THE ROLE OF NATURAL KILLER CELLS IN PANCREATIC DUCTAL ADENOCARCINOMA

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ABSTRACT

Natural Killers are innate immune cells, actively involved in the detection and killing of malignant cells. Their role in pancreatic cancer is not yet understood, in part because of the paucity of infiltrative cells, compared other immune cells. Here, we have demonstrated that circulating NK cells undergo phenotypic changes, leading to functional impairment, characterised by downregulation of the main activating receptors, such as DNAM-1, NKG2D, NKp30 and NKp46. However, they retain ADCC ability and show a mature phenotype. Moreover, the analysis of the cytokines production indicates a shift to a more suppressive phenotype, highlighted by the expression of the suppressive cytokine IL-10. These changes are even more emphasized in the infiltrative compartment, with the almost complete loss of ADCC ability and the acquirement of a more “regulatory” phenotype, similar to decidual NK cells during early phases of pregnancy. Our results suggest that this could be partially induced by tumour cells, through the release of soluble form of ligands and/or the chronic cell-to-cell contact. Nevertheless, pancreatic cancer is potentially a target for NK cells’ killing activity, as demonstrated by the expression of activating ligands. In conclusion, NK cells actively participate in shaping the immune response against pancreatic cancer and they could be exploited either as a target in immune therapy and/or as biomarkers to better stratify patients, in a patient-tailored treatment settlement.

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LIST OF ABREVIATIONS

αSMA	Alfa-smooth muscle actin
ADCC	Antibody-dependent cellular cytotoxicity
AIS	Adaptive immune system
AJCC	American joint committee on cancer staging system
AMP	Antimicrobial peptide
APC	Antigen presenting cell
APP	Acute phase protein
ASR	Age-standardized incidence rate
BM	Bone marrow
BMI	Body max index
BR-PDAC	Borderline resectable pancreatic duct adenocarcinoma
CA	Carbohydrate antigen
CAF	Cancer associated fibroblast
CAR-NK	Chimeric antigen receptor-NK cells
CCL	C-C motif ligand
CD	Cluster of differentiation
CDC	Complement dependent cytotoxicity
cDCs	Conventional dendritic cells
CEA	Carcinoembryonic antigen
CLP	Common lymphoid progenitor

CMP	Common myeloid progenitor
CRP	C-reactive protein
CT	Computer tomography
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T lymphocyte antigen 4
CXCL	C-X-C motif ligand
DAMPS	Damage-associated molecular patterns
DC	Dendritic cells
DM	Diabetes mellitus
ECM	Extra cellular matrix
EGF	Epidermal growth factor
EMT	Epithelial-mesenchymal transition
ERCP	Endoscopic retrograde cholangiopancreatography
ESMO	European society for medical oncology
EUS	Endoscopic ultrasound
FAEE	Fatty acid ethyl ester
FAP	Fibroblast activated protein
FBP1	Fructose bisphosphatase 1
FGF	Fibroblast growth factor
FLT3L	Fms-like tyrosine kinase 3 ligand
FNA	Fine-needle aspiration
FOLFIRINOX	Leucovorin/fluorouracil/irinotecan/oxaliplatin
Foxp3	Forkhead box protein 3

GIST	Gastrointestinal stromal tumour
GM-CSF	Granulocyte macrophage colony stimulating factor
GMP	Granulocytic monocytic precursor
GTPase	Guanosine triphosphatase
hCMV	human Cytomegalovirus
HD	Healthy donor
HDN	High density neutrophile
HGF	Hepatic growth factor
HLA	Human leukocyte antigen
HMGB1	High mobility group box 1
HSC	Haematopoietic stem cell
ID2	Inhibitor of DNA binding 2
IDO	Indolamine 2,3-dioxygenase
Ig	Immunoglobulin
IGF	Insulin-like growth factor
IHC	Immunohistochemistry
IIS	Innate Immune System
IL	Interleukin
ILC	Innate lymphoid cells
INF	Interferon gamma
iNK	Immature Natural Killer
IRF2	Interferon regulatory factor
ISGPS	International study group on pancreatic surgery

KIR	Killer Ig-like receptor
LAMP-1	Lysosomal associated membrane protein 1
LDNs	Low density neutrophils
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
mDCs	Myeloid dendritic cells
MDSCs	Myeloid derived suppressor cells
MFI	Mean fluorescent intensity
MHC	Major histocompatibility complex
miRNA	Micro RNA
MMP	Metalloprotease
mNK	Mature Natural Killer
MTOC	Microtubule organising centre
NCAM	Neural cell adhesion molecule
NCCN	National comprehensive cancer network
NCR	Natural cytotoxicity receptor
NF-κB	Nuclear factor B
Nfil3	Nuclear factor interleukin-3
NK	Natural Killer
NKP	Natural Killer progenitor
NKT-cell	Natural Killer T cell
NLR	Nod-like receptors
OGM	Organoid grown media

OR	Odd ratio
OS	Overall survival
PAMPS	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cells
PCNA	Proliferating cell nuclear antigen
PD-1	Programmed cell death protein 1
PDAC	Pancreatic ductal adenocarcinoma
pDC	Plasmacytoid dendritic cell
PDGF	Platelet derived growth factor
PDX	Patient derived mouse xenograft
PGE2	Prostaglandin E2
PPPD	Pylorus-sparing pancreatic-duodenectomy
PPV	Positive predictive value
PRR	Pattern-recognition receptor
PSC	Pancreatic stellates cell
PV	Portal vein
qPSC	Quiescence pancreatic stellate cell
RAG	Recombinase activating gene
RCT	Randomized control trial
RFS	Recurrence free survival
RLR	RIG-I-like receptors
ROS	Reactive oxygen species
Runx3	Runt-related transcription factor 3

SAA	Serum amyloid A
SHH	Sonic hedgehog
sLea	Sialyated Lewis A-blood group antigen
SMA	Superior mesenteric artery
SMV	Superior mesenteric vein
SNARE	Soluble N-ethylmaleimide sensitive factor (NSF) attachment protein receptor
SV	Splenic vein
TAM	Tumour associated macrophage
TCF-1	T cell factor 1
TCR	T cells receptor
TF	Transcriptional factor
TGF	Transforming growth factor
TIGIT	T cell immunoglobulin and ITIM domain
TIL	Tumour infiltrating lymphocyte
TIR	Toll-interleukin 1 receptor
TLR	Toll-like receptor
TME	Tumour microenvironment
TNF	Tumour Necrosis Factor
TRAIL	Tumour necrosis factor (TNF)-related apoptosis-inducing ligand
VEGF	Vascular endothelial growth factor
WCRF	World cancer research fund
Zeb2	Zinc-finger E homeobox-binding 2

Chapter 1. Introduction

1.1 Pancreatic duct adenocarcinoma (PDAC)

The pancreas is a retroperitoneal gland, located in the supra mesocolic compartment, behind the stomach and lying on the spine (L1-L2). It is an essential organ, and it serves two main functions: 1. endocrine function and control of blood sugar and 2. exocrine function and digestion of food. Diseases of the pancreas are common and include diabetes, pancreatitis and cancer.

Histologically, the pancreas comprises an acinar and ductal component, which together constitute the exocrine part of the gland. This is responsible for the production of digestive enzymes, such as trypsin, lipases and amylase among others. Pancreatic islets or *islets of Langerhans* represent the endocrine part, consisting of alpha, beta, delta and gamma cells. Glucagon and insulin, synthetized respectively by alpha and beta cells, are the key regulators of glucose homeostasis, whereas delta and gamma cells are involved in gastrointestinal motility and regulate digestion and absorption. The endocrine and the exocrine components of the pancreas are surrounded by connective tissue elements, vessels and nerves, which have a supporting function.(1, 2)

Most of the tumours affecting the pancreatic gland, arises from the exocrine part, and specifically from the ducts. In fact, pancreatic ductal adenocarcinoma (PDAC) represents almost 80% of all pancreatic malignant tumours.

1.1.1 Epidemiology and risks factors

PDAC is the 12th most common tumour in the world and the 7th most common cause of death for cancer. Worldwide, geographical variations in the incidence probably reflects exposure to different risk factors, but it could also depend on access to diagnosis in the developing countries.

Among Asian populations, the highest incidences of PDAC are observed in Armenia, Japan and Kazakhstan, where the age-standardized incidence rates (ASR) per 100,000 population are 11.9, 10.6 and 9.3 respectively in men, and 7.3, 6.7 and 5 respectively in women. On the other hand, ASRs are relatively low in the southern part of Asia, such as the State of Palestine, Bhutan and China. In China, the aged-standardized incidence and mortality rates for men are 4.5 and 4.3 per 100,000, and 2.8 and 2.7 for women.(3)

Worldwide, Europe has the second highest incidence of pancreatic cancer in terms of new cases, but the highest as age-standardized incidence rate (men: 9.3 per 100,000; women: 6.3 per 100,000). Men are more affected in Central and Eastern Europe (9.9 per 100,000), while the highest incidence among females is found in Western European countries (7.2 per 100,000), particularly in Hungary, which globally holds the highest incidence in women (9.1 per 100,000), followed by Slovakia, Czech Republic and Serbia.(4, 5) However, it is expected that by 2040, the incidence in Western European countries will reach Eastern and Central, either in terms of new cases or in mortalities.(6) Mortality overlaps the incidence rate, with the highest among men in the Central and Eastern countries (7.9 per 100,000), while the Westerns show the highest in the female population (6.3 per 100,000). Overall, the Russian Federation

holds the highest mortality from pancreatic cancer.(5) In Europe, the average 5-year survival is 6% in the male population and 8% in the female, with Croatia and Belgium showing the best survival in men (11%) and women (12%), respectively.(7)

In the United Kingdom (UK), PDAC is the 12th most common tumour in males and the 9th in females. Incidence has been steadily increasing since the early 1990s. Moreover, the incidence of PDAC has been projected to rise by 6% between 2014 and 2035. This will be larger for males rather than female population: for males, pancreatic cancer European age-standardised incidence rate in the UK is projected to rise from 15 to 23 cases per 100,000 (7%) by 2035, while in females, the rate is projected to rise from 15 to 18 cases per 100,000 (4%). This trend is probably influenced by the increasing prevalence of different risk factors, namely obesity, age and other yet to be defined causes.(8)

The mortality rate is very close to the incidence rate and thus, for most patients a diagnosis of PDAC is fatal. This is mostly due to a lack of specific symptoms and/or signs that prevents early detection, so most of the pancreatic tumours are diagnosed at a locally advanced and unresectable or metastatic stage. Unfortunately, characteristics of the tumour do lend themselves well to screening tests and currently there is no available test. There has been interest in the development of diagnostic decision tools to aid general practitioners in early diagnosis,(9, 10) but a lack of accuracy means they have no use in clinical practice. There are early works using biomarkers but this has not been tested in normal population.(11)

As with other cancers, PDAC has a multifactorial origin, which is the result of the interaction between environmental factors and genetic predispositions.

- **Age and gender.** Age is a major risk factor for pancreatic cancer, demonstrated by the fact that the incidence strongly increases in older people. The median age at diagnosis in United States is 71 years old in both sexes, while in the UK, almost half of the cases diagnosed from 2012 to 2014, were made in people older than 75 years. In the US, men are generally more affected by pancreatic cancer, and this does not differ according to age. In Europe and UK, the incidence is similar in both sexes, pancreatic cancer is expected to rise more in the male population rather than the female.(8, 12) This susceptibility observed in men could be related to differences in environmental exposure or endogenous hormones, or probably a combination of the two.
- **Nicotine.** Cigarette smoking has been associated with a 6-fold increased risk of developing PDAC.(13) Nicotine, the main chemical component of cigarette smoke, is adsorbed in the upper gastrointestinal tract and causes genetic mutations in pancreatic cells. It has also implied in chemo-resistance, tumour growth and metastasis, by influencing the interaction between tumour-associated stroma and pancreatic cancer cells through paracrine hepatic growth factor (HGF) signalling and/or through Src pathway.(13-15)
- **Obesity**. In 2015, the World Cancer Research Fund (WCRF) published a systematic review demonstrating a strong relation between obesity and PDAC.(16) In support of this, a recent meta-analysis and a pooled study report 10% and a 14% increase risk for each 5 kg/m^2 increment in BMI, respectively. In particular, the increasing incidence of high BMI in early childhood seems to be related to higher pancreatic risk and mortality.(17) The mechanism remains to be completely understood, but obesity may promote carcinogenesis at

different levels: adipocytes produce pro-inflammatory cytokines such as tumour necrosis factor (TNF)-alpha, interleukin (IL)-6 and C-reactive protein (CRP), inducing a chronic inflammatory state; hormones such as Insulin-like growth factor 1 (IGF-1), oestrogens, insulin and leptin are upregulated, making the environment favourable to tumour growth. In particular, the endocrine pancreatic gland tries to compensate insulin resistance by increasing insulin production. Hyperinsulinemia has been showed to be a risk factors for colon cancer, endometrium, pancreatic and renal cancer.(16)

- **Diabetes mellitus**. The association between diabetes mellitus (DM) and pancreatic cancer has been extensively analysed since 1833, when DM was first described. However, the presence of risk factors associated with both diabetes and pancreatic cancer, such as obesity and age, may confound the analyses. A recent pooled analysis from the Pancreatic Cancer Cohort Consortium demonstrated that a 2- to 8-year duration of DM has been associated with increased risk of developing the tumour (OR=1.79; 95% CI: 1.25-22.55),(18) while the association between the use of oral hypoglycaemic agents and the risk of pancreatic cancer seems controversial.(19-22)
- **Dietary habits**. Association between dietary habits and risk of PDAC shows conflicting results. High consumption of red or processed meat, fructose and dairy products have been proposed to be directly involved, but other studies have shown null correlations. Studies that tried to show a protective effect of coffee and tea consumption had the same contradicting results.(3, 16) The relation between 25-hydroxy-vitamin D [25(OH)D] and risk of pancreatic is also unclear.(23, 24)

- **Alcohol intake and chronic pancreatitis**. Alcohol consumption and related chronic pancreatitis are known risk factors for pancreatic cancer. Alcohol metabolites such as acetaldehyde and fatty acid ethyl esters (FAEEs) play a significant role in carcinogenesis: the chemical injury promotes a long-term inflammation in the gland, characterised by the release of cytokines, reactive oxygen species and cyclooxygenase 2, which induce genomic damage and cell proliferation, eventually leading to malignancy. This results in a 7.2-fold increased risk of developing the tumour.(25) The role of chronic pancreatitis as risk factor has been demonstrated by numerous studies. Among others, *Duell et al.* showed that a 2 years' history of chronic pancreatitis increases the risk of cancer by 3-fold (OR: 2.71; 95% CI: 1.96-3.74), and the risk is even greater in younger patients (<65 years) (OR: 3.91, 95% CI: 2.53-6.04) compared to older patients (OR: 1.68, 95% CI: 1.02-2.76; p=0.006).(26)
- **Genetic predisposition.** A familiar predisposition is present in approximately 10% of the cases. Among these, just 5-10% can be related to hereditary cancer syndromes or inherited forms of pancreatitis caused by high-risk mutations. Thus, in almost 90% of the families in which there is a history of pancreatic cancer, a genetic cause is not found.(27) Table 1.1 summarises the most frequent familiar cancer syndromes.

