



Exploring natural killer cell immunology as a therapeutic strategy in lung cancer

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Abstract: Cytotoxic immune cells are key in the control of tumor development and progression. Natural killer (NK) cells are the cytotoxic arm of the innate immune system with the capability to kill tumor cells and surveil tumor cell dissemination. As such, the interest in harnessing NK cells in tumor control is increasing in many solid tumor types, including lung cancer. Here, we review the pre-clinical models used to unveil the role of NK cells in immunosurveillance of solid tumors and highlight measures to enhance NK cell activity. Importantly, the development of NK immunotherapy is rapidly evolving. Enhancing the NK cell response can be achieved using two broad modalities: enhancing endogenous NK cell activity, or performing adoptive transfer of pre-activated NK cells to patients. Numerous clinical trials are evaluating the efficacy of NK cell immunotherapy in isolation or in combination with standard treatments, with encouraging initial results. Pre-clinical studies and early phase clinical trials suggest that patients with solid tumors, including lung cancer, have the potential to benefit from recent developments in NK cell immunotherapy.

Keywords: Natural killer cells (NK cells); immunosurveillance; lung cancer

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Natural killer (NK) cells, the cytotoxic component of the innate immune system, were named following the observation that they could kill tumor cells *in vitro* over thirty years ago (1). Now, in the age of immunotherapy, NK cells have re-emerged as a therapeutic strategy that can be harnessed for tumor cell control, an interest reflected by a substantial increase in the number of publications in this field (*Figure 1A*). Indeed, the therapeutic weaponization of NK cells is mature in solid tumors such as melanoma, however their utilization is emerging in lung and burgeoning in indications such as pancreatic cancer (*Figure 1B*). In this review, we interrogate why NK cells are a key cytotoxic immune mediator in controlling the dissemination of solid tumors. We explore NK cell research

beyond the lung cancer field, that is currently investigating enhanced NK cell cytotoxicity as an immunotherapeutic approach. These insights will enable us to strategize the key elements of NK cell therapy, which may fast-track their implementation in the treatment of lung cancer.

NK cells

NK cells are lymphocytes of the innate immune system, primarily functioning as first responders to stressed cells. Functional human NK cells are identified by flow cytometry as CD3⁻CD56⁺ and are categorized as cytotoxic (CD56^{dim}CD16⁺) or cytokine producing (CD56^{bright}CD16⁻) (2-5). They can directly induce target cell death through

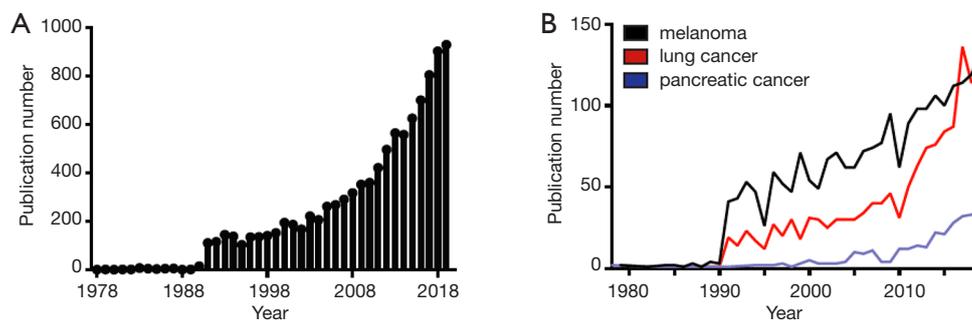


Figure 1 NK cell publication rate. (A) Publications describing ‘NK cells’ and ‘cancer’ since the initial discovery of NK cells in 1978. (B) Publications of ‘NK cells’ and melanoma (black), lung cancer (red) or pancreatic cancer (purple) since 1978. Data sourced from Web of Science.

the secretion of the pore forming molecules Perforin and Granzyme B (6) and indirectly induce apoptosis through the release of tumor necrosis factor- α (TNF- α) and Fas cell surface death receptor (FAS) ligand (7,8). Moreover, NK cells play a broad immunomodulatory role through the secretion of cytokines, interleukins and interferons to mobilize the innate and adaptive immune response to the site of infection or disease (9-12).

NK cells play an important role in both the circulation and as resident cells in tissues. Their crucial function in barrier immunity is reflected by the recurrent infection and inflammation observed in the absence of NK cells (13,14). Increased numbers of NK cells reside in tissues at the interphase between the body and the environment (15), with a high number of tissue resident NK (trNK) cells in the lungs (16). The large trNK cell population are reliant on cytokines, such as Interleukin 15 (IL-15) for their survival and development, which is secreted by bronchiolar epithelial cells in the lung (17). Unlike the majority of trNK cells, NK cells that reside in the lung are predominantly in the cytokine producing state (18,19), likely due to prolonged IL-15 exposure (20,21). Therefore, the trNK cells that reside in the lung are a key cytokine-producing compartment in the immune microenvironment contributing to immunosurveillance of the lung.

The cytotoxic activity of NK cells is dictated by a balance between activating and inhibitory signals, both in the form of ligands present on the interacting cell, or cytokines in the surrounding milieu (22). This activity primarily centers on the recognition of self through Major Histocompatibility Complex Class-I (MHC-I; mouse) or Human Leukocyte Antigen (HLA; human), which are ubiquitously expressed on all healthy tissue (23). Expression of MHC-I on healthy

cells confers an inhibitory signal to NK cells via specific receptors. In humans, HLA are detected by receptors such as the killer immunoglobulin-like receptors (KIRs) and leukocyte immunoglobulin-like receptors (LILRs), while in mice MHC-I is detected by the family of c-type lectins (Ly49), whereby engagement of these receptors inhibits a cytotoxic response (24). Consequently, the lack of MHC-I on the cell surface therefore signals NK cell activation and induces a cytotoxic response (*Figure 2*). Therefore in cancer cells, the downregulation of MHC-I, a well-described mechanism of cytotoxic T cell evasion, results in the induction of the NK cell response (25). In addition to the absence of MHC-I, activation of the NK cell cytotoxic response includes a number of ligand interactions (26,27). Primary activating receptors include Natural Killer group 2D (NKG2D) and recognize MHC-I-related proteins expressed on stressed cells [MHC-I chain related protein A and B (MICA/MICB) and UL16 binding proteins 1–6 (ULPB1–6); human. H60, murine UL16-binding protein-like transcript (Mult1) and retinoic acid early inducible 1 (Rae1); mouse] (28). Additionally, the natural cytotoxicity receptor (NCR) family, including NKp30, NKp44 and NKp46, react to a broad spectrum of ligands inducing NK cell activation (9,26,27). Taken together, the activation of NK cells is a complex balance of inhibitory and activating signals to initiate a cytotoxic response to stressed cells.

