmic domain that after phosphorylation convey inhibitory signals to NK cells (78). The CD94:NKG2C/E/F are activating receptors non-covalently associated to the ITAM-containing adaptor molecule DAP12 that transduces activating signals into the cytoplasmic compartment. NKG2D by binding to the DAP10 adaptor molecule, forms a receptor complex that transmits activating signals via the ITAM domain of DAP10 (58). All the genes encoding for CD94 and the NKG2 proteins are clustered in the NK cell gene complex (NKC) on chromosome 12p12-p13 (79).

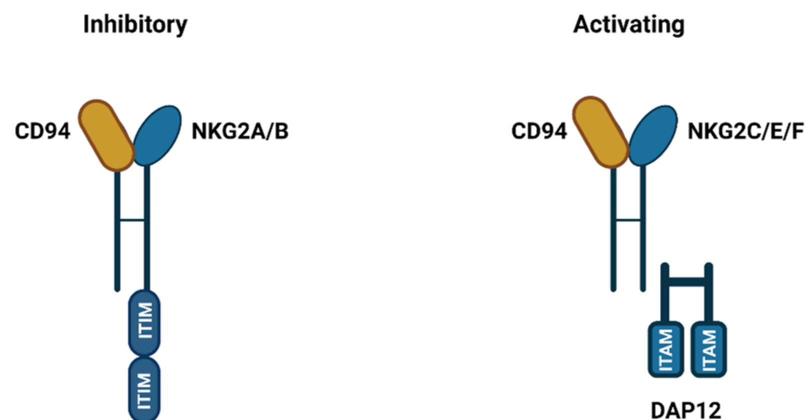

Figure 5. CD94/NKG2 receptors. CD94/NKG2A mediates an inhibitory signal to the NK cells via its cytoplasmic ITIMs. NKG2C, E and F are activating receptors that promote NK cells effector functions upon ligand interaction.

Three members of this family are particularly relevant to the control of the NK cell functions. The inhibitory CD94:NKG2A and the activating CD94:NKG2C receptors, both recognizing the nonclassical MHC-I molecule HLA-E (78), enable NK cells to avoid the attack of normal healthy cells, but also to mediate the recognition of aberrant, virally infected cells (78). In particular, NKG2C has been proposed as a mean to drive the expansion of peculiar NKG2C+ cell subsets in response to viruses, such as CMV (80). Of note, the presence/absence of NKG2A expression marks different maturational stages within the terminal differentiation of NK cells, as this receptor is preferentially expressed

on CD56^{bright} and CD56^{dim}KIR^{-} NK cells, while it tends to disappear in NK cells acquiring the expression of KIRs and of the terminal differentiation marker, CD57 (81,82).

The third relevant member of the NKG2 family is represented by NKG2D. This receptor is known to recognize ligands with structural homology to MHC-I molecules, such as the polymorphic stress-induced ligands MHC-I chain-related (MIC)-A/B and UL16 binding proteins (ULBP1–6) (78). These ligands are rarely expressed in healthy tissues but induced by various cellular stresses, such as DNA damage, heat shock, or cellular transformation (60). Therefore, NKG2D plays a crucial role in the triggering of anti-tumor and anti-viral NK cell functions.

1.4.5. Receptors belonging to the Nectin/Nectin-like (nec/necl) family.

The nec/necl family represents a group of adhesion molecules capable of interacting/recognizing intrafamily members. Some of these molecules are involved in the control of T and NK cell functions, representing actual activating or inhibitory receptors, such as CD226 (DNAX accessory molecule-1, DNAM1), CD96 (T cell-activated increased late expression, TACTILE), and T-cell immunoglobulin and ITIM domain (TIGIT). They bind nectin proteins, CD112 (Nectin-2), and CD155 (Poliovirus receptor, PVR), and they have been identified as crucial regulators of antitumor NK cell function (83). DNAM-1, for instance, has been shown to have a role in mediating recognition of tumor cells, NK cell responses against tumors and NK cell migration. The interaction of DNAM1 with its ligands induces the recruitment of tyrosine kinase fibroblast endothelial kinase (Fyn) and serine threonine protein kinase C (PKC), together with actin polymerization and activation of other surface receptors, thus consenting a stable interaction of NK cells with target cells (84).

TIGIT and TACTILE receptors, on the other hand, contain an ITIM cytoplasmatic domain that inhibits NK cell functions and counteracts DNAM-mediated activation signals (83).

1.4.6. NK cell effector functions

The primary mechanism of NK cell-mediated cytotoxicity is based on granule exocytosis. Consequent to recognition of target cells by NK cells, a lytic immunological synapse is formed at the site of cell-to-cell contact and the preformed lytic granules, equipped with perforin and granzymes, are released by NK cells into the synaptic cleft (60). The release of the granules' content involves the polarization of the microtubule-organizing center (MTOC) and the subsequent transfer of the granules from microtubules to filamentous actin in proximity of the immunological synapse (85). Perforin is a glycoprotein able to polymerize and form pores in the cell membrane of target cells, therefore, the granules can fuse with the plasma membrane and enable the release of granzymes into the target cell. Once entered, granzymes, which are serine proteases, trigger cell apoptosis through caspase activation (86,87).

Degranulation can be induced either upon the engagement of activating receptors by their cognate ligands on target cells, or upon the binding of CD16 to the Fc portion of antibodies coating a target cell.

A second mechanism of cytotoxicity is a perforin-independent apoptosis induced by Fas ligand (FasL, CD95L/APO-1L)- and TNF-related apoptosis-inducing ligand (TRAIL)-mediated engagement of death-inducing receptors on target cells (88). Death receptors (DRs) are members of the TNF receptor super family and are expressed on a wide variety of cell types where they have a broad range of apoptotic and non-apoptotic functions depending on the cell context. Six DRs have been identified in humans, including Fas (CD95/APO-1), TNF-R1, TRAIL-R1 (DR4), TRAIL-R2 (DR5/APO-2), DR3 (TRAMP/APO-3) and DR6. These receptors are stimulated by the binding of ligand molecules FasL (CD95L), TNF, TRAIL and TL1A to Fas, TNF-R1, DR4/5 and DR3, respectively (89).

While DRs are expressed on many cell types, death receptors ligands are expressed exclusively on specific immune cells, including NK cells and cytotoxic T lymphocytes (CTLs). The expression of FasL is strictly regulated and is induced in activated NK cells and in primed effector T cells (60,83).

The association of Fas with FasL leads to the activation of the initiator caspase-8, promoting apoptosis via extrinsic and mitochondrial pathway (intrinsic) in target cells (90).

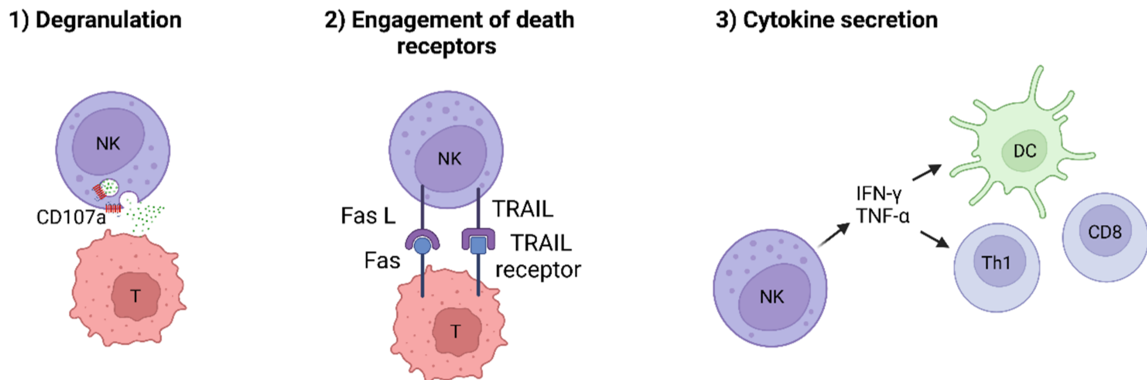

Figure 6. Antitumoral effector functions of NK cells. NK cells promote antitumor response directly through degranulation (1) and death receptor-mediated (2) cell lysis and indirectly with the release of cytokines such as IFN-γ and TNF-α (3).

At variance with FasL, TRAILis constitutively expressed in some subsets of innate lymphocytes and represents an important effector molecule also in NK cells, as it can induce apoptosis in TRAIL-sensitive tumor cells (56). Its interaction with the respective receptors TRAILR1 or TRAILR2 on target cells results in receptor oligomerization and triggering of a pro-apoptotic signal through caspase cascade (60).

1.4.7. NK cells as a source of cytokines and chemokines

In addition to target cell killing, upon activation, NK cells release a variety of cytokines, primarily IFN-γ and TNF-α, that support innate and adaptive immune responses against tumors (59). IFN-γ, the sole member of the type II IFN family, is mainly secreted by NK cells, activated $CD8^+$ T cells, Th1 $CD4^+$ T cell, NKT cells and macrophages. It is a pleiotropic cytokine capable of orchestrating several mechanisms that support protective functions, including macrophage and dendritic cell activation and secretion of IL-12, $CD4^+$ T cell polarization toward a Th1 phenotype, antigen processing and presentation, in addition to promoting Ig production and class switching in B cells (45,91).

TNF-α contributes to the shaping of adaptive immune responses by enhancing B cell proliferation(60). Furthermore, it can cause tumor necrosis by damaging tumor-associated vasculature (92).

Upon activation, NK cells also serve as chemokine producers, primarily secreting CCL3 (MIP-1α), CCL4 (MIP-1β), CCL5 (RANTES), XCL1 (lymphotoxin), and CXCL8 (IL-8) which promote the recruitment of effector lymphocytes and myeloid cells to inflamed tissues (93).

1.5. NK cells in cancer and cancer immunotherapy

NK cells' effector functions are crucial for cancer immunosurveillance and for controlling disease progression, as they not only mediate direct tumor cell killing, but also exert their anti-tumor functions indirectly, by reshaping the tumor microenvironment and promoting the recruitment and activation of immune cells.

The primary role played by NK cells in mediating anti-tumor responses has been evidenced by the association of higher cancer susceptibility and metastases formation with impaired NK cell activity in preclinical and clinical studies (94–97). In addition, NK cell infiltration into tumors has been found to positively correlate with patient prognosis in different types of tumors, including melanoma, colorectal carcinoma, and NSCLC (43,51,98,99). Lastly, the efficiency of therapies based on tumor-targeting antibodies, employed for cancer treatment, has been described to partially rely on NK cell-mediated ADCC.

These represent some of the reasons why there has been a growing interest in investigating NK cell-based therapies in preclinical and clinical settings for cancer treatment.

Two main strategies have been pursued for NK cell-based cancer immunotherapy. The first strategy involves the activation and enhancement of the cytotoxic activity of endogenous NK cells directly in-vivo, while an alternative approach consists of the ex-

vivo expansion, and activation or genetic engineering of NK cells for subsequent infusion to patients as cellular therapies (100).

NK cells utilized for the preparation of cellular therapies can be either autologous (i.e. from the recipient patient) or allogenic (i.e. from healthy donors). The use of autologous NK cells has overall shown limited efficacy (101). Indeed, in patients with melanoma, renal cell carcinoma and metastatic breast cancer autologous NK cells were not effective at inducing relevant clinical responses (102,103). Such limited efficacy can be imputable to the inhibition of infused NK cells by self-HLA signals from tumor cells, but it may also be related to the poor quality of patients' NK cells, consequent to the putative systemic suppressive effects of the tumor (101). The discovery that the interaction of inhibitory KIRs with self-MHC class I molecules, expressed on the surface of tumor cells, plays a critical role in controlling NK cell cytotoxicity, drew a growing interest towards the use of allogeneic NK cells for cancer therapy (101,104). The beneficial effects of NK cells of allogenic origin have been extensively studied in the context of allogeneic hematopoietic stem cell transplantations (HSCT) for the cure high-risk leukemias. In these transplanted patients, a subset of NK cells, developed from the graft, expressed KIRs not recognizing recipient HLA-I alleles (i.e. showing a KIR-HLA mismatch). These alloreactive NK cells, which fail to recognize inhibitory KIR-ligands on recipient tumor cells, showed enhanced anti-leukemia effect and ability to protect from graft-versus-host disease (GvHD), likely eliminating host DC that could stimulate alloreactive T cells (105–107).

Likewise, the infusion of allogenic NK cells deriving from peripheral blood or generated from umbilical cord blood, has demonstrated to possess the potential to be applied for the treatment of solid tumors (108,109).

The limited persistence of infused NK cells in peripheral blood due to their rapid recognition and clearance by the host immune system is still one of the major challenges in adoptive NK cell therapy (110,111). However, persistence can be improved by administering lymphodepleting chemotherapy to the recipient prior to NK cell infusion (112). For this approach, fludarabine and cyclophosphamide represent the most

commonly used regimens (113). Preliminary studies showed that preconditioning with lymphodepletion may extend the persistence of infused allogenic NK cells from few days to 14–21 days, although a reduced number of cells may still be detectable in circulation for extended timepoints (112,114,115).

Additionally, short-term gains in persistence that reduce the need for frequent NK cell infusions can be made through cytokines that enhance NK cell survival. Cytokines such as IL-2 and IL-15 have been used to supplement patients undergoing adoptive cell therapy to support NK cell survival, expansion, and persistence (110,113,116,117).

Most NK cells express the low affinity receptor for IL-2 (IL-2Rβ) and lack the high affinity IL-2Rα. For this reason, NK cells require the administration of high IL-2 doses for their activation in the patients, with increased risk of side effects due to IL-2 toxicity (118). Moreover, the administration of IL-2 can favor the activation of the IL-2Rα^{+} Tregs, thus counteracting and limiting its clinical effects (111). Indeed, although IL-2 infusions in patients with metastatic melanoma, renal cell carcinoma and AML that received allogenic NK cell therapy led to in-vivo NK cell expansion, it was not effective at inducing remission in most patients (118).

IL-15 therapy has emerged as a promising alternative to IL-2 therapy. IL-15, when bound to the IL-15Rα chain, binds to IL-2Rβ with high affinity. Administration of recombinant IL-15, which works primarily after binding to membrane IL-15Rα molecules, resulted in expansion of NK cells and tumor regression in various preclinical cancer models, including melanoma, colon carcinoma and lung adenocarcinoma (100,119). Despite this, recent clinical trials have reported that cytokine support by systemic IL-15 resulted in reduced clinical activity compared to IL-2. The disappointing outcome was justified by the observation that IL-15 can potentially induce recipient CD8 T cell activation and consequently accelerate donor NK cell rejection (117).