Table 1.1 Selected Genetic Syndromes with Associated Pancreatic Cancer Risk

Syndrome	Gene	Estimated Cumulative Risk	Estimated Increased Risk compared to general population
Peutz-Jeghers syndrome	STK11	11%-36% by age 65-70 years	132-fold
Familial atypical multiple mole and melanoma syndrome (FAMMM)	CDKN2A	14% by age 70 years 17% by age 75 years	20-fold to 47-fold
Hereditary nonpolyposis colorectal cancer	MLH1, MSH2, MSH6	4% by age 70 years	9-fold to 11-fold
Hereditary breast and ovarian cancer syndrome	BRCA1, BRCA2	1.4%-1.5% (women) and 2.1%-4.1% (men) by age 70	2.4-fold to 6-fold
Hereditary Pancreatitis	PRSS1, SPINK1, CFTR	40%-53% by age 70-75 years	26-fold to 87-fold
Familial pancreatic cancer	unknown	≥3 first-degree relatives with pancreatic cancer: 7-10% by age 70 years 2 first-degree relatives with pancreatic cancer: 3% by age 70 years	≥3 first-degree relatives with pancreatic cancer: 32-fold 2 first-degree relatives with pancreatic cancer: 6.4-fold 1 first-degree relatives with pancreatic cancer: 4.6-fold

Adapted from National Comprehensive Cancer Network. Pancreatic Adenocarcinoma, Version 1.2021. URL: https://www.nccn.org/professionals/physician_gls/pdf/pancreatic.pdf. Accessed Date: 23rd October 2020

1.1.2 Clinical aspects

Signs and symptoms of pancreatic cancer are related to the location of the disease within the gland. Approximately 60%-70% of PDAC arises from the head of the pancreas, while 20%-25% involves the body/tail and a remaining 5% involves the entire gland.(28) Therefore, in most cases the first manifestations are due to the obstructive effect of the mass on the biliary tree and on the drainage of the exocrine pancreas. Unfortunately, these occur late in the stage of the disease and early symptoms are usually very unspecific or more often, lack completely.

Symptoms suspicious of the presence of a compressive mass in the head of the pancreas are usually a sudden onset of painless obstructive jaundice, with dark urine, pale stools and pruritus, upper gastroduodenal obstruction and steatorrhea. These are often accompanied or anticipated by less specific symptoms such as weight loss, loss of appetite with nausea and vomiting, epigastric and abdominal pain, and back pain. Sudden onset of diabetes is also strongly suspicious of pancreatic cancer, especially in patients >55 years old with no risk factors for diabetes.

Tumours localised in the body/tail of the pancreas are usually diagnosed in more advanced stages than those located in the head of the pancreas, lacking the signs of the compressive effect on the bile and/or pancreatic duct.

1.1.2.1 Diagnosis and staging

Radiological studies are the gold standard in the diagnosis and staging of pancreatic neoplasia.(28, 29) All patients with suspected pancreatic disease should undergo a

pancreatic-protocol computed tomography (CT) scan. This is obtained by submillimetre axial sections, acquired during pancreatic and portal phase of contrast enhancement. In particular, the radiological report should describe the relation between the tumour and the arterial vessels (celiac axis, main hepatic artery and superior mesenteric artery [SMA]), and the venous vessels (portal vein [PV], superior mesenteric vein [SMV], and splenic vein [SV]). This improve the prediction of resectability, which has been demonstrated to be between 70% to 85%.(30-32)

Usually, normal pancreatic tissue enhances better than tumour tissue, therefore PDAC will often appear as a hypodense mass within the pancreas. More rarely, it can appear as a cystic or necrotic lesion. Other signs of malignancies are pancreatic duct dilation associated or not with bile duct dilation (double duct sign), pancreatic atrophy or deformity of the pancreatic burdens.

High-quality CT scan is able to detect >2cm pancreatic lesion with a sensitivity of almost 90%-100%, which is reduced to 60% for smaller lesions. However, despite technological improvements, the ability to detect small hepatic or peritoneal metastatic deposit is still poor.(30, 33) Ideally, timing between the last high-quality CT scan and surgery should not exceed the 4 weeks.(34)

Magnetic Resonance Image (MRI) has not demonstrated any superiority compared to CT scan in the diagnosis of pancreatic cancer.(35) Currently, its role is limited to suspicious pancreatic mass not visualised at CT scans, and to identify liver metastasis.(36, 37)

Tissue confirmation of PDAC is required before the administration of neoadjuvant chemotherapy or for palliative management, in case of locally advanced

disease not suitable for surgery, or metastatic disease, whereas it is not necessary before surgery in clearly resectable or borderline-resectable tumours.(38) **Fine-needle aspiration (FNA) biopsy** under **Endoscopic-Ultrasound (EUS)** guidance has been demonstrated to be the safest, most reliable and most cost-effect approach to obtain pre-treatment tissue, in comparison to percutaneous CT guided-FNA biopsy or endoscopic retrograde cholangiopancreatography (ERCP).(39, 40) EUS can access the entire length of the pancreas, and this characteristic makes it a powerful tool to identify lesions too small to be detected by CT scan: in a recent systemic review by *Krishna et al.*, EUS was demonstrated to be able to identify 20 mm lesions in 70% of indeterminate CT scan; moreover, EUS shows a sensitivity of >80% to detect lesions ≤10 mm, compared to 50% of positron emission tomography (PET).(41, 42)

NCCN guidelines emphasize the need of multidisciplinary consultation at a large volume centre to establish the most appropriate diagnostic management and subsequently the best therapeutic approach. They also define radiological the criteria to determine the extension of the disease, to match the best therapeutic approach. Once metastatic disease has been ruled out, PDAC is classified as resectable, borderline or unresectable, according to vascular involvement.(43)

The American Joint Committee on Cancer Staging System (AJCC) defines four stages of disease according to three variables: tumour size (T), regional lymph nodes involvement (N) and distal metastasis (M). The proper radiological staging of PDAC includes the evaluation of the local extent of the mass within the pancreas and the surrounding tissue, including the main vessels, the description of lymph nodes involvement and the presence of metastatic disease. This system provides useful

information on the short- and long-term prognosis, and it is the worldwide accepted system to generate survival data.(44)

1.1.2.2 Biomarkers: the role of Carbohydrate Antigen (CA) 19-9

CA 19-9 is a sialyated Lewis A-blood group antigen (sLea), first described in colorectal cancer and subsequently identified in other gastrointestinal tumours, as gastric, oesophageal and biliary, both in the tissue and in the serum (45). It represents an abnormal product of the disialyl Lewis-a protein due to the epigenetic silencing since early stages of the carcinogenesis, of the 1,4-fucosyl transferase gene, whose enzyme is responsible for the antigen epitope production. Normally, this protein is widely expresses on the gastrointestinal epithelium, where it plays an important role in cell-to-cell adhesion, being a ligand for E-selectin, but also in immune surveillance, as it is a ligand for monocytes and macrophages (46). Of notice, 7%-10% of the population is Lewis antigen-negative ($Le^{\alpha-\beta-}$ genotype), characterised by the loss of the 1,4-fucosyl transferase enzyme. This leads to the complete absence of CA 19-9.

Among all biomarkers, CA 19-9 is the gold standard in the management of PDAC, although with some limitations. In patients being evaluated for symptoms suspicious of pancreatic cancer, elevated CA 19-9 serum levels have a positive predictive value (PPV) of just 0.5%-0.9%, which is similar to the PPV tested in the asymptomatic population. Of notice, an increased level of CA 19-9 has also been noted in a significant number of patients affected by other tumours involving the hepatobiliary system.(46) Due to its low sensitivity and poor PPV, CA 19-9 is considered to have no role as a screening tool for pancreatic cancer in the general population.

For these reasons, several molecules have been proposed as alternative of CA 19-9 or in conjunction with it. Briefly, carcinoembryonic antigen (CEA) and carbohydrate antigen 125 (CA 125) in combination with CA 19-9 can predict the outcome of patients undergoing surgery and chemotherapy,(47, 48) while serum periostin (POSTN) and CA 242, could improve the diagnostic power of CA 19-9. (49) Another study shows that the serum concentration of CA 50, 199 and 242 could distinguish between benign and malignant pancreatic tumours, (50) while MIC-1, MIA and serum glycoproteins are able to detect pancreatic cancer at early stages and also differentiate between benign and malignant conditions. (51, 52)

Lately, several circulating miRNA molecules have been also investigated for their ability to detect pancreatic tumours. In particular, miRNA-21-5p, one of the most aberrantly expressed in pancreatic tumours has showed a high accuracy in the diagnosis, with a sensitivity of 77% and specificity of 80%.(53) Preliminary in vitro results have also showed that fibrosis-related miRNAs such as let-7d, probably expressed by pancreatic stellates cells (PSCs), could become a useful serum biomarkers. (54) Although all these new molecules show potentials in the management of PDAC, they have not been validated in proper large clinical trial, and thus they cannot be introduced in clinical use.

1.1.2.3 Management of resectable pancreatic disease

Only 20% of the pancreatic cancer are found to be resectable according to NCCN criteria, and for this fortunate small percentage of patients, surgical resection followed by adjuvant chemotherapy is the standard treatment.(55)

Most of the PDAC are localised in the head of the pancreas or in the uncinate process, therefore a classic Whipple's procedure or a pylorus-sparing pancreatic-duodenectomy (PPPD) are the most frequent operations performed. Comparing to the PPPD, the Whipple's procedure tends to be more aggressive, as it includes the resection of the third distal part of the stomach and the pylorus. However, a recent meta-analysis of randomized control trials and a propensity score-matched analysis comparing the two procures, have shown that PPPD presents a greater risk to develop delayed gastric emptying after the procedure.(56-58)

Tumours localised in the neck/body/tail of the pancreas would benefit of a distal pancreatectomy, with or without spleen resection.

1.1.2.4 Management of borderline resectable disease

While upfront surgery for resectable disease is universally recommended, the management of borderline resectable pancreatic cancer (BR-PDAC) is still under debate. According to the consensus guidelines from the International Study Group on Pancreatic Surgery (ISGPS), a borderline tumour involving venous vessels or adjacent organs do not benefit from neoadjuvant treatment, if the surgical resection is feasible and an R0 can be achieved. On the other hand, in case of arterial involvement, ISGP

always recommends neoadjuvant treatments, either for BR or unresectable disease Arterial resections are not routinely recommended, but under exceptional cases it can be offered, if within experimental protocols.(59)

Traditionally, chemoradiation schemes, based on gemcitabine or 5-FU protocols along with 50-54 Gy of radiation, had been administered, but the secondary resection rates did not reach 30%.(60, 61) In recent years, the introduction of FOLFIRINOX (FOL=folinic acid (leucovorin), F=fluorouracil (5-FU), IRIN=irinotecan, OX=oxaliplatin) scheme and its modification as neoadjuvant therapy for BR-PDAC, has demonstrated to be highly effective, with a resection rate variably improved to 50%-80%.(62-64) Unfortunately, so far there are no randomized control trials (RCTs) comparing the two approaches, but several studies definitely indicate FOLFIRINOX as the best therapeutic option(62, 64, 65). On the other hand, a recent multicentric phase III RCT (PREOPANC trial), comparing preoperative chemoradiation vs. surgery followed by adjuvant gemcitabine in resectable and borderline resectable patients, has demonstrated a better OS and DFS, as well as an improved R0 rate, in those patients diagnosed with a borderline disease, who received the neoadjuvant treatment.(66)

The advantages of neoadjuvant chemotherapies could be related to a secondary patient selection according to tumour biology: clearly, a progressive disease under neoadjuvant treatment reflects an aggressive tumour, which would have not benefited from surgery since the presentation. Also, neoadjuvant treatment could help avoiding arterial resections, which, despite being sometimes technically feasible, still hold a high rate of morbidity and mortality.(67) Moreover, the oncological benefit of arterial resection is not clear, as these patients experience earlier local recurrence or metastatic disease.(68, 69)

Despite technological progress, the accuracy of high-resolution CT scan is still limited in the neoadjuvant setting, not being able to discriminate between proper tumour involvement of the artery and tissue fibrosis.(70)

1.1.2.5 Adjuvant therapy

Adjuvant chemotherapy is standard treatment for PDAC, as the disease holds a high rate of local and metastatic recurrence, despite an R0 been achieved.

Treatment schemes vary according to tumour stage and biology and patient performance status, so no definite standard treatment has been established.
Available options include gemcitabine or 5-FU, alone or in combination with leucovorin or capecitabine. In particular, the results of ESPAC-4 phase III randomized trial showed that gemcitabine in combination with capecitabine significantly improved median survival, compared to gemcitabine monotherapy (28 months vs 25.5 months respectively: HR, 0.82; 95% CI, 0.68-0.98; p=0.032).(71)

Recently, the phase III PRODIGE24/CCTG PA.6 trial has compared gemcitabine monotherapy versus FOLFIRINOX, as adjuvant therapy in patients with good performance status. After a median follow up of 30.5 months, the OS in the FOLFIRINOX arm was 54.4 months, compared to the 35 months of gemcitabine (HR=0.66; 95% CI, 0.49-0.89). FOLFIRINOX was superior also in preventing recurrence, being the DFS 21.6 months, compared to 12.8 months (HR 0.59; 95% CI, 0.47-0.74).(72)

Chemoradiation could also be an option, but to date no studies have clearly demonstrate superiority compared to chemotherapy alone.

Currently, there is no worldwide agreement on the post-operative follow up of PDAC patients. The guidelines approved by European Society for Medical Oncology (ESMO) focus on symptoms, nutrition and psychological support,(28) while the American NCCN guidelines recommend a more aggressive approach, with serial CA 19-9 measurement and TAC-CT scan every 3 to 6 months, for the first 2 years after resection. The aim of the American protocol is to recognise early recurrence and thus be able to start salvage therapies.(43)

1.2 The Immune system

The Immune System (IS) is a complex organisation of different cells and soluble molecules, which has co-evolved with microbes since the appearance of multicellular organism on Earth, about 750 million years ago. The primary role of the immune system is to defend the host not only from bacterial, viral or fungal infection, but also from chemical and traumatic injuries. In vertebrates, the IS can be differentiated into two main branches, the **Innate Immune System (IIS)** and the **Adaptive Immune System (AIS)**.

