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## Natural killer cells in pancreatic cancer stroma

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### Abstract

Pancreatic cancer remains one of medicine's largest areas of unmet need. With five-year survival rates of < 8%, little improvement has been made in the last 50 years. Typically presenting with advance stage disease, treatment options are limited. To date, surgery remains the only potentially curative option, however, with such late disease presentation, the majority of patients are unresectable. Thus, new therapeutic options and a greater understanding of the complex stromal interactions within the tumour microenvironment are sorely needed to revise the dismal outlook for pancreatic cancer patients. Natural killer (NK) cells are crucial effector units in cancer immunosurveillance. Often used as a prognostic biomarker in a range of malignancies, NK cells have received much attention as an attractive target for immunotherapies, both as cell therapy and as a pharmaceutical target. Despite this interest, the role of NK cells in pancreatic cancer remains poorly defined. Nevertheless, increasing evidence of the importance of NK cells in this dismal prognosis disease is beginning to come to light. Here, we review the role of NK cells in pancreatic cancer, examine the complex interactions of these crucial effector units within pancreatic cancer stroma and shed light on the increasingly attractive use of NK cells as therapy.

**Key Words:** Pancreatic cancer; Natural killer cells; Tumour microenvironment; Pancreatic cancer stroma; Stromal cells

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**Core Tip:** Increasing evidence has found natural killer (NK) cells to be crucial players in the prognosis and progression of cancer. Whilst pancreatic cancer remains one of medicine's largest areas of unmet need, NK cells may prove to be an exciting new therapeutic option for pancreatic cancer patients. Here we provide an overview of the complex interactions between NK cells and pancreatic cancer stroma, suggest a role for NK cells as prognostic biomarkers and highlight exciting new NK cell-based treatment options which may transform the therapeutic landscape of pancreatic cancer.

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## INTRODUCTION

### Pancreatic cancer

Pancreatic cancer is a malignancy with a dismal prognosis. Set to become the second leading cause of cancer-related death worldwide by 2030, little progress has been made in the treatment of pancreatic cancer over the past five decades[1,2]. Surgery remains the only potentially curative option, however, with the majority of patients typically presenting with advance stage disease, most cases are unresectable. Further to this, with approximately 80% of surgery patients relapsing, frequently within two years, pancreatic cancer has solidified itself as one of medicine's most urgent areas of unmet need[3].

### Pancreatic ductal adenocarcinoma tumour microenvironment

Characterised by its strong desmoplastic reaction, the pancreatic ductal adenocarcinoma (PDAC) tumour microenvironment (TME) plays a crucial role in disease progression[4,5]. Primary tumour sites display extensive fibrosis characterised by overexpression of extracellular matrix proteins (such as laminin, collagen and fibronectin) and activation of fibroblastic cells. Multiple cell types, both cancer and stromal, are present in the pancreatic TME, including; pancreatic stellate cells (PSCs), myeloid-derived suppressor cells (MDSCs), tumour associated macrophages (TAMs) and regulatory T-cells (Tregs), amongst many other cell types[6,7]. The dense fibrosis associated with PDAC (largely orchestrated by PSCs) results in tumour hypoxia, a feature characteristic of PDAC, which is exacerbated by the secretion of anti-angiogenic factors (such as endostatin and angiostatin) by both pancreatic cancer and stellate cells[6,8]. Development of a hypoxic TME has been linked to disease aggressiveness and progression, as well as chemotherapy resistance. Importantly, in addition to developing resistance to chemotherapeutics, the pancreatic TME is highly immunosuppressive, limiting the efficacy of the immune-mediated cancer surveillance [6]. MDSCs release reactive oxygen species (ROS) and reactive nitrogen species which have been shown to inhibit T cell proliferation and migration into the TME. In addition, release of immunosuppressive cytokines including interleukin (IL)-10 and transforming growth factor beta (TGF- $\beta$ ) sustains the development of Tregs which further modulate the TME[9]. Tregs secrete immunosuppressive cytokines such as IL-10 and TGF- $\beta$  which recruit additional immunosuppressive cells to the TME and stimulate the transition of CD4<sup>+</sup> T cells to FoxP3<sup>+</sup> regulatory cells, facilitating immune evasion[6,10]. Through the release of IL-10 and TGF- $\beta$ , tumour associated macrophages are also able to induce T-cell anergy leading to the development of an immune-privileged microenvironment[11]. Finally, pancreatic cancer cells can downregulate Fas, resulting in resistance to CD8<sup>+</sup> T cell-induced Fas/FasL apoptosis[6,12]. Key components of the PDAC TME are shown in Figure 1.

## NATURAL KILLER CELLS

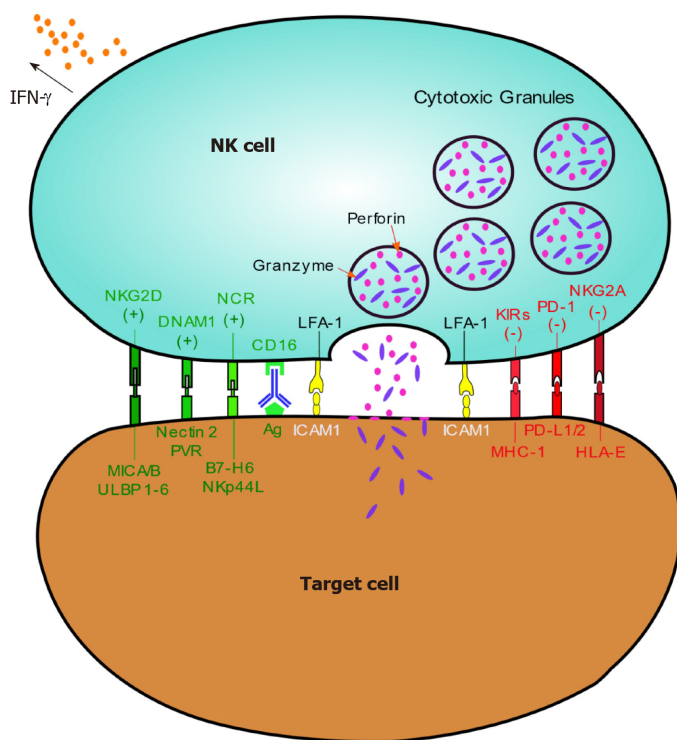
NK cells are large granular lymphocytes which are key components of the innate immune system, and are poorly understood compared to other lymphocytes (T and B



**Figure 1 Pancreatic ductal adenocarcinoma tumour microenvironment.** Upon activation, pancreatic stellate cells secrete an abundance of extracellular matrix proteins including collagen, fibronectin, laminin, and hyaluronic acid, leading to dense desmoplasia. In addition, fibroblastic cells (CAFs) become active, and immune suppressive cells (myeloid-derived suppressor cells, regulatory T-cells and tumour associated macrophages) are sequestered to the tumour microenvironment (TME). Secretion of anti-angiogenic factors, in addition to dense desmoplasia, results in the development of a hypoxic TME. Cancer stem cells are also observed in pancreatic ductal adenocarcinoma.