Besides limited persistence, the clinical application of NK cell-based adoptive therapies for the treatment of solid tumors presents additional challenges. NK cell function is often impaired in solid tumors due to the presence of immunosuppressive cells such as Tregs,

Myeloid Derived suppressor Cells (MDSC), Tumor Associated Macrophages (TAM), Tumor Associated Fibroblasts (TAF), and tumor cells (110,120). Through the production of soluble factors, such as Transforming growth factor-β (TGFβ), PGE2, and IDO, these cells inhibit NK cell cytokine secretion, degranulation and metabolism, thus greatly impacting their antitumor activity (100,121–123). Moreover, tumor cells can also inhibit NK cell-mediated killing by modifying the expression of ligands for activating or inhibitory NK-Receptors, releasing suppressing factors, or altering their cytoskeletal structure during the formation of the immunological synapsis with NK cells (124–126).

Additionally, hypoxia is another considerable barrier to NK cell cytotoxic activity in the tumor microenvironment, as it mediates the downregulation of activating receptors, promotes the degradation of granzyme B through autophagy, and decreases NK cell cytokine secretion (120,127).

Finally, effective NK cell trafficking and infiltration into tumors is still challenging. In most solid tumors, the frequency of NK cells among total tumor-infiltrating immune cells is low, thus resulting in a low ratio between NK cells and tumor cells. In addition, NK cells infiltrating tumors tend not to be found in direct contact with tumor cells, where they can exert their cytotoxic activity, but, instead, they locate within the stroma in proximity to blood vessels (128).

Another important issue regards the possibility for NK cell-based therapies to prevent or contrast the metastatic disease. Several reports suggest a role for NK cells in the control of the metastatic dissemination (28). However, the information on how NK cells could interact with cancer stem cells (CSC), or with circulating tumor cells and cell clusters is rather limited. Some studies have indicated that NK cells can kill CSC, or eliminate tumor cells that have undergone the process of Epithelial-to-mesenchymal transition and have acquired mesenchymal and pro-metastatic properties (129). However, it remains poorly investigated the effect that NK cells can exert on the tumor cell clusters, which are thought to protect and deliver tumor initiating cells to the premetastatic niches.

Various approaches have been proposed for overcoming the barriers that limit the use of adoptively transferred NK cells in solid tumors. These approaches were essentially focused on three major aims:

a) Improving NK cell expansion and activation.

b) Improving NK cell trafficking and tumor targeting.

c) Overcoming tumor-induced NK cell inhibition.

Genetic engineering has been proposed to improve the trafficking and tumor targeting of NK cells. Tumor targeting has been achieved by engineering NK cells for CARs able to recognize specific tumor antigens or ligands for activating receptors. CAR-engineered NK cells recognizing EGFR, ErbB2/HER2, EGFRvIII and EpCAM have been tested in preclinical and clinical studies for the treatment of breast cancer brain metastases, glioblastoma and breast carcinoma, showing promising antitumor activity and a good safety profile (130–132). In addition, to improve NK cell trafficking and retention in tumors, NK cells have been engineered to express certain chemokine receptors either through DNA- or mRNA-based transfection (133). CXCR4 expression on NK cells was shown to improve their trafficking toward UG87 glioblastoma tumors that secrete CXCL12/SDF-1α (134). Likewise, in the context of ovarian cancer, inducing the expression of CXCR1 on NK cells was shown to significantly improve their tumor targeting and thus the anti-tumor responses of NK against both subcutaneous and intraperitoneal ovarian cancer xenografts (135).

To overcome tumor-induced NK cell inhibition, combination therapies with other targeted drugs, including checkpoint inhibitors such as anti-PD-1/PD-L1 antibodies, are being investigated (136). NK cell engineering has also been proposed to shield adoptive NK cell therapies from the suppressive effects of TGF-β, through the expression of a dominant negative form of TGF-β type II receptor (TGF-βRII) that efficiently blocks TGF-β signaling and maintains the cell surface expression of activating receptors and cytotoxicity in NK cells (137). For instance, the expression in NK cells of a CAR composed of a TGF-βRII extracellular and transmembrane domains combined with the

intracellular domain of NKG2D converted the TGF-b-mediated immunosuppressive signal into increased cytotoxicity while preventing the downregulation of NKG2D surface expression (138).

A further approach that has been studied to improve NK cell activity against solid tumors regards the tailoring of ex-vivo expansion and activation protocols to increase the actual number of cells available for infusion and to create a product capable of sustained in-vivo anti-tumor activity. Diverse methods for the ex-vivo expansion and activation of NK cells have been investigated. The most common ones include overnight and long-term culture of NK cells with cytokines alone or together with feeder cells such as K562 and Epstein–Barr virus-transformed lymphoblastoid cell lines (EBV-LCL) (139). Currently, most clinical studies involving adoptive NK-cell therapies employ IL-2 activated NK cells (139). This activation method has displayed variable outcomes, as a great variability among NK cells from different donors has been reported (140,141). Additionally, it has failed to induce NK cells with a sustained in-vivo antitumor activity (139). While the use of feeder cells for the expansion/activation of NK cells has resulted in NK cell products with increased expression of NCRs, TRAIL and NKG2D, and superior tumor cytotoxicity compared with IL-2-activated NK cells, safety concerns have been raised regarding their use, as they involve the employment virus-infected tumor cells(142,143).

The discovery of a new subset of NK cells endowed with memory-like functionality in the context of certain viral infections has led to the development of novel expansion protocols aimed to obtain in-vitro "memory-like" cells with increased persistence and functionality (144–146). In this regard, Cooper et al (147) first reported in 2009 that the brief exposure of murine NK cells to a cytokine cocktail comprising IL-12, IL-18 and IL-15 followed by a 7 to 22-day resting period, induced a memory-like differentiation of NK cells leading to the amplification and prolongment of their responsiveness upon restimulation with cytokines. This study provided first evidence that certain activation/expansion protocols could enable the differentiation of NK cells into a subset exhibiting innate immune memory. Following-up on these observations, Romee et al (144) confirmed that this memory-like differentiation could be also induced on human

peripheral blood NK cells by a short-term pre-activation with cytokines followed by a 7-day resting period in low-dose IL-15. Since then, the efficacy of these NK cells, termed cytokine-induced memory-like (CIML) NK cells, as anti-tumor effectors has been primarily tested in the context of hematological malignancies, where they have been shown to exhibit a greater responsiveness after restimulation with several leukemia cell lines, as well as with primary acute myeloid leukemia blast, compared to control NK cells (148). Besides their increased effector functions, both preclinical and clinical studies have revealed that they display improved persistence in-vivo, thus overcoming one of the major hurdles in NK cell-based adoptive cell therapies. Indeed, results from a recent clinical trial involving AML patients revealed that these cells were able to induce complete responses in 87% of patients and persisted for up-to 2 months in circulation (149). However, the mechanism driving the responsiveness and persistence of CIML NK cells have not been yet fully elucidated. Studies indicate that these cells undergo a still poorly characterized epigenetic reprogramming leading to their altered phenotype and functionality (150,151). In addition, previous studies revealed that metabolic changes occur within CIML NK cells, including upregulation of nutrient transporters (i.e. CD71, CD98, GLUT1 and GLUT3), and a metabolic shift from an oxidative to a glycolytic state (152). This metabolic reprogramming was shown to be essential to support their increased functional activity (152). Interestingly, the antitumor ability of CIML NK cells has been recently tested in preclinical studies in the context of ovarian carcinoma and melanoma, providing evidence of their potential suitability for the treatment of solid tumors (153,154).

Phenotypical characterization of these cells performed both in-vivo and in ex-vivo studies have indicated that CIML-NK cells represent a heterogeneous population that also includes poorly responsive cells, posing the issue on whether and how CIML-NK effectors could be optimized (155). Moreover, although their anti-tumor potential has been well-characterized in the treatment of hematologic malignancies both in preclinical and clinical studies, their efficacy against solid tumors and particularly against tumor cells driving tumorigenesis and metastatic dissemination remains to be fully investigated.

2. AIMS OF THE PhD PROJECT

The main goal of this project was to evaluate the antitumor effect of CIML NK cells against NSCLC cells, particularly focusing on the effects on potential tumorigenesis and stemness properties of tumor cells using a model of NSCLC tumor spheroids. To achieve this objective, the project has been organized on three aims, as follows:

1) The first aim of this project was to obtain a detailed phenotypical and functional characterization of CIML NK cells to better define traits associated with the memory-like properties and to identify the driving CIML-NK cell subset. In this project frame we also performed comparative analyses between CIML- and IL-2-activated-NK cells, which are still routinely employed in preclinical studies or in clinical trials.

2) The second aim was to assess the reactivity of CIML NK cells against tumor cells derived from different NSCLC subtypes (i.e. Adenocarcinoma, Squamous cell carcinomas, and Large cell carcinomas) to determine their possible therapeutic potential in NSCLC. Even more importantly, the purpose was also to evaluate the ability of CIML NK cells to control the CSC compartment, exploiting a model of in vitro generated NSCLC tumor spheroids which are enriched in $CD133^+$ cells.

3) The third aim was to determine whether CIML NK cells can benefit from combination with Tri-specific engagers, targeting the $CD133^+$ population in NSCLC cells, and enhancing NK cells via triggering of CD16 and IL-15 receptor.

3. MATERIALS AND METHODS

3.1 Materials

Table 1. Biological and Chemical Reagents

Reagent	Catalog number	Vendor
RPMI 1640 media with L-glutamine	SH30027.LS	Cytiva
Penicillin-Streptomycin (10,000 U penicillin and 10 mg/ml streptomycin in 0.9% NaCl)	P0781	Sigma-Aldrich
Trypsin-EDTA	25-200-056	Fisher Scientific
Heat-Inactivated FBS	16140-063	Gibco
AB Serum	H6914	Millipore Sigma
DMEM/F-12, HEPES	11-330-032	Gibco
Dulbecco's Phosphate Buffered Saline (DPBS); w/o Ca2+, w/o Mg2+	SH3002803	Hyclone
Trypan Blue Solution, 0.4%	15250061	ThermoFisher Scientific
Dimethyl sulfoxide (DMSO), cell culture grade	D2438-50ML	Millipore Sigma
Corning® Costar® Ultra-Low Attachment Multiple Well Plate	CLS7007-24EA	Sigma-Aldrich
B-27™ Supplement (50X), minus vitamin A	12587010	Gibco
Mouse EGF, Recombinant Protein	PMG8041	Gibco
Human FGF-basic, Recombinant Protein	PHG026	Gibco
Ficoll® Paque Plus	17-1440-03	Cytiva
NK Cell Isolation Kit, human	130-092-657	Miltenyi
Pre-Separation Filters	130-041-407	Miltenyi
LS Columns	130-042-401	Miltenyi
Interleukin-2, human recombinant protein (Proleukin)		Clinigen
Interleukin-15, human recombinant protein	BT-015	R&D
Interleukin-12, human recombinant protein	200-12	PeproTech
Interleukin-18, human recombinant protein	B001-5	MBL
Fixation/Permeabilization Solution Kit with BD GolgiStop™	554715	BD Biosciences
Protein Transport Inhibitor (Containing Brefeldin A)	555029	BD Biosciences
Carboxyfluorescein succinimidyl ester (CFSE)	13-0850-U500	Tonbo
Annexin V Binding Buffer (1X)	TNB-5000-L050	Tonbo
Incucyte® Caspase-3/7 Dye for Apoptosis	4440	Sartorius

Table 2. Antibodies used in cytofluorimetric analysis

Specificity	Fluorochrome	Clone	Amount/sample	Catalog No.	Vendor
CD3	FITC	REA613	2μL	130-113-138	Miltenyi
CD3	BV510	UCHT 1	2μL	563109	BD Biosciences
CD3	V500	SP34-2	3μL	560770	BD Biosciences
CD19	PE-CF594	HIB19	3μL	562294	BD Biosciences
CD19	FITC	LT19	1μL	130-091-328	Miltenyi
CD19	BV711	SJ25C1	4μL	363022	BioLegend
CD16	FITC	REA423	1μL	130-113-392	Miltenyi
CD16	PE	REA423	1μL	130-113-393	Miltenyi
CD16	APC-Vio770	REA423	2μL	130-113-390	Miltenyi

CD16	BV510	3G8	3µL	563830	BD Biosciences
CD56	PE-Cy7	N901	3µL	A21692	Beckman Coulter
CD45	PE	HI30	5µL	555483	BD Biosciences
CD69	nonconjugated	FST3	2 µg/mL		Our laboratory
CD107a	eFluor660	H4A3	2µL	50-1079-42	eBioscience
CD107a	BV711	H4A3	3µL	328640	BioLegend
CD113/1	PE	REA753	1µL	130-110-962	Miltenyi
CCR7	nonconjugated	150503	10µg/mL	MAB197	R&D
CXCR1	nonconjugated	42705	25 µg/mL	MAB330	R&D
CXCR6	nonconjugated	K041E5	25 µg/mL	2380010	Sony Biotechnology
CXCR4	nonconjugated	44717	25 µg/mL	MAB173	R&D
CXCR3	Nonconjugated	49801	25 µg/mL	MAB160	R&D
CX3CR1	PE	2A9-1	3µL	D070-5	MBL
DNAM-1	Nonconjugated	F22	2 µg/mL		Our laboratory
NKp44	Nonconjugated	Z231	2 µg/mL		Our laboratory
NKp46	Nonconjugated	BAB281	2 µg/mL		Our laboratory
NKp80	Nonconjugated	MA152	2 µg/mL		Our laboratory
NKG2D	Nonconjugated	BAT221	2 µg/mL		Our laboratory
NKG2A	Nonconjugated	Z199	2 µg/mL		Our laboratory
NKG2A	APC	REA110	1µL	130-113-563	Miltenyi
KIR2D	PE	NKVFS1	1µL	130-123-710	Miltenyi
KIR2D	FITC	NKVFS1	5µL	130-098-689	Miltenyi
KIR3DL1/DL2	FITC	REA970	1µL	130-116-177	Miltenyi
IFN-γ	PE	B27	1µL	554701	BD Bioscience
IFN-γ	Pacific Blue	B27	1µL	3132630	Sony Biotechnology
PD-1	PE	PD1.3.1.3	1µL	130-117-384	Miltenyi
TIM-3	nonconjugated	F38-2E2	1:400	345004	BioLegend
TIGIT	nonconjugated	MBSA43	1:400	16-9500-82	Invitrogen
Granzyme B	Alexa Fluor 647	GB11	3µL	515406	Biolegend
Isotype control	Alexa Fluor 647	MOPC-21	3µL	400136	Biolegend
Fixable viability dye	Ghost violet V540		1:1000	13-0879-T100	Tonbo
Fixable viability dye	Ghost violet UV450		1:1000	13-0868-T100	Tonbo
Fixable viability dye	FVS450		1:1000	562247	BD Biosciences
Annexin V	APC		5µL	20-6409	Tonbo
Goat Anti-Mouse IgG1, Human	PE		1:200	1070-09	Southern Biotech
Goat anti-Mouse IgG2b	Alexa Fluor™ 647		1:800	A-21242	Invitrogen

3.2 Cell lines and cell culture

The human lung cell line A549 was originally isolated from lung tissue of a white, 58-year-old male with lung adenocarcinoma. These cells are hypotriploid human alveolar

basal epithelial cells with the modal chromosome number of 66, occurring in 24% of cells (156,157)

The human H661 cell line was isolated from a 43-year-old male with large cell carcinoma lymph node metastasis (158). These cells exhibit no gross structural DNA abnormalities. The human cell line H3122 is derived from a 52-year-old male patient with primary bronchoalveolar adenocarcinoma prior to treatment. While the human squamous cell carcinoma cell line SW900 is derived from a Caucasian 53-year-old male with primary squamous cell carcinoma (157).