Phylogenetically, the IIS appeared approximately together with the first multicellular organisms and evolved in conjunction. In fact, some components of the IIS are widely conserved in mammals, insects and plants (for example the Toll-like Receptors and their intracellular domains, the Toll-Interleukin Receptors), suggesting that they must have first appeared in a common progenitor. It represents the first barrier against pathogens, and it is so efficient that most organisms were able to survive evolution through it alone. (73) The main components of the IIS are:

1. The epithelial barriers: skin and mucosal surfaces.
2. Humoral effectors, such as the complement system, antimicrobial peptides (AMPs) or acute phase proteins (APPs)
3. Cellular effectors:
    a. Myeloids cells, such as *mononuclear phagocytes* (macrophages and dendritic cells), and *polymorphonucleates* (neutrophils, basophils and eosinophils)

b. Innate lymphoid cells (ILC), subdivided in three main groups according to the cytokines they produce. NK cells are a subclass of ILCs.

The Innate Immune System response depends on the recognition of specific microbial molecules by superficial cell receptors. One of the best characterised of these molecules is the endotoxin lipopolysaccharide (LPS), first reported by Pfeiffer in 1892. (73, 74) The disseminated response to LPS is the main responsible of the septic shock, which is characterised by fever, hypotension and metabolic acidosis, and eventually leads to multiorgan failure. At the same time, its presence is necessary for the recognition of some Gram-negative bacteria, at least at an early stage. (73)

The AIS emerged late, approximately 350-500 million years ago, in jaw fishes (i.e., sharks), and represents a furthermore specific protection. Moreover, it is characterised by “memory”, that is the ability to mount a more vigorous response at each exposure to the same pathogen. The foreign molecules able to trigger an adaptive immune response are called **antigens**, which are recognised by the two main cellular components of the AIS: T lymphocytes (T cells) and B lymphocytes (B cells), and the latter’s humoral products, the **antibodies**.

The two systems, the IIS and the AIS, work in synergy. Very briefly, cells from the IIS, the so-called Antigen-Presenting Cells (APCs), among which the most important are the dendritic cells, process and present the antigen to the cellular components of the AIS and activate them. This leads to the production of antibodies by the B cells, which recognise extracellular microbes, and the elimination of the

infected cells by the T cells. Conversely, the AIS secretes chemokines and cytokines which stimulate and enhances the IIS, making it more efficient.

The appearance of AIS and its merge with the ISS is one the greatest mysteries in biology.

1.2.1 The Innate Immune System: an overview

The IIS comprises the first weapons that the host puts in place to defend itself against pathogens. In this perspective, the integrity of the skin and the mucosae of the gastrointestinal, respiratory and urogenital tracts is an essential part of the defence. Normal epithelial architecture is guaranteed by appropriate cell-cell interactions and cell-ECM (Extra Cellular Matrix) interaction. Either the skin or the mucosal epithelia constantly secrete a viscous fluid, the *mucus*, enriched in glycoproteins and/or peptides which have a direct toxic effect on pathogens, such as lysozyme.

During the first phases of inflammation, several proteins important to boost host defence are synthetized, mainly by the liver. APPs are differentiated in positive or negative, according to the trend of their concentration in the plasma.

Albumin is the main negative APP: its concentration decreases during inflammation, either for loss due to gastrointestinal or renal damage, or for a reduce hepatic synthesis, in favour of other positive APPs or pro-inflammatory proteins.

C-reactive protein (CRP) and **serum amyloid A** (SAA) are the most important APPs in humans. CRP is secreted by the liver under the stimulation of the cytokines IL-1 and IL-6. In normal condition, CRP concentration in plasma is very low, but can increase up to 1000-fold during inflammation. SAA is a pentraxin protein, able to regulate the inflammatory response and to attract leucocytes to the site of the inflammation. (75-77)

1.2.1.1 Toll-like Receptors

In the early days of immunology, the IIS was thought to unspecifically recognise microbes, compared to the more sophisticated AIS. This concept was overcome with the discovery of germ-line encoded pattern-recognition receptors (PRRs) on the surface of IIS cells, whose Toll-like receptors (TLRs) are the prototype.

Toll is a protein involved in the developing of dorsoventral polarity of *Drosophila melanogaster's* embryos. In the early 1990s, the cytosolic domain of this protein was found to be homologous to the cytosolic domain of IL-1R type 1 (IL-1R1), and both proteins showed to work through nuclear-factor-κB (NF-κB) signalling. After the discovery of TRLs, different types of PRRs had being identified on many cells, including non-immunological cells, for example RIG-I-like receptors (RLRs) and Nod-like receptors (NLRs).(78)

TLRs are type 1 transmembrane proteins: the extracellular domain is rich in leucine and mediates the recognition of pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs); the transmembrane domain consists of a single α-helix sequence; the intracellular domain, named Toll-interleukin 1 (IL-1) receptor (TIR), transmits the signal transduction. So far, 10 TRLs have been identified in humans and 12 in mice, the first 9 being conserved in both species. TRL10 is not functional because of retrovirus insertion, but its sequence is similar to TRL1.(79, 80)

TRLs recognise different types of PAMPs from bacteria, viruses, parasites and fungi, and are located in different cellular comparts, such as the cellular membrane (TRL1-2-4-5-6), or intracellularly in the endosome or lysosome (TRL 3-7-8-9).(81) The

localisation affects the type of PAMPs recognised: the first group is able to detect microbial membrane components, such as lipids and lipoproteins, the second group detects nucleic acids.(78) TRLs are able to create heterodimers, expanding in these way the pools of molecules that can be detected. For example, TRL2 heterodimerizes with TRL1 to sense triacylated lipopeptides, and with TRL6 to sense diacylated lipopetides, while TRL2 alone recognises non-lipopeptides from different pathogens.(80) Importantly, TRLs are known to recognise a wide range of endogenous proteins: degradation products of extracellular matrix, heat-shock proteins, chromatin-DNA and RNA complexes and high-mobility group box 1 (HMGB1).(82)

1.2.1.2 Cell-mediated innate immunity

Myeloid cells are a heterogeneous group of cells which originate in the bone marrow, from a multipotent haematopoietic stem cells (HSC).They are the most represented leukocytes in the peripheral blood.(83) Their ultimate differentiation leads to two main subtypes of myeloid cells: monocytes, from which macrophages and dendritic cells originate, and granulocytes (neutrophils, basophils and eosinophils). Myeloid cells are involved in host's protection through several mechanisms: direct phagocytosis of pathogens, elimination of dying cells, and tissue remodelling. Importantly, they represent the link between innate and adaptive immune system, being able to elaborate and present the antigen that will ignite the T- and B-cells response.

NK cells are a class of innate lymphoid cells, which exhibit a strong antigen-independent cytotoxic activity. For this reason, they are one of the first cell line

recruited in case of injury, cellular transformation or infection.(84) NK cells originate from the bone marrow and are a relatively rare population in the peripheral blood, accounting for 5-15% of the whole leukocytic population.(85) They are the main object of this thesis; therefore their development, activity and functionality will be thoroughly described in the next chapters.

Macrophages derive from circulating monocytes, undergone their ultimate differentiation in the tissue they are recruited by. For this reason, they are not a uniform population, but a broad spectrum of cells whose phenotype reflects the microenvironment in which they have developed: Küpffer cells in the hepatic sinusoids, stellate microglial cells in the central nervous system, alveolar macrophages in the lungs, osteoclasts in the bone and mesangial macrophages in the kidneys.(73, 83, 86) Functionally, they can be differentiated in two groups, with opposite roles: the "classically activated" macrophages, or M1, which promote inflammation under INFγ stimulation, secrete high level of IL-12, and have a strong killing effect on infected and tumoral cells; and the "alternatively activated" macrophages, or M2, which have an anti-inflammatory effect, express scavenger receptors, produce high level of IL-10 and low IL-12 and promote tissue repair. *In vivo*, M1 and M2 represent the two extremes of a grey scale of phenotypically different macrophages.(87)

In the bone marrow, monocytes differentiate into **dendritic cells** (DCs), which exist in two phenotypes, conventional DCs (cDCs, or mDCs, myeloid DCs) and plasmacytoid DCs (pDCs), with different functions and location.

cDCs represent the largest population of dendritic cells and can be considered the most important connection between innate and adaptive immune system. They are present in peripheral tissue as “migratory DCs” (Langerhans cells or interstitial DCs) or in lymph nodes, as “lymphoid DCs”. In absence of stimuli, both populations are kept in an immature, steady states. Antigens such as PAMPs or DAMPs are recognised by TLRs expressed on quiescent cDCs; they are therefore phagocyted and this starts the maturation process of cDCs, which leads to the expression of MHC molecules, downregulation of antigen-capture activity, and expression of co-stimulatory molecules, such as CD80 and CD86. Then, mature cDCs loaded with the antigen, are able to migrate to lymph nodes, where ultimately, they will present the peptide to naïve T-cell. Antigens can also be recognised and presented to T cell directly in the lymph nodes, by lymphoid cDCs. (88, 89)

pDCs are circulating cells, which under inflammatory stimuli migrate to lymph nodes, particularly in T-cell enriched area, the spleen and mucosal-associated lymphoid tissues. (76, 90) They play a key role in viral infection, producing a large amount of INFγ and IL-10.(91) Differently from cDCs, pDCs are less efficient APCs: firstly, they express low level of MHC-II molecules and costimulatory molecules; secondly, they lack in two lysosomal proteases, involved in antigen processing. (90, 92) For this reason, recently it has been speculated that their main role could be “modulating” the immune response, by releasing cytokines and chemokines.

Granulocytes are myeloid cells characterised by specific nuclear morphology and granules in the cytoplasm. **Neutrophils** are the largest granulocytic population

and the first cells recruited at the site of infection or damage. It is estimated that in adults, 10^{11} neutrophils are produced every day.(93) Once released in the blood stream, they hold a very short half-life, around 24 hours, even though a recent study has calculated a life-span of almost 6 days.(94) Despite being the largest population in the blood, neutrophils are rare in peripheral tissues, where they exist at a steady state, in particular skin, lung and gastrointestinal epithelia, but not in the brain or the gonads.

In cancer patients, circulating neutrophils have a dual role. N1 or high-density neutrophils (HDNs), have a strong anti-tumour effect: they release pro-inflammatory cytokines, such as IL-12, TNFα, CCL3, CXCL9 and CXCL10, which are recruiter for cytotoxic $CD8^+$ T cells. Conversely, N2 or low-density neutrophils (LDNs) promote tumour development and metastases progression, producing various pro-cancerous molecules: vascular endothelial growth factor (VEGF), which promotes neo-angiogenesis and metastases; metalloproteases (MMP), in particular MMP-9, which promote tumour progression by remodelling the ECM; hepatocyte growth factor (HGF), which promotes tumour progression in lung adenocarcinoma.(95)

- At later stage of tumorigenesis, cancer is able to switch N1 anti-tumoral neutrophils to N2, probably through transforming growth factor TGF-β signal.(96) This could explain why at early stage of cancer development, neutrophils depletion has detrimental effect on prognosis, while at later stage is protective.

1.2.2 The adaptive immune system: an overview

Compared to the innate immunity, the adaptive immune system presents several important differences:

1. While the innate immune response is fully functional without previous exposures to antigens and can mount a vigorous defence in hours, the adaptive response needs several days before initiating a response to first exposure. This because lymphocytes clones need to be expanded and differentiated in functional cells.
2. Repeated exposures to the same antigen allow the AIS to respond more rapidly and efficiently. It means that the AIS keeps memory of the antigens it came in contact with. In the IIS, there is little or no change in the quality of the immune response.
3. The AIS can potentially recognise millions of different molecules from pathogens or from injured/dead cells, including the self, due to the somatic genetic rearrangement that takes place during T-cells selection. In contrast, it is estimated that the IIS can recognise approximately 1000 different peptides.

T and B cells represent the two cellular compartments of the AIS, and they originate from lymphoid organs. The antibodies produced by B cells represent the humoral components.

Lymphoid organs are present in all vertebrates and are classified as primary (the bone marrow and the thymus) or secondary (the spleen and the lymph nodes), according to their function.

T and B-cells are released by the bone marrow as pro-T and pro-B lymphocytes. Then, pro-T-cells migrate to the thymus where they undergone their final selection and maturation, while B-cells migrate to the lymph node or/and the spleen for final differentiation. Both mature T- and B-cells are activated in secondary lymphoid organs, either by direct contact with pathogens' peptides or by the interaction with APC from the IIS. Once activated, they are able to travel to the target tissue and exert their function.(97)

1.2.2.1 T-cells receptor and the major histocompatibility complex (MHC)

T-cells receptor (TCR) is a heterodimeric protein expressed on the cell surface of T-cells, which has evolved to recognise antigens bound to major histocompatibility complex (MHC) class I or II. 90-95% of T-cells are $\alpha\beta$-TCR, which means that TCR is composed by two transmembrane polypeptide chains (α and β chains), covalently linked to each other by a disulphide bridge between extracellular cysteine residues. 5-10% of the T-cells present a different conformation of TCR, named $\gamma\delta$-TCR, generated by extrathymic compartment and primarily localised in the gastrointestinal tract.(76, 98) A small subset of $\alpha\beta$-TCR can be $CD4^-/CD8^-$ (double negative) and are defined as NKT cells. They recognise antigens presented on CD1d molecules, instead of MHC,

and seem to have a regulatory role, secreting a wide range of cytokines as INFγ, IL-4 and TNF, among others.(99)

The high variability of TCR receptor is due to the somatic genetic rearrangement that occurs during T-cells maturation in the thymus. 3 germline genes encode for TCR, named V (variable), D (diversity) and J (joining). α chains are encoded by V and J, while β chains contain also D segments. The enzymes responsible for the V(D)J rearrangement are recombinases composed by two proteins, encoded by the gene recombinase-activating genes 1 and 2 (*RAG1* and *RAG2*).(100, 101)

αβ-TCR combined with CD3 on the cell surface of T-cells in a complex able to recognise antigens presented by MHC class I or II.

MHC molecules are surface glycoproteins that bind peptides originated from the cleavage of endogenous proteins (MHC class I) or from exogenous proteins, phagocytised by APCs (MHC class II). This determines the subclass of T-cells that can recognise the antigen and therefore the type of adaptive immune response: cytotoxic T-cells expressing $CD8^+$ match with MHC class I, while T helper $CD4^+$ match with MHC class II.

MHC class I, also defined as HLA class I (HLA-A, HLA-B, HLA-C), are three extremely polymorphic molecules, expressed in almost all the nucleated cells. They are responsible for self-antigens tolerance, for the defence against viral infection and tumour recognition. They are composed by a heavy chain (α chain) bound to non-polymorphic protein, named β_2-macroglobulin.(102)

MHC class II (HLA-DR, HLA-DQ, HLA-DP) consist of two polymorphic α and β chains, constitutively expressed on B-cells, dendritic cells, monocytes and

macrophages. They can also be induced on other cells under INFγ stimulation, allowing them to present antigens and behave as APCs.(102)

1.2.2.2 T Lymphocytes maturation and differentiation

T-cells develop in the bone marrow from a haematopoietic stem cell (HSC) in common with B-cells. Under the influence of cytokine-driven signals, the most important of which is IL-7, this common progenitor is induced to differentiate towards the T lineage and undergo proliferation and expansion, before being released in the blood.(97) The immature pro-T-cells migrates to the thymus for the final selection and maturation. At this stage, these cells do not express any TCRs, and are $CD4^-CD8^-$ (double negative).