cells) belonging to the adaptive immune system. Acting as the first line of defence against viral infected and malignant cells[13,14], NK cells are classified as  $CD56^+CD3^-$  cells. This classification can be further sub-divided into two main effector populations: immunomodulatory  $CD56^{bright}CD16^-$  cells which regulate their function through cytokine release [specifically interferon (IFN)- $\gamma$ ], and cytotoxic  $CD56^{dim}CD16^+$  effector cells[14]. Activation of NK cells relies on the balance of signals received from inhibitory and activating cell surface receptors (Figure 2)[13]. Inhibitory receptors, comprised of killer cell immunoglobulin-like receptors (KIRs) and C-type lectin-like receptors, including natural killer group 2 member A (NKG2A), specifically recognise major histocompatibility complex (MHC) class 1 molecules. These ligands are highly expressed on non-transformed 'self' cells, and consequently prevent harmful activation of NK cells against host cells. Conversely, malignant cells often downregulate the expression of surface MHC-1 molecules to evade detection by T cells. This 'missing self' signal prevents the inhibition of the NK cells, resulting in cytotoxic efficacy[13,14]. NK cells can also be negatively regulated by checkpoint proteins such as programme death 1 (PD-1), which binds to its' ligands programme death ligand 1 and 2 (PDL-1, PDL-2)[13]. Activating receptors include the type 1 transmembrane natural cytotoxicity receptors (NCRs) NKp46 (which is exclusive to NK cells), NKp30 and NKp44 (which are also expressed on T cells), the C-type lectin-like receptors NKG2C and NKG2D, the activating KIRs and the DNAX accessory molecule 1 (DNAM1)[13-15]. Ligands for the activating KIR receptors include the HLA-C2 and HLA-A ligands, however, the interaction between ligand and receptor is less well understood for activating KIRs than for their inhibitory counterparts[16]. NKG2D receptors recognise stress-induced proteins on transformed and virally infected cells. These include the MHC class 1 related genes MICA and MICB and UL-16 binding proteins[16]. Whilst recognising nectin adhesion molecule and the poliovirus receptor, DNAM1 also interacts with the  $\beta 2$  integrin leukocyte function-associated antigen 1 (LFA-1) which is involved in the formation of the immunological synapse[15]. It is important to note that in addition to independent activation, specific combinations of activating receptors are synergistic, increasing the overall signal received by the effector cell and consequently, increasing its cytotoxic response[17].

$CD16^+$  NK cells, in addition to direct receptor-ligand binding, express a propensity to carry out antibody-dependent cellular cytotoxicity (ADCC)[18].  $CD16$  (Fc $\gamma$ RIII) is a transmembrane receptor which can bind the Fc region of IgG1 and IgG3 antibodies,



**Figure 2 Activation and cytotoxicity of natural killer cells.** Natural killer (NK) cells recognise a multitude of ligands on both healthy and transformed cells. Inhibitory receptors (red) recognise 'self-antigens' on healthy tissue preventing activation. However, these molecules are lost on aberrant cells as a result of viral transformation or malignancy ('missing-self') leading to NK cell activation. Alternatively, NK cells may become active through engagement of activating receptors (green) via stress ligands expressed on transformed cells. Binding of leukocyte function-associated antigen 1 to ICAM1 stabilises the immunological synapse between NK and target cells and ensures effective cytotoxicity. Upon activation NK cells release cytotoxic granules which contain perforin and granzymes to initiate target cell death via necrotic or apoptotic pathways. NK cells can also execute antibody dependent cellular cytotoxicity through Fc engagement of the CD16 receptor. Finally, NK cells secrete cytokines, such as interferon- $\gamma$ , facilitating crosstalk between the adaptive and innate immune system, resulting in dendritic and T cell recruitment.

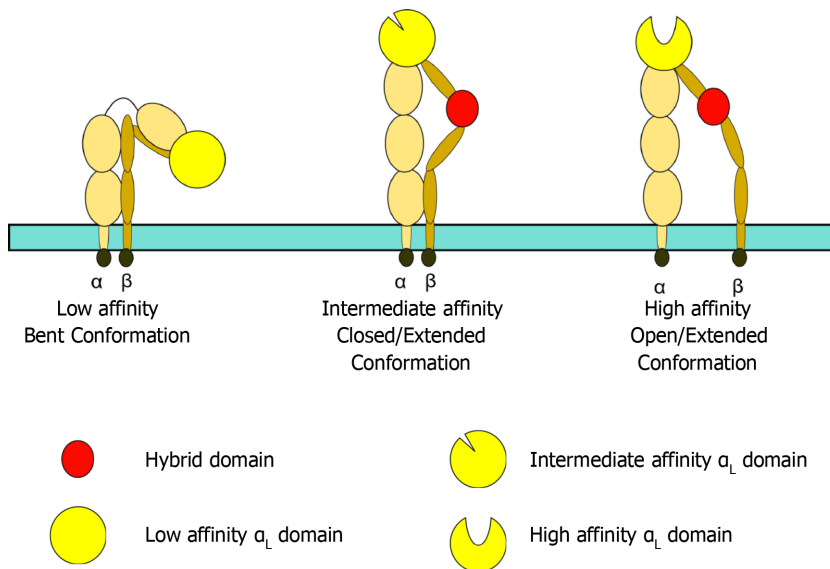
enabling NK cells to kill immunoglobulin labelled cells[16], a crucial concept for monoclonal antibody-based therapies[13].

### NK Cytotoxicity

Following initial interactions between activating/inhibitory receptors and target cells, the balance of signals received by the NK cell determines its activation status. If activated, NK cells must form an immunological synapse with the target cell. Formation of this synapse enables stable adhesion, polarisation of cytotoxic granules and subsequent lysis of the target cell. This is achieved through binding of the  $\beta 2$  integrin, LFA-1[17]. Consisting of two chains,  $\alpha_L$  and  $\beta_2$ , LFA-1 is a heterodimer whose reactivity to its ligands (ICAM family members) can be modified via conformational changes. Specifically, a bent conformation exhibits a low affinity for its ligands, intermediate affinity can be achieved through a closed/extended conformation, whilst an open/extended conformation results in high-affinity binding (Figure 3)[17,19,20].