A549 H3122, H661 and SW900 cells were cultured in 75 cm2 cell culture flasks in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin/streptomycin (100 U/mL penicillin, 10 µg/ml streptomycin) further referred as complete RPMI (cRPMI). All cell lines were maintained under standard cell culture conditions (37°C, humidified atmosphere, 5% CO2) and were regularly tested for mycoplasma contamination. Passaging of adherent cells was performed at 70% to 80% of confluent monolayer using 1mL of 0.05% trypsin/ethylenediaminetetraacetic acid (EDTA) solution after a washing step with 1x Dulbecco's Phosphate Buffered Saline (DPBS). The reaction was stopped by adding 9 mL of fresh media. After a centrifugation step for 5 min at 300 x g, the cell pellet was resuspended in 10 ml media, followed by cell counting (section 3.2.2). Cell Suspensions were passaged twice a week by mixing the resuspended pellet after a centrifugation step (5 min, 300 x g) in a ratio of 1:4 with fresh medium. The fourth to fifth passaged cells were used for further experiments. (i.e., generation of spheroids, NK cell co-cultures)

3.2.2 Cell counting and viability.

Cell counting was performed by diluting cells 1:1 with trypan blue to discriminate alive and dead cells (trypan blue positive cells). 10uL of the dilution solution were then added to a hemacytometer and cells were counted.

3.2.3 Generation of tumor spheroids

Tumor spheroids were formed by culturing 4 x 10^3 tumor cells in serum-free cell culture medium composed of DMEM/F-12 (Sigma-Aldrich) supplemented with B-27 without vitamin A, 20 ng/mL of epidermal growth factors (EGF), 20 ng/mL basic fibroblast growth factor (bFGF), 5 µg/mL heparin and 1% penicillin/streptomycin into ultra-low attachment 6 or 96-well microplates with round bottom. Tumors cells were incubated under humidified atmosphere of 5% CO2 and 95% air at 37°C for 3 to 7 days to ensure the formation of tumor spheroids.

3.3 NK cell isolation from peripheral blood

3.3.1 PBMC separation from peripheral blood

Peripheral blood from healthy donors was provided by the transfusion center of the *Ospedale Policlinico San Martino* following approved internal operational procedures (IOH78). Additionally, peripheral blood from healthy donors was obtained from STEMCELL. Written informed consent from the donors was provided according to the Declaration of Helsinki. Immediately upon receipt, blood was brought to room temperature, the total volume recorded and diluted 1:1 (v/v) with 1xPBS.

Then, peripheral blood mononuclear cells (PBMCs) were obtained by centrifugation on a Ficoll–Paque density gradient at 400 x g for 30 min with slow acceleration and break-off. The PBMC layer was gently recovered and washed two times using 50mL of 1xPBS. Afterward, 100uL were aliquoted into an Eppendorf tube, diluted 1:10 and stained with trypan blue before counting the number of viable PBMCs under the microscope using a hemocytometer.

3.3.2 NK cell isolation

Human NK cells were purified from PBMCs by magnetic negative selection, with a human NK cell isolation kit (MACS-Miltenyi Biotec) following the manufacturer's protocol. PBMCs were washed with 1xPBS EDTA buffer (Miltenyi), resuspended in the

presence of an antibody cocktail and incubated for five minutes at 4°C. Then an appropriate amount of magnetic microbeads was added and incubated for further 10 minutes at 4°C. The volumes of the two reagents (antibody cocktail and microbeads) were optimized as indicated by the manufacturer based on the number of PBMCs. The purification of NK cells was obtained through passage into LS columns on a magnetic stand. The eluted fraction containing NK cells was collected and analyzed by flow cytometry (section 2.2.4), gating NK cells as viable CD19-CD3-CD56+CD16+ cells. The overall purity of the isolated NK cells was ≥ 90%.

3.3.3 Generation of CIML-, IL15- and IL-2 NK cells

After isolation (Day zero; D0) NK cells were cultured for seven days following one of three different activation conditions as described below and represented on figure 7:

a) *IL-2NK cells*: D0 NK cells were culture in cRPMI supplemented with 100 U/mL recombinant human IL-2 (Proleukin, Novartis Basilea, Switzerland). The medium was refreshed every two days.

b) *IL-15NK cells:* D0 NK cells were culture in cRPMI supplemented with 1ng/mL recombinant human IL-15 (IL-15 media) at a final volume of 200uL/well (Proleukin, Novartis Basilea, Switzerland). Half of the media (100uL) was replaced every 2 days with 100uL of fresh IL-15 media.

c) *CIML NK cells*: To generate CIML NK cells D0 NK cells were preactivated for 16± 2 hours in cRPMI in the presence of a cocktail of IL-12 (10ng/mL), IL-18 (50ng/mL), and IL-15 (1ng/mL) at a cell concentration of $3x10^6$/mL in 96 well round bottom plates (200uL/well). After 16 hours, cells were recovered and washed 3 times in cRPMI without cytokines and cultured for 6-7 days in IL-15 media. Cells were maintained in culture by replacing half of the media (100uL) with fresh IL-15 media every 2 days.

In all three cases, cells were seeded in 96-well round bottom plates at a density of $3x10^6$/mL. Cells at days 6-8 were used for functional and phenotypical studies.

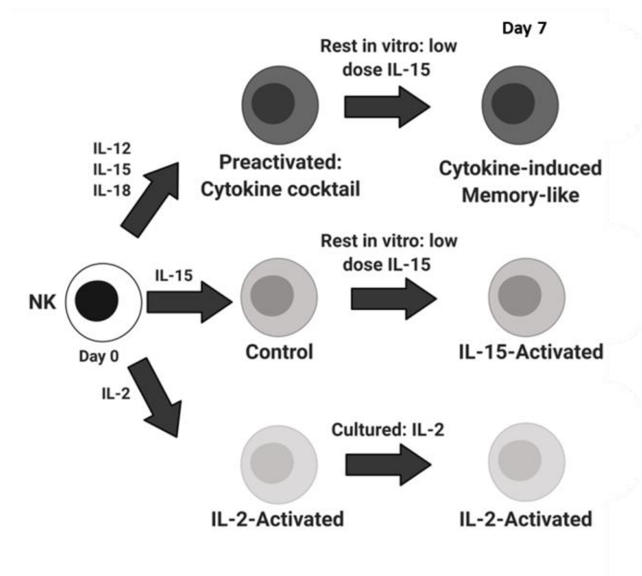

Figure 7. NK cell culture conditions to generate the three tested NK cell products.

3.4 Phenotypical analysis.

NK cell products (D0 and D7 NK cells) were phenotypically characterized by flow cytometric analysis. Details of all antibodies used for flow cytometry are found in Table1 a. Surface staining was performed in sorter buffer (2% FBS in PBS) for 30 min at 4°C followed by two washes in sorter buffer. All the employed antibodies were titrated before use to determine the optimal antibody concentration. Flow cytometry samples were acquired on a LSR Fortessa flow cytometer (BD Biosciences) or a Galios flow cytometer (Beckman Coulter). Acquired data were analyzed in FlowJo v.10.7 (BD Biosciences) or Kaluza (Beckman Coulter) and gated on live CD19–CD3–CD56+ single cells (NK cells).

3.5 Functional assays

3.5.1 CD107 and IFNY intracellular staining

Co-coltures of adherent cells or spheroids with CIML, IL-15 and IL-2 NK cells were performed in 96-round bottom plates at an effector:target (E:T) ratio of 2:1 in the presence

of CD107a antibody. After 1 hour, Brefeldin A (1:1000, GolgiPlug, BD Biosciences) and monensin (1:1500, GolgiStop, BD Biosciences) were added to the co-cultures, and cells were cultured for 5 additional hours. Next, cells were recovered, washed once with PBS and stained with fixable viability stain 450 (BD Biosciences) or ghost UV450 viability dye (Tonbo) for 20 minutes at 4°C. Following, cells were stained with surface NK markers (CD56, CD16, NKG2A and mix KIRs), and then fixed and permeabilized with Fixation and Permeabilization Kit (BD Biosciences, New Jersey USA). Cells were incubated with anti-IFNγ antibody, followed by flow cytometric analysis. NK cells were gated as viable, CD56+ single cells.

3.5.2 Flow cytometry-based killing assay.

Before performing the co-cultures, tumor cells ($1x10^6$ cells/mL) were labeled with 2 μM of CSFE in PBS for 10min at RT and afterward free dye was quenched by addition of complete media.

Flow-based killing assays were performed by co-culturing IL-15, IL-2, CIML or CIML +IL-2 cells NK cells in U bottom 96-well plates (Corning Incorporated) for 24 hours with spheroids formed using CFSE-labeled SW900, A549 or H661 at E:T ratios of 8:1, 4:1, 2:1, and 1:1, as shown in Figure 8. Next, cells were recovered, spheroids were disaggregated by briefly incubating them with Stem Cell Dissociation Reagent (Stem Cell) and stained with BV540 fixable viability dye (Tonbo) in PBS for 20 minutes (dead cell marker). Cells were then washed once in sorter buffer and subsequently in annexin binding buffer. Cells were set at a concentration of <1x106 cells/100uL in annexin binding buffer and stained with 5uL of annexin V-APC, following the manufacturer's instructions (Tonbo Biosciences). All samples were acquired on a flow cytometer as described above. Dead target cells were defined as CSFE+ Annexin V+ and/or dead cell marker (DCM)+ and specific cytotoxicity was calculated as:

((% dead experimental- % dead target only) ÷ (100% - % dead target only)) x 100%.

The effect of NK cells on the CD133+ population within the spheroids was determined by staining tumor cells after the co-culture with ant-CD133/1 antibody at 4C. Before running

samples on flow cytometer, 100uL of counting beads were added to normalize the number of cells/volume. CD133 positive cells were gated on viable, CSFE+ single cells.

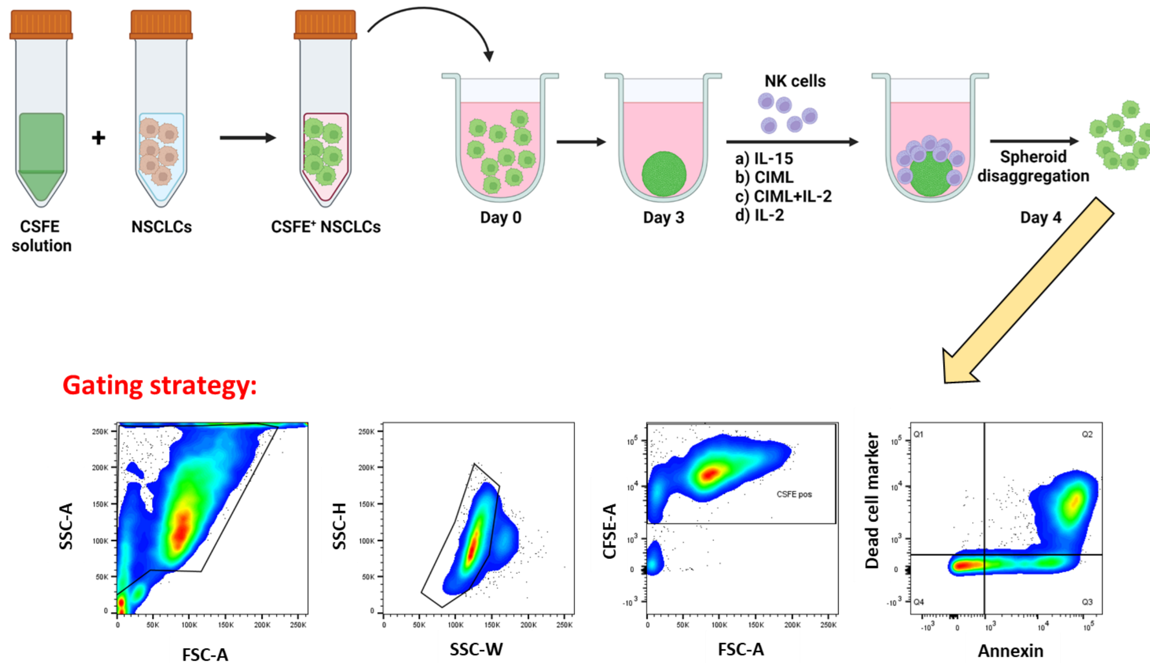

Figure 8. Schematic representation of the flow cytometry-based killing assay. NSCLC cells were labeled with CSFE on day 0 and seeded in ultra-low attachment U bottom 96-well plates. Once the spheroids reached a solid state, NK cells were added at different E:T ratios. Next, spheroids were disaggregated, and stained with viability dye (dead cell marker, DCM) and annexin V. Cytofluorimetric analysis of DCM and annexin V was performed by gating on single cells and CSFE positive cells.

3.5.3 IncuCyte measurement of spheroid killing

Killing of tumor spheroids was evaluated in real time using the IncuCyte® S5 platform (Essen Bioscience, Sartorius). SW900, A549 and H661 spheroids were generated following the procedures described in section 2.1.3 and cultured in ultra-low attachment 96 well round bottom microplates. When spheroids reached a solid state (day 3-4), 80,000 NK cells (IL-15, CIML, IL-2 or CIML+ IL2 NK cells) were added to each well containing a single-spheroid.

Tumor cell apoptosis in the spheroids was monitored for 48hours during the co-culture by including in the media the Caspase 3/7 Green detection dye (Sartorius). A schematic representation of the assay is shown in Figure 9. Images of each well were acquired every hour using a 4x objective lens and analyzed by IncuCyte Controller v2020A (Essen Biosciences). The means of the technical replicates for each condition (n=3) were compiled for N=3-4 donors.