In the thymic cortex, pro-T-cells, now defined as thymocytes, undergo proliferation, gene rearrangement, leading to the expression of a functional $\alpha\beta$TCR or $\gamma\delta$TCR and subsequentially assembly with CD3, and expression of both CD4 and CD8 markers (double positive). Before leaving the cortex for the medulla, double positive thymocytes are positively selected for self-MHC molecules, retaining those cells which show low affinity for self-MHC/peptides complexes. This process implies the interaction with the specialised thymic epithelium. In the medulla, cells are negatively screened for autoreactivity: those cells binding with high affinity to self-MHC/peptide complex are not permitted to mature (central tolerance). Finally, the double positive pro-T-cells will lose one of the CD4/CD8 marker according to the type of MHC molecules they recognise: cells matching with MHC class I will express only CD8, and cells matching with CD4 will express only CD4 (single positive).(103, 104) Eventually, less than 5%of the pro-T-cells will complete the whole maturation process.(102)

In the blood and in secondary lymphoid organs, αβ-TCR $CD4^+CD8^-$ (T helper or T_H) are the largest majority of T-cells, while cytotoxic αβ-TCR $CD4^-CD8^+$ represent 30%-40%.(102)

From a naïve T_H cell (**T_H0**), several functionally different cells can originate, depending on the type of cytokine stimulation they receive. The first two groups of T_H identified were defined as T_H1 and T_H2.(105)

T_H0 differentiates into **T_H1** under the stimulation of IL-12 secreted by activated APCs, and INFγ secreted by NK cells. These cells play a pivotal role in the elimination of intracellular pathogens, promoting the proliferation of $CD8^+$T-cells, via IL-2 secretion.(106, 107) Also, T_H1 secrete INFγ in turn, enhancing the phagocytic activity of mononuclear phagocytes and NK cells cytotoxic activity.(108)

The cytokines IL-4 and IL-2 promote the differentiation towards **T_H2.** The role of T_H2 is against extracellular microbes, including helminths, and in promoting of antibodies secretion. Importantly, they are a source of the anti-inflammatory cytokine IL-10, which inhibits T_H1 and innate immune cells. (97, 109)

T_H17 differentiate in response to IL-6, IL-21, IL-23 and TGF-β. They are the main source of the potent pro-inflammatory cytokines IL-17F and IL-22, leading to a massive inflammatory response in the tissue, including recruitment of granulocytes, B-cells, memory cells and NK cells. They are thought to be one of the main characters in autoimmune disease, transplant reactions, and allergies.(110)

T_{reg} are potent immune-suppressive T-cells that play a central role in the maintenance of dominant self-tolerance, as well as the foetal-maternal tolerance during pregnancy.(111) There are characterised by the surface expression of CD4 and

CD25 and by the expression in the nucleus of the forkhead box protein 3 (Foxp3). These cells secrete the immune-suppressive cytokines IL-10 and TGF-β, but can also act directly through cellular contact.(112) T_{reg} exert their suppressive effect on many immune cells, including APCs, B-cells, $CD4^+$ and $CD8^+$ T-cells, NK cells and NKT cells.(111)

1.2.2.3 B lymphocytes

B-cells are the 15% of the circulating leukocytes in humans, and are defined by their production of immunoglobulin.(102) B-cells develop in the bone marrow upon IL-7 stimulation from surrounding stromal cells. During their development, they acquire antigen specificity, and their antigen receptor is assembled in a manner very similar to TCR.(97) They are then secreted as naïve cells in the peripheral blood and complete their development in the secondary lymphoid organs. Several subpopulations of B-cells have been identified, according to the expression of different surface markers.

B-cells can be activated by the antigens in two ways. Polymorphic antigens, such as LPS, can directly activate B-cells (T-cell independent activation), probably because they cross-link multiple immunoglobulin receptors on the cell surface. This phenomenon is clearly demonstrated in murine models and has been demonstrated in humans as well.(97) Unfortunately, this pathway creates only a weak memory, making difficult to create vaccines against polysaccharides molecules.(102)

During the T-cell dependant activation, $CD4^+$T- and B-cells interact in order to reciprocally enhance the response to the antigen. B-cells can act as APCs, which means that they are able to internalise the antigens, process it and mount it on an MHC

class II molecule, to finally present it to T-cells. This process leads also to the surface expression on B-cells of important co-stimulators, such as CD80 and CD86. This complex is recognised by $CD4^+$T-cells, which are activated, and in turn induce the final maturation of B-cells in plasma cells, through the interaction of CD40 to CD40L on T-cells. The pattern of cytokines secreted by activated $CD4^+$T-cells defines the type of immunoglobulins that will be produced: IL-10 causes switching from IgG1 to IgG3, IL-13 induces IgE, TGF-β induces IgA and INFγ induces IgG2.(102, 113)

B-cells memory is characterised by isotype switching, through DNA rearrangement and somatic mutations in the antigen-biding region of heavy and light chains. A memory response to antigens is characterised by the rapid production of high affinity IgG, IgA and IgE, and is fundamental for the success of vaccines strategies.(114)

1.3 NK cells

NK cells represent a class of innate lymphoid cells (ILCs) actively involved in recognizing and killing infected and malignant cells.

ILCs develop in the bone marrow (BM) from a common lymphoid progenitor (CLP), which gives origin also to T and B-cell. The precursors then migrate into the infected or injured tissues, and finalise their maturation into three different classes, defined according to cytokines production:(115)

1. ILC1: INFγ and TNFα.
2. ILC2: IL-4, IL-5, IL-13
3. ILC3: IL-17, IL-22 and GM-CSF
4. NK cells: granzymes, perforin and INFγ

Functionally, ILCs 1-2-3 can be considered the innate counterpart of $CD4^+$ T-cells T_H1, T_H2 and T_H17 respectively, while NK cells share functional and phenotypic characteristics with cytotoxic $CD8^+$ T-cells.(116) Also, some Authors have speculated that ILCs could be evolutionary precursors of T-cells, as transcription factors such as T-bet, Gata3 and RORγT are highly preserved in both ILCs and T-cells. Despite these similarities, ILCs have not been isolated from invertebrates or jawless fishes.(117) As part of the cellular components of IIS, ILCs differ from T-cells as they do not express acquired antigen receptors or undergo clonal differentiation and expansion once activated.

1.3.1 Origin and differentiation

The complexity and plasticity of early phases of haematopoiesis in humans have made difficult to define a linear-restricted NK cells progenitor, at least in humans.(118) Therefore, NK cells development has been better defined in murine models.

An important study from *Galy et al.* identified a subpopulation of HSC in the BM, defined by the co-expression of CD34, CD45RA and CD10, that could give rise to pro-T cells, pro-B cells, NK cells and DCs, under particular cultural conditions.(119). HSC are typically located in particular “nice” of the BM, located in proximity of the sinusoid vessels, and in contact with the surrounding stromal cells, which support the development through the release of pivotal cytokines such as IL-7 and IL-15.(120) Subsequently, each step of NK cells development has been classified according to the set of markers expressed on the surface of the cell. In this way, three stages, corresponding to three different phenotype, have been identified: NK cells precursors (NKP), immature NK cells (iNK), and mature NK cells (mNK).(120, 121) The progression from one step to the next one is a continuum, with often an overlapping of markers’ expression. However, this distinction has a practical usage when planning a gating strategy with flowcytometry. Figure 1-1 summarises all the markers expressed at each stage, in mice.

Briefly, the passage from common lymphoid progenitor (CLP) to a NK progenitor is typically marked by the expression of CD122, the receptor for IL-2.(122) The stages A, B and C that characterised the immature phase, are typically marked by the expression of NKG2D, CD161 and NKp46, respectively. CD161, also named Killer cell lectin-like receptor subfamily B member 1 (NK1.1 or KLRB1) is a type 2 membrane

protein expressed also by NKT-cells, important for the cytolytic function. NKG2D and NKp46 will be discussed in 1.3.4. The expression of the integrins CD51 and CD49b indicates the enter into the mature stages D and E. The maturation is then completed once in stage F, mNKs express the leukosialin CD43, CD11b and the Killer cell Lectin-like Receptor G1 (KLRG1), which enables the migration to secondary lymphoid organs.(120)

The cytokine profile secretion differs according to the stages: while as NKPs, NK cells secret GM-CSF, IL-5 and IL-13, as iNKs they produce IL-22, CXCL8 and TNFα. The ability to produce INFγ is acquired later, during the passage between immature and mature NK cells.(123, 124)

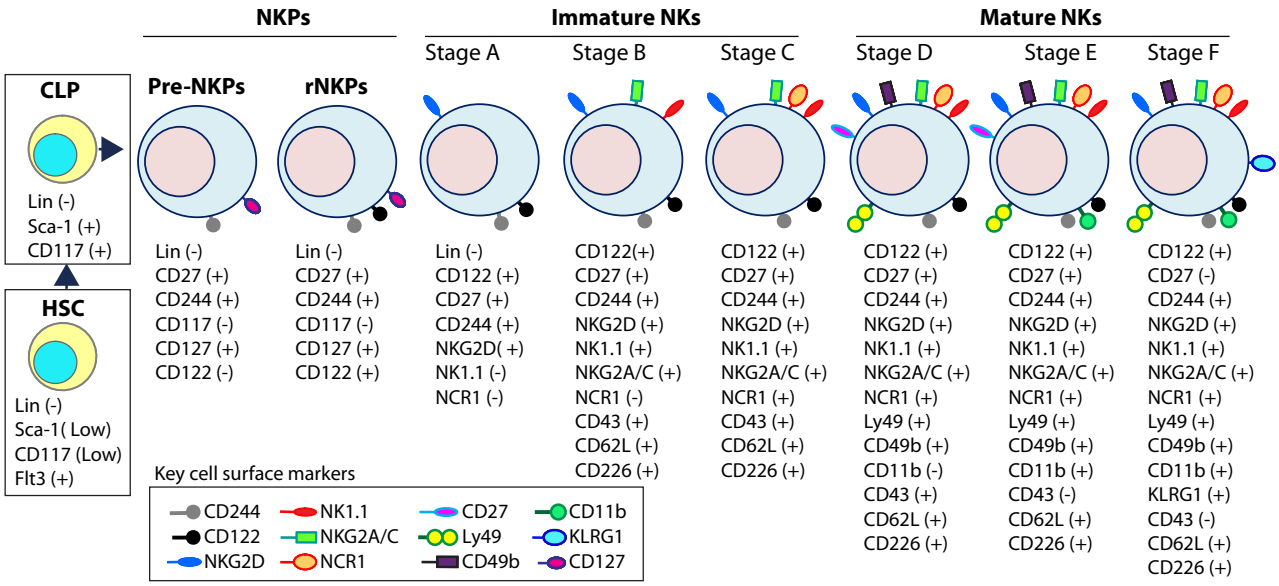


Figure 1-1.1 Murine model of expression markers in different developmental stages.

Haematopoietic stem cells (HSCs), defined as Lineage neg (Lin^-) Sca^+CD117^+, differentiate into common lymphoid progenitors (CLPs), $Lin^-Sca^{low}CD117^{low}Flt3^+$. The expression of CD127 (IL-7Rα), CD27 and CD244 indicates the commitment into pre-NK cell precursors (Pre-NKPs), which progress into refined-NKPs acquiring the expression of CD122 (IL-2Rβ). Immature NK cells (iNKs) are marked by the expression of NKG2D, followed by NK1.1 (stage A), NKG2A/C (stage B) and NCR1 (stage C). Mature NK cells are characterised by the expression of Ly49 (stage D), CD27/CD11b (stage E), and finally KLRG1 (stage F).

Adapted from Abel et al. (120) Open access.

More recently, a 5 stages theory has been developed to clarify NK ontology in humans (118, 120): in *stage 1*, $CD34^+$ progenitors acquire CD45RA and CD10; in *stage 2*, CD10 is lost and CD117 (c-kit) acquired; in *stage 3*, progenitors are now restricted towards the NK lineage, under IL-15 and microenvironment stimulation, CD34 is downregulated and LFA-1 is expressed; *stage 4* is then characterised by the expression of CD94 and CD56 ($CD56^{bright}$ NK cells). An ultimate differentiation (*stage 5*) will be marked by downregulation of CD94 and CD56 and the expression of CD16 and KIRs. These cells will represent the $CD56^{dim}$ pool.(125) Figure 1.1-2 summarises the known markers expressed during NK development in humans.

This model has been recently challenged by data showing that NK cells can also be generated from CMPs and granulocytic-monocytic precursors (GMPs) isolated from cord blood.(126, 127) These findings are supported by the evidence that NK cells can display functions in common with DCs, like MHC class II upregulation and antigen-presentation. Vice versa, DCs can exert cytotoxic activity, as NK cells.(118) Interestingly, some organ-specific NK cells subpopulation can also derive from differentiation of resident multilineage progenitors, under the influence of local stromal cells.(126, 128)

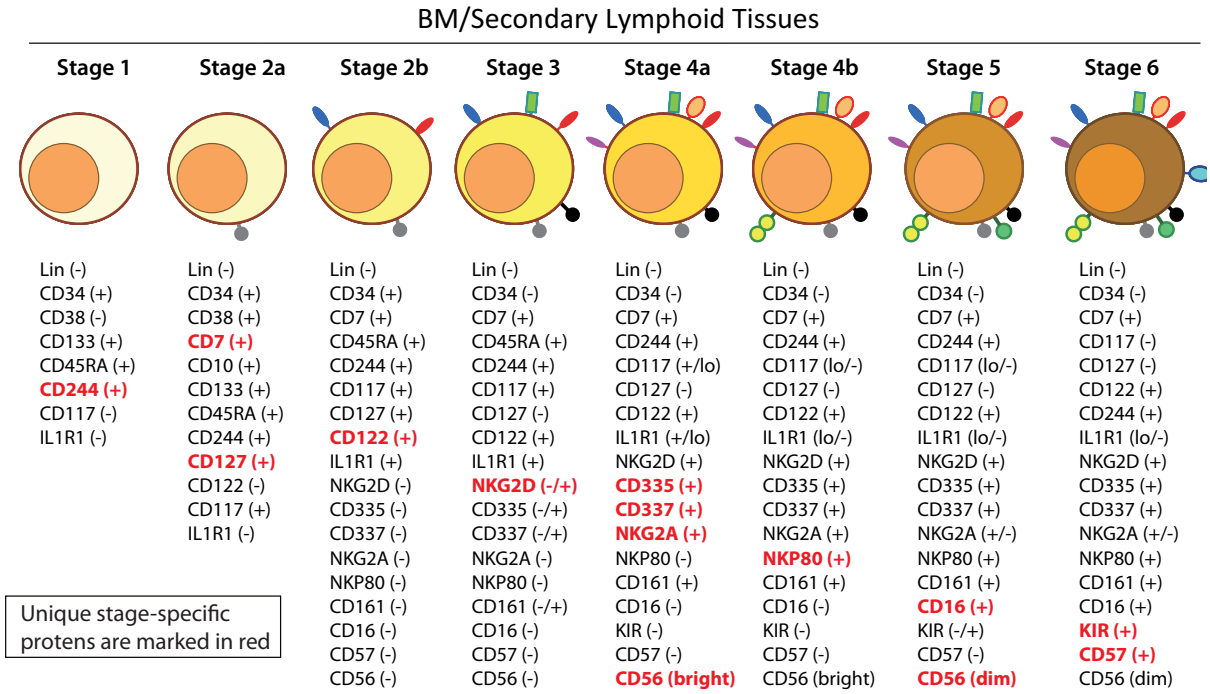


Figure 1.1-2 Human model of expression markers in the bone marrow and lymph nodes.