In contrast to T cells, NK cells do not require inside-out signals (such as chemokines and T cell receptor activation) to stimulate LFA-1 binding and can signal autonomously[15,21]. Following integrin activation, a signalling network is employed to facilitate the convergence of cytotoxic granules to the microtubule organising centre (MTOC) which is subsequently polarised towards the target cell, allowing degranulation (Figure 4)[21-26]. Actin remodelling is also carried out at the immunological synapse in response to NK cell activation, facilitating docking of the cytolytic granules [27]. Integrin-linked kinase, paxillin, Pyk2 and RhoGEF7 signalling cascades are employed for the polarisation of MTOC, with Cdc42, CLIP-170, Par6 and APC components of this signalling cascade being key for granule polarisation[21,28]. During this process, the mitochondria and Golgi complex have also been shown to polarise towards the immunological synapse[29,30]. Following polarisation of the cytotoxic granules, the pore-forming protein, perforin, and the serine proteases, granzymes, are exocytosed leading to target cell death via perforin induced necrosis, or granzyme stimulated caspase-dependent apoptosis[28,31,32]. Non-granule dependent cytotoxic mechanisms include the binding of death ligands such as TNF-related





**Figure 3 Leukocyte function-associated antigen 1 conformation.** Leukocyte function-associated antigen 1 (LFA-1) affinity can be altered by its conformation. When bent, LFA-1 exhibits low affinity for its ligand, ICAM1. Intermediate affinity is achieved through a closed/extended conformation, whilst an open/extended conformation results in high affinity binding to ICAM1 and generation of an effective, stable immunological synapse.

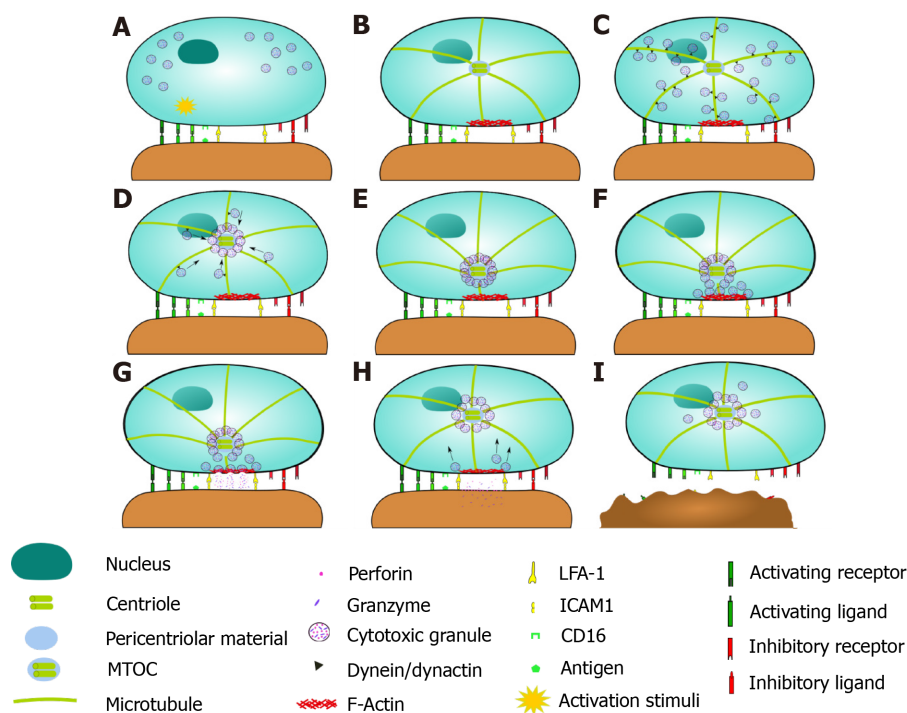
apoptosis-inducing ligand and FasL to their receptor on target cells result in extrinsic apoptosis *via* caspase-8 activation[33] and release of IFN- $\gamma$ [13,34,35].

### NK cells in cancer

Initially identified as a result of their ‘natural’ cytotoxicity towards both syngeneic and allogeneic tumour cells, NK cells have widely demonstrated potent anti-tumoral cytotoxicity[36–38]. However, they are highly heterogeneous both between cancer types and intra-tumourally[36]. Moreover, the variable functional status of NK cells is seen to greatly impact their anti-tumoral efficacy[36]. Upregulation of the inhibitory receptor NKG2A has been associated with NK cell exhaustion and poorer prognosis in patients with liver cancer[39], whilst PD-1 engagement of NK cells has been shown to block the polarisation of lytic granules and impair outside-in integrin signalling[40]. Increased expression of PD-1 on NK cells has also been associated with poorer overall survival in patients with hepatocellular carcinoma[41]. Conversely, high proportions of functionally active NK cells result in favourable outcomes in many cancer types [42]. Retrospective flow cytometric assessment of peripheral blood samples from metastatic prostate cancer patients (Gleason scores between 6–9) followed by univariate Cox regression analysis demonstrated a significant correlation between expression of the activating receptors NKp46 and NKp30, and longer overall survival [42]. Similarly, immunostaining of tissue samples from 98 patients with gastric cancer (stage 1–4) combined with multivariate analysis revealed a positive correlation between NKG2D expression in tumour infiltrating lymphocytes and prolonged OS (hazard ratio 0.34)[43]. With the propensity to exhibit potent anti-tumoral activity, NK cell-based immunotherapy research has flourished in the past few years[36].

### NK cells as prognostic markers in PDAC

NK cell number has been found to convey prognostic significance. Through flow cytometric analysis of PBMCs from resectable PDAC patients (stage Ib–III) both pre- and post-surgery, Hoshikawa *et al*[44] demonstrated a positive correlation between the percentage of NK cells in peripheral blood and recurrence-free survival, with patients who exhibited high NK levels expressing later disease recurrence. Moreover, univariate and multivariate analysis using the Cox proportional hazard regression model demonstrated NK cell frequency to be the only favourable prognostic factor for recurrence-free survival. Additional factors tested included tumour stage, N status, radicality, age and gender. Importantly, no additional circulating mononuclear cells were included in this analysis. NK cell infiltrate within the TME (assessed by gene expression profile) was also found to be associated with later disease recurrence, however, this was not statistically significant[44]. Assessment of a larger cohort of patients would improve the power of this assessment and may provide further insight into the role of tumour infiltrating NK cells in PDAC.



**Figure 4 Cytotoxic granule convergence and microtubule organising centre polarisation.** A: Following receptor stimulation leading to natural killer (NK) cell activation, leukocyte function-associated antigen 1 engages with its ligand ICAM1 on the malignant cell, forming a stable immunological synapse; B: F-actin accumulates and polymerises at the immune synapse, forming a filamentous mesh which modulates the release of cytolytic granules. Tubulin microtubules then form from the microtubule organising centre (MTOC); C and D: Cytotoxic granules converge on the microtubules (C) and are polarised towards the MTOC where they converge (D); E: This granule movement is dependent on dynein/dynactin motor function. Dynamic rearrangement of the microtubules facilitates polarisation of MTOC towards the immunological synapse; F and G: This polarisation is stimulated via Integrin-linked kinase, paxillin, Pyk2 and RhoGEF7 signalling. Following polarisation to the immunological synapse, a subsection of cytotoxic granules fuse with the plasma membrane (F) (a process largely regulated by Munc 13-4 and Rab27a) and undergo degranulation via either complete or incomplete fusion (G); H: Cytotoxic granules which do not degranulate are recycled and are hypothesised to remain converged at the MTOC to facilitate serial NK cell killing. Granules which undergo incomplete fusion are rapidly recycled through clathrin mediated endocytosis of granule membrane proteins, further facilitating serial killing; I: Finally, the malignant cell undergoes perforin induced necrosis or granule dependent apoptosis. NK cells detach from the malignant cell and move on to the next target.