Cancer surveillance

The ability of NK cells to target stressed cells plays an important role in the surveillance of tumor initiation and disseminating tumor cells (29). This is evident in humans where increased cancer prevalence is correlated with

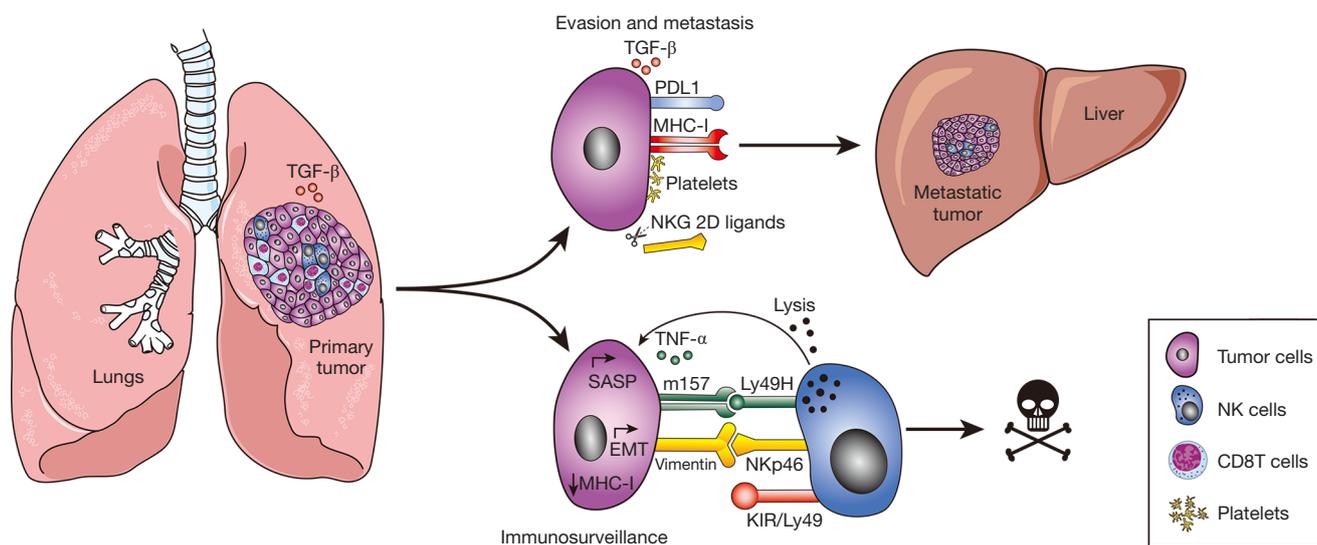


Figure 2 Tumor cell evasion and surveillance by NK cells. Primary tumor cells that metastasize harness mechanisms to actively evade immunosurveillance. This includes the secretion of TGF- β , expression of PD-L1 and MHC-I ligands, platelet association and the secretion of NKG2D ligands, inhibiting the NK cell cytotoxic response. Conversely, primary tumor cells that are effectively killed by NK cells express activating ligands to NK cell receptors through the upregulation of SASP and EMT gene regulation pathways (i.e., TNF- α , vimentin, m157) or downregulate the inhibitory ligand MHC-I, initiating lysis of target cells. TGF- β , transforming growth factor beta; PD-L1, programmed cell death ligand 1; MHC-I, major histocompatibility complex 1; SASP, senescence associated secretory phenotype; TNF- α , tumor necrosis factor alpha; EMT, epithelial to mesenchymal transition; KIR, killer immunoglobulin-like receptor.

NK cell deficiency (30,31). This observation is further supported by a long-term study whereby increased NK cell activity negatively correlated with the likelihood of developing cancer (32). Recently, the association between NK cell infiltration of solid tumors has been found to be prognostic. By selectively combining immune gene signatures (33,34), Cursons *et al.* distilled an NK signature to deconvolute NK cells, cytokines and ligands present in the tumor microenvironment (TME). When applying the NK signature to melanoma datasets, an increased NK score stratified patients with improved survival, suggesting that NK cell infiltration is prognostic in this setting (35). A similar approach has been adopted by others, whereby a signature based on NK cell and dendritic cell infiltration also stratified patient survival in melanoma, head and neck squamous cell carcinoma (HNSCC) and lung adenocarcinoma (LUAD) (36). In line with transcriptional studies, NK cell infiltration was quantified using CD57 immunostaining, which also revealed a positive correlation with prognosis in gastric (37) and colorectal (38,39) cancer patients. Together, these findings suggest that NK cells play a critical role in cancer immune surveillance and prognosis.

The tumor microenvironment can suppress NK cell activity through secreted factors to evade immunosurveillance (Figure 2). Transforming growth factor beta (TGF- β), a multifunctional cytokine, inhibits the activity of NK cells (40). The binding of TGF- β to its cognate receptor on the NK cell surface (TGF- β -receptor II; TGF β RII) provides signals that inhibit the mammalian target of rapamycin (mTOR) pathway. This results in reduced NK cell proliferation, survival and activity (41). In conditional knockout mice, whereby *TGF β RII* deletion is restricted to *NKp46*-expressing NK cells (*NKp46-Cre/TGF β RII^{Δ/Δ}*), the inhibitory effect of TGF- β on NK cells was ablated (42). It has also been found that members of the TGF- β superfamily, such as Activin-a, similarly inhibit NK cell activity. Inhibition of Activin-a signaling through the use of Follistatin, an activin binding and neutralizing glycoprotein, reduced the metastatic burden in B16F10 melanoma cells in an intravenous (i.v.) transplantation mouse model (43). These findings highlight the importance of the TGF- β pathway in NK cell activity. Indeed, NK cells are not only regulated by the TGF superfamily in the TME. Adenosine, an anti-inflammatory metabolite,

has also been found to negatively influence NK cell proliferation and maturation through the engagement of the adenosine A2A receptor (A2AR) (44). In addition to inhibitory signaling pathways, there are also physiological barriers for NK cells in the TME of solid tumors (45,46). In the microenvironment of colorectal liver metastasis, for example, NK cells have been found to undergo apoptosis due to the acidic conditions (47). Therefore, tumor cells reside in a TME which is inhospitable to NK cell survival and activity, constraining immunosurveillance. Counteracting the effect of the TME could be an alternative approach to re-engaging innate defense processes.