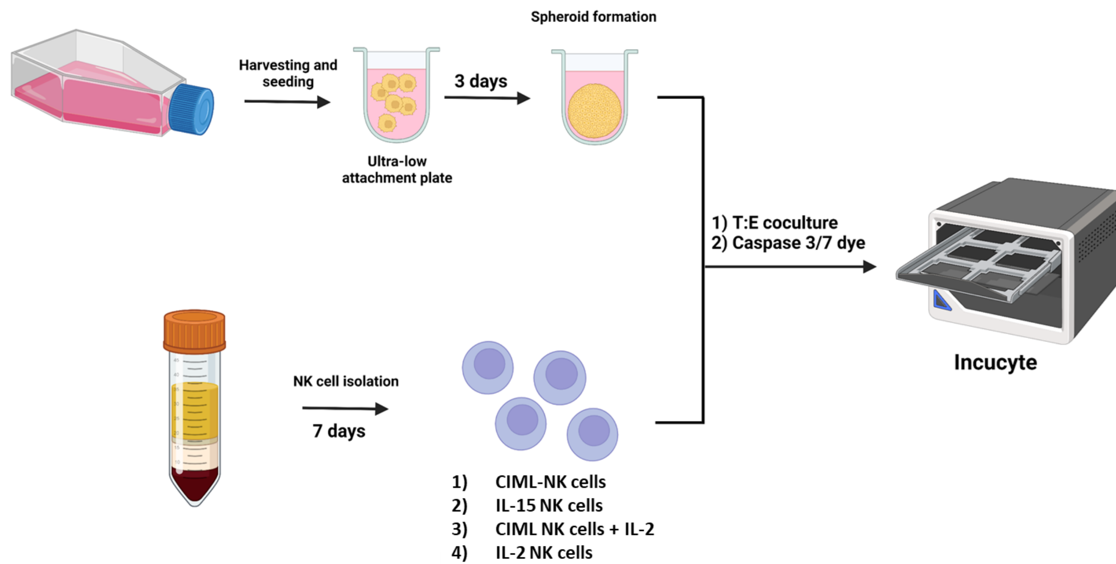

Figure 9. Schematic representation of the real-time Incucyte-based killing assay. NSCLC cells were seeded in ultra-low attachment U bottom 96-well plates. Once the spheroids reached a solid state, NK cells were added at different E:T ratios in the presence of caspase 3-7 dye. Next, real-time imaging for a period of 48 hours was performed by using the Incucyte system.

3.6 Statistical analysis

All statistical analyses were done using GraphPad Prism Software (V.9, San Diego, California USA). Used statistical tests as well as sample sizes are indicated in the figure legends. If not indicated differently, data are shown as mean±SD. Statistical significance was defined as *P<.05; **P<.01; ***P<.001.

4. RESULTS

4.1. Generation and characterization of CIML NK cells: identification of the CD56^{bright} cell subset as a major player of CIML functions and anti-tumor activity.

CIML NK cells were generated by first isolating NK cells from peripheral blood derived from healthy donors. Next, freshly isolated NK cells were stimulated for 16 hours with IL-15+IL-12+IL-18, followed by a 7-day resting period in the presence of low-dose IL-15 to support NK cell survival, following the procedures outlined in section 3.3.3. As control, NK cells were cultured in the presence of low dose IL-15. The generation of CIML NK cells was routinely confirmed by evaluating, at day 7 of culture, the expression levels of IFN-γ after a 6-hour cytokine (IL-12/IL-18) stimulation and comparing it to the one obtained after stimulation of control NK cells (Figure 10). Additionally, memory-like differentiation was determined by measuring the surface expression levels of NK cell-associated markers including CD56, CD16, NKG2D, and NKp44 (Figure 11A-D). In line with previous reports (144), we observed that memory-like differentiation consistently correlated with an increased IFN-γ production upon restimulation of NK cells with a cytokine cocktail over control NK cells, and this was employed as a readout of CIML NK generation (Figure 10A and B).

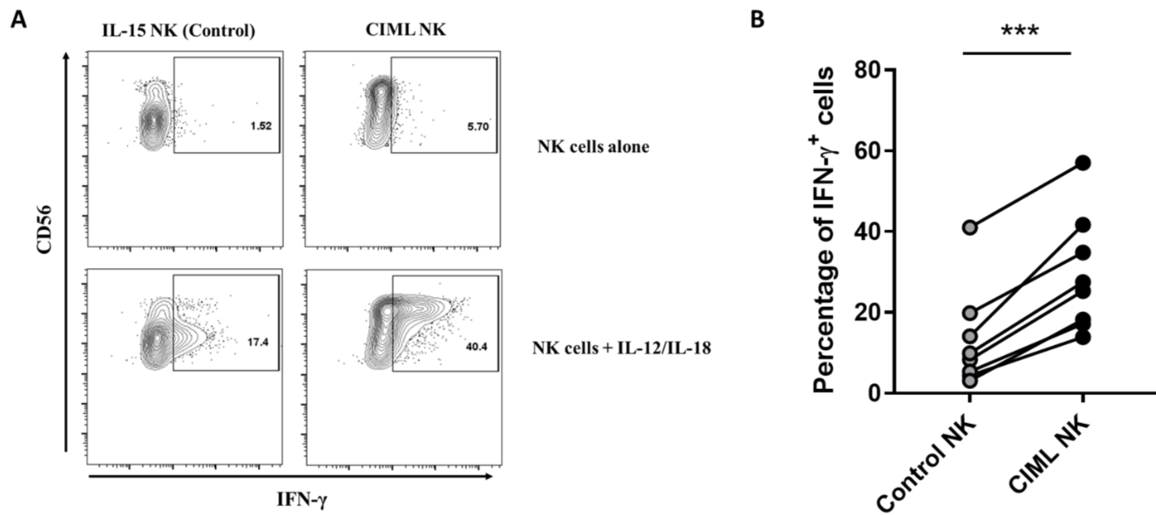

Figure 10. Frequency of IFN-γ+ NK cells. IFN-γ release by day-7 IL-15 (control) and CIML NK cells without stimulation (NK cells alone) or after a 6-hour stimulation with a cytokine cocktail (IL-12/IL-18). A) Representative flow cytometry plots showing the expression of IFN-γ by $CD56^+$ NK cells. Percentage of IFN-γ+ IL-15 (Control NK) and CIML NK cells gated on CD56+ cells (N=8 donors). Statistical significance was determined using a paired two-tailed T test (*, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001).

We also noted that CIML NK cells are characterized by a high-level expression of CD56, NKG2A, and NKp44, and a reduced expression of CD16, and this was considered in the evaluation of CIML NK cells after preparation.

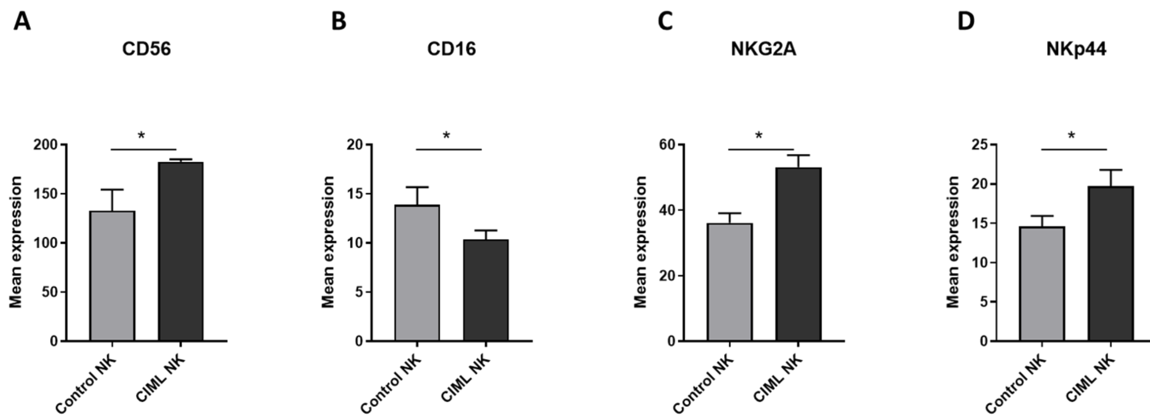

Figure 11. Phenotypical evaluation of CIML NK cells. Day 7 NK cells (IL-15 or CIML NK cells) were analysed for the expression of NK cell–associated markers by flow cytometry. Histograms display the mean expression ± SEM of CD56 (A), CD16 (B), NKG2A (C) and NKp44 (D) in CD56+ NK cells (N=5-6 donors). Statistical significance was determined using a paired two-tailed T test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$).

Once the protocol for the generation of CIML NK cells was validated, we performed an extensive cytofluorimetric analysis of the obtained NK cells with the aim to evaluate their phenotypical profile, thus identifying possible characterizing cell subsets and assessing their contribution to the functional CIML features.

The phenotype of CIML NK cells at day 7 of culture was compared to that of freshly isolated NK cells (D0), or to NK cells that had been cultured for 7 days in the presence of IL-2 (IL-2 NK cells). This latter condition was used as a benchmark, given that IL-2 is still a commonly employed cytokine in clinical protocols for NK cells' stimulation or to sustain their in-vivo activation and survival upon administration to patients, although its use has shown only modest therapeutic effects (113,117,140). NK cells cultured in the presence of low dose IL-15 (IL-15 NK) were also included in our studies as control group, since this represents the conventional reference condition of CIML NK cells (144,148).

To assess the contribution of major NK cell subsets to CIML functions, a phenotypical characterization was carried out based on the expression of CD56, CD16, NKG2A, and KIRs. Results revealed that CIML NK cells undergo a significant expansion of the CD56^{brigh}CD16^{dim} subset. In line with previous findings, this expansion was not exclusively observed in CIML NK cells, but also in the other tested culture conditions (140,141,159), although it was significantly higher in CIML NK cells, in which it constituted in some donors up to 60% of the total NK cells (Figure 12A-C).

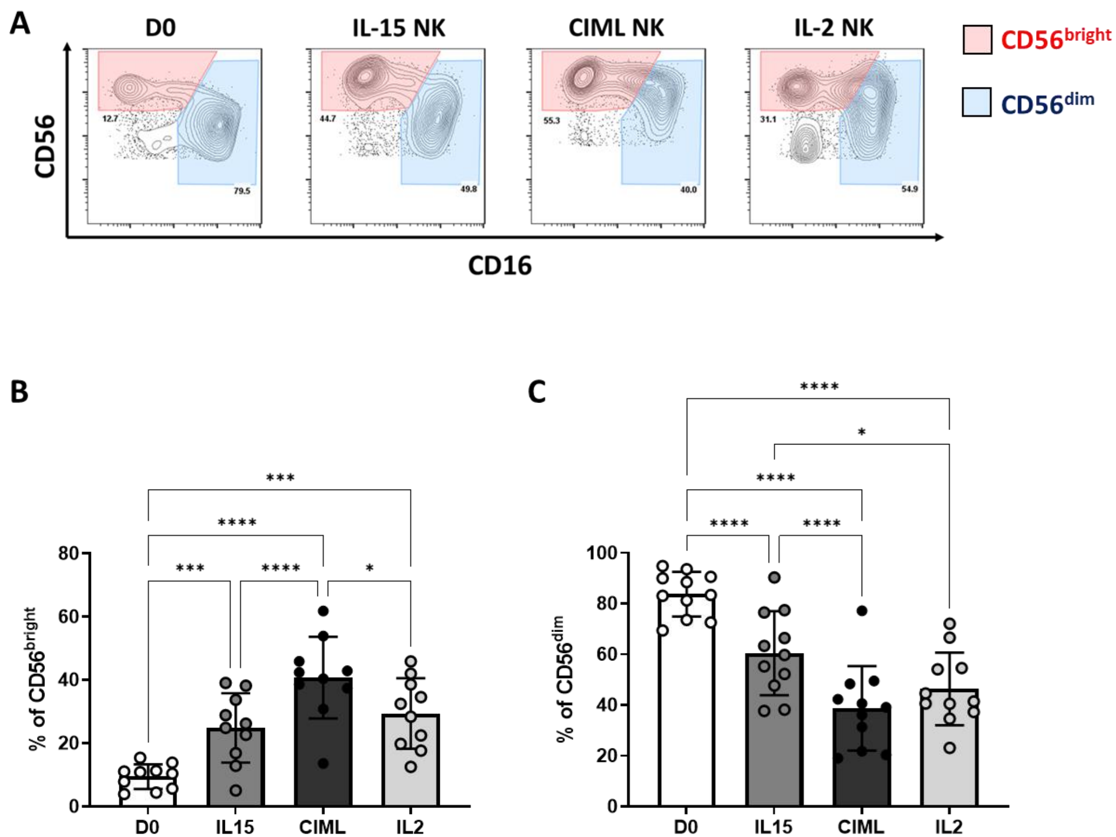

Figure 12. Phenotypical characterization of freshly isolated NK (D0), IL-15 NK, IL-2 NK and CIML NK cells by flow cytometry. (A) Representative flow cytometry plots of CD56 versus CD16 showing the gating of CD56^{bright} (red) and CD56^{dim} (blue) subsets. (B,C) Frequency of CD56^{bright} and CD56^{dim} subsets prior stimulation and in the different NK cell products at day 7 (IL-15, CIML and IL-2). Histograms show mean ± SD. Statistical

significance was determined using a one-way ANOVA with post-hoc Tukey test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$).

In agreement with these results, CIML NK cells showed a preferential expansion of the $NKG2A^{+}KIR^{-}$ cell subset, which typically characterizes immature $CD56^{bright}$ cells. It is noteworthy that in CIML NK cells, the $NKG2A^{-}KIR^{+}$ cell population nearly disappeared compared to freshly isolated NK cells, providing evidence that this more mature subset does not participate in the generation of CIML NK cells (Figure 13).

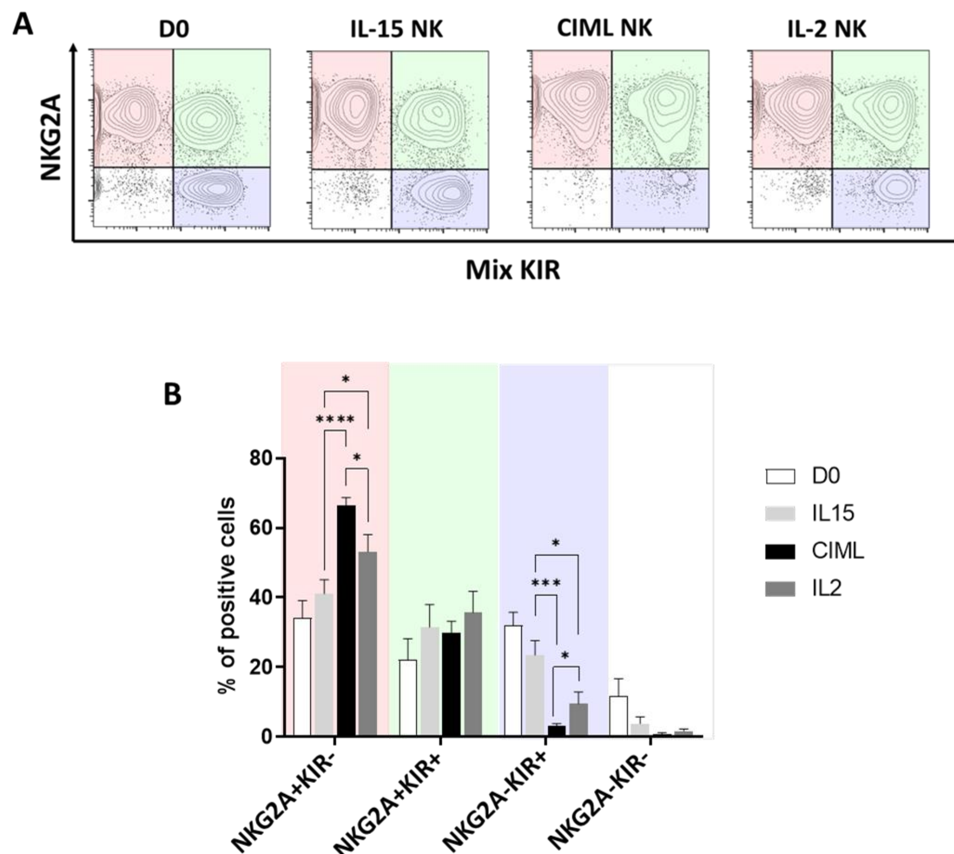

Figure 13. Characterization of NK cell subsets based on the expression of NKG2A and KIRs (Mix KIR). A) Representative flow cytometry plots of NKG2A and KIR gated on

CD56+ cells on the different NK cell products. B) Frequency of NKG2A+KIR-, NKG2A+KIR+, NKG2A-KIR+ and NKG2A-KIR- populations gated on CD56+ cells for the different NK cell products. Histograms display the mean ± SD (N=6 donors). Statistical significance was determined using a Two-way ANOVA test, mixed effects model with Tukey post hoc test. (*, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001).