Finally, gene set enrichment analysis revealed that patients with enriched type I and II IFN signatures within tumour tissues had later disease recurrence. Type I and II IFNs are closely associated with NK cell function. Type I IFNs induce NK cell activation both directly, through binding to type I IFN receptor, or indirectly, through stimulating dendritic cell release of IL-15, whilst type II IFNs (namely IFN- $\gamma$ ) are produced by activated NK cells[45,46]. Moreover, IFNs can induce CXCL10 release, leading to the further recruitment of NK cells to the tumour tissue[44]. Thus, enriched IFN signatures were also concluded to be a positive prognostic biomarker in PDAC [44].

Yang *et al*[47] also suggest prognostic implication of NK cells in PDAC. High densities of NK cells in peripheral blood samples (analysed by flow cytometry) were found to correlate with poor overall survival in patients with advanced PDAC (stage III/IV) when accompanied by a high neutrophil: lymphocyte ratio (obtained from routine hospital data). NK cell number (HR 1.45), as well as patient age (HR 1.34), neutrophil to lymphocyte ratio (HR 1.48) and absence of metastasis (HR 0.72) were found to be independent prognostic markers following univariate and multivariate Cox regression analysis. It is perhaps prudent to note that subsequent ELISAs to measure release of IFN- $\gamma$  and TNF- $\alpha$  from NK cells as well as serum IL-2 Levels, a known activator of NK cells, demonstrated lower levels of all three markers in patients with high NK cell densities, although only IFN- $\gamma$  reached significance. These results suggest a distinct subtype of NK cells with impaired function in PDAC patients and thus prognostic significance may rely not just on NK cell numbers, but on functional subtypes[47]. The results obtained within this study focus solely on peripheral blood circulating NK cells. Further work to classify functional NK cell subtypes within PDAC tumour tissue would provide more conclusive insights into the prognostic significance of these effector cells in PDAC.

### NK cells in murine models of PDAC

NK cells have demonstrated potent anti-tumoral efficacy in murine models of PDAC. Using a transgenic mouse model in which oncogenic transposons for Kras<sup>G12V</sup> and myristoylated Akt2 were introduced into p53<sup>fl/fl</sup> mice *via* intra-pancreatic injection and electroporation, along with plasmids for Cre recombinase and sleeping beauty transposase, Brooks *et al*[48] demonstrated that neo-adjuvant PD-1 blockade plus adjuvant CD96 inhibition in combination with gemcitabine prevented relapse and facilitated long term remission following resection surgery. To further investigate the role of immune cells in neo-adjuvant and adjuvant treatment of PDAC, mice were injected (intraperitoneal) with anti-CD8 and anti-NK.1.1 depletion antibodies. Pre-operatively, depletion of both CD8<sup>+</sup> T cells and NK cells significantly reduced survival when compared to the control. Furthermore, adjuvant NK but not CD8<sup>+</sup> depletion was found to impair survival and resulted in an increase in local disease recurrence. *In vitro* luciferase cytotoxicity assays confirmed NK cell cytolytic efficacy against tumour derived cancer cells, a finding consistent with the results obtained for depletion experiments. Thus, targeting both T and NK cells through immune checkpoint inhibition may confer long term survival benefits in metastatic cases of PDAC[48]. Similarly, in a Kras<sup>LSL-G12D</sup> p53<sup>LSL-R172H</sup> Pdx1-Cre (KPC) model of PDAC, NK cell-based adoptive transfer immunotherapy was found to significantly delay tumour growth [49]. In addition, immunostaining of KPC tumours at end time-points revealed significantly elevated areas of necrosis in mice treated with adoptive transfer of NK cells compared to control mice, demonstrating the cytotoxic efficacy of NK cell treatment, a finding replicated through *in vitro* flow cytometry cytotoxicity assays[49].

Xenograft models of PDAC have also been used to demonstrate the efficacy of *ex vivo* expansion of NK cells. NOD *scid* gamma (NSG) mice injected subcutaneously with MiaPaca2 cells demonstrated a significant reduction in tumour growth when treated with adoptive transfer of *ex vivo* expanded NK cells (versus control group)[50]. This finding demonstrated both successful trafficking of NK cells to the tumour site and tumour control following intravenous injection, suggesting that NK cells may prove an effective systemic treatment in xenograft models of PDAC[50]. It is prudent to note that the nature of this model requires the mice included within the study to be immuno-compromised, and this must be taken into consideration when reviewing the data presented. Employing additional models would add further validity to the therapeutic impact of *ex vivo* expanded NK cells in murine models of PDAC[50].

Notorious for their resistance to typical anti-proliferative/cytotoxic therapies, cancer stem cells are an important subpopulation involved in cancer progression, metastasis and recurrence. Despite their notoriety, CSCs are preferentially targeted by NK cells in both the autologous and allogeneic setting; an effect which was found to be NKG2D dependent[51]. Metastatic, intra-pancreatic and subcutaneous models of PDAC were established in NSG mice using the PANC-1 cancer cell line. Mice treated with adoptively transferred NK cells were found to have substantial reductions in tumour volume when compared to untreated mice. Flow cytometric analysis also demonstrated a significant reduction in CSC populations in mice treated with adoptive NK cell transfer (denoted by aldehyde and CD24 expression). Moreover, immunostaining revealed co-localisation of NK cells and CSCs within tumour tissues. Ames *et al*[51] conclude that NK cells possess the ability to identify and preferentially target CSCs in solid tumours. As such, further work investigating the impact of systemic adoptive transfer of NK cells on CSC populations may yield exciting new therapeutic insights into the treatment of this dismal prognosis disease.