Six stages have been identified in humans. Stage 1 are pre-NK cell precursors and express CD244. Stage 2a and 2b include NK cell progenitors and express c-kit (CD117) and low level of IL-1R1. Stage 3, or immature NK cells, is characterised by higher level of IL-1R1, NKG2D, NKp46 (CD335), NKp30 (CD337) and CD161 (NK1.1). Stage 4a and 4b define the mature phases of NK development and are marked by the expression of NKp80, NKG2D, NKp46, NKp30, CD161 and, importantly CD56, at their peak ($CD56^{bright}$). The final stage 5 is described as $CD56^{dim}$, $CD16^{+}$ and $KIRs^{+}$. The later stage 6 defines “memory” NK cells after antigen exposure, marked by high expression of NKG2C.

Adapted from Abel et al. (120) Open access.

As expected, the differentiation, expansion and maturation of NK cells is strictly regulated by multiple transcriptional factors (TFs), which act in synergy. Most of these transcriptional factors have been verified in mouse model. The deletion of the gene encoding for a TF could lead to a partial or complete loss of NK cells in terms of number, or could translate in an impair function, either as cytotoxic ability or in cytokines production. This depends on the stage of maturation in which the deletion takes place.

T cell factor 1 (TCF-1, encoded by *Tcf7*) is involved in early stages of differentiation: *Tcf*$^{-/-}$ mice show a drastic reduction of the number of NK cells in the bone marrow regardless of the stage of maturation. Moreover, the NK cells overexpress granzyme B and show a pro-apoptotic profile, which impairs their functionality.(129, 130)

The **Nuclear factor interleukin-3** (Nfil3, encoded by *E4bp4*) is important only in the very early phase of NK cells maturation in the BM, before the division between NK progenitors and ILC precursors. This is demonstrated by the complete loss of NK cells in terms of numbers and in cytotoxic function, including INFγ production, in *E4bp4*$^{-/-}$ mice. Instead, if the deletion happens in later stages of NK cells differentiation, this have barely any effects. In turn, Nfil3 regulates other important TFs such as Inhibitor of DNA binding 2 (Id2) and Eomes.(131, 132)

ID2 is expressed since early stages of NK cells differentiation but is particularly important in the final maturation process. In fact, *Id2*$^{-/-}$ mice have a normal number of cells in BM, but lack in mature CD27/CD11b NK cells.(133)

Other TFs involved in early stages of NK cells maturation are the members of the Ets family, such as **Ets-1** and **Myeloid Elf-1 Like Factor** (MEF), **Notch** and

STAT5.(133) In particular STAT5 (encoded by *Stat5a* and *Stat5b*) is necessary for the proper convey of the signal generated by the engagement of IL-15R, which is required for the development and homeostasis of NK cells. Also, STAT5 regulates other TFs and genes, as T-bet.(134, 135)

T-bet belongs to the T-box family TFs (encoded by *Tbx21*) and together with **Eomes**, are involved in late stages of maturation.(135) While the lack of one of these two TFs has no impact on the amount of NK cells in the BM, the blood stream and the peripheral tissues show a dramatic deficiency of NK cells. Interestingly, if both the TFs are lacking, all organs are affected, implying a sort of compensation between the two.(135)

Runt-related transcription factor 3 (Runx3), interferon regulatory factor (IRF2) and Zinc-finger E homeobox-binding 2 (Zeb2) are TFs expressed in late stages and are important for NK maturation.(133) A defect of these TFs is translated in an impaired NK development and phenotypes. In particular, **Runx3** directly regulates the expression of IL2-R (CD122) and the expression of genes up-regulated in response to IL-2 and IL-15.(136) A defect in **IRF2** in the BM reduces the presence of NK cells expressing CD11b and CD43. Moreover, these cells show a pro-apoptotic phenotype and an impaired cytotoxic activity.(137) Finally, when **Zeb2** is missing, NK cells do not progress over the $CD11b^{+}/CD27^{+}$ stage.(138)

1.3.2 Circulating NK cells

Mature circulating NK cells are defined as $CD34^+/CD45RA^+/CD3^-/CD56^+$, but according to the intensity of CD56 expression, they can be further subdivided into $CD56^{bright}$ and $CD56^{dim}$. These represent two subpopulations on NK cells, different in their function and in their anatomical site.

CD56, also known as NCAM (Neural Cell Adhesion Molecule), belongs to the Ig superfamily glycoproteins and is widely expressed in the central and peripheral nervous system, where it mediates cell-to-cell and cell-to-ECM.(139) As result of alternative splicing of its encoding gene, it exists in three main isoforms (NCAM-120, NCAM-140, NCAM 180). In the haematopoietic tissues, it is found not only on NK cells, where it represents the main marker, but also on activated $CD8^+$ T-cells, $\gamma\delta$-T cells, monocytes and dendritic cells.(140)

$CD56^{dim}$ subpopulation represents 90% of the entire NK cells in the peripheral blood and in the spleen. They usually co-expresses CD16 (**$CD16^{pos}$**) and are highly cytotoxic, as demonstrated by the large quantity of preformed perforins, granzymes and cytolytic granules in their cytosol, and by the expression of activating receptors such as NKp30, NKp44 and NKp46. For long time they were thought to play a secondary role in the secretion of INFγ (141), compared to $CD56^{bright}$, but recently it has been demonstrated that in fact they are able to secrete a good amount of INFγ, but in a very short time frame (2-4 hours) after the engagement of different activating receptors such as NKG2D, CD16, 2B4 and DNAM-1. Therefore, $CD56^{dim}$ are able to immediately kill the infected or tumoral cell and simultaneously release INFγ upon

target cell recognition, until the $CD56^{bright}$ are able to take over the production, approximately 16 hours from the stimulation.(141, 142)

$CD56^{bright}$ represents the remaining 10% of NK cells in the peripheral blood. They do not usually express CD16 (**$CD16^{neg}$**) and are mainly localised in lymph nodes. They are the main source of INFγ, but can also secrete other cytokines, according to the combination of interleukins secreted by the environment. For example, IL-15 plus IL-18 represents the best combination to trigger INFγ secretion, while IL-12 combined to IL-15 rouses an optimal production of IL-10; TNFα, TNF-β, IL-1 and IL-13 are secreted in response to IL-2 secretion from T lymphocytes, influencing the antigen-specific immune response and acting as a link between innate and adaptive response. In conclusion, $CD56^{bright}$ show a "regulatory" phenotype.(143, 144) The secretion and role of INFγ will be further discussed in chapter 1.3.3.1.

Table 1.2 summarises the main phenotypic differences between the two NK subpopulations. The relationship between these two phenotypically different subgroups is still under debate, but recent data have showed that most certainly, $CD56^{bright}$ are precursors of $CD56^{dim}$.(118, 145, 146) An interesting theory speculates that $CD56^{bright}$ could also represent a reservoir for $CD56^{dim}$, which in pathological conditions have a high turnover and need to be quickly replaced.(147)

NK cells can be further characterised according to the simultaneous expression of CD56 and CD16. **CD16** recognises the F_c fragment of IgG (FcγRIII) and this interaction triggers the *antibody-dependent cellular cytotoxicity* (ADCC, see chapter 1.3.3). CD16 is also found on neutrophils, macrophages, monocytes and mast cells.

$CD56^{bright}CD16^{neg}$ and $CD56^{dim}CD16^{pos}$ are the two main subpopulations in the blood and have been previously discussed. According to this division, other two subgroups could be identified:

1. **$CD56^{bright}CD16^{pos}$**. These cells could represent an intermediate stage of the development of $CD56^{bright}$ into $CD56^{dim}$. They are found to be increased in breast cancer, where they correlate with the stage of the disease.(148) Interestingly, compared to the $CD56^{bright}CD16^{neg}$ counterpart, they show a more active phenotype, demonstrated by the overexpression of NKp46, NKG2D and KIRs. This is translated in a more efficient degranulation ability and improved cytokines production.(149, 150). The treatment with immune check points inhibitors seems to upregulate this subgroup, as demonstrated in melanoma patients treated with ipilimumab, a mAb against CTLA-4.(151)
   $CD56^{dim}CD16^{dim/neg}$ are usually found in the healthy population and phenotypically are thought to be precursor of the $CD56^{dim}CD16^{pos}$ NK cells: they have a lower expression of the maturation marker CD57, compared to the $CD56^{dim}CD16^{pos}$, as well as lower NKG2A, DNAM-1 and KIRs, but interestingly, they are more efficient in degranulation.(152) Another interesting hypothesis speculates that these cells could actually represent $CD56^{dim}$ cells that have lost CD16 expression after degranulation or because of cleavage from metalloproteases. This could explain why the vast majority of these cells expresses CD107a, a marker of degranulation.(149, 153)

1.3.2.1 Memory NK cells

NK cells have showed to adapt through epigenetic remodelling and can even form long-lasting immunological memory, a characteristic that makes NK cells unique among the innate immune cells. The first evidence came from the identification of subsets of NK cells developed in response to hCMV infection. These NK cells exhibit a distinctive pattern of receptors, consisting in increased expression of CD94/NKG2C, CD2, CD57 and reduced expression of NKG2A, NKp46, CD16 and Siglec-7. These cells, named "**Memory NK cells**", show peculiar characteristics. hCMV induced-memory NK cells persist from several months to years in the peripheral bloods, even in absence of detectable infection, while usually NKs' estimated half-life is 14 days.(154, 155) Importantly, despite lower expression of CD16, memory NK cells are able to initiate a more efficient and amplified response to Ab-mediated stimulation, characterised by a greater degranulation and increased secretion of INFγ and TNFα.(156) The molecular mechanism relies on a significant epigenetic demethylation in the IFNG regulatory region, similar to what occurs in memory T_H1 cells.(157) Finally, memory NK cells seem to be resistant to the immune suppressive microenvironment in cancer patients. In particular, they are resistant to MDSC-mediated and T_{reg}-mediated suppression, as they express lower levels of IL-37R, IL1R8, TIGIT and PD-1, but higher level of Tim-3.(158-160)

These characteristics make memory NK cells good candidates for cancer immunotherapy, as shown in a recent study in which CAR-adaptive NK cells demonstrate a greater antitumoral effect compared to other NK cells subsets.(161)

Table 1.2 Phenotypic differences between $CD56^{bright}$ and $CD56^{dim}$ NK cells

	$CD56^{bright}$	$CD56^{dim}$
CD56	++	+
CD16	±	++
Inhibitory receptors		
KIR	−	+
ILT2	−	+
CD94/NKG2A	++	±
Activating receptors		
NKp46	++	+
CD117(c-kit)	++	−
Cytokine receptors		
IL2R$\alpha\beta\gamma$	+	−
IL2R$\beta\gamma$	++	+
IL1RI	++	+
IL18R	++	+
Chemokine receptors		
CCR7	++	−
CXCR3	++	±
CXCR1	−	++
CX3CR1	−	++
Adhesion molecules		
CD2	++	±
CD11c	++	±
CD44	++	+
CD49e	++	+
CD54	++	±
CD62L	++	±
CD11a	+	++
Other molecules		
CD57	−	+
CD160	−	++
CD55	++	+
CD59	++	+
HLA-DR	+	−

Adapted from Poli A et al. (147). With permission, license number 4977000735412

1.3.3 NK cells cytotoxic mechanism

NK cells' primary functions include recognition of self-antigens of cell surface and elimination of infected, damaged or transformed cells. These rely on a delicate balance between activating and inhibitory signals, mediated by a variety of germ line-encoded receptors (Table 1.3).

Normal cells belonging to the "self" express MHC I molecules which trigger a robust inhibitory signal, mainly through the CD94/NKG2A complex and KIRs receptors. This signal is able to put the NK cells at rest. One of the main features of tumour cells is the loss of MHC I molecules, which prevents the inhibitory signals to be ignited on NK cells. This, along with the expression on tumour cells of activating ligands such as MICA-B and/or ULBP 1-6 ligands, can turn on the NK cell and to rise a potent response which ultimately leads to the elimination of the target cell.

Table 1.3 NK cells activating and inhibitory signals

Type	Receptors	Ligands	Species
Activating receptors	NKG2D	Mouse: Rae1a-e, MULT-1, H60 Human: MIC-A/-B, ULBP1–4	Mouse/ human
	CD94-NKG2C	Mouse: Qa1b Human: HLA-E	Mouse/ human
	Ly49D	Mouse: H-2D^{d}	Mouse
	Ly49H	Mouse: m157 of MCMV	Mouse
	KIR2DL4	Human: HLA-G	Human
	KIR2DS1	Human: HLA-C2	Human
	KIR2DS2	Human: HLA-C1	Human
	KIR2DS3	Unknown	Human
	KIR2DS4	Human: HLA-A11	Human
	KIR2DS5	Unknown	Human
	KIR3DS1	Human: HLA-Bw4	Human
	NKp30	Human: B7H6, BAT3, pp65 of HCMV, PfEMP1 of *Plasmodium falciparum*, viral HA	Human
	NKp46	Heparin, viral HA and HN	Mouse/ human
	NKp44	Viral HA and HN, PCNA, proteoglycans	Human
	NKR-P1C		Mouse
	NKR-P1F	Mouse: Clr-g, Clr-c	Mouse
	NKR-P1G	Mouse: Clr-g, Clr-f	Mouse
	DNAM-1	Mouse and human: CD112, CD155	Mouse/ human
Inhibitory receptors	Ly49A	Mouse: H-2D^{b,d,k,p}, H-2M3	Mouse
	Ly49C	Mouse: H-2D^{b,d,k} H-2K^{b,d,k} m157	Mouse
	Ly49I	Mouse: H-2k^{b,s,q,v}	Mouse
	Ly49P	Mouse: H-2D^{d,k}	Mouse
	KIR2DL1	Human: HLA-C2	Human
	KIR2DL2	Human: HLA-C1	Human
	KIR2DL3	Human: HLA-C1	Human
	KIR3DL1	Human: HLA-Bw4	Human
	KIR3DL2	Human: HLA-A3,-A11	Human
	NKR-P1A	Human: LLTI	Human
	NKR-P1B NKR-P1D	Mouse: Clr-b	Mouse
	CD94-NKG2A	Mouse: Qa1b Human: HLA-E	Mouse/ human
	ILT2 (CD85j)	Human: HLA-A, -B, -C, HLA-G1, HCMV UL18	Human
	CD244(2B4)	Mouse and human: CD48	Mouse/ human

Adapted from Paul S. et al,(162). Open access.