Given the marked expansion of CD56^{bright} cells, we next tried to characterize in more detail their phenotype, and to evaluate their potential contribution to the "CIML functional traits". To this scope, we analyzed the expression levels of major activating and inhibitory check-point receptors in the bulk NK cells, and within the CD56^{bright} and CD56^{dim} subsets upon exposition to the different culture conditions (Figure 14A-C).

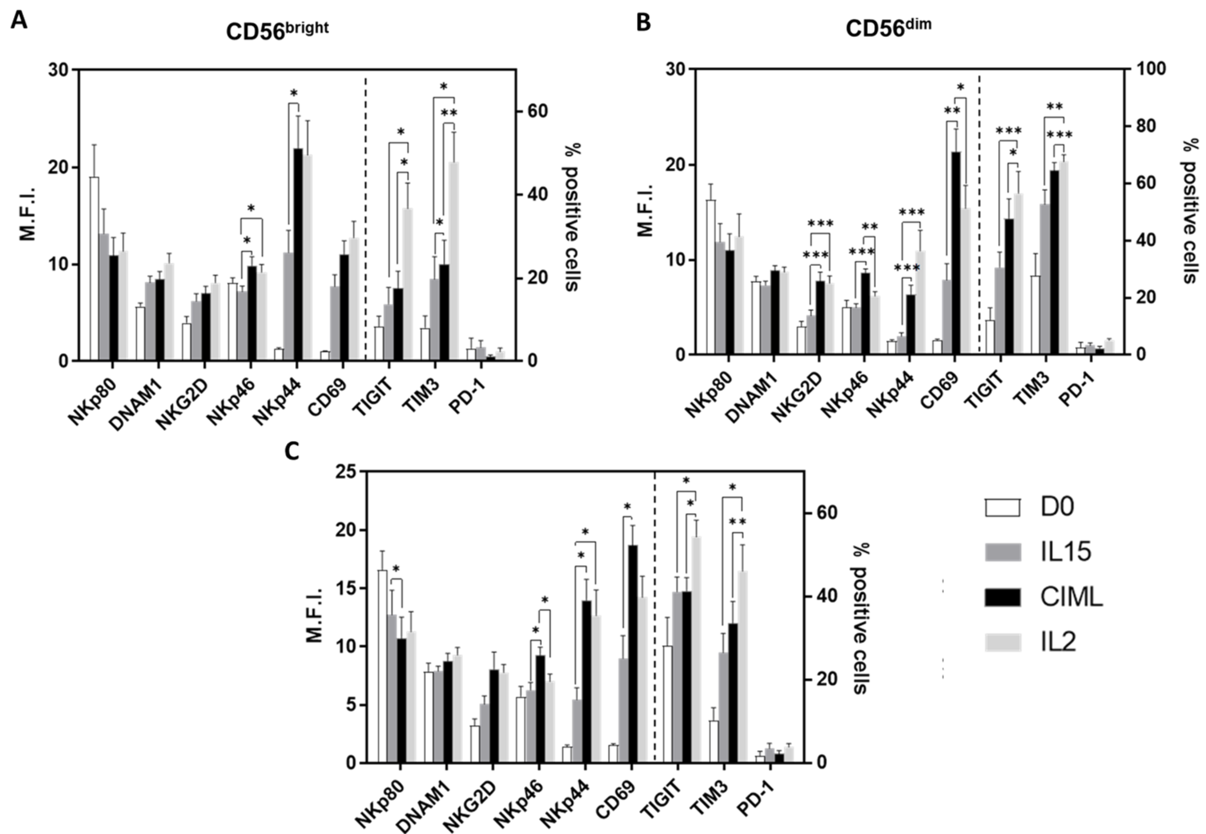

Figure 14. Evaluation of the expression of different activating receptors and inhibitory checkpoints. Expression levels of NK cell receptors on CD56bright (A), CD56dim (B) subsets, and total NK cells (C) are shown as mean fluorescence intensity (M.F.I) or percentage (%) of positive cells. Histograms show mean ± SD (N=6-9 donors). Statistical significance was determined using a two-way ANOVA mixed effects model paired with Tukey post-hoc test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$).

We found that CIML NK cells, compared to D0 and IL-15 NK cells, exhibited higher levels of NKp44, NKp46 and CD69 and lower expression of NKp80, whereas the expression of these activating receptors was comparable to those seen on IL-2 NK cells. Among the $CD56^{dim}$ and $CD56^{bright}$ subsets, the latter one showed the highest expression levels of activating receptors NKp44 and NKp46 in all the three tested conditions, and this difference was particularly pronounced in CIML NK cells. Major differences between CIML- and IL-2-NK cells were observed by the analysis of three inhibitory checkpoint receptors particularly relevant to NK cells, PD-1, TIM-3, and TIGIT. In NK cells derived from the three culture conditions tested in this study, PD-1 was lowly expressed, although it seemed to be somewhat lower in CIML NK cells compared to the other products.

The expression levels of both TIGIT and TIM-3 were more pronounced than those of PD-1. The assessment of these receptors revealed that, indeed, even though CIML NK cells expressed higher levels of TIM-3 and TIGIT than IL-15 NK cells, as a result of their higher activation status, their expression in both $CD56^{bright}$ and $CD56^{dim}$ subsets was significantly lower than that of IL-2 NK cells.

Another important point to be studied to evaluate the therapeutic potentiality of CIML-NK cells regards the ability of these cells to respond to chemotactic stimuli. To this intent, we

measured the expression of the chemokine receptor repertoire by cytofluorimetry on the different NK cell products. Flow cytometric analysis demonstrated that CIML NK cells upregulate the expression of chemokine receptors CXCR4 and CXCR3 compared to D0, IL-2 and IL-15 NK cells, and that these receptors were most abundant in $CD56^{dim}$ and $CD56^{bright}$ subsets, respectively (Figure 15A-C)

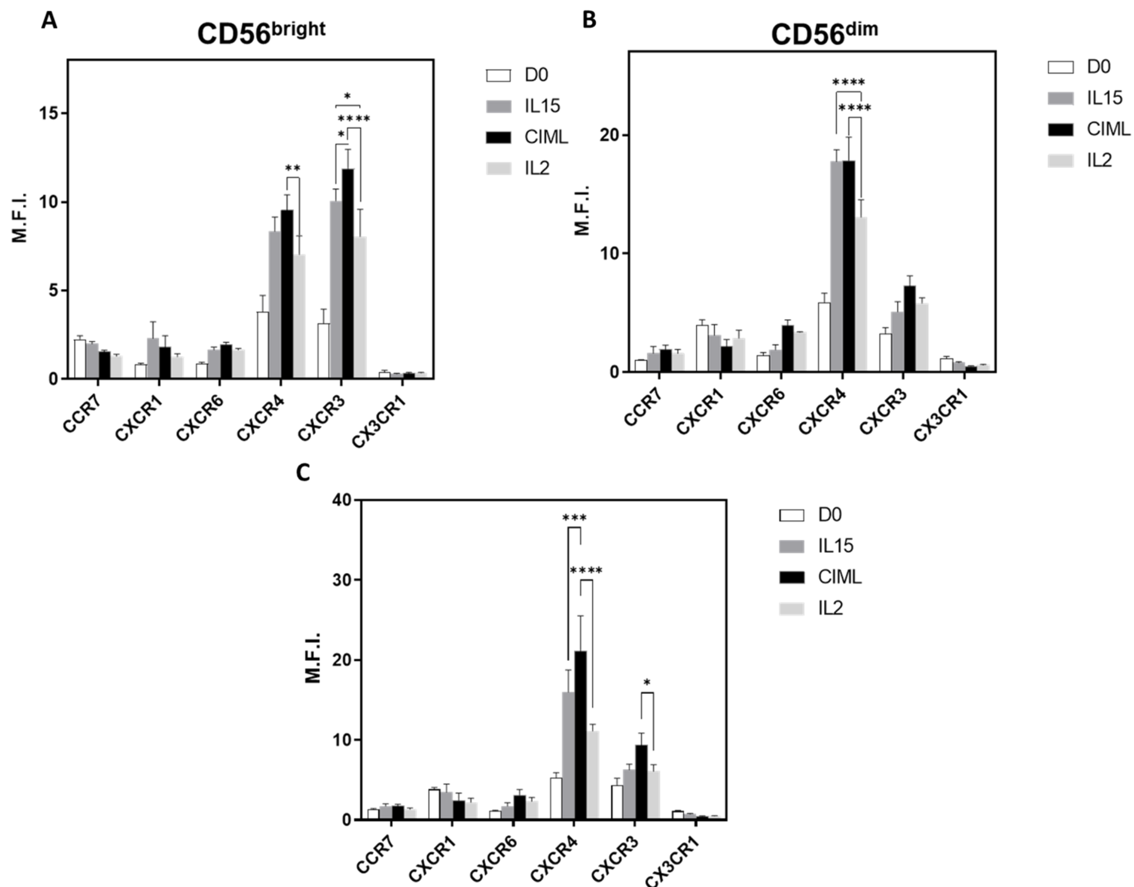

Figure 15. Evaluation of the expression of different Chemokine receptors on the different NK cell products. Expression levels were assessed on $CD56^{bright}$ (A), $CD56^{dim}$ (B) and on total NK cells (C). Expression levels are reported as mean fluorescence intensity (M.F.I) ± SD (N=5 donors). Statistical significance was determined using a two-way ANOVA, mixed effect model paired with Tukey post-hoc test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$).

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Overall, our data concerning the phenotypical characterization of CIML NK cells indicated that the CD56^{bright} cell subset may drive a functional advantage of CIML NK cells over the classical IL-2-stimulated NK cells. Indeed, this cell subset within CIML NK cells showed a marked expression increase of specific activating and chemokine receptors, accompanied by a reduced surface abundance of TIGIT and TIM-3 inhibitory checkpoints.

Next, to determine whether CIML NK cells have a functional advantage over IL-15 and, mainly, over IL-2 NK cells, and the potential contribution of the CD56^{bright} and CD56^{dim} subsets, we characterized the effector functions of these NK cell products against NSCLC targets. Specifically, we evaluated the effector cells for their ability to release IFN-γ and cytotoxic granules in response to four different NSCLC cell lines. These target cells were derived from various tumors, representing the most common histotypes of NSCLC: A549 (adenocarcinoma – ADC), H3122 (ADC), SW900 (squamous cell carcinoma – SCC), H661 (large cell carcinoma – LCC). Following a 7-day differentiation period, we performed a standard 6-hour co-culture of effector and target cells and evaluated the expression of IFN-γ and CD107a, as a surrogate marker for cytotoxic degranulation, in the effector cells by flow cytometric analysis (Figure 16). In all three NK cell preparations, the CD56^{bright} subset proved to be the main driver of the effector response both in terms of degranulation and cytokine production, being the highest response displayed by CIML CD56^{bright} NK cells.

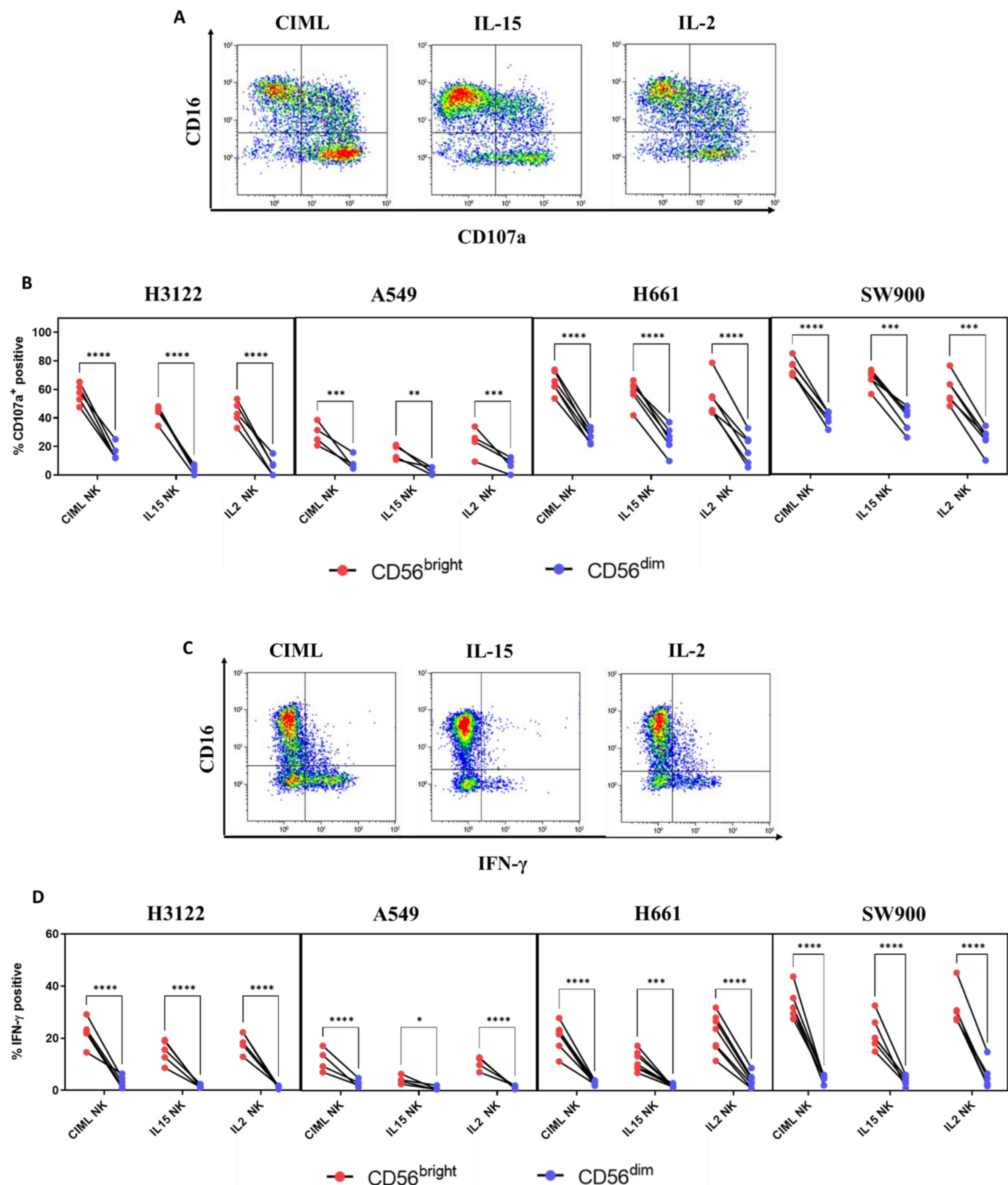


Figure 16. Functional characterization of CD56^{bright} and CD56^{dim} cells in CIML-, IL-15 and IL-2 NK cells. NK cells at day 7 were co-cultured with NSCLC cells (E:T ratio of 1:1) for 6 hours, then the expression of the degranulation marker CD107a and the production of IFN-γ were evaluated on CD56^{bright} and CD56^{dim} NK cells by flow cytometry. A and C)

Representative flow cytometry plots showing the expression of CD16 vs CD107a and IFN-γ gated on total viable NK cells. B and D) Frequencies of CD107 and IFN-γ positive cells in $CD56^{bright}$ (red) and $CD56^{dim}$ (blue) NK cells. (N=4-5 donors). Significance was determined by using a two-way ANOVA paired with Sidak's post-hoc test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$).