### Tumour induced NK cell dysfunction and immune evasion in PDAC

Pancreatic tumour cells have developed several methods of NK-cell immune evasion. Murine models of pancreatic cancer specifically designed to express MYC at the Rosa26 Locus, either with or without the *Lsl-Kras*<sup>G12D</sup> allele, were used to determine the role of MYC in pancreatic tumour progression. MYC expression was found to drive pancreatic cancer development and accelerate disease progression in precursor lesions initiated by KRAS. Moreover, bulk RNA sequencing of end stage tumours revealed reduced expression of T-cell, B-cell and NK cell markers in tumour tissue, suggesting that immune cell infiltration may be regulated by MYC. GeneGo analysis revealed that allelic activation of both KRAS and MYC resulted in significant downregulation of type I IFN, an effect that was found to be dependent on repressional binding of MYC-interacting zinc finger protein, MIZ1. Deletion of MIZ1 resulted in restoration of NK and B cell infiltration into tumour tissues, an effect ablated upon antibody dependent blockade of type I IFN[52]. Similarly, the oncoprotein Sloan-Kettering Institute has been shown to inhibit SMAD association with the acetyltransferases CBP and p300,

which are key regulators of inducible expression of NKG2D ligands on cancer cells, and facilitate repression of gene transcription *via* histone deacetylases. This results in downregulation of NKG2D ligands on tumoral cells, reducing NKG2D dependent cytotoxicity and facilitating immune evasion[53]. In addition, tumoral cells are seen to exhibit intricate crosstalk with NK effector cells and have been shown to induce functional deregulation[54]. Co-culture with the pancreatic cancer cell line MiaPaca2 was found to induce NK cell anergy *via* fibrinogen-like protein 2 and was characterised through decreased expression of DNAM-1, IFN- $\gamma$  and CD107a and increased expression of PD-1[55]. Importantly, when co-cultured, anergic NK cells were seen to induce anergy in naïve NK cells, reducing IFN- $\gamma$  and CD107a expression[55]. Further work investigating the reversal of NK cell anergy, both tumour-dependent and bystander anergy may lead to novel therapeutic insights, and provide a baseline from which to further challenge PDAC immune evasion.

MHC class-I chain-related molecules A/B (MICA/B) are crucial ligands for the activating receptor NKG2D. Several studies have demonstrated immune evasion as a result of MICA/B shedding by tumour cells. Duan *et al*[56] demonstrated that high glucose levels, which are widely correlated with pancreatic cancer, could facilitate immune evasion. Specifically, *in vitro* assays demonstrated a decrease in NK cell induced lysis of pancreatic cancer cell lines (demonstrated by lactate dehydrogenase release assays) when cultured in high glucose conditions. Moreover, western blot and quantitative real-time polymerase chain reaction (PCR) analysis revealed that this decrease in function was a result of reduced expression of MICA/B in cancer cell lines at both the protein and mRNA level. Mechanistically, high glucose was found to inhibit AMP-activated protein kinase signalling, leading to upregulation of the polycomb group protein (PcG) Bmi1, and subsequent promotion of GATA2 expression. This augmentation inhibited expression of MICA/B on pancreatic cancer cells and led to their immune evasion[56]. Similar shedding of the NKG2D ligand has been observed in response to hypoxia. High levels of HIF1 $\alpha$  have been correlated with decreased expression of MICA/B on pancreatic tumour cells and also with increased internalisation of the activating receptor NKG2D, suggesting a dual role for the hypoxic TME in NK cell dysfunction[57]. Specifically, immune-histochemical analysis of PDAC patient tumour tissues revealed a significant correlation between MICA surface expression and high HIF1 $\alpha$ . Moreover, immune-fluorescent staining of NK cells isolated from patient PBMCs demonstrated clear internalisation of the activating NKG2D receptors as well as MICA/B[57].

Receptor expression is found to be largely augmented in pancreatic cancer patients. Flow cytometric analysis of participant blood samples revealed that expression of the activating receptors DNAM-1 (CD226) and CD96 were significantly reduced in pancreatic cancer patients (stage I-IV) when compared to healthy controls. This downregulation was suggested to lead to NK cell dysfunction and tumour evasion.[58] Downregulation of NKG2D in pancreatic cancer patients has also been correlated to reduced cytotoxicity of the effector cells. *In vitro* blockade of NKG2D using neutralising antibodies was found to significantly decrease killing of MiaPaca2, BxPC3 and Capan2 cell lines by IL-15 stimulated NK cells[59]. This finding highlights the functional importance of NKG2D expression on NK cell function and may prove to be a crucial marker of NK cell efficacy against malignancy.

Additionally, flow cytometric analysis of cell surface markers following *in vitro* co-cultures of NK cells derived from healthy PBMCs and pancreatic cancer cell lines revealed downregulation of the NK activating receptors NKG2D, NKp30, NKp46 and DNAM-1. ELISA analysis suggested that this dysfunction was induced as a result of matrix metalloproteinase 9 (MMP9) and Indoleamine 2,3 dioxygenase (IDO) signalling cascades.[60] In addition to downregulation of activating receptors, exposure to MMP9 and IDO also led to decreased TNF- $\alpha$  and IFN- $\gamma$  production by NK cells, an effect that was reversed upon blockade of MMP9 and IDO using tissue inhibitor of metalloproteinases 1 and 1-Methyl-DL-tryptopan, respectively[60]. This finding was replicated in a comprehensive study of surface receptor expression and cytotoxic granule positive cells in pancreatic (stage II and IV), gastric (stage 0-IV) and colorectal cancer (stage I-IV) patients. Using flow cytometry, Peng *et al*[61] identified significant downregulation of the activating receptors NKG2D, NKp30, NKp46 and DNAM-1 on NK cells identified in peripheral blood samples from pancreatic cancer patients when compared to healthy controls, whilst expression of the inhibitory receptor KIR3DL1 was significantly upregulated. Moreover, the percentage of perforin positive circulating NK cells was found to be significantly reduced in pancreatic cancer patients. Taken together, these alterations evidence the dysfunction of NK cells observed in malignancy.



Impairment in degranulation was also identified by Jun *et al*[62]. In an *in vitro* flow cytometry-based degranulation assay, PBMCs derived from malignant patients, non-malignant patients or healthy controls were mixed with target cells before staining with CD107a. NK cells derived from pancreatic cancer patients showed significantly impaired degranulation when compared to non-malignant and/or healthy control samples. Despite demonstrating impaired cytotoxic capabilities, no significant difference in NK cell IFN- $\gamma$  production was observed between cancer patients and healthy controls. Importantly, multivariate analysis revealed that tumour-induced NK cell dysfunction correlated with disease stage, suggesting progressive impairment of NK cells with advanced-stage disease. Thus, as previously suggested, NK functional status may prove an attractive prognostic marker in PDAC.

### **NK cell interaction with stromal cells**

NK cell-stromal cell interactions have been shown to result in significant cellular dysfunction and exclusion of NK cells from tumour tissues, suggesting that NK cells can be educated by the TME[47,63]. As crucial players in the development of the pre-metastatic niche, extracellular vesicles are key to the progression of PDAC and contain large numbers of immune regulatory factors including TGF- $\beta$ , nectin-2 and PVR. Flow cytometric analysis following *in vitro* co-culture of NK cells and extracellular vesicles demonstrated downregulation of multiple NK cell receptors and cytokines, specifically, IFN- $\gamma$ , TNF $\alpha$ , CD107a and NKG2D, resulting in gross cytotoxic impairment (demonstrated through tumour sphere cytotoxicity assays) and NK cell dysfunction. This impairment was further associated with the activation of the TGF- $\beta$ -Smad3/4 signalling pathway[64]. It should also be noted that NK cell dysfunction is heavily regulated by the soluble factors and cytokines secreted by both tumoural and stromal cells, with TGF- $\beta$ , IDO, MMPs and interleukins proving largely responsible for this impairment (Figure 5)[50,60,65-67].