In addition to an inhibitory microenvironment, tumor cells directly evade NK cell surveillance through the altered regulation of ligands and their association with platelets (28). NKG2D is a key activating receptor on NK cells (48). Through the downregulation of NKG2D ligands, tumor cells can evade the cytotoxic NK cell response (*Figure 2*) (49,50). Specifically, cancers can shed NK cell activating stress ligands through proteolytic processes, removing the ligands from their cell surface (51). Importantly, antibody mediated inhibition of NKG2D ligand shedding resulted in enhanced NK cell activity, highlighting the possibility of countering this evasion mechanism (51). The importance of NKG2D ligand availability is exemplified in Cursons *et al.* where bioinformatic approaches demonstrated that NK cell infiltration was ineffective in the absence of activating ligands (35). Furthermore, platelets have long been associated with cancer metastasis, forming a protective 'barrier' on disseminating tumor cells. This mechanism effectively shields tumor cells from NK cell immunosurveillance as well as facilitating metastatic dissemination (52,53). Co-incubation of platelets with tumor cells has identified that platelets can impair NK cell activity by a myriad of mechanisms, including the secretion of TGF- β (54), transfer of functional MHC-I to the tumor cell surface (55) and initiating NKG2D ligand shedding by tumor cells (56). Platelets thereby hinder NK cell function both directly and indirectly to facilitate tumor cell evasion of NK cell cytotoxicity and surveillance.

Systemic barriers to NK cell activity

NK cells are not only impacted by the TME or the tumor cells themselves, but have also been shown to be effected by systemic environmental and therapeutic agents. Lung cancer has a strong association with cigarette smoke

exposure, which has been demonstrated to directly impact NK cell function. Likewise, chemotherapy, a first line therapy for the majority of lung cancer patients, has also displayed an impact on NK cell activity.

Cigarette smoke exposure significantly alters the lung immune environment (57). Increased metastatic burden has been observed in smoke exposed mice i.v. injected with the melanoma cell line B16-M05 (58). This increased burden was directly attributed to poorly functional NK cells due to an inhibitory effect of smoke exposure (58). Cigarette smoke reduces the production of IL-15, thereby impairing NK cell cytolytic capabilities (59,60). The influence of cigarette smoke has long-term impacts on NK cells, with reduced numbers of circulating NK cells present in the lungs of cigarette smokers years after cessation (61). Taken together, NK cells are sensitive to the effects of cigarette smoke in both reducing their functionality and numbers in lung tissue.

In the setting of cancer, it is important to be aware that chemotherapy can also impact NK cell activity. Administration of chemotherapy and other cancer treatment modalities, such as radiotherapy and cytostatic drugs, elevates the expression of stress signals on the surface of tumor cells, which increases their visibility to NK cells (62). However, *in vitro* studies investigating the effect of cancer therapeutics on NK cell activity have highlighted potential adverse effects. Microtubule inhibitors, such as Paclitaxel and Vinblastine, were found to reduce the ability of NK cells to lyse the leukemia K562 cell line in culture (63), suggesting that chemotherapy has deleterious effects on NK cell activity. This effect has also been observed in clinical studies in non-small cell lung cancer (NSCLC). Patients treated with Paclitaxel initially exhibited reduced NK cell activity upon receiving treatment. However, during the course of treatment NK cell activity was restored (64), suggesting the inhibitory effect of chemotherapy on NK cells may be transient. Whether chemotherapy negatively or positively influences NK cell activity also appears to be dependent on dosage, adding additional layers of complexity to this process (62). Therefore, when considering the cancer setting it is important to take into account the effect of the chemotherapeutic agent in increasing NK cell activating signals, which must outweigh the negative effect of chemotherapy on the NK cells themselves.

Lung cancer

Given the utility of NK cells in the control of tumor cell

dissemination in solid tumors, it is likely that NK cells also play a key role in the control of lung cancer cells. Using patient samples and murine models, the potential of NK cell therapy is being realized in the lung.

NK cell infiltration

It has recently been identified that NK cell infiltration positively correlates with survival in many solid tumors (35,36). While the degree of NK cell infiltration in the primary tumor does not necessarily dictate the potential efficacy of NK cell therapy, it does provide an indication of the current level of control NK cells exert on lung cancer. Patients with NK cell defects are prone to developing tumors (65), while NK cells in the peripheral blood of cancer patients have poorer cytotoxicity compared to healthy individuals (66). These studies highlight the systemic interaction between NK cells and tumor cells in the patient.

In lung cancer patients, NK cell infiltration has been assessed in specimens through immunostaining and flow cytometry of primary material. Although reports of NK cell infiltration and patient outcome are mixed for NSCLC; from no correlation (67-69), to prognostic (70-72); squamous cell carcinoma (LUSC) does seem to display increased infiltration compared to LUAD (69,72-74). NK cells infiltrating NSCLC appear to have reduced cytotoxic function (68,75,76), likely due to a tumor microenvironment rich in TGF- β (42). Encouragingly, while tumor-infiltrating NK cells exhibit reduced cytotoxic function, no difference in cytokine production between NK cells infiltrating the tumor or adjacent lung tissue has been identified (76). This suggests that NK cells play an important role in the immune microenvironment of NSCLC.