Analyzing the unfractionated populations, overall, CIML-NK cells showed enhanced IFN-γ release, compared to control IL-15 NK cells, while they did not exhibit a significant advantage over IL-2 NK cells (Figure 17A).

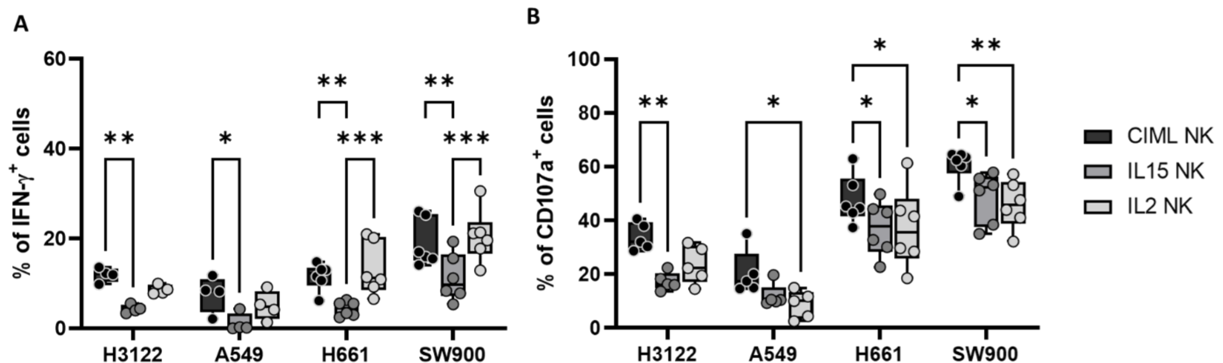


Figure 17. Functional characterization of CIML NK cells compared with IL-15 and IL-2 activated NK cells. CIML, IL-15 and IL-2 NK cells were stimulated for 6 hours with H3122, A549, H661 or SW900 cells afterward, the expression of IFN-γ (A) and CD107a (B) by NK cells (CD56+ cells) was measured by flow cytometric analysis. Only significant P values are shown (N=5-6 donors). Two-way ANOVA with Turkey post-hoc was used to compare groups (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$).

On the other hand, and more importantly, CIML NK cells showed a higher cytotoxic degranulation than IL-15- or IL-2-induced NK cells when re-stimulated with NSCLC targets (Figure 17B). These findings were consistent with the higher levels of granzyme B detected

in CD56^{birhgt} CIML NK cells in comparison to CD56^{bright} IL-15 and IL-2 NK cells (Figure 18B).

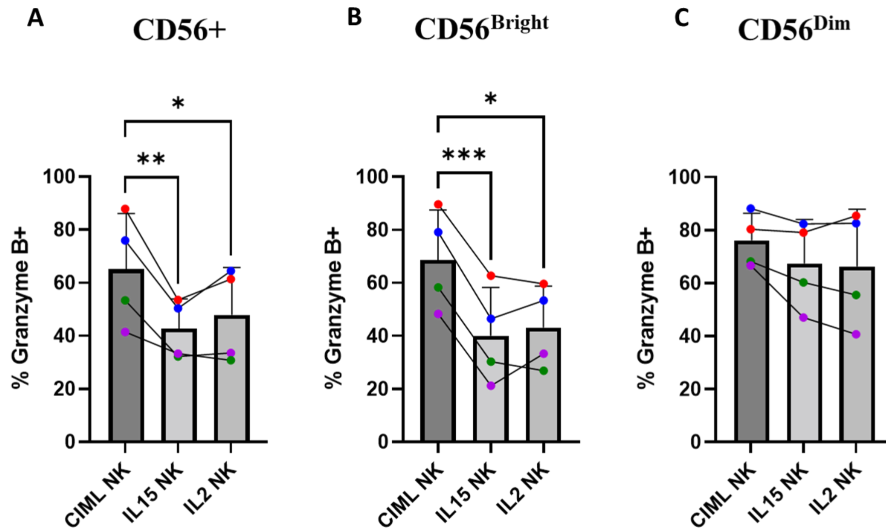


Figure 18. Evaluation of Granzyme B expression by the different NK cell products. The expression levels of Granzyme B (GzmB) were assessed on fixed and permeabilized day-7 NK cells. Cells were gated on Total NK cells (A), CD56^{bright} (B), or CD56^{dim} cells (C). Graphs show mean± SD (n=4 donors). Comparisons between groups were performed using one-way ANOVA with Turkey post-hoc test. (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$).

The aforementioned results clearly indicate that CIML NK cells have a superior cytolytic response over IL-2 and IL-15 NK cells against different NSCLC cell lines representative of the three histotypes, with the CD56^{bright} subset serving as the main driver of CIML-mediated functions.

4.2. NSCLC cells derived from 3D spheroid clusters demonstrate reduced NK cell stimulation capabilities.

NSCLC possesses a high metastatic potential, and this is a major factor determining poor prognosis and reduced survival time (24). Therefore, one key aim of our study was to ascertain whether CIML-NK cells can be suitable for controlling metastatic dissemination, targeting cancer stem cells (CSC) in NSCLC. It has been reported that clusters of circulating tumor cells forming spheroid-like structures drive metastatic dissemination in serval tumors, including NSCLC (30,34). By forming these clustered structures, tumor cells mediate immune evasion and therapeutic resistance, ensuring the protection and the "delivery" of CSCs to the premetastatic niche. To assess NK cells' reactivity against clustered cancer cells, we generated NSCLC tumor spheroids derived from four different NSCLC cell lines by culturing them in ultra-low adherence plates in the presence of bFGF and EGF, following a previously described protocol (160) reported in the methods section 3.2.3 .

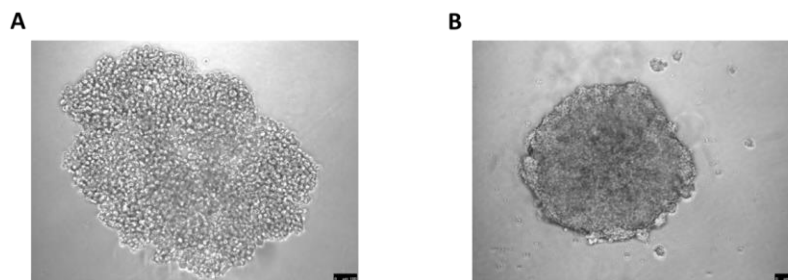
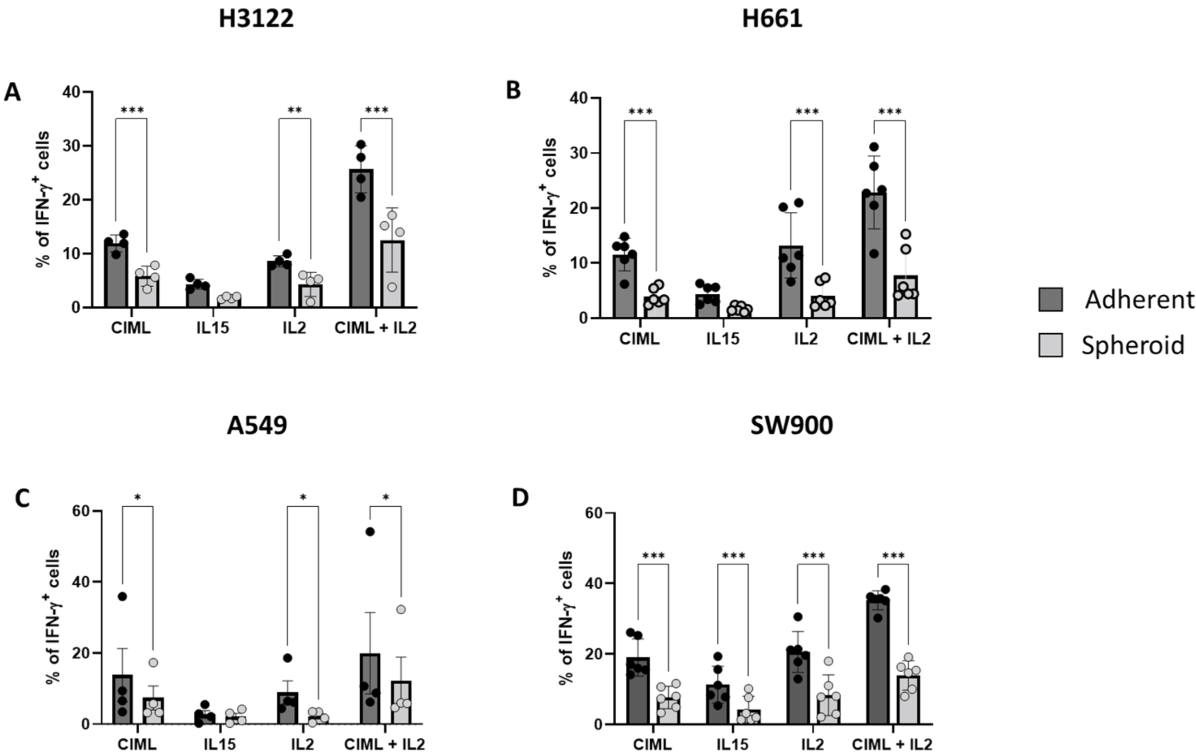


Figure 19. NSCLC spheroids. Representative images of (A) A549 and (B) SW900 derived spheroids. NSCLC cells were cultured for 3-7 in ultra-low adherence plates in DMEM/F-12 media supplemented with B-27, EGF, bFGF and heparin.

Solid-state spheroids, as shown in figure 19, were formed after 2-7 days of culture under the above-mentioned conditions. Upon reaching this solid state, they were disrupted, and single

cells derived either from the spheroids or from adherent cultures were used in standard 6-hour co-culture experiments to assess NK cell reactivity.

We observed that the functional response of CIML, IL-2, and IL-15 NK cells, evaluated in terms of cytotoxic degranulation and IFN-γ production, significantly decreased when target cells were switched from adherent to 3D growth conditions. This data indicates that tumor cells forming these structures are endowed with a superior ability to evade NK cell-mediated responses (Figure 20 A-H).



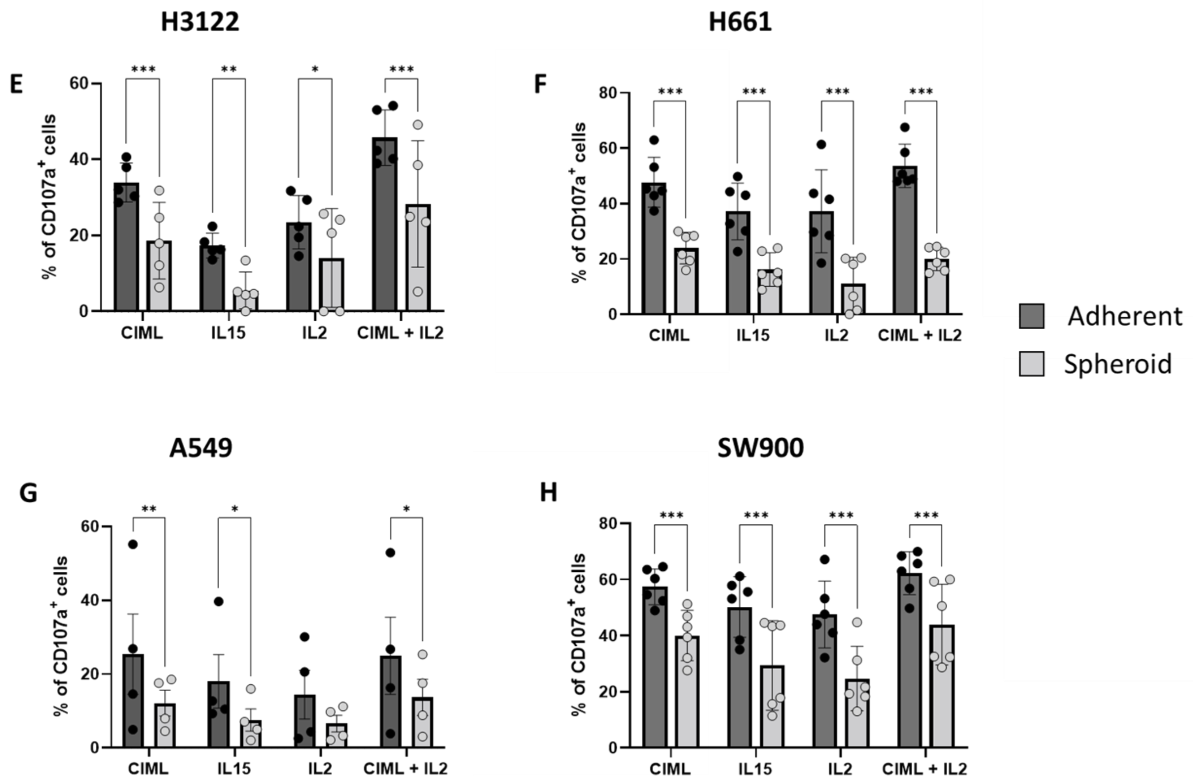


Figure 20. NK cells show a decreased reactivity to spheroids than to adherent cells. Degranulation, measured by CD107a (A, B, C and D), and IFN-γ production (E,F, G and H) by CIML-, IL-15-, IL-2- and CIML+ IL-2 NK cells upon coculture for 6 hour with adherent 2D cells and cells derived from 3D spheroids of SW900, H3122, H661 and A549 determined by flow cytometry. Histograms show mean ± SD (N=4-6 donors). Statistical significance was determined using a two-way ANOVA paired with Sidak's post-hoc test (*, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001).