NK cells mediate their cytotoxic effect through different mechanisms:

1. Degranulation of pre-formed vesicles containing granzymes and perforin
2. Expression of death receptors ligands
3. Secretion of exosomes
4. Secretion of cytokines.

Lytic granule-mediated killing is the main cytotoxic mechanism of NK cells. The production and storage of the lytic granules containing granzymes and perforins is strictly regulated and controlled, in order to prevent the early intracellular activation of these powerful enzymes.(163) Granzymes are a family of five serine proteases and represent the main components of NK cells' secretory granules.(164) They are able to induce the activation of caspases, a group of proteases playing a key role in programmed cell death, mitochondrial dysfunction and apoptosis in the target cells. While stored in the granules, their activity is inhibited by acidotic pH and by the association with chondroitin sulphate proteoglycan serglycin.(165) **Granzyme B** is the best characterised. It activates caspase-3 and caspase-7 by cleaving Asp residues, but also it targets cytoskeleton and nuclear proteins.(166) The other granzymes are:

- **Granzyme A**, a trypsin-like enzyme which cleaves preferably basic residues Arg or Lys, inducing cell death independently from caspases activation. It targets mitochondrial membrane and electron transportation, and nuclear DNA. It is also able to activate reactive oxygen species (ROS).(167)
- **Granzyme K** shows an activity similar to granzyme A but targets also pre-mRNA binding proteins. (168)

- **Granzyme M** cleaves Meth or Leu residues. It induces cell death targeting nucleolar phosphoprotein nucleophosmin. Its activity is independent by caspase. (169)
- **Granzyme H and granulysin.** Not much is known about these two granzymes. The first seems to show a mechanism similar to granzyme B, while the second seems to induce the disruption of the lysosomal compartment in the targeted cells.(163)

The degranulation of the lytic organelles into the targeted cells implies four main steps.

1. Once NK cell is activated, a lytic immunological synapse develops at the point of contact between the two cells, under the control of the adhesion molecules LFA-1 and CD11a/CD18. Moreover, the cytoskeleton of the NK cell undergoes rearrangement of the actin microfilaments, in order to focus the secretory granules.
2. The reorganisation of the cytoskeleton continues, and the microtubule-organising centre (MTOC) polarizes the lysosomes towards the synapse.
3. The lysosomes are transported to the synapse along the microtubules using dynein motor proteins, and docked to the cellular membrane to start the fusion.
4. The membrane of the lysosome fuses with the cellular membranes of the NK cell with the help of GTPase of the Rab family, Munc proteins and the formation of the SNAREs complex. The granules are released into the synaptic cleft and then fused with the plasma membrane of the target cell. Their content is eventually released.

During the entire degranulation process, NK cells express lysosomal-associated membrane protein-1 (LAMP-1, also known as **CD107a**), which is therefore used as a marker of actively degranulating NK cells.(170)

Perforins are stored into the lysosomes together with the granzymes. Once released into the extracellular space, the monomers can aggregate and form pores onto the plasma membrane of the infected/transformed cell, allowing the granzymes to access the target cell.(171) This mechanism of delivery seems to be quicker and predominant in comparison to the endocytic release of granzymes, which instead could represent a backup delivery to enhance the performance of the NK cell.(172)

The second important mechanism of cytotoxicity involves death receptors apoptosis, in particular FasL and TRAIL. **Fas ligand** (FasL, CD95L) is type II transmembrane protein belonging to TNF family.(173) In NK cells, FasL is stored in granules different from those containing granzymes and perforins, which implies that probably also the mechanism of transportation and surface externalisation is different.(174) Once expressed on the cell surface, FasL interacts with its receptor CD95, activating the apoptosis cascade via caspases 8 and 10.(175) Metalloproteinases, often released into the microenvironment by tumour cells, can interfere with this interaction cleaving the ligand from tis receptor.(176) Soluble FasL is inactive, thus this could represent an immune escape strategy. NK cells treated with recombinant IL-18 exert their antimetastatic effect through Fas-FasL pathway.(176)

TRAIL is a type II transmembrane protein homologue to FasL and TNF, usually expressed on stimulated NK cells, but also on monocytes, dendritic cells and macrophages.(177, 178) The interaction with its ligand induces apoptosis in tumour

cells or virus-infected cells. IL-12, which is a powerful inducer of INFγ, is also able to upregulate TRAIL expression on NK cells in liver, spleen and lungs. This improves the control over metastatic cells by NK cells, a mechanism in which TRAIL is a key player.(179) In contrast with FasL, soluble TRAIL is able to exert the killing activity, and potentially is able to kill target cells in proximity with NK cells.(163) In humans, four TRAIL receptors have been identified: of these, only TRAIL-R1 and TRAIL-R2 are able to induce apoptosis.(180, 181)

NK cells can form one synapse with one target cell at the time. Once the target cell has been killed, the NK cell needs to separate from the target and reconstitute the granzymes storage, as 10% of the granules are released per killing event. (163) The detachment from the dying cell is a well-regulated process, which implies the downregulation or the shedding of activating receptors.(182) However, while synthetizing de-novo granzymes and perforins granules, NK cells are able to continue the killing activity exploiting the death-receptor mechanisms. This implies a plasticity in modulating the killing effect according to the activity.(163)

The secretion of exosomes is a secondary mechanism of cytotoxicity in anti-tumour immunity. Either resting or activated NK cells secrete exosomes in the microenvironment, characterised by the expression of NK markers and activating receptors, such as CD56, NCRs and NKG2D. Also, these exosomes are loaded with FasL and perforins, and are showed to have cytotoxic activity against cancer cell lines.(183, 184)

1.3.3.1 Chemokine and cytokine production

Importantly, upon activation NK cells produce and secrete a plethora of chemokines, such as MIP-1α, MIP-1β, IL-8 and RANTES (CCL5),(141) and cytokines, such as INFγ, TNFα, IL-5, IL-10, IL13 and GM-CSF.(185, 186) Among them, **INFγ** is the most important to control infections and for antitumor immunity. NK cells secrete INFγ in response to stimulation from exogenous cytokines produced by DCs, macrophages and neutrophils, such as IL-12, IL-15 and IL-18, (187) and upon target cell activation (see chapter 1.3.2). In particular, the co-engagement of the activating receptors NKG2D, B24 and LFA-1 is required to induce INFγ and TNFα production and secretion.(141) In contrast, the activation of CD16 alone is adequate to stimulate INFγ and TNFα secretion, reflecting the importance of antibody-mediated cytotoxicity developed by the adaptive immune system. Interestingly, the secretion of INFγ and TNFα succeed the secretion of the proinflammatory chemokines MIP-1α, MIP-1β, IL-8 and RANTES, for which the engagement of a single activating receptors NKG2D, CD16 or 2B4 is sufficient. This demonstrates that the expression and secretion of INFγ are more strictly regulated and require a more complex engagement of different activator receptors, with probably different thresholds.(141) However, it has been demonstrated that the secretion of INFγ can also be rapid, as the INFγ locus in NK cells is constitutively demethylated. This means that INFγ locus exists in a more open configuration and does not need chromatin remodelling, making this locus readily accessible to transcriptional factors.(188)

INFγ exerts his antitumoral effects by modulating caspases, FasL and TRAIL expression(189), but importantly it is a potent T-cell stimulator. In particular, it can

regulate T_H polarization inducing activated $CD4^+$ T-cells to differentiate into T_H1, while suppressing T_H2 and T_H17 differentiation.(190) INFγ can also promote T-cells infiltration by inducing expression of the CXCL9, and can induces IgG expression by B-cells.(187)

TNFα is a multifunctional pro-inflammatory cytokine expressed by several cells belonging either to the innate and to the adaptive immune system. TNFα induces apoptosis, upon interaction with TFNR1 receptor, expressed in almost all nucleated cells.(191) The other receptor, TNFR2 is expressed on T cells, NK cells and endothelial cells, and is thought to have a more regulatory role.(191, 192) In particular, the interaction of TNFα with TNFR2 on NK cells, enhances the effect of IL-2, potentiating the cytotoxicity and the production of INFγ.(193) Despite its pro-inflammatory role, TNFα seems to promote tumour growth, cellular migration and invasion when released in the microenvironment, and high levels of TNFα have been associated with poor prognosis in breast cancer.(194, 195)

IL-10 is secreted by almost all immune cells, and by tumour cells as well.(194, 196, 197) Its role is still controversial: on one hand it has a well-recognised anti-inflammatory activity, downregulating CD28 on CTL and suppressing the secretion of other cytokines such as IL-2, INFγ, GM-CSF by dendritic cells and T_H1. On the other side, IL-10 improves NK cells cytotoxic activity, but at the expense of reducing the secretion of TNFα and INFγ.(196)

Importantly, $CD56^{bright}$ are the primary source of IL-10, in response to exogenous cytokines, in particular IL-12 in combination with IL-2 or IL-15. The

secretion of IL-10 upon cell contact stimulation has not been observed yet.(141) Also, the “strength” of IL-12 modulates the NKs’ response: a low release of IL-12 by dendritic cells, induces the secretion of INFγ, while a high concentration of IL-12 stimulates the release of IL-10.(198)

IL-10 has been related to tumour growth and progression in solid cancers, such as gastric and breast cancer.(194, 197) In this context, the chronic release of IL-10, not only by tumour cells but also by Tumour Associated Macrophages (TAMs) in the microenvironment, impairs NK cells cytotoxic activity and stimulates the recruitment of T_{reg}, MDSCs and neutrophils, which have a detrimental effect on NK functions.(194, 199) However, IL-10 is also able to downregulate MHC class I expression on tumour cells, thus busting tumour recognition.(200) Hence, it is clear that the secretion and the effect of IL-10 on NK cells depend on a very delicate balance of a combination of different cytokines and environmental factors.

1.3.4 Activating and inhibitory receptors and their ligands

1.3.4.1 NKG2 family receptors.

NKG2 is a group of seven lectin-type receptors expressed on the surface of NK cells, $\gamma\delta$-T cells and $CD8^+$ T cells, which can have either activating or inhibitory functions.(201) They usually recognise nonclassical MHC class I molecules, in particular HLA-E. An exception is NKG2D, which instead binds MHC class I homologous MICA/MICB (MHC class I polypeptide-related sequence A/B) and ULB-family proteins.

Both **NKG2A** and **NKG2C** form heterodimers with CD94, with opposite effects. Despite they both recognise HLA-E, CD94/NKG2A has an inhibitory effect on NK cells cytotoxic activity, while CD94/NKG2C activates NK cells. Moreover, CD94/NKG2A has a higher affinity for HLA-E, and it is early expressed on NK cells during their development.(202, 203) On the other hand, there are no supporting data on a role of the expression of CD94/NKG2C during NK cells differentiation.(201) As previously described for memory NK cells, individual infected with hCMV show an increased number of CD94/NKG2C$^+$ NK cells, which persists for years even in absence of viremia. Also, when NK cells are co-cultured with hCMV infected fibroblasts, the expansion of CD94/NKG2C$^+$ subset is demonstrated.(204) All these findings led to the conclusion that CD94/NKG2C$^+$ NK cells have a pivotal role in controlling the long lasting hCMV infection.

First described in 1991, **NKG2D** is type II trans-membrane protein with an extra-cellular C-type lectin-domain, associated with DAP10 homodimer.(205) Its encoding gene *Klrk1* is located on human chromosome 12, within the “NK complex”, which includes CD69, CD94 and the NKG2 family receptors. NKG2D shares just 21% of its sequence with the other receptors of NKG2 family, and also shows a different function.(206-208) Besides NK cells, NKG2D is also expressed on CD8$^+$ T-cells, NKT, $\gamma\delta$-T-cells and some autoreactive CD4$^+$ T-cells(209), and it elicits a strong activating cytolytic signal, which overcomes the inhibitory signal from MHC class I interaction. This leads to the killing of the target cell and the induction of cytokines production, such as TNFα and INFγ.(210-212)

During development, NKG2D is one of the first receptor expressed, and its level increases in parallel with the maturation of the cell. In human, its role in the development of NK cells is difficult to establish, but in mice it has been demonstrated that NKG2D has an impact on the expression and responsiveness of many other receptors.(209) The assumption is that NKG2D plays a role in the early “education” of NK cells, setting the threshold of activating and inhibiting signals towards locally expressed ligands, a process similar to positive and negative selection of T-cells.(213) For example, in mice, NK cells lacking NKG2D show a reduced level of c-kit, DNAM-1 and other inhibitory signals, making these cells more reactive to murine CMV infection.(214) Despite in humans there are no data confirming a similar behaviour, it has been noticed that during the first trimester of pregnancy, the maternal decidual NK cells population evolves from a $NKG2D^{neg}$ to a $NKG2D^{pos}$ phenotype, and this is accompanied by a dramatic change in the KIR repertoire and a switch toward an anti-inflammatory function.(215)

In peripheral blood, the engagement of NKG2D to its ligands induced by acute stress stimuli, can overcome the inhibitory effect of MHC class I and strongly promotes the cytotoxic activity of NK cells. Also, NKG2D ligation is able to reduce the activating thresholds of other activating receptors, either in mice and humans, including CD16, NKp46 and 2B4.(115) Conversely, a chronic exposure to its ligands has the paradoxical effect to downregulate NKG2D receptor, through endocytosis and degradation, and to impair the engagement of other receptors sharing the $FC\varepsilon RI\gamma$ signal adaptor, like CD16, NKp46 and NK1.1. The ultimate effect is the induction of an hypo-responsive cell, which could be advantageous in certain physiological conditions

such as pregnancy, but could also be exploited as a mechanism of tumour escape.(209, 216)

NKG2D recognises the two MHC class I-related chain A and B proteins (**MICA** and **MICB**) and the UL16-binding proteins, **ULBP1-6**. NKG2D ligands are usually absent in homeostatic conditions and are expressed on the surface of epithelial cell in response to stressful events such as infections, particularly viral, cellular damage or tumour transformation and progression. Their expression is highly regulated at different levels: transcriptional and post-transcriptional, RNA splicing and post-translational.(217)