Recently, infiltration of NK cells into SCLC has been quantified by flow cytometry. The study compared the proportion of CD8⁺ T cells and NK cells in endobronchial ultrasound-guided transbronchial needle aspirate (EBUS-TBNA) SCLC tumor material and matched peripheral blood mononuclear cells (PBMCs) from a cohort of SCLC patients (77). Strikingly, while the proportion of CD8⁺ T cells remained unchanged between PBMC and SCLC samples, NK cells were consistently reduced in SCLC, suggesting active reduction of this cytotoxic immune cell type. The analysis further applied the NK infiltration signature (35) to the recently stratified SCLC subtypes, ASCL1, NEUROD1, YAP1 and POU2F3 (78). The POU2F3 subtype displayed a consistently increased immune

signature, while the ASCL1 subtype had a broad spectrum, irrespective of patient stage (77). This suggests the presence of heterogeneity in immune infiltration between the SCLC subtypes, similar to that observed in the NSCLC subtypes.

Non-small cell lung cancer

Beyond studying the lung as a secondary site of metastasis, investigation of lung cancer cells themselves and their ability to evade or be targeted by NK cells is essential in evaluating their therapeutic potential. In lung cancer, the most exhaustive investigation of NK cell activity has been explored in LUAD, the most prevalent subtype of NSCLC. This is largely due to the availability of syngeneic murine models with an intact immune environment (Table 1).

Similar to patient samples (73,75), quantification of NK cells infiltrating murine models of LUAD reveal a decreased frequency as the primary tumor develops in the lung (83,84). However, the key role NK cells play in early tumor development (29) and the control of metastatic dissemination (82) are becoming apparent (Figure 2).

Epithelial to mesenchymal transition (EMT) is a process that promotes solid tumor dissemination and has been found to be associated with a high NK cell signature score in melanoma (35). In the A549 human LUAD cell line, TGF- β -induced EMT (86) was found to modulate the expression of NK-activating ligands, leading to increased susceptibility of NK cell killing (82). Indeed, EMT-induced A549 cells metastasize *in vivo* when injected into the flanks of NK deficient CB17SC-beige mice (79). Strikingly, in *Rag*^{-/-} mice, depletion of NK cells using the anti-ASGM1 antibody resulted in increased spontaneous lung metastasis of EMT-induced A549 cells; with no impact on primary tumor growth (82). Furthermore, the murine Lewis Lung Cancer (LLC) cell line transplanted into immune competent C57Bl/6 mice metastasized only when NK cells were depleted (82). This evidence suggests that NK cells are key cytotoxic effectors in the control of metastatic dissemination in LUAD models. A critical factor in metastatic dissemination is the process of EMT, providing a double-edged sword to NK cells. TGF- β in the primary tumor microenvironment inhibits NK cell activity (42) and induces EMT in LUAD cells enabling metastatic dissemination, thus modulating NK ligand expression making the tumor cells more vulnerable to NK immunosurveillance.

Consistent with the effects of TGF- β on NK cells (42), the microenvironment of LUAD has been shown to be

Table 1 Murine models investigating NK cell activity in lung cancer

Mouse model	Immune status	Finding	Reference
CB17SC-beige	No T, B, NK cells	EMT-induced A549 spontaneously metastasize	Reka <i>et al.</i> (79)
NOD scid gamma (NSG)	No T, B, NK cells	JQ1 and Ricolinostat combination treatment exhibited cytostatic effect in SCLC; required NK cells for tumor cell clearance	Liu <i>et al.</i> (80)
<i>Pfp</i> ^{-/-} / <i>Rag2</i> ^{-/-}	No T, B cells; perforin expression	Spontaneous metastasis of SCLC cells occurs in the <i>Pfp</i> ^{-/-} / <i>Rag2</i> ^{-/-} mouse model and not in NK proficient mice	Sodeur <i>et al.</i> (81)
<i>Rag</i> ^{-/-}	No T, B cells; NK cells depleted with anti-ASGM1	EMT-induced A549 metastasize in NK depleted model only	Chockley <i>et al.</i> (82)
C57Bl/6	Immune competent; NK cells depleted with anti-ASGM1 or anti-NK1.1	Lewis lung carcinoma (LLC) cell line spontaneously metastasized in NK-depleted setting	Chockley <i>et al.</i> (82)
<i>Pfp</i> ^{-/-}	Deficient in perforin	No impact on subcutaneous growth of SCLC cells; significant increase in metastatic dissemination	Best, Hess <i>et al.</i> (77)
<i>Cish</i> ^{-/-}	Immune competent; NK cells missing inhibitory feedback loop (94)	Enhanced control of metastatic dissemination in SCLC model	Best, Hess <i>et al.</i> (77)
<i>Nfil3</i> ^{-/-}	No NK cells	Enhanced metastasis in LLC cell line	Cong <i>et al.</i> (29)
<i>NKp46-Cre/Mc1r</i> ^{Δ/Δ}	No NK cells (91)	No impact on subcutaneous growth of SCLC cells; significant increase in metastatic dissemination	Best, Hess <i>et al.</i> (77)
<i>NKp46-Cre/TGFβRII</i> ^{Δ/Δ}	Immune competent; conditional deletion of TGFβRII on NK cells (34)	Enhanced control of metastatic dissemination in SCLC model	Best, Hess <i>et al.</i> (77)
<i>Keap1</i> ^{Δ/Δ} / <i>Pten</i> ^{Δ/Δ} , Adeno-Cre	Immune competent	NK cells decrease as lung adenocarcinoma develops	Best <i>et al.</i> (83)
<i>Kras</i> ^{G12D/+} / <i>p53</i> ^{Δ/Δ} Lenti-Cre	Immune competent; NK cells depleted with anti-NK1.1	NK depletion did not impact lung adenocarcinoma development; NK cells localized to the tumor stroma	Schmidt <i>et al.</i> (84)
<i>Kras</i> ^{G12D/+} / <i>p53</i> ^{Δ/Δ} / <i>rtTA</i> ^{T/+} , Lenti-Cre+TRE-m157	Immune competent; NK cells depleted with anti-NK1.1	Doxycycline induction of m157 resulted in increased NK cell infiltration, reversed by NK cell depletion.	Schmidt <i>et al.</i> (84)
<i>Kras</i> ^{G12D/+} / <i>p53</i> ^{Δ/Δ} , Adeno-Cre	Immune competent; NK cells depleted with anti-NK1.1	Trametinib and Palbociclib combination treatment induced senescence in tumors; NK cell dependent tumor cell death	Ruscetti <i>et al.</i> (85)