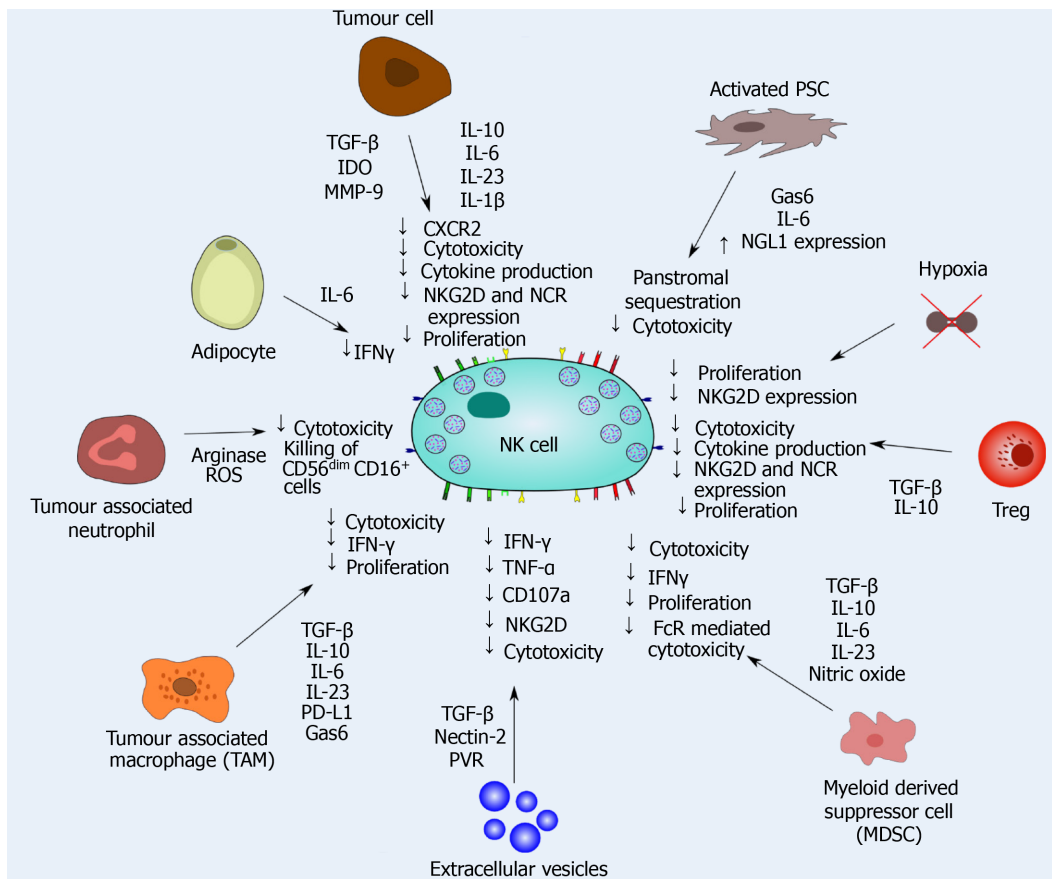
NK cell interaction with suppressive immunoregulatory cells has a significant impact on cytotoxic effector function. Both MDSCs and TAMs produce IL-23, IL-6, IL10 and TGF- $\beta$  which results in the downregulation of IFN- $\gamma$ , perforin and IL-12 production by NK cells, decreasing cytotoxicity and NK cell proliferation within the TME[65]. TAMs also inhibit NK cell function through cell-cell interactions. M2 macrophages express PDL-1 which binds to PD-1 expressed on NK cells. This checkpoint protein prevents NK cell engagement and induces downregulation of the activating receptors NKG2D, natural cytotoxicity receptors and DNAM1, leading to reduced cytotoxicity[67]. Immunosuppressive Tregs release IL-10 and TGF- $\beta$  into the TME. This cytokine release decreases NK cell cytotoxicity through the downregulation of activating receptors and decreased production of anti-tumour cytokines such as IFN- $\gamma$ [65].

Moreover, MDSCs have also been shown to augment FcR mediated NK cell functions. Adoptive transfer of MDSC in a Panc02-EGFR<sup>+</sup> murine model of pancreatic cancer significantly inhibited the efficacy of monoclonal antibody therapy, with mice receiving Cetuximab + MDSCs expressing significantly larger tumour volumes than did mice treated with Cetuximab + splenocytes. Furthermore, NK cells co-cultured with tumour derived (melanoma) MDSCs were found to express significantly reduced phospho-ERK than did those cultured alone (as measured by flow cytometry). Thus, it was concluded that MDSCs inhibit FcR mediated signal transduction, resulting in impaired cytokine production, ADCC dysfunction and reduced anti-tumour activity. An effect that was found to be, at least in part, in response to MDSC nitric oxide production[68].

Likewise, tumour-associated neutrophils have been shown to impair NK cell function through the release of arginase and ROS[67]. *In vitro* co-culture assays using PBMCs derived from healthy donors demonstrated that activated granulocytes (stimulated with Phorbol 12-myristate 13-acetate) produced ROS including hydrogen peroxide, which was found to be cytotoxic to CD56<sup>dim</sup> CD16<sup>+</sup> NK cells, but not CD56<sup>bright</sup> CD16<sup>-</sup> subsets, suggesting that interactions with stromal cells may result in differential effects on cytotoxic effector subset[69].

Tumour-associated macrophages and cancer-associated fibroblasts have also been shown to produce growth arrest-specific gene 6 (Gas6) in the pancreatic TME. As a negative regulator of the immune system, the Gas6-AXL pathway is seen to prevent NK cell activation. Orthotopic syngeneic models of pancreatic cancer in which tumour cells derived from KPC mice were transduced with zsGreen/Luciferase and orthotopically injected into the pancreas of immune-compromised mice were used to investigate the role of Gas6 in NK cell activation and pancreatic cancer development. Pharmacological blockade of Gas6 signalling using neutralising antibodies was found to inhibit pancreatic cancer metastasis. Moreover, immunohistochemical analysis of





**Figure 5 Natural killer cell dysfunction caused by tumoral and stromal cells in pancreatic ductal adenocarcinoma.** Natural killer (NK) cell interactions with multiple stromal and tumour cells significantly impacts the cytotoxic efficacy of NK cells in pancreatic ductal adenocarcinoma. Transforming growth factor - $\beta$ , interleukin (IL)-10, IL-6, IL-23 and IL-1 $\beta$  release significantly dampens NK cell cytotoxicity and function, and inhibits intratumoural proliferation of NK cells. NK cell mediated cytokine release is also inhibited within the immunosuppressive tumour microenvironment. Finally, chemokine release may also sequester NK cells in the panstromal compartment, preventing engagement with tumour cells.