4.3. CIML-NK cells show superior ability to attack spheroids; IL-2 boosts CIML NK anti-tumor activity

We next assessed whether CIML NK cells could display higher responsiveness to spheroid-derived tumor targets. (Figure 21 A-B). The effector functions of CIML NK cells were also

evaluated by measuring the production of IFN-γ and surface levels of CD107a upon co-culture with the selected targets.

CIML NK cells have been reported to upregulate CD25, which can substantially increase their affinity for IL-2 and potentially contribute to enhancing their effector function against tumor targets (161). Following this rationale, we investigated whether the additional over-night exposure to low-doses of IL-2 (50 IU/mL) could further augment CIML NK cell reactivity. Compared to IL-2-activated and control IL-15 NK cells, CIML NK cells displayed superior cytotoxic degranulation against all of the four analyzed NSCLC targets, and a slightly higher IFN-γ production. Interestingly, the obtained results showed that CIML NK cells' responsiveness was further increased by the IL-2 boost, particularly in terms of IFN-γ production, which was approximately two-fold higher than CIML NK cells (Figure 21 A). In general, we observed that SW900 spheroids stimulated a higher response on all NK cell products, while A549 induced the lowest response.

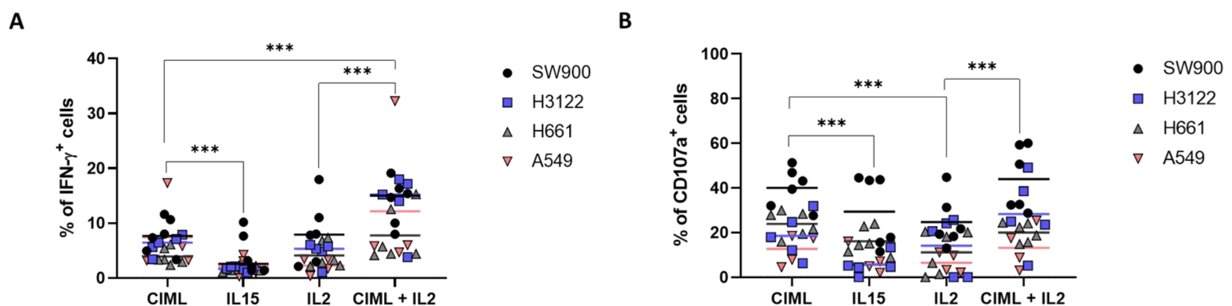


Figure 21. Functional characterization of NK cell products against NSCLC spheroids. CIML-, IL-15, IL-2 and CIML NK cells booted with IL-2 (CIML +IL2) were stimulated for 6 hours with H3122, A549, H661 or SW900 cells derived from spheroids. Afterward, the expression of IFN-γ (A) CD107a (B) by NK cells (CD56+ cells) was measured by flow cytometric analysis (N=4-6 donors). A two-way ANOVA with Tukey post hoc test was used to compare groups. (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$).

To determine if the higher NK cell functionality of CIML NK cells, and, particularly, of CIML NK cells boosted with IL-2, correlated with an augmented tumor cell killing, we tested the killing efficiency of the different NK cell products. These experiments were conducted using target tumor spheroids derived from A549 and SW900 cell lines, which are representative of the two main NSCLC histotypes, adenocarcinoma and squamous carcinoma, respectively. After co-culturing NK cells with NSCLC spheroids, we analyzed tumor cell killing by means of two diverse approaches; a flow cytometry-based assay, schematized in figure 8, which allowed the assessment of the killing efficiency at different effector: target (E:T) ratios (Figure 22 A-D), and a real-time live cell imaging Incucyte analysis (schematized in figure 9). This latter approach allows to quantitatively evaluate target cell killing over time by performing the co-culture in the presence of a caspase 3/7 detection reagent (Figure 23 A-D).

Flow cytometric analysis of the killing ability of the different NK cell products revealed that CIML NK cells displayed a significant superior killing of NSCLC cells clustered into spheroids compared to control IL-15 and IL-2 NK cells. This difference appeared to be particularly accentuated at the lowest effector:target ratios tested in the experiment (Figure 22 B,D).

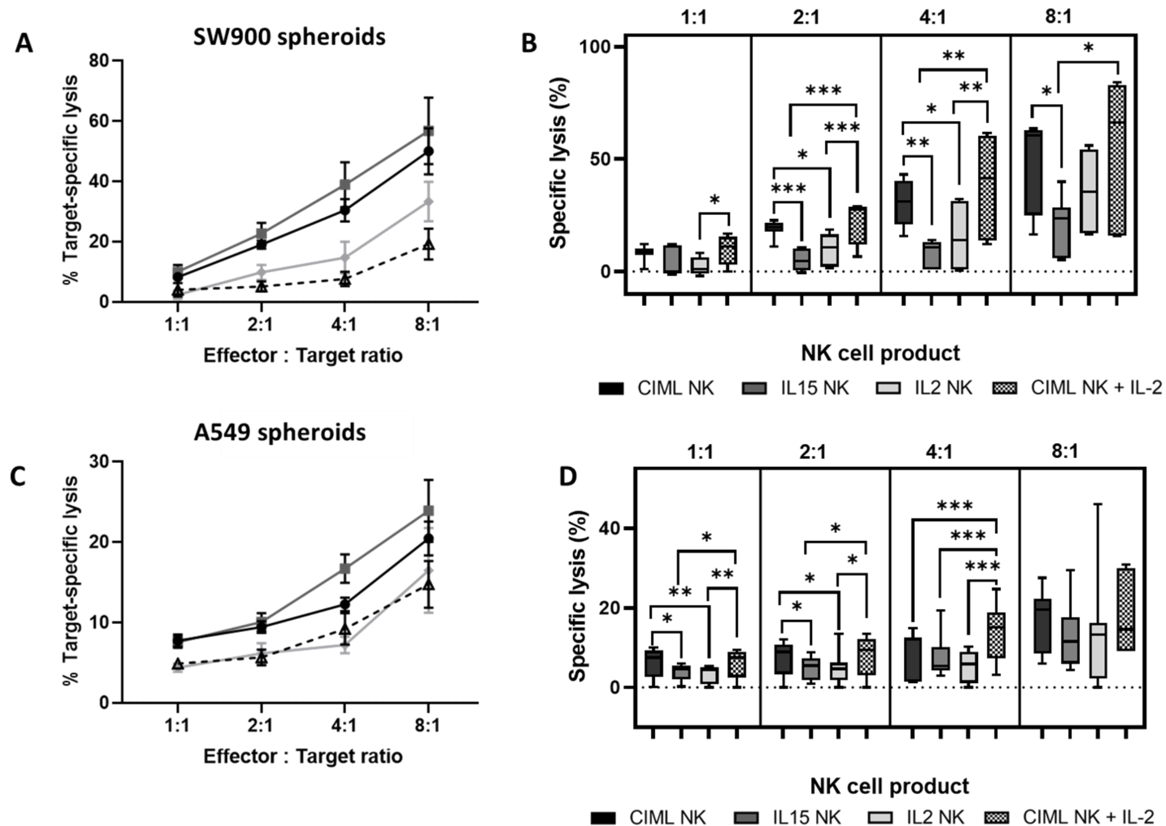


Figure 22. Killing activity of the NK cells products against NSCLC spheroids. Percent of specific lysis of SW900 (A,B) and A549 (C,D) spheroids labeled with CSFE at 24 hours upon co-culture with the different NK cell products at different E:T ratios, by staining with Dead cell marker and Annexin V, followed by flow cytometric analysis. B and D) Data for the different E:T ratios displayed as whisker plots with median ± minimum to maximum value (N=4 donors). Groups were compared using a two-way ANOVA with Tukey post hoc test. (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$).

Notably, the killing ability of tumor spheroids mediated by CIML NK cells was significantly improved by their boosting with IL-2. This corroborates our initial hypothesis that the upregulation of CD25 can be harnessed to further potentiate the effector functions of CIML NK cells.

These outcomes were validated by real time measurement of the killing activity through the Incucyte system. Indeed, as shown in Figure 23, through this assay we confirmed the enhanced antitumor response of CIML NK cells over IL-2 and IL-15 NK cells against SW900 and A549 tumor spheroids seen by flow cytometry analysis, and how CIML NK cell functionality was boosted by overnight IL-2 supplementation.

A careful analysis of the caspase 3/7 activity over time, which is an indirect evaluation of tumor cell killing within the single spheroids, showed that a difference in the killing activity between CIML NK boosted with IL-2 versus IL-15 and IL-2 activated NK cells was already appreciable at early time points (after 4 hours) and was maintained throughout the entire measurement period (48 hours).

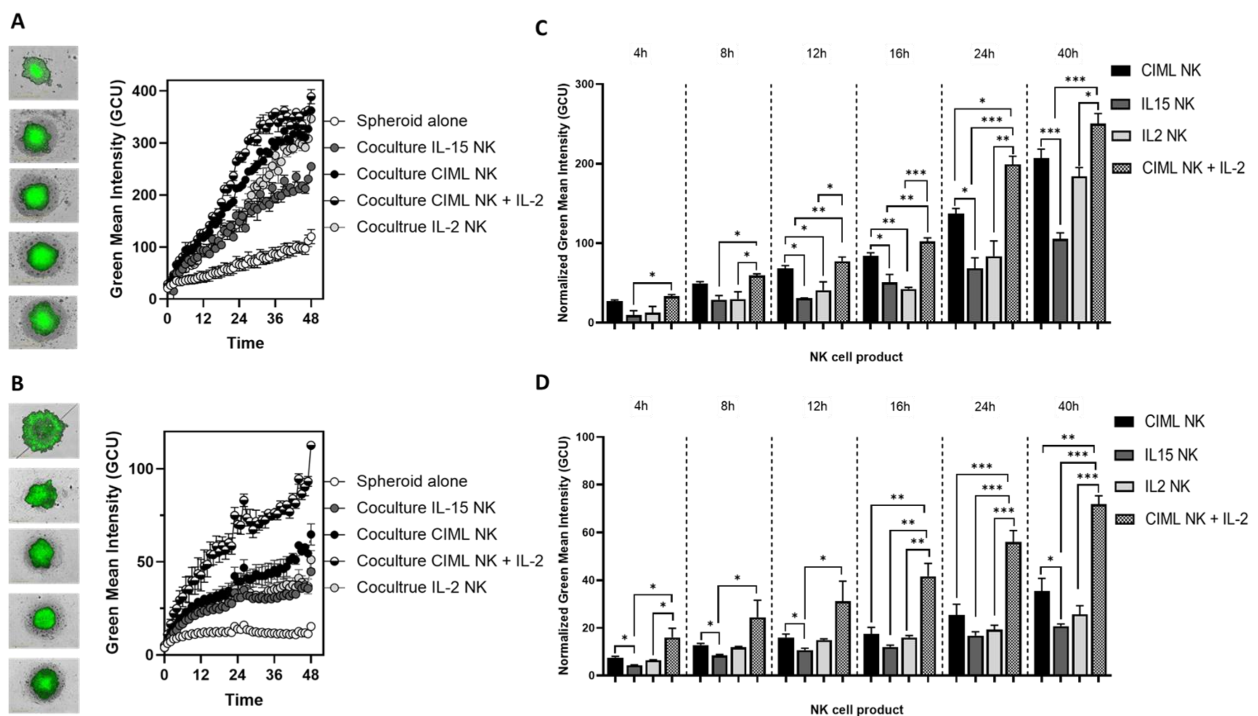


Figure 23. Real time-killing analysis of NK cell killing of NSCLC spheroids.

SW900 and A549 spheroids were co-culture with the different NK cell products (E:T ratio 8:1) in the presence of caspase 3-7 green dye in a 96-well plate. Wells were imaged every

hour for 48 hours to quantify the caspase 3-7 activation (green signal) within the spheroids using the Incucyte system. Representative images of SW900 (A) and A549 (B) spheroids co-cultured with the different NK cell products displaying caspase activation (at time=48 hours). A, B) Quantification of caspase activation (green mean intensity) in spheroid boundary at indicated time points of co-culture. Graph show mean ± SEM of three donors ran in triplicates. C, D) Target-specific caspase 3-7 activity at different time points of the co-culture displayed as histograms showing mean ± SEM. A using a two-way ANOVA with Tukey post hoc test was used to compare groups. (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$).

4.4. **CIML-NK cells can decrease the CSC content from 3D tumor cell clusters.**

Several groups have reported that NSCLC cells grown as spheroids exhibit an increased percentage of $CD133^{+}$ cells versus the counterpart grown under adherent conditions (160,162,163). As demonstrated by Bertolini et al. (26), $CD133^{+}$ cells represent a subset of tumor cells with CSC properties, displaying an elevated tumorigenic potential and considered responsible for therapeutic resistance in NSCLCs. Indeed, higher levels of $CD133^{+}$ cells have been found to correlate with poor prognosis in NSCLC patients since these cells are commonly resistant to standard platinum-based chemotherapy.

To determine whether $CD133^{+}$ CSC could be sensitive to NK cell killing, we evaluated the percentage of viable $CD133^{+}$ cells within the spheroids before and after co-culture with the different NK cell products. The obtained results demonstrated that CIML-NK cells were particularly effective at reducing the CSC content in NSCLC spheroids derived from SW900 and A549 cell lines (Figure 24 A-B). Flow cytometric analysis showed that the frequency of $CD133^{+}$ cells upon co-culture with CIML NK cells markedly decreased compared to

untreated spheroids. Remarkably, such a decrement of $CD133^+$ cells was not induced co-culturing the spheroids with either IL-2 or IL-15 NK cells.

The findings reported in this section are of particular interest since they provide evidence that in-vitro induction of memory-like differentiation could confer NK cells with an enhanced antitumor activity against a tumoral subpopulation imputable for key pro-tumoral mechanisms, including metastatic spreading, drug-resistance and tumor relapse, and the rationale for the implementation of novel combinatorial therapies.