detrimental to NK cell function in murine models. To critically study the TME, tumors must develop from lung epithelia in immune-competent murine models. To this end, the *Kras*^{G12D/+} model of LUAD (87) has been utilized with or without the acceleration afforded by loss of *p53* (88,89) to investigate the effect of TME on NK function (29,84). Administration of lentivirus encoding Cre recombinase directly into the lung results in spontaneous tumor development in *Kras*^{G12D/+} (K) and *Kras*^{G12D/+}/*p53*^{fl/fl} (KP) mice, where NK cell activity can be analyzed. In lungs bearing KP tumors, NK cells were identified in the tumor stroma and displayed a reduced capacity to produce cytokines when stimulated *ex vivo* (29,84). Analysis of the TME in the tumors of K mice resulted in the identification a TGF- β -specific decrease of key NK cell effector proteins and glycolytic function (29). Furthermore, depletion of NK cells using anti-NK1.1 did not impact KP tumor progression (84), suggesting the reduced function and stromal location of NK cells renders them non-functional in the LUAD microenvironment. Moreover, a transcriptional program regulated by SOX2, a key transcription factor identified in KP metastases, results in the downregulation of MHC-I ligands. Critically, this results in selective ‘pruning’ of SOX2⁺ metastatic cells by NK cell killing, naturally reducing the heterogeneity of metastases in this model (57). In addition, forced expression of the NK cell-activating ligand m157 (activating the NK receptor Ly49H) on established KP lesions, resulted in enhanced infiltration and activation of NK cells, further boosted by the stimulating cytokine IL-15 (84). These findings suggest that the combination of enhanced NK ligand expression together with stimulation of NK activating pathways are capable of invigorating the NK cell response to target LUAD.

An alternative approach of harnessing NK cells to kill LUAD has been to repurpose the enhanced killing ability of NK cells to senescent cells. Using *KRAS*-mutant LUAD models, Ruscetti *et al.* investigated the potential of NK cell cytotoxicity to senescent cells (85). NK cells are attracted to senescence associated secretory phenotype (SASP) chemokines involved in recruitment (CCL2, CCL4, CCL5, CXCL10, CX3CL1) and cytokines to induce proliferation (IL-15, IL-18, TNF- α) (90-92) released by senescent cells (93). *KRAS*-mutant LUAD cells induced to senescence through the combined inhibition of the MAPK (Trametinib) and CDK4/6 (Palbociclib) pathways induced enhanced cytotoxicity of NK cells (85). Strikingly, in the autochthonous KP model, the combination of Trametinib and Palbociclib induced tumor shrinkage in

an NK cell dependent manner (85). SASP induced by oncogenic *KRAS* has been well documented (93) and now provides a compelling strategy to enhance NK mediated cytotoxicity independent of immune-activating agents (84). Furthermore, this study identifies one context in which NK cells can effectively target the primary tumor, suggesting that the SASP transcriptional program could be key to enticing NK cells to the primary lesion. It is plausible that the microenvironment of senescent cells switches from TGF- β enriched to TNF- α dominant, a key cytokine in the SASP phenotype and integral to the NK cell response (85).

Overall, studies using LUAD models prove that substantial NK cell cytotoxicity can be leveraged to target both the primary and metastatic sites. Though NK cells poorly infiltrate LUAD (75,83,84), the activity of NK cells have great therapeutic potential (29,82,84,85). This provides support for the potential of NK cells in targeting LUSC, where there is greater evidence of NK cell infiltration in patient samples (72,74) and is more predictive of patient outcome (70,71). Therefore, NSCLC provides a promising avenue for the exploration of NK immunotherapy.

Small cell lung cancer

There is promising pre-clinical evidence to suggest that NK cell therapy may be highly suited in the treatment of SCLC. Key to the induction of NK cell cytotoxicity, MHC-I expression is reduced in SCLC primary biopsies and cell lines compared to other cancers, including NSCLC (94). This observation, potentially explaining poor T cell immunotherapy responses in SCLC (95), suggests that NK cell-based cytotoxicity may be crucial to SCLC control. Critically, primary tumor cell lines derived from the murine SCLC model, *p53* ^{$\Delta\Delta$} /*Rb1* ^{$\Delta\Delta$} (96), similarly displayed reduced MHC-I expression (97). This cell line model was further used to identify that epigenetic regulation controls MHC-I expression, which was reversible following inhibition of Ezh2, a member of the polycomb repressive complex (PRC2), sensitizing SCLC tumors to T cell mediated killing (97). This elegantly highlights the vulnerability of tumor cells to the cytotoxic arms of the immune system and that evasion of the MHC-I-mediated CD8⁺ T cell response results in susceptibility to NK cell cytotoxicity.

Given that SCLC is a highly metastatic disease, it is tantalizing to hypothesize that NK cells play an important role in SCLC progression. Indeed, metastatic dissemination of SCLC has been identified as an immune-

regulated process through the use of immune-deficient murine models (81). Subcutaneous transplantation of human SCLC cell lines (OH1, OH3, H69, H82) rarely results in spontaneous metastasis in murine models deficient in B and T cells. However, in the *Pfp^{-/-}/Rag2^{-/-}* immune-compromised mouse model, deficient of B and T cells with additional loss of perforin (Table 1), SCLC spontaneously metastasized in this NK-impaired model (81). The role of NK cells in metastatic immunosurveillance of SCLC has recently been corroborated in genetically engineered mouse models (77). Using an immune-competent metastatic SCLC model, cell lines generated from a primary *p53^{ΔΔ}/Rb1^{ΔΔ}* SCLC tumor were injected i.v. into syngeneic immune-competent recipients, resulting in liver metastasis. Genetic depletion of NK cells using the *NKp46-Cre/Mcl1^{ΔΔ}* model (98,99), resulted in a significant increase of SCLC metastasis, while T cell depletion had no effect (77). Furthermore, genetically enhanced NK cells; *Cisb^{-/-}* (100) and *NKp46-Cre/TGFβRII^{ΔΔ}* (42) with no “brakes” on NK cell activation, or NK cells activated with cytokines (super IL-2) were superior in regulating metastatic dissemination of SCLC cell lines (77). Together, these findings indicate that the baseline regulatory capacity of NK cells can be enhanced therapeutically to control metastatic dissemination of SCLC.