NK cell infiltrates revealed a significant increase in the number of NKp46<sup>+</sup> NK cells in lung metastasis in mice treated with anti-Gas6 therapy when compared to the control. This finding was also observed in the tumour draining lymph nodes; however no significant difference was observed in NK cell infiltration in primary tumour sites between treatment groups[70]. As such, Gas6 blockade may prove a promising new therapeutic target for the treatment of metastatic lesions in pancreatic cancer.

NK cells have been shown to be excluded from tumour tissue as a result of NK-stromal cell interactions. Through *ex vivo* immunostaining and Ariol image analysis of tissue micro-arrays, Ene-Obong *et al*[4] demonstrated that infiltrates of CD56<sup>+</sup> natural killer cells were significantly lower in the juxtatumoural compartment of PDAC tissues (stage I-III) when compared to panstroma. This finding suggests PSCs sequester NK cells in the panstromal compartment of the TME, preventing NK induced cancer cell death. Similarly, Lim *et al*[50] demonstrated that NK cell frequencies in malignant tissue from PDAC patients (stage I-IV) were as low as < 0.5% (as assessed by flow cytometry). This low infiltration was attributed to reduced expression of the CXCR2 receptor on NK cells which resulted in poor chemotaxis into tumour tissues.

Known orchestrators of the TME, pancreatic stellate cells are crucial regulators of immune cell infiltrates in pancreatic cancer and have been shown to promote tumour progression through the development of an immunosuppressive environment[5,71-73]. In an orthotopic mouse model of pancreatic cancer in which either Panc02 cells alone or in combination with activated PSCs were injected into the pancreas of C57BL/6 mice, Li *et al*[74] report a significant reduction in the number of NK cells in tumour tissue in mice co-transplanted with PSCs when compared to mice injected with Panc02 cells alone. This finding is consistent with *ex vivo* analysis of human PDAC samples[4]. Further work is needed to demonstrate the mechanistic link between PSCs and NK cell tumoural exclusion.

In a recent study, Francescone *et al*[75] demonstrated dynamic crosstalk between PDAC cancer cells, CAFs and immune cells. Specifically, in *in vitro* co-culture assays

CAFs were found to ectopically express the neural presynaptic protein NetrinG1 (NG1) whilst its binding partner, NGL1 was identified on PDAC cells. This interaction was found to convey protection on cancer cells from NK cell-driven elimination, a fact quantified through CRISPR/Cas9 NG1 knockout which resulted in decreased expression of the immunosuppressive cytokines TGF- $\beta$ , IL-8 and IL-6, and restored NK cell anti-tumour activation. This finding was reproduced in murine orthotopic models of PDAC in which NGL1 was knocked out of syngeneic mouse cells. This work provides a potential novel target in PDAC in which the immunosuppressive nature of PDAC induced by CAF cells can be reverted.

Finally, *in vitro* and xenograft models of PDAC in NOD SCID mice demonstrated that stromal TGF- $\beta$  signalling stimulates CAF cells to secrete IL-6, which in turn suppresses NK cell function as assessed through NK cell killing assays. Concomitantly, stromal TGF- $\beta$  limits NK cell function itself[76]. *In vivo* TGF $\beta$  blockade using the anti-mouse TGF $\beta$ R2 mAb, 2G8, was found to reduce tumour progression, an effect that was reversed upon NK cell depletion[76]. Furthermore, a conditional KRAS (G12D) model of pancreatic cancer combined with a high-fat calorie diet demonstrated that stromal adipocytes produce IL-6, reducing NK cell function through IL-6 mediated downregulation of IFN- $\gamma$  production. Thus, it has been suggested that adipocytes and PSCs may provide tumour sanctuaries within the TME through the immunosuppression of NK cells[77,78].

Taken together these findings demonstrate the complex interactions between NK cells and the PDAC microenvironment. Despite their cytotoxic efficacy against malignancy, the dysfunction induced by stromal cells greatly augments the natural anti-tumoral activity of NK cells. As such, a deeper understanding of these interactions is crucial to fully unlock the potential of NK cells in the treatment of PDAC.

## NK CELLS AS THERAPY IN PDAC

Despite the known dysfunction of NK cells associated with PDAC, these cytotoxic effector units display evident cytotoxic capabilities and as such have received much attention as a potential immunotherapeutic tool in the treatment of PDAC. One of the immunotherapeutic routes explored is the use of NK cells as cellular immunotherapy [79]. When compared to T cells, the use of NK cells as cellular immune-therapeutics express several advantages. Firstly, NK cells present a much lower risk of on-target/off-tumour toxicity, rendering NK cells a relatively safe treatment option. Moreover, neurotoxicity, cytokine release syndrome and graft-versus-host disease are much less likely to occur in chimeric antigen receptor (CAR)-NK treatment than observed with the use of CAR-T therapy. Secondly, due to this reduced risk of graft-versus-host disease from allogeneic NK sources, NK cells have the potential to be derived from multiple sources, including cell lines such as NK92. This facilitates the development of a truly 'off-the-shelf' cellular immunotherapeutic, eliminating the need for personalised therapeutics and the subsequent challenges these treatments invoke[79]. Thirdly, CAR-NK cells retain their natural cytotoxic capabilities and consequently can eliminate malignant cells in both a CAR dependent and independent manner[79,80].

Several studies have focused on harnessing the anti-tumoral efficacy of NK cells against PDAC. Lee *et al*[81], rationally designed a CAR-NK to specifically target folate receptor alpha (FR $\alpha$ ) and death receptor 4 (DR4) which were found to be highly expressed on tumour cells. In addition, the CAR-NK was loaded with an apoptosis-inducing death ligand to further induce anti-tumoral cytotoxicity. Treatment with the FR $\alpha$ /DR4 targeting NK cells significantly increased tumour-selective apoptosis and NK cell infiltration in tumour tissue in sub-cutaneous models of pancreatic cancer. Similarly, Xia *et al*[82] investigated the efficacy of a Robo1 bi-specific CAR-NK cell treatment in combination with  $^{125}\text{I}$  seed radiotherapy in orthotopic murine models of pancreatic cancer. Expressed on pancreatic cancer cells, Robo-1 is a member of the axon guidance receptor family which has been found modulate T-cell chemotaxis into the TME. Tumour size was significantly reduced in mice treated with combination therapy when compared to  $^{125}\text{I}$  seed treatment alone. Moreover, tumours of mice receiving combination therapy displayed a significantly higher greyscale value than did  $^{125}\text{I}$  treatment alone. Thus, it was concluded that bi-specific CAR-NK cells may prove a promising immunotherapeutic when combined with  $^{125}\text{I}$  seed therapy in treatment of pancreatic cancer. Identification of novel antigens for therapy are useful strategies for cell-based therapy[83,84].