MICA and MICB are two transmembrane glycoproteins encoded by the highly polymorphic genes *MICA* and *MICB* respectively. They are the best characterised NKG2D ligands, and, compared to ULBPs, they are more expressed in human tumours. Also, MICA is usually more frequent and abundant than MICB. MICA alone is able to trigger a strong cytolytic effect of NK cells, but is also a co-stimulator of $CD8^+$ T-cells.(216, 218) For this reason, it plays an important role in tumour surveillance, at least at early tumour stage. With tumour progression, soluble forms of MIC proteins (s-MICs), are shed by the tumour cells in the microenvironment, causing downregulation of NKG2D and severe impairment of NK cells. In fact, a high concentration of s-MICs, but also s-ULPBs, has been correlated with poor prognosis and metastasis in many solid tumours, including pancreatic adenocarcinoma.(218)

ULBPs are a group of six MHC-class I related proteins, defined by their ability to recognise and bind cytomegalovirus protein UL16. ULBP 1-2-3 and -6 are GPI-linked proteins, which shared 55%-60% of their sequences, while -4 and -5 are transmembrane proteins.(211, 219) The interaction between ULBPs and their ligand,

not only activate NK cells cytotoxicity, but also induce expression of cytokines and chemokines, such as TNFα, TNFβ and INFγ, which recruit other inflammatory cells, like macrophages, neutrophils and adaptive immune cells.(211) On the other hand, the soluble forms are highly immunosuppressive, downregulating the expression of NKG2D, as seen for MICs.(217, 218, 220)

Therefore, chronic expression of NKG2D ligands represents a mechanism of tumour immune evasion, and could be targeted for immunotherapy at different levels: blocking tumour expression and shedding (221), preventing their interaction with the receptor with monoclonal antibodies (222), removing from plasma through absorption apheresis to precondition patients for cell therapy(223), or up-regulating NKG2D on NK and T cells.(219)

1.3.4.2 DNAM-1 receptor and its ligands

DNAX accessory molecule-1 (**DNAM-1** or **CD226**) is an Ig-superfamily transmembrane glycoprotein, associated with the adhesion integrin LFA-1. It is expressed on many leucocytes, including NK cells, $CD8^+$ T-cells, B cells, macrophages and platelets.(224) Its importance in immune surveillance is demonstrated by the increased incidence of fibrosarcoma and papilloma tumours in mice deficient for DNAM-1, compared to the wild-type.(225)

DNAM-1 recognises the poliovirus receptor (**PVR** or **CD155**) and Nectine-2 (**CD112**), two stress-induced proteins expressed on many epithelial cells, including tumour cells. CD155 seems to have greater affinity for DNAM-1 than CD112, and both are also ligands for TIGIT and CD96, however with the opposite effect to inhibit

activation. The interaction between DNAM-1 and CD155 is fundamental for triggering NK cells cytotoxicity against cancer cells and cytokines production, but it is not sufficient. In fact, its role is more similar to a costimulatory on already activated NK cells via the activating receptors NKp46, 2B4 or NKG2D.(226)

In mice, DNAM-1 expression defines two distinctive subsets of circulating NK cells, DNAM-1^{+} and DNAM-1^{-}, which could represent the mouse analogue to the human $CD56^{bright}$ and $CD56^{dim}$ subset, respectively. In fact, DNAM-1^{+} are mostly located in the BM and in the peripheral lymph nodes, while DNAM-1^{-} in the bloods and lung. Moreover, DNAM-1^{+} NK cells produce a large number of cytokines and chemokines, such as INFγ, IL-6, GM-CSF and also express IL-15 related genes. On the other hand, DNAM-1^{-} NK cells express higher rate of NKR-related genes and produce large amount of MIP1, and could represent a further differentiation of DNAM-1^{+} NK cells.(227)

As seen for NKG2D, the chronic expression of its ligands by cancer cells, downregulates the expression of DNAM-1 on the surface of NK cells. This not only reduces the ability to mount a vigorous cytotoxic activity against cancer cells, but also impairs monocytes' recruitment and $CD4^{+}$ $CD8^{+}$ T-cells co-stimulation, representing therefore a further mechanism of immune escape.(228, 229)

1.3.4.3 Natural Cytotoxicity Receptors (NCRs) and their ligands

The NCRs family was originally identified on the surface of NK cells almost 20 years ago, and subsequently identified also on $CD8^+$ T cells, γδ-T cells, ILC1 and on a subtype of ILC3 cells. NCRs comprise three type I transmembrane proteins, named NKp46, NKp40 and NKp30, encoded by the genes *NCR1*, *NCR2* and *NCR3* respectively.(230) These receptors recognised a wide range of membrane, intracellular and soluble ligands, which altogether tailor the activation/suppression activity of NK cells (Table 1.4).

NKp46 is 46 KDa transmembrane protein expressed in all mammalian species. In humans it is expressed on $CD56^{dim}CD16^{pos}$ and $CD56^{bright}CD16^{neg}$ NK cells, ILC1 and ILC3, γδ T cells and intraepithelial TLs. The interaction with its ligands induces calcium release and the secretion of INFγ and TNFα by NK cells.(230, 231)

NKp44 is a 44KDa protein expressed by all primates but not found in mice. In humans, it is typically expressed on $CD56^{bright}$ NK cells, in particular in response to IL-2, IL-15 and IL-1β stimulation. It is also found on plasmacytoid dendritic cells, ILC1 and ILC3, and γδ-T cells.(230) NKp44 recognises either membrane ligands, such as heparan sulphate glycosaminoglycans, intracellular ligands expressed by tumour cells, such as Proliferating Cell Nuclear Antigen (PCNA), or ligands expressed on virus-infected cells, as reported in HIV infection.(232) Interestingly, NKp44 recognises also the Platelet -Derived Growth Factor-DD (PDGF-DD), secreted in the microenvironment by tumour cells, which induces degranulation and cytokines secretion by NK cells.(233) Conversely, the interaction with PCNA suppresses NK cells cytotoxic activity and INFγ secretion.(234)

NKp30 is a 30KDa protein, expressed on mature NK cells independently by their status. It exists in three different isoforms, NKp30a, NKp30b, NKp30c. While the first two isoforms ignite NK cytotoxic activity, NKp30c has been showed to elicit the secretion of the immunosuppressive cytokine IL-10 and to decrease the production of INFγ and TNFα, representing a possible mechanism of tumour escape. It has also been correlated with poor survival in patients affected by GIST, in treatment with imatinib (235, 236)

The discovery of NCRs and their ligands has raised many questions, in particular how it is possible that a receptor can recognise ligands so different in structure and origin. Also, each ligand can elicit a different strength of activating/inhibitor signal, according to the tissue and the primary insult that has roused the inflammatory response. Interestingly, NCRs expression is not suppressed by soluble ligands, as seen for other NK cells activating receptors like NKG2D or DNAM-1. This polyfunctionality makes NK cells response adaptable to the microenvironment and can be exploited as therapeutic target in cancer immunotherapy.(230)

Table 1.4 Expression of Natural Cytotoxicity Receptors and their ligands

Receptor	Gene	Cellular expression	Ligands	Ligand-induced signal
NKp46	*NCR1*	NK cells, ILC1, NCR$^+$ ILC3, Small intestine TCR$\alpha\beta^+$ CD8$^+$ IEL, Small intestine TCR$\alpha\beta^-$ innate-like IEL, TCR$\gamma\delta^{low}$CD3$^-$, Expanded peripheral blood γδ T cells (Vδ1$^+$), NK, NKT and T lymphomas, Cord blood T cells cultured in IL-15	Heparan sulfate (HS) gylcosaminogylcans (GAGs) HA (hemagglutinin) of influenza virus HA of human vaccinia virus HN of avian Newcastle disease virus, Sendai virus and human parainfluenza virus (DBL)-1 α domain of *Plasmodium falciparum* erythrocyte membrane protein (PfEMP1) Vimentin expressed on cells infected with *Mycobacterium tuberculosis* Unidentified ligand expressed by *Fusobacterium nucleatum* Unidentified ligand expressed by pancreatic β-cells *C. glabrata* Epa proteins Complement Factor P (properdin)	Activation Activation Activation Activation Activation Activation Activation Activation Activation Activation
NKp44	*NCR2*	NK cells, ILC1, ILC3, Plasmacytoid dendritic cells, Small intestine TCR$\alpha\beta^+$ CD8$^+$ IEL, Expanded peripheral blood γδ T cells (Vδ1$^+$), Cord blood T cells cultured in IL-15	HS GAGs Syndecan-4 (*in cis*) HA of Influenza virus HN of avian Newcastle disease virus, Sendai virus and human parainfluenza virus PDGF-DD Nidogen-1 PCNA NKp44L expressed on tumor cells, bystander CD4$^+$ T cell during HIV infection, or cartilage-derived chondrocytes Domain III envelope protein from West Nile and Dengue viruses Unknown ligand(s) on *Mycobacterium tuberculosis, M. bovis, Nocardia farcinica and Pseudomonas aeruginosa*	Activation Inhibition Activation Activation Activation Inhibition Inhibition Activation Activation Unclear
NKp30	*NCR3*	NK cells, CD8$^+$ T cells, Expanded peripheral blood γδ T cells (Vδ1$^+$), ILC2 Cord blood T cells cultured in IL-15	HS GAGs HA of human vaccinia virus pp65, main tegument protein of human cytomegalovirus (DBL)-1 α domain of Plasmodium falciparum erythrocyte membrane protein (PfEMP1) BAT3/BAG6 B7-H6 Galectin-3 β-1,3 glucan	Activation Inhibition Inhibition Activation Activation Activation Inhibition Activation

Adapted from Barrow AD et al, (230). Open Access.

1.3.4.4 NK cells inhibitory receptors: Killer Ig-like receptors (KIRs)

KIRs are a family of type I transmembrane glycoprotein expressed on the surface of NK cells, which specifically interact with MHC class I expressed on all the nucleate cells. This binding strongly inhibits the cytotoxic activity of NK cells, putting the cell at rest. According to the “missing-self” theory, an infected or transformed cells activates NK cells by losing or downregulating MHC class I molecules, therefore preventing the inhibitory signals to be triggered.(237)

KIRs are also key regulators of the “education” of NK cells, which implies the selection of those NK cells expressing inhibitory KIRs (iKIRs) that strongly recognise HLA class I molecules.(238) The strength of the inhibition will affect the efficiency of the future NK cell, as NK cells not expressing KIRs are usually anergic.

The expression of KIRs occurs at late stages of differentiation, while immature NK cells usually express CD94/NKG2A heterodimers. At intermediate stages, both receptors could be present, but eventually mature NK cells will express at least one KIR or more rarely CD94/NKG2A only.(239) This process is pivotal to maintain the self-tolerance, but also allows NK cells to detect the loss of even a single HLA class I.(240) The comparison of inhibitors receptors profile between homozygotic twins using mass cytometry, showed that KIRs repertoire is control by genetic factors, but the whole phenotypic profile of NK cells is affected by external pressures exerted by environmental factors or unpredicted random phenomena.(241)

KIRs are encoded by extremely polymorphic genes, therefore the final NK cells repertoire is exceptionally variegated, reflecting the same polymorphism seen for HLA genes.(238) But an important role seems to be played also by environmental factors,

in particular viral infection. For example, hCMV infection is able to shape the iKIRs repertoire enforcing epigenetic changes, resulting in the expansion of an NK cells subset specifically expressing the heterodimer CD94/NKG2C, CD57 and KIR2DL, an iKIR specific for HLA-C.(156, 242)

The nomenclature of KIRs describes their structure and function: a KIR receptor could contain two or three extracellular domains (KIR2D or KIR3D), while its cytoplasmatic tail could be long (L) or short (S). This latter reflects also the function of the receptor, the L having inhibitory function, while the S is activating.(237, 238)

Interestingly, there is a huge variation in the frequency of expression of activating KIRs among the populations worldwide. For example, less than 20% of the Japanese population express KIR2DS2, while the same is expressed in over 80% of the Australian Aboriginals, demonstrating a strong selection pressure on specific population, probably due to environmental factors, such as viral infection or others not known yet.(243)

Recently, KIRs have been tested as target for cancer immunotherapy, with encouraging results. A phase I clinical trial testing Lirilumab, an IgG4 mAb against KIRD2L, has proven the efficacy, safety and tolerability in treating patients affected by Multiple Myeloma. In combination with Lenalidomide, an NK cells stimulator, this mAb is able to disrupt the interaction between the receptor and its ligand, making NK cells reactive against myeloma cells.(244) The mAb IPH4102 directed against KIR3DL2 has been reported to be safe and effective in treating patients affected by cutaneous T cell lymphoma, in whom KIR3DL2 is overexpressed.(245) These promising results need be confirmed in large clinical trials.

1.3.4.5 Immune checkpoint inhibitors

Immune checkpoints are predominantly inhibitory signals, by which the immune system regulates itself in order to prevent overreactions against the pathogens and environmental damages, and to prevent self-harming. Tumour cells are able to trigger these inhibitory signals to escape recognition and elimination by the immune system. *In vitro* and *in vivo* experiments have demonstrated that mAbs against immune checkpoints are able to restore the ability of immune cells to mount a defence against the tumour. Therefore, several immune checkpoints inhibitors have now been developed and tested in humans as immune therapy against different cancer, the first of which was melanoma.(246) Unfortunately, PDAC does not respond as expected to immune checkpoints inhibitors antibodies, either as monotherapy or in combination with other checkpoints, but promising results have been showed if used in combination with chemo or radiotherapy.(247)

CTLA-4 is an inhibitory receptor homologous to CD28, with which it competes for binding the ligands CD80 (B7-1) and CD86 (B7-2). However, CTLA-4 has a greater avidity and affinity for these ligands, rather than CD28. It is usually expressed on activated NK cells, at least in mice, and on T_{reg} and its engagement suppresses the INFγ production in response to DC.(248) In a murine model, anti-CTLA-4 therapy was able to restore NK activation and degranulation in tumour microenvironment, in parallel with the depletion of T_{reg}. Moreover, the combined therapy of IL-15/IL-15Rα complex with anti-CTLA-4, potentiates the control over the tumour.(249)

Programmed cell death protein 1 (**PD-1**) is a receptor belonging to the CD28/CTLA-4 family, typically expressed on T cells, B cells and macrophages. In healthy humans, PD-1 is expressed on 25% of circulating NK cells, in particular on $CD56^{dim}/NKG2A^{-}/KIR^{+}/CD57^{+}$ mature NK cells, while $CD56^{bright}$ do not express PD-1.(250) Its ligands, PD-L1 and PD-L2, are usually upregulated in many tumours and the binding with PD-1 on T cells strongly inhibits TCR signalling and thus T cells' effector functions, and represent a mechanism of immune escape.(251, 252) NK cells expressing PD-1 are considered memory-like NK cells, and they show an exhausting phenotype characterised by reduced cytotoxic activity and cytokines secretions.(250, 253, 254)

TIGIT is an inhibitor receptor belonging to the CD28/CTLA-4 family, expressed either on T- and NK cells, which in NK cells suppresses killing activity through the ITIM domain, either in mice or in humans.(255, 256) It competes with DNAM-1 and **CD96**, another inhibitory receptor expressed on NK cells, in binding CD155 and CD122. Differently from CD96, it is also able to decrease the secretion of INFγ, either from NK cells or $CD8^{+}$ T cells. TIGIT is highly expressed on infiltrating NK cells in a number of tumour models, as colon cancer cell lines, breast and fibrosarcoma.(256, 257)