The contribution of NK cells to therapeutic outcomes is not limited directly to their activation. NK cells have been found to be vital to tumor cell clearance when drug combinations have cytostatic effects (80). The combination of cell cycle inhibitor (Ricolinostat ACY-1215) and epigenetic regulator (JQ1) were investigated in PDX from chemo-refractory SCLC patients and human SCLC cell lines (80). Strikingly, the anti-tumor effect of combination treatment that was present in T cell deficient nude mice was abolished in immune-deficient recipient NOD scid gamma (NSG) mice (Table 1). The key difference between these immune-deficient mouse models is the presence of NK cells in nude mice, which were found to mediate cytotoxicity due to increased MHC-II expression on tumor cells following combination therapy (80). This finding highlights the importance of NK cells in improving the outcome of conventional therapeutic strategies. Taken together, these findings highlight the significance of NK cell regulation in SCLC, due to reduced MHC-I expression and enhanced metastatic dissemination. NK cell immunotherapy is therefore a promising therapeutic modality for SCLC patients that requires further investigation in a clinical

setting.

NK cell therapy

Mounting evidence for the beneficial role of NK cells in the detection and control of cancer and a greater understanding of NK cell biology have driven a renaissance in drug discovery for NK cell immunotherapy (Table 2). NK cell immunotherapy can be approached from two directions: the activation of endogenous NK cells currently circulating or resident within tissues or tumors; or the administration of activated autologous or allogeneic NK cells.

Activation of NK cells

The stimulation of endogenous NK cells can be achieved through administration of cytokines or cell signaling peptides that potentiate cytotoxic immune cell function and homeostasis. Cytokines that activate the immune system, such as interleukin-2 (IL-2) and IL-15 activate signaling cascades that enhance the activity of NK cells (100). Clinically, activation of the immune system using cytokine therapy has been used primarily in melanoma and renal cell carcinoma, where IL-2 and IFN- α have been the primary therapeutic modality until recent advances in clinical practice (109). The therapeutic use of cytokines, however, promotes a systemic response and can, in certain cases, reduce immune cell activity. Indeed, in stem cell transplants with adjuvant ultra-low dose of IL-2 cytokine therapy, regulatory T cell activity was enhanced (110). However, the systemic nature of cytokine therapy carries the risk of side effects, such as fever, resulting from overactivation of the immune system (111). To mitigate these risks, considerable effort has been applied to improve cytokine therapy (112,113). For example, recombinant IL-2R α linked to an NKG2D binding protein (OMCP-mutIL-2) (114). This modified cytokine activates IL-2 signaling only in NKG2D-expressing cells, NK cells, alleviating the majority of off-target effects from systemic cytokine use (114).

The principal cytokine for NK cell development and homeostasis, IL-15, is also receiving therapeutic interest (109,115). To improve the biological activity of IL-15, it is complexed with its' receptor, IL-15R α , which significantly enhances its availability (116). This IL-15/IL-15R α complex displays enhanced efficacy compared to IL-15 alone and rapidly induces NK cell activation (116). In a first-in-human trial, administration of the IL-15/IL-15R complex as recombinant human IL-15 (rhIL-15), resulted in a robust

Table 2 Summary of selected clinical trials investigating NK cell-mediated treatment in cancer

Tumor type	Phase	NCT identifier	NK Therapy	Combination	Enrollment	Finding	Reference
AML or MDS	I/II	NCT01898793	CIML-NK infusion	ALT-803	140	Planned endpoint: phase I: maximum tolerated dose of CIML-NK cells; phase II: Remission rate	Last update: August 2020
AML	II	NCT02782546	CIML-NK infusion	ALT-803	60	Planned endpoint: leukemia free survival rate	Last update: August 2020
Advanced AML	I	NCT01385423	Haploidentical Donor NK cell therapy	Intravenous rhIL-15	26	Observed outcome: 36% patients had robust <i>in vivo</i> NK cell expansion at day 14, and 32% achieved CR	Cooley et al. (101)
AML	II	NCT02395822	Haploidentical Donor NK cell therapy	Subcutaneous rhIL-15	17	Observed outcome: NK cell expansion at day 14 was seen in 27% of the patients, and 40% achieved remission	Cooley et al. (101)
NSCLC	IB/II	NCT02523469	ALT-803	Nivolumab	58	Observed outcome: tolerable dose of IL-15 super-agonist established and first findings of activity of IL-15 treatment in NSCLC patients	Wrangle et al. (102)
Metastatic solid tumors	I	NCT03388632	rhIL-15	Nivolumab and/or Ipilimumab	50	Planned endpoint: safety, toxicity profile, dose-limiting toxicity and maximum tolerated dose	Last update: March 2020
Relapsed/refractory mature T-cell malignancies	I	NCT03905135	rhIL-15	Avelumab	30	Planned endpoint: maximum tolerated dose	Last update: July 2020
HCC	II	NCT01246986	Galunisertib	None, Sorafenib or Ramucirumab	204	Observed outcome: Galunisertib was active in patients	Giannelli et al. (103)
Metastatic or locally advanced solid tumors	I	NCT02517398	M7824 (anti-PD-L1/TGFβRIII fusion protein)	-	600	Observed outcome: M7824 saturated peripheral PD-L1 and sequestered TGF-β1, 2 and 3 in the plasma, with early signs of tumor control	Strauss et al. (104)
Relapsed SCLC	II	NCT03554473	M7824 (anti-PD-L1/TGFβRIII fusion protein)	Topotecan or Temozolomide	67	Planned endpoint: efficacy (PR or CR)	Last update: August 2020
Advanced NSCLC	III	NCT03631706	M7824 (anti-PD-L1/TGFβRIII fusion protein)	-	584	Planned endpoint: comparison to pembrolizumab—PFS and OS.	Last update: September 2020
Metastatic HNSCC	IB/II	NCT02643550	Anti-NKG2A	Cetuximab	140	Observed outcome: overall benefit, majority of patients responding favorably (31% PR, 54% stable disease) with an acceptable safety profile	Andre et al. (105)
Advanced solid tumors	I/II	NCT02671435	Monalizumab	Duvalumab	383	Planned endpoint: drug limited toxicities, safety profile, ORR	Last update: July 2020

Table 2 (continued)