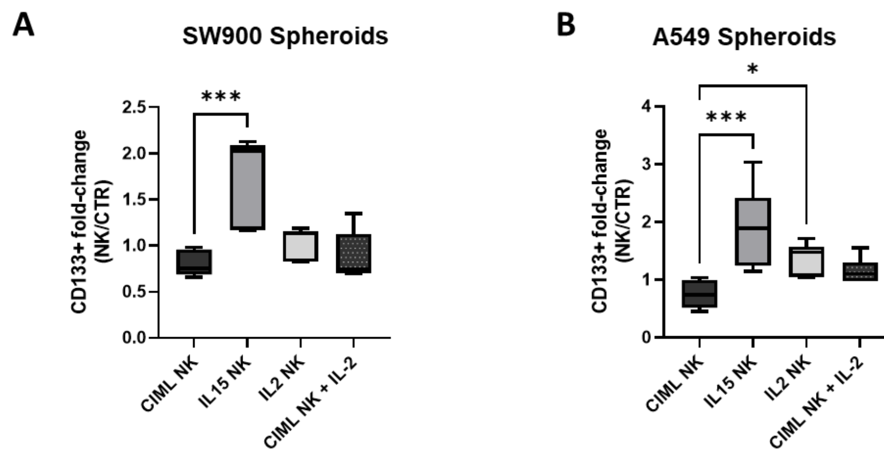


Figure 24. $CD133^+$ cells are sensitive to CIML NK cells. SW900 (A) and A549 (B) spheroids were co-cultured for 24 hours with the different NK cells products (1:1 E:T ratio). Next, spheroids were desegregated, stained with viability dye and CD133 and analyzed by flow cytometry. The levels of CD133 are reported as fold-change to control, untreated spheroids. Data is shown as whisker plots with median ± 5 to 95 percentiles. Comparisons between groups were assessed by one-way ANOVA with Tukey post hoc test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$).

4.5. CD16-IL15-CD133 TriKE increases the ability of CIML-NK cell to eliminate CSCs.

NK cell activation and killing activity can be promoted by antibody-dependent cellular cytotoxicity (ADCC), which involves the engagement of the CD16 Fc low affinity receptor

(FcγRIII). To better target NK cells to malignant cells, the use of bispecific or trispecific killer engagers (BiKE and TriKE, respectively), containing an anti-CD16 single-chain variable fragment (scFv) linked with other scFvs specific for antigens on the surface of target tumor cells have been proposed (164–167). These types of molecules can ensure the engagement of the CD16 receptors with a high affinity and the formation of immunologic synapses between NK cells and target cells, thereby enhancing and directing the cytotoxic killing of cancer cells.

To explore the feasibility of a combination therapy based on CIML NK cells along with TriKE or BiKE, we evaluated whether the addition of an engager targeting the $CD133^+$ population could further enhance the ability of CIML to eliminate CSC within NSCLC spheroids. To this aim, we co-cultured CIML NK cells or CIML NK cells supplemented with IL-2 with single-spheroids in 96 well plates. The co-culture was performed either in the absence or in the presence of a TriKE binding CD16 and CD133 and bearing the IL-15 moiety providing the survival signal required by NK cells (1615133 TriKE) (165).

In this experiment we showed that a relative low dose of TriKE (50nM) was sufficient to enhance the cytotoxic action of CIML NK cells against $CD133^+$ SW900 cancer cells, leading to a further reduction of the content of this subpopulation of tumor cells in SW900 spheroids, providing evidence that CIML NK cells can benefit from combination with bispecific/trispecific engagers (Figure 25A).

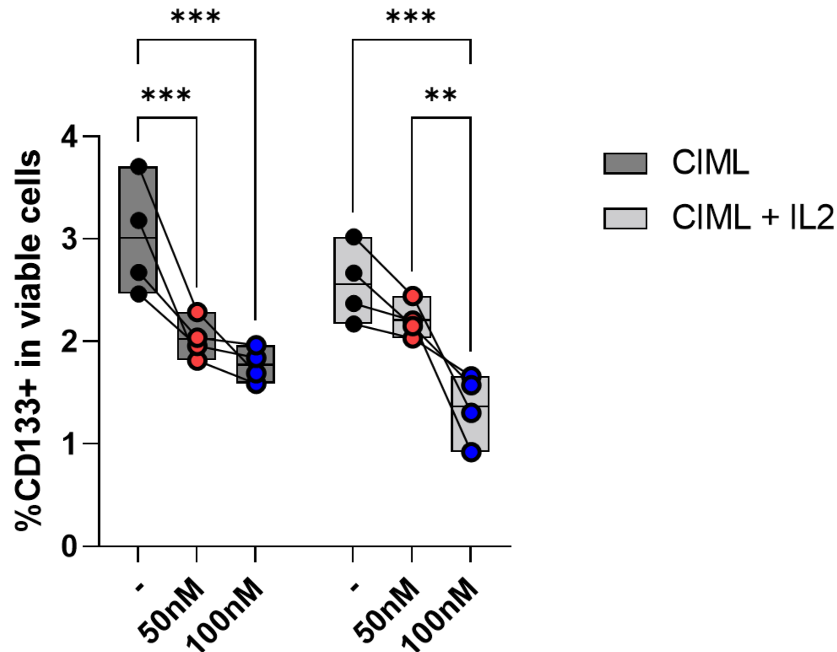


Figure 25. Effect of CIML NK cells and TriKEs targeting CD133 on CD133+ cells of SW900 spheroids. SW900 spheroids were co-cultured with CIML NK cells or CIML-NK cells boosted with IL-2 for 24 hours in the presence 50 or 100nM of 1615133 TriKE. The expression of CD133 was assessed by flow cytometry on desegregated spheroids. Graphs are floating bars displaying mean, and maximum and minimum values, with additional dot plots representing individual donors. (N=4). Significance between groups was determined using a two-way ANOVA with Tukey post hoc test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$).

5. DISCUSSION AND CONCLUSION

5.1 Discussion

The 5-year survival rate of patients diagnosed with NSCLC, particularly those with advanced disease, has sought only moderate improvement in recent years despite the clinical implementation of innovative therapies, such as targeted therapies and immune-check point inhibitors (ICIs), pointing out the urgent need for more effective treatment options able to control tumorigenesis and tumor spreading (1,5,13). Indeed, even though ICIs have demonstrated high effectiveness, only around 10–15% of patients exhibit sustained long-term benefits in response to ICIs, and in up-to 30% of cases tumor progression occurs after an initial response (23).

Adoptive cell therapy has shown the potential to facilitate the treatment of oncological diseases by introducing immune cells endowed with potent effector functions. NK cells are particularly attractive among the various adoptive cell therapies due to their suitability for allogenic use and no need for previous antigen identification. Moreover, NK cell therapies have been successfully used for the treatment of hematological malignancies, not only inducing remission, but also displaying a good safety profile (106,107). Despite the promise of NK cell-based therapies, their efficacy in the context of NSCLC and other solid tumors still needs to be proven.

Here we investigated whether CIML NK cells, recently tested in a phase 1 clinical trial of patients with relapsed AML (149), could mediate an enhanced anti-tumor response against NSCLC cells, with a main focus on their effect on tumorigenesis and stemness properties. Two recent studies have provided first evidence that CIML NK cells exhibit a more robust and effective anti-tumor activity than conventional NK cells preactivated with IL-15, offering the potential to overcome some of the current challenges of NK cell-based therapies in solid tumor settings (153,154). Our findings further support these observations, showing that allogenic CIML NK cells have an increased reactivity against various NSCLC cell lines representative of the three main histotypes. In addition, we provide first evidence that CIML NK cells display improved responsiveness against NSCLC over IL-2 activated NK cells,

which have been standardly used in clinical trials investigating safety and efficacy of NK cell-based therapies (117,140).

Phenotypical analysis of CIML NK cells revealed that they undergo an expansion of the $CD56^{bright}$ subset, although this expansion was not restricted to CIML NK cells. Indeed, in line with what has been previously reported (140,141,159), we also observed an expansion of $CD56^{bright}$ cells in NK cells after 7 days of culture in the presence of either IL-15 or IL-2. However, this phenomenon was particularly marked in CIML NK cells. In IL-2 activated NK cells, for instance, the $CD56^{bright}$ expansion was not constantly observed, but highly varied between donors. A donor-dependent expansion has also been reported by Huenecke et al. and Koehl et al in NK cells upon IL-2 activation (141).

In agreement with the observed expansion of the $CD56^{bright}$ subset, CIML NK cells were characterized by an immature $NKG2A^{+}$ KIR^{-} profile, with the more mature $NKG2A^{-}$ KIR^{+} population nearly disappearing compared to freshly isolated NK cells or IL-15 and IL-2 NK cells at day 7.

In contrast to the common thought that $CD56^{bright}$ cells are mainly cytokine-producing cells with limited cytotoxic activity (52), we observed that the $CD56^{bright}$ subset in CIML NK cell was the main driver of their effector function, exhibiting enhanced IFN-y production and cytotoxic degranulation against NSCLC targets. As reported in previous studies by Wagner et al. (159) and Koehl et al. (140), following stimulation with IL-15 and IL-2 we also observed that the effector functions of $CD56^{bright}$ cells were significantly boosted, increasing their contribution to the anti-tumor response. However, the increment of the $CD56^{bright}$ functionality was mostly accentuated in CIML NK cells. Phenotypic profiling showed that $CD56^{bright}$ CIML NK cells expressed comparable levels of activating receptors (NKp44, NKp46 and CD69) to those observed in $CD56^{bright}$ IL-2 activated NK cells. On the other hand, there was a sharp difference in the expression of checkpoint receptors (TIGIT and TIM-3) between IL-2 activated NK cells and CIML NK cells, with the latter one showing a considerably lower expression of these receptors. This event, accompanied with a comprehensive reduced expression of KIRs, may explain the improved reactivity of CIML NK cells against NSCLC cells.

To develop a successful NK cell-based treatment it is essential that these cells could efficiently traffic to tumors upon infusion to patients, thereby to target and kill malignant cells at the tumor site. We found that the CIML NK cell product is characterized by high surface levels of chemokine receptors CXCR4 and CXCR3, with the CXCR4/CXCL12 axis known to play a key role in mediating NSCLC metastasis formation in brain, liver and bone marrow (168). This elevated expression of CXCR4 on CIML NK cells might favorably impact their antitumor properties in patients with metastatic NSCLCs, offering the potential to improve the treatment of hard-to-treat or untreatable metastatic tumors. Indeed, since CXCL12 is highly expressed in primary NSCLC as well as in the metastatic tumors, increased expression of CXCR4 on the surface of CIML NK cells can facilitate their accumulation at the major metastasis sites (168).
Concerning the expression of CXCR3, it is important to highlight that this chemokine receptor has been associated with NK cell recruitment to the lung, thus owing an intrinsic advantage to CIML NK cells for lung cancer treatment (169). However, future studies on how the chemokine receptor patterns acquired by CIML NK cells can affect their ability to migrate towards target tissues should be conducted in vivo using appropriate NSCLC models.

It has been recently stated that by organizing into clusters, tumor cells are more prone to evade host immunity and to develop drug-resistance, thus facilitating tumor progression and spreading (29,34).
Tumor cell clusters can be found in circulation in patients with different types of solid tumors, such as NSCLC, even at the early stages, where they are thought to mediate metastatic spreading (34). Thus, the development of in vivo and in vitro models of circulating tumor cell clusters may be of particular relevance in the identification of therapeutic strategies that can mediate an anti-metastatic effect. Tumor spheroids have been widely utilized as a useful tool for screening in vitro the efficacy of antitumor treatments, given that they well-recapitulate the structural organization of tumors (170). Several groups, including Fortunato et al. (160), have shown that spheroids generated from NSCLC cells show similarities with circulating tumor cell clusters, like high metastatic potential and enrichment in the $CD133^+$ CSC content. In agreement with these findings, we observed an increment in the number of

CD133^{+} CSCs (data not shown) in NSCLC upon culturing as spheroids, and we further noticed that cancer cells forming these structures were less capable of stimulating NK cell effector functions than cells maintained under adherent culture conditions. The reactivity decrease by over 2-fold was not due to a reduction of the surface contact between NK and target cells shifting from adherent to spheroid conditions since disaggregation of spheroids prior co-culture did not result in an improvement of NK cell functionality. This latter observation suggests that phenotypical changes in these cells could possibly mediate their evasion from NK cell reactivity. Additional studies aimed at characterizing the mechanisms mediating resistance to NK cell responses in tumor spheroids may be highly desirable to develop strategies that improve the antitumor function of NK cells against solid tumors.

Our results corroborated the relevance of using 3D cultures as in vitro models for testing NK cell antitumor activity, as the use of tumor cells kept under adherent conditions may significantly over-estimate NK cell responsiveness.

Despite NK cells overall showed a reduced efficacy against 3D tumor cell cultures, CIML NK cells exhibited a superior effector function compared to the other NK cell products tested in this study, as determined by evaluating cytotoxic degranulation and tumor cell killing activity, providing additional evidence of their suitability as cellular therapies for the treatment of solid tumors.

CD133^{+} tumor cells in NSCLC have been associated with high tumorigenicity and poor prognosis (26). These cells have been shown to be refractory to standard chemotherapeutic agents and to mediate tumorigenesis and metastatic spread in xenograft models of NSCLC. Our results indicate that CIML NK cells are effective at eliminating CD133^{+} CSCs, accountable for tumor progression and relapse, thus paving the way for more effective therapeutic strategies harnessing CIML NK cells as single therapy or in combination with standard therapies like chemotherapy or radiation therapy capable to only target actively proliferating tumor cells, and thus showing a reduced efficacy at preventing and suppressing metastatic disease. To further confirm the clinical relevance of our findings, in-vivo studies characterizing the anti-metastatic potential of CIML NK cells, alone or in combination with current therapeutic strategies in NSCLC would be highly desirable.

5.2 Conclusions

The present study explored the anti-tumor activity of CIML NK cells in Non-Small Cell Lung Cancer (NSCLC). In brief, the main findings obtained are as follows:

- The CD56^{bright} subset in CIML NK cells proved to be the main driver of the effector functions against NSCLC, in terms of both cytotoxic degranulation and cytokine production.
- Overall, CIML NK cells exhibited a superior response against NSCLC targets than IL-2 NK cells. This could be explained in part by the lower levels of check-point receptors found in CIML NK cells.
- NK cell reactivity was reduced by switching from classical two-dimensional (2D) adherent cell culture models to 3D spheroid models of NSCLCs
- CIML NK cells displayed a superior killing ability of NSCLC tumor spheroids than IL-2 and IL-15 NK cells. CIML NK cell killing activity can be further boosted by short-term supplementation with low-doses of IL-2.
- CIML NK cells were particularly effective at reducing spheroids' CD133^{+} tumor cells, and their effect was enhanced by combining them with a Tri-specific engager targeting CD133.

Taken together, the studies in this thesis have provided new evidence on the effectiveness of CIML NK cells as cellular therapies for the treatment solid tumors, in general, and for NSCLC in particular. Additional studies exploring in-vivo their anti-tumor effects in the context of NSCLC using relevant murine models could be valuable to further validate the findings of this study giving important hints for the implementation of CIML NK cell-based clinical studies to prevent NSCLC tumor progression.

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