**Table 1** Current clinical trials employing natural killer cells therapy in pancreatic cancer

Primary therapy	Additional intervention	Tumour type	Phase	NCT number	Status	Ref.
NK cell infusions	Irreversible electroporation	Advanced pancreatic cancer	I/II	NCT02718859	Completed	[90, 91]
Dendritic cell activated, cytokine induced killer treatment	S-1 (drug)	Advanced pancreatic cancer	I/II	NCT01781520	Completed	[87, 92]
BiCAR NK cells (ROBO1 CAR-NK cells)		Pancreatic cancer	I/II	NCT03941457	Recruiting	[93]
<i>Ex-vivo</i> expanded autologous NK cells (SNK01)	Trastuzumab; Cetuximab	Advanced solid tumour; metastatic cancer; HER-2+ breast cancer; HER-2 positive gastric cancer; HER-2 protein overexpression; oesophageal cancer; ovarian cancer; endometrium cancer; bladder cancer; pancreatic cancer; colorectal cancer; NSCLC; EGFR+ NSCLC; head and neck squamous cell carcinoma; triple-negative breast cancer; cervical cancer; sarcoma	I/IIa	NCT04464967	Not yet recruiting	[94]
High activity NK cells		Pancreatic cancer	I/II	NCT03008304	Completed	[95]
Activated NK cells		Lung cancer; breast cancer; colon cancer; pancreatic cancer; ovarian cancer	I/II	NCT03634501	Recruiting	[96]
FT500-an allogenic, iPSC derived NK cell immunotherapy	Nivolumab; pembrolizumab; atezolizumab; cyclophosphamide; fludarabine; IL-2	Advanced solid tumours; lymphoma; gastric cancer; colorectal cancer; head and neck cancer; squamous cell carcinoma; EGFR positive solid tumour; HER2 positive breast cancer; hepatocellular carcinoma; small-cell lung cancer; renal cell carcinoma; pancreas cancer; melanoma; NSCLC; urothelial carcinoma, cervical cancer; microsatellite instability; merkel cell carcinoma	I	NCT03841110	Recruiting	[97]
FT500-101 allogenic NK cell immunotherapy		Advanced solid tumours; lymphoma; gastric cancer; colorectal cancer; head and neck cancer; squamous cell carcinoma; EGFR positive solid tumour; HER2 positive breast cancer; hepatocellular carcinoma; small-cell lung cancer; renal cell carcinoma; pancreas cancer; melanoma; NSCLC; urothelial carcinoma, cervical cancer; microsatellite instability; merkel cell carcinoma	Observational study	NCT04106167	Recruiting	[98]
FATE-NK100 (donor-derived <i>ex-vivo</i> activated immunotherapy)	Trastuzumab; Cetuximab	HER2+ gastric cancer; colorectal cancer; head and neck squamous cell carcinoma; EGFR <sup>+</sup> solid tumours; advanced solid tumours; HER2 positive breast cancer; hepatocellular carcinoma; NSCLC; renal cell carcinoma; pancreatic cancer; melanoma	I	NCT03319459	Active, not recruiting	[99]
Autologous dendritic cell vaccine loaded with personalised peptides to stimulate innate and adaptive immune response <i>via</i> activating T and NK cells	Standard of care; Nivolumab	Pancreatic adenocarcinoma	Ib	NCT04627246	Recruiting	[100]
ACE1702 cellular therapy (anti-HER2 NK cells)	Cyclophosphamide; Fludarabine	Locally advanced solid tumours; metastatic cancer; solid tumour; HER-2+ gastric cancer; HER-2 + metastatic breast cancer	I	NCT04319757	Recruiting	[101]
NK cells	Bortezomib	CML; pancreatic cancer; colon/rectal cancer; multiple myeloma; carcinoma-NSCLC	I	NCT00720785	Recruiting	[102]
Cytokine-induced killer cells	Tegafur; Gimeracil; Oteracil potassium	Advanced cancer	II	NCT03002831	Terminated	[103, 104]
Anti-MUC1 CAR-pNK cells		Hepatocellular carcinoma; NSCLC; pancreatic cancer; triple negative invasive breast carcinoma; malignant glioma of the brain; colorectal carcinoma; gastric carcinoma	I/II	NCT02839954	Unknown	[105]

Autologous NK/NK T cell immunotherapy	Breast cancer; glioma; hepatocellular carcinoma; squamous cell lung cancer; pancreatic cancer; colon cancer; prostate cancer	I	NCT00909558	Suspended	[106]
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NK cell: Natural killer cell; CAR: Chimeric antigen receptor; NSCLC: Non-small cell lung cancer; CML: Chronic myeloid leukemia; iPSC: Induced pluripotent stem cells.

In addition to CAR therapies, cytokine supplement either alone or in combination with additional therapeutics has demonstrated significant benefit in both preclinical studies and clinical trials. In combination with the immune priming CD40 agonist, supplementation of IL-15 in orthotopic and KPC mouse models of pancreatic cancer resulted in a significant increase in immune cell infiltration into tumour tissue (assessed through multi-colour flow cytometry), particularly of NK and CD8<sup>+</sup> T cells. This infiltration resulted in enhanced anti-tumour effect and significantly improved long term survival (log-rank tests;  $P \leq 0.0001$ ) [85]. Furthermore, Lin *et al* [86] demonstrated that allogeneic NK cell transfer in combination with irreversible electroporation significantly increased median progression-free and overall survival in stage III PDAC patients and increased median overall survival in stage IV patients. Moreover, multiple allogeneic transfers correlated with better prognosis in stage III patients. Similarly, treatment with a dendritic cell/cytokine-induced killer cell vaccine was found increase both overall and progression-free survival in advanced pancreatic cancer patients [87]. Taken together these results highlight the therapeutic potential of NK cell-based therapies. Current clinical trials employing NK cell therapy in pancreatic cancer (identified through entering the search terms 'natural killer cells' and 'pan-creatic cancer' at [clinicaltrials.gov](https://clinicaltrials.gov)) are highlighted in Table 1.

## CONCLUSION

NK cells are powerful effector units, which, when harnessed, can confer striking therapeutic benefit. With the innate effector units demonstrating strong efficacy against malignancy, both in preclinical studies, and clinical trials, the potential for NK cell therapy in PDAC is only just beginning to come to light. Additional research is needed to fully elucidate the role of NK cells in pancreatic cancer and deconvolute the intricate relationships between NK and stromal cells within the TME, facilitating co-targeting of tumour stroma [73,88]. Through defining these relationships, novel functional and mechanistic insights into this devastating disease can be achieved [89] and the full therapeutic potential of NK cells can be harnessed.

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