Table 2 (continued)

Tumor type	Phase	NCT identifier	NK Therapy	Combination	Enrollment	Finding	Reference
Malignant solid tumors	I/II	NCT02843204	Allogeneic adoptive transfer	Pembrolizumab	110	Observed endpoint: the addition of NK cell therapy improved outcome over Pembrolizumab on its own	Lin et al. (106)
Advanced solid tumors	I	N/A	IL-2 activated NK-92 cell line	-	15	Observed outcome: some lung cancer patients in trial responded to treatment	Tonn et al. (107)
HER2+ glioblastoma	I	NCT0383978	NK-92/5.28.z cells: CAR-NK specific to HER2	-	30	Planned endpoint: adverse events, maximum tolerated/feasible dose, period of detectability, cytokine profile	Last update: September 2020
CD19+ B cell lymphoma	I	NCT03056339	iC9/CAR.19/IL15-Transduced CB-NK cells: cord blood CAR-NK specific to CD19	-	11	Observed outcome: 73% patients had a response; of these, 7 (4 with lymphoma and 3 with CLL) had a CR	Liu et al. (108)

"Last update" refers to the website update on clinicaltrials.gov as of publication of this review. CR, complete response; PR, partial response; PFS, progression-free survival; OS, overall survival; ORR, overall response rate; NSCLC, non-small cell lung cancer; AML, acute myeloid leukemia; MDS, myelodysplastic syndrome; HCC, hepatocellular carcinoma; SCLC, small cell lung cancer; HNSCC, head and neck squamous cell carcinoma; CIML, cytokine induced memory-like.

expansion of circulating NK cells in acute myeloid leukemia (AML) patients (NCT02395822, NCT01385423) (101). Patients additionally exhibited cytokine release syndrome, which required additional immune-modulatory treatment. Though no objective response was observed, the trial has established a clinical potential of rhIL-15 in enhancing NK cell activity (101). Currently, rhIL-15 is being investigated in combination with T cell immunotherapy to further explore its therapeutic use (Nivolumab and/or Ipilimumab in metastatic solid tumors: NCT03388632; Avelumab in T-cell malignancies: NCT03905135). An alternative IL-15 complex, ALT-803 (IL-15/IL-15R α /IgG1Fc/N72D) is a super-agonist of IL-15 and substantially increases the levels of circulating CD56^{bright} NK cells (117). In a Phase Ib clinical trial, ALT-803 combined with Nivolumab (NCT02523469) was well-tolerated in metastatic NSCLC patients with increased numbers and activity of NK cells (102). Promisingly, of 11 subjects who relapsed with Nivolumab alone, 3 subjects exhibited a partial response to the combination with ALT-803, suggesting that the IL-15 super-agonist may be efficacious in NSCLC (102). Furthermore, the combination of subcutaneous ALT-803 injection with adoptive transfer of NK cells (NCT01898793, NCT02782546) enhances NK cell activity without severe adverse events (118), highlighting the potential utility of this treatment modality. Taken together, IL-15-based cytokine therapy holds promise in the adjuvant treatment of lung cancer.

A key mediator of NK cell activity is TGF- β , whose therapeutic inhibition has the potential to enhance the activity of NK cells. Currently, clinical trials are assessing the efficacy of TGF- β pathway inhibition using small molecules (galunisertib) in hepatocellular carcinoma (NCT01246986) (103), or biologics (M7824) in metastatic or locally advanced solid tumors (NCT02517398) (104), relapsed SCLC (NCT03554473) and advanced NSCLC (NCT03631706). The small molecule galunisertib (LY2157299) is an inhibitor of the TGF- β receptor (TGF- β 1 receptor type I) and results in downregulation of SMAD2 phosphorylation, consistent with inhibition of the TGF- β pathway (119,120). In a Phase II clinical trial, galunisertib treatment is manageable and active in hepatocellular carcinoma patients (103,121). The biologic molecule M7824 is a bifunctional anti-PD-L1/TGF β R2 fusion protein, with enhanced anti-tumor response compared to targeting each molecule in isolation (122,123). Strikingly, in Phase I clinical trials, M7824 saturated peripheral PD-L1 and sequestered TGF- β 1, 2 and 3 in the plasma, with early

signs of tumor control (104). Although the M7824 trial in relapsed SCLC (NCT03554473) and advanced NSCLC (NCT03631706) are yet to report findings, the efficacy of TGF- β signaling pathway inhibition is promising. It is likely that the systemic inhibition of TGF- β signaling enhances NK cell activity, however this has not yet been described in the current trials.

The systemic activation of NK cells can also be achieved through checkpoint inhibition of NKG2A, the cognate receptor for MHC-I recognition. As described above, the presence of MHC-I results in an inhibitory signal in NK cells, ligating with the NKG2A/CD94 complex on the NK cell surface. Recently, a monoclonal humanized anti-NKG2A IgG4-blocking antibody, Monalizumab, has been developed to block the interaction between MHC-I and NKG2A, thus ablating this significant inhibitory pathway in NK cells (105). Significantly, in murine pre-clinical studies anti-NKG2A was found to synergize with anti-PD-L1 T cell immunotherapy and vastly improve survival (105). Though NKG2A is additionally expressed on CD8⁺ T cells and anti-NKG2A enhances CD8⁺ T cell activity, tumor control was dependent on the presence of NK cells as highlighted by the loss of tumor control in combination with the NK depletion antibody, anti-asailo-GM1 (105). This finding further highlights the phenomenon of antibody-dependent cellular cytotoxicity (ADCC), where NK cells contribute to the efficacy of T cell immunotherapy (10,124,125). Inhibition of NKG2A has been assessed in the clinic in combination with cetuximab in HNSCC patients (NCT02643550). Promisingly, in significantly pre-treated patients (platinum-based chemotherapy, T cell immunotherapy, cetuximab alone) this strategy provided overall benefit, with the majority of patients responding favorably (31% partial response, 54% stable disease) with an acceptable safety profile (105). Ongoing trials of Monalizumab in combination with Duvalumab (metastatic colorectal cancer: NCT02671435) (126) and cetuximab (colorectal cancer: NCT02671435) additionally support the use of NK cell checkpoint blockade immunotherapy in solid tumors. It is tantalizing to speculate that NK cell immunotherapy in lung cancer is now within reach.

Adoptive transfer of NK cells

An alternative approach to the systemic activation of NK cells is to directly introduce activated NK cells to a patient, known as adoptive transfer. Generation of NK cells suitable for adoptive transfer can be procured from multiple sources,

including peripheral blood from healthy adults, stem cells from cord blood, induced pluripotent stem cells and NK cell lines. Isolated NK cells are activated by exposure to stimulatory signals such as cytokines (127) or permanently augmented by genetic manipulation (22).

Adoptive transfer of a patients' own NK cells (autologous transfer) enables *ex vivo* stimulation and expansion prior to re-administration as a therapeutic modality. Clinical trials employing autologous NK cell transfer have been performed in hematopoietic and solid tumors. In a clinical study of metastatic melanoma and renal cell carcinoma, adoptive transfer of *in vitro* activated autologous NK cells identified persistent activated NK cells in the circulation (128). Although the transferred NK cells displayed high cytolytic potential when expanded *in vitro*, the transferred NK cells required further *in vivo* activation with IL-2, suggesting that autologous transfer should be used in combination with systemic cytokine therapy (128). A study in advanced NSCLC patients treated with *ex-vivo* expanded autologous NK cell therapy in combination with docetaxel was similarly performed to evaluate feasibility of autologous transfer (129). No therapeutic benefit was obtained from the combination, likely due to poor NK cell activity *in vivo*, however this was not assessed in the study (129). Though autologous NK cells can be expanded and activated *in vitro*, the limited clinical success suggests that this approach is not a feasible treatment modality. This is possibly due to the recognition of the self-MHC-I (130), or the requirement for additional *in vivo* stimulation (128). Taken together, these findings suggest that autologous transfer of NK cells is not an optimal therapeutic approach in the clinic.

Adoptive transfer of 'foreign' NK cells (alloreactive transfer) results in MHC-I mismatch and an efficient immune response. Alloreactive transfer of KIR ligand incompatible donor NK cells were found to efficiently target AML cells and provided a prolonged protective response (131). Following this initial discovery, clinical trials exploring the adoptive transfer of mismatched alloreactive NK cells as a form of immunotherapy have minimal toxicity and exhibited preliminary success in hematopoietic malignancies and solid tumors including NSCLC (132,133). Strikingly, in late-stage NSCLC, repetitive infusions of alloreactive donor NK cells resulted in partial response or disease stabilization in the majority of patients (111), highlighting the potential efficacy and safety in the lung cancer setting. Furthermore, a clinical study in advanced NSCLC patients investigated the effect of allogeneic NK cell transfer combined with pembrolizumab

(NCT02843204) (106). This study identified enhanced NK cell activity in patients receiving NK cells, together with improved survival compared to patients receiving pembrolizumab alone (106). Together, these findings provide preliminary clinical evidence that alloreactive mismatched NK cell transfer can provide clinical benefit in lung cancer, laying the foundation for more extensive clinical investigation.

To expand the therapeutic use of alloreactive NK cells, human NK cell lines have been generated as a renewable source of NK cells. The human NK cell line NK-92, originally derived from a lymphoma patient (134), is highly cytotoxic against a variety of cancer types (135,136) and has received Food and Drug Administration (FDA) approval for use in clinical trials (137). The NK-92 cell line has been used as a source of NK cells for adoptive transfer and modified for improved efficacy and target specificity. Examples of modification include genetic manipulation and cytokine activation prior to adoptive transfer. In a Phase I trial interrogating advanced solid tumors, infusion of NK-92 cells performed particularly well in the lung cancer patient cohort (107). Three of four lung cancer patients (SCLC and NSCLC) in the study displayed mixed response or prolonged stable disease in response to the adoptive transfer of IL-2 activated NK-92 cells (107). It is hypothesized that NK cells reside in the lung, prior to circulating following intravenous administration (138), which could explain the favorable response in lung cancer patients in the Phase I trial (107). While there are numerous benefits of utilizing cultured NK cells, there are however also limitations to their use. To ensure the cells do not divide uncontrollably within the patient, NK cell lines are irradiated prior to adoptive transfer and therefore have a limited lifespan and efficacy period. However, these early clinical trials suggest that the beneficial effects of NK cell autologous transfer can last well beyond their initial infusion.

To enhance the benefits of NK-92 infusion, genetic manipulation can be additionally performed to enhance NK cell activity and target specificity. A genetic method that has recently gained traction is the introduction of chimeric antigen receptors (CAR) on the surface of NK cells that act as targeting ligands to increase affinity to specific antigens (22). This technology used has been developed and optimized in T cells (CAR-T cells) and has been successfully applied to NK cells (9). Modification of the NK-92 cell line with CAR specific to the antigen HER2 are currently being trialed in HER2⁺ Glioblastoma patients (NCT03383978). Additionally, CAR-NK cells targeting the CD19 antigen

(anti-CD19 CAR-NK), prominent in B cell lymphoma, have been engineered from HLA-mismatched NK cells derived from cord blood (108). In a Phase I and II trial of 11 patients with CD19⁺ B cell malignancies, the majority of patients had complete remission following anti-CD19 CAR-NK therapy (108). Significantly, patients undergoing CAR-NK cell immunotherapy did not experience significant adverse events that have been observed following CAR-T therapy (139), and the anti-CD19 CAR-NK cells persisted for at least 12 months (108). These studies lay the foundations for successful implementation of CAR-NK therapy, and likely with the success of CAR-T cells, we envisage broad implementation of CAR-NK therapy in the cancer setting.

The application of NK therapy as a standalone agent or in combination with other therapeutic modalities is a rapidly evolving field that is producing promising results. Pre-clinically, NK cells have been shown to play a crucial role in metastatic tumor surveillance in both NSCLC and SCLC. Although these findings have yet to fully be realized in a clinical setting, they highlight a potential therapeutic modality in a field with limited treatment options and a low survival rate. Indeed, the application of NK cell immunotherapy has significantly progressed in recent years, with the most recent clinical trials showing tremendous potential. Although the clinical focus of NK cell therapy is largely in hematopoietic malignancies, the research outlined in this review highlights the conceivable progression of NK cell immunotherapy in the treatment of lung cancer.

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