LAG-3 is an inhibitory checkpoint which binds MHC class II on target cells. it is demonstrated to suppress immune response in Hodgkin's lymphoma, stomach, breast and lung cancer, in which it is expressed on almost 41% of the TILs.(258, 259) On NK and T cells it inhibits INFγ production.(260)

TIM-3 is a type I transmembrane inhibitory receptor, which binds galectin-9, high mobility group box 1 protein (HMGB1) and carcinoembryonic antigen-related cells adhesion molecule 1 (CEACAM-1). Other than NK cells, TIM-3 is expressed on T_H1

lymphocytes, dendritic cells and macrophages.(261) It is also highly expressed on a number of tumours, such as GIST, lung, stomach melanoma and acute myeloid leukaemia. In pancreatic cancer, its expression is promoted by cancer-associated fibroblasts, IL-2, IL-12, IL-15, IL-18 and TNFα secretion by the TME.(260) NK cells expressing TIM-3 showed an impaired and exhausted phenotype, with a lower production of cytokines, including INFγ.(262) Moreover, they correlated with poor prognosis, at least in gastric and lung cancer.(263, 264) Recently, it has been demonstrated that TNFα is able to induce TIM-3 expression on NK cells, by activating the NF-kB signalling pathway, in oesophageal cancer. Moreover, NK cells expressing TIM-3 produces less INFγ and their presence among the TILs correlates with poor prognosis.(265)

Importantly, while KIRs and NKG2A are constitutively expressed on mature NK cells, the expression of other checkpoints is inducible.(266) In the last decades, much effort has been focused in developing mAbs blocking checkpoints inhibitors, in order to restore NK and T cells functionality. Some of these mAb are now being tested in clinic trials, either as monotherapy or in combination with other mAbs and/or radio-chemotherapy. *Henriksen et al.* has recently published a systematic review about ongoing clinical trials targeting checkpoints inhibitors in pancreatic cancer.(247)

1.4 The immune response in cancer

1.4.1 The immune editing theory

Inflammation is an active part of the neoplastic milieu since the first stage of carcinogenesis. If at least at early stages, the acute inflammatory response to neoplastic cells is able to limit the spread of the disease, chronic inflammation detected at later stages is probably one of the main contributors to tumour progression, in particular promoting genomic and epigenetic instability, immune invasion, angiogenesis and metastatic dissemination.(267) In this sense, tumour associated immune cells are either able to control and to contribute to neoplastic development. This has been largely demonstrated by the association between a higher incidence of cancer and other diseases induced by chronic inflammation in response to infections, autoimmunity or tissue damage from toxic agents, like for example smoke.

In early 2000, *Dunn et al.* introduced the concept of “immunoediting”, a theory that tries to explain not only how tumour cells are able to escape the sophisticated surveillance of the immune cells, but also how the immune response is partially responsible for shaping the tumour growth.(268)

The first phase of immunoediting is the **elimination** of the transformed cells and overlaps with the concept of immune surveillance. The transforming cells disrupt the local microenvironment, which responds attracting cells of the innate immune system to repair the damage, through the release of chemotactic cytokines. Therefore, NK cells, NKT, $\gamma\delta$-T cells, macrophages and dendritic cells reach the site of tumour cells,

are activated and start producing the most important molecule that drives the next steps: INFγ. In particular NK cells are able to recognise cancer cells in two ways: (1) the loss of MHC class I on cancer cells imbalances the inhibitory effect of KIRs receptors towards an activated phenotype, and (2) through the expression of NK activated ligands, such as MICA/MICB or ULBPs. Moreover, NK are not only induced to eliminate the cancer cells, but they also secrete cytokines and chemokines which enhance the recruitment of other immune cells: CCL5 and CXCL1 promote the recruitment of conventional DC to the microenvironment; INFγ and TNFα promote the recruitment and the activation of $CD8^+$ T cells, monocytes and neutrophils and boost macrophage phagocytosis. In turn, these cells enhance NK cytotoxic activity.(269-271) Also, INFγ has a powerful apoptotic effect on tumour cells and an angiostatic action on the microenvironment, preventing the blood supply to the tumour.

The death of cancer cells releases a large number of antigens, which are drained to the lymph nodes, where they are presented by activated dendritic cells to $CD4^+$ T cell. In turn, $CD4^+$ T cells facilitate the activation of tumour specific cytotoxic $CD8^+$ T cells. The adaptive immune cells are now involved in the elimination of remaining cancer cells. The final goal is the complete elimination of all the transformed cells.

Despite the efficiency of the initial immune infiltrate, some tumour cells can survive and establish an active balance with the elimination pressure from the lymphocytes and INFγ. This is called the **equilibrium** and in humans could last years. During this phase, the cytotoxic pressure from immune systems continues, but leads to a positive selection of less immunogenic cancer cells, which eventually accumulate more and more genetic instabilities and epigenetic changes. This finally leads to the

final stage which is the tumour **escape**. Here, the tumour cells overwhelm the elimination by the immune system, start to proliferate indiscriminately and become able to develop strategies to promote their own growth, in particular shaping the inflammatory response in the microenvironment, towards an immune suppressive phenotype: M2 macrophages and T_{reg} are recruited and secrete the immune suppressive cytokines IL-10 and TGFβ; also, they inhibit IL-12 secretion from DCs, dampening the presenting antigen activity and eventually excluding NK and T cells from the cancer milieu.(271-274)

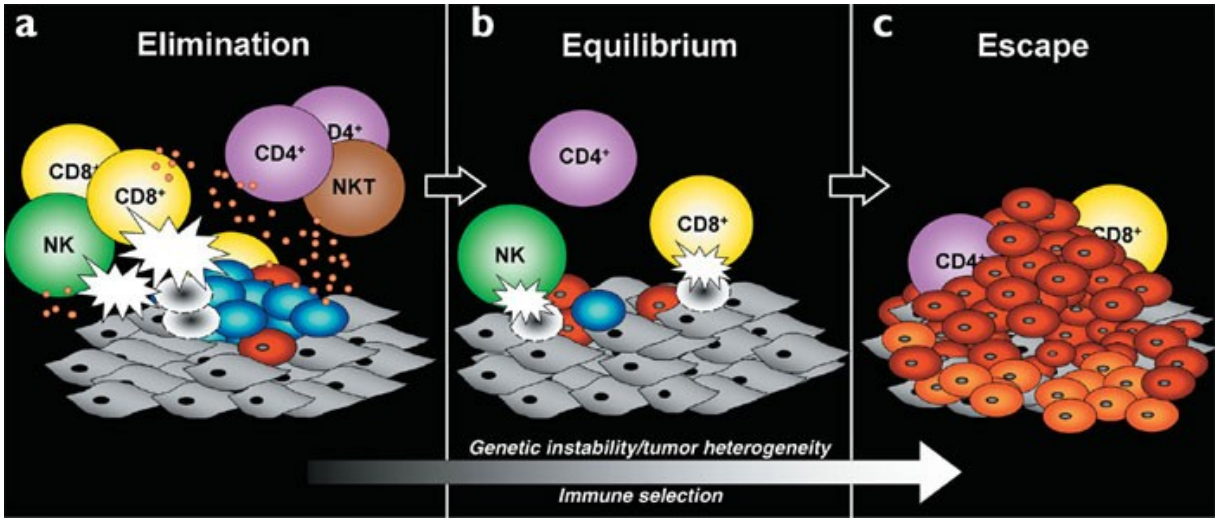


Figure 1.1-3 The three Es of immunoediting theory.

The first stage of the immunoediting theory is a) **Elimination: t**his phase involves either the innate and the adaptive immune systems, and the production of cytokines (small orange circles). The efficacy of the immune system usually leads to the complete elimination of the transformed cells. If some cancer cells survive, they enter in the **Equilibrium** phase (b), in which the cytotoxic pressure leads to a positive selection of less immunogenic cancer cells. The accumulation of genetic instability and epigenetic changes lead to the final **Escape** (c): tumour cells now proliferate indiscriminately and shape the inflammatory response towards an immune suppressive phenotype to promote their own growth.

Adapted from Dunn et al.,(268). With permission, license number 4977120882616

1.4.2 Tumour microenvironment

It has become clear that the tumour microenvironment (TME) is not only a bystander in oncogenesis, but it is a source of cellular and molecular components that promotes tumour progression, metastasis and chemo resistance.(275-278) It is also now evident that tumours differ in the microenvironment composition. If this is a cause or a consequence of the chronic inflammation is impossible to establish, but definitely there is a complex correlation between the two compartments, which contributes and shapes tumour progression.(279)

The dense stromal reaction which characterises the microenvironment of primary solid tumours, also named **desmoplastic reaction**, is a peculiar characteristic of pancreatic cancer. Importantly, increased thickness of the fibrotic stromal compartment correlates with poor prognosis.(280) Conversely, in healthy individuals, the stromal environment protects against tumorigenesis, and this apparently contributes to longevity and survival.(281) However, if under certain conditions the normal epithelium acquires a malignant phenotype, the adjacent stroma is converted in a pathological entity that co-evolves with the cancer cells. In such conditions, stromal cells are educated to accelerate disease progression by synthesizing a wide range of cytokines, chemokines, growth factors and proteinases.(282, 283) All these molecules induce ECM remodelling and regulate immune cells infiltration and immune escaping. Thus, in response to cancer progression, the TME acquires a plastic phenotype that paradoxically accommodates the growing tumour, since the early stages.

The TME has a complex three-dimensional structure, in which bidirectional communication between stromal cells and their surrounding matrix plays a critical role

in tissue homeostasis and cancer progression.(284) The main cellular component is represented by fibroblasts. Fibroblasts associated with cancer have been named Cancer Associated Fibroblasts (CAFs).

Fibroblasts are non-epithelial and non-immune cells with likely a mesenchymal lineage origin. In normal adult tissue, they are located in the connective tissue, and primarily exist in a quiescent state. CAFs are a heterogeneous population of irreversible activated fibroblasts, playing a dual role in oncogenesis, either promoting or fighting the cancer cells at the same time.(277, 285) They are recruited by cancer cells and infiltrating immune cells, through the secretion of a wide range of different molecules, which differ according to the tumour type. Among other, in vitro studies have shown a key role of fibroblast activation of TGF-β, PDGFs, epidermal growth factors (EGFs), fibroblasts growth factors (FGFs) and sonic hedgehog (SHH). (282)

CAFs promote tumour progression on different levels:

1. Acting on immune infiltration, promoting an immunosuppressive phenotype, or retaining activated immune cells
2. Promoting angiogenesis
3. Re-organizing the ECM, thus promoting cancer cell invasion and metastasis,
4. Maintaining cancer stem cells, through paracrine signalling

On the other hand, CAFs can also have a protective role against cancer. In fact, they secrete immunomodulatory cytokines, such as IL-10, TNFα, IFNγ and IL-6, that recruit and activate macrophages, T lymphocytes and NK cells. This has also been demonstrated in clinical studies, which show that the level of expression of markers of

CAFs, such as collagen-I, αSMA and fibroblast activated protein (FAP), correlate with good prognosis, in lung and breast cancer.(286, 287)

In PDAC, quiescence pancreatic stellate cells (qPSCs) are the main source of CAFs. In normal pancreas, they are located in the periacinar space, along with interlobular fibroblasts. They show characteristics in common with hepatic stellate cells (HSCs): they store vacuoles of vitamin A in their cytoplasm and they both have a stellate morphology.(283) Their main role is probably connected with ECM turnover, as they can produce metalloproteases, if stimulated. For this reason, qPSCs are thought to be the main effector of fibrosis in chronic pancreatitis.(288, 289) Unfortunately, in humans qPSCs lack of specific markers.

Once activated during carcinogenesis, qPSCs lose their stellate shape and their vitamin A vacuoles, acquire a spindle-shape morphology and start expressing αSMA and FAP. (290) This new phenotype gives them the ability to proliferate and migrate, and to produce a wide spectrum of proteins, directly involved in many stages of tumour progression. In fact, they develop the ability to modulate inflammatory response, to stimulate angiogenesis and to promote metastatic spread, co-migrating with cancer cells into the blood stream, creating the soil for the metastasis in the target organ. (291, 292)

There is evidence that epithelial-mesenchymal transition (EMT) could play a role in recruiting cells similar to CAFs. EMT is a process that allows epithelial cells to lose their rigid architecture and develop a more flexible mesenchymal-like structure. This implies loss of polarity and down regulation on E-cadherin. This results in a new cellular phenotype, characterised by a spindle-shape form, increased motility and expression of typical mesenchymal markers, such as desmin, vimentin and fibronectin.(293) In *in*

vivo studies on human pancreatic cancer samples, these markers are overexpressed in poorly differentiated areas and correlate with worse overall survival and with a metastatic susceptibility. (282, 294, 295)

Myeloid-derived Suppressor Cells (**MDSC**) are a pivotal component of the microenvironment and represent the most important suppressive lymphocytes of the tumour infiltrate.(296)

MDSC are usually divided in polymorphonuclear-MDSCs (PM-MDSC), cells similar to neutrophils with a relatively modest suppressive activity, and monocytic-MDSC (M-MDSC), which are similar to monocytes but with a stronger suppressive activity.(297, 298) The location of these two subclasses is also different: while PM-MDSC are usually located in secondary lymphoid organs, M-MDSC are found in the tumour and in metastatic tissues, where they are induced to differentiate into tumour-associated macrophages (**TAMs**).(299) These cells represent almost 50% of the infiltrating leukocytes.(300)

The recruitment of TAMs involves not only chemotactic molecules belonging to the CC family, such as CCL2, CCL11, CCL16 and CCL21, but also hypoxia and high concentration of lactic acid seem to play an important role, in particular inducing phenotypic differentiation toward a suppressive phenotype. In fact, TAMs show functional properties similar to M2 macrophages (see chapter 1.2.1.2), in their ability to secrete immune suppressive cytokines, such as IL-6, IL-8 and IL-10. However, this has been recently questioned, as TAMs show a more plastic phenotype, with characteristics peculiar of M1 macrophages as well.(301, 302)

TAMs play a key role in tumour growth, invasion and escape in many solid tumours, and their presence in the infiltrate negatively correlate with prognosis.(300) TAMs are also able to escalate EMT and angiogenesis, by secreting IL-6 and IL-8, while the secretion of IL-10 and TGF-β promotes tumour escape. Also, they express checkpoint inhibitors such as PD-1, and Siglec-10, which interacts with CD24 on tumour cells inhibiting the phagocytic function.(303, 304) Moreover, TAMs can prevent phagocytosis of tumour cells by expressing receptors recognising "self" ligands on cancer cells, such as SIRPα, a ligand of CD47, and LILRB1, which recognises MHC class I. These mechanisms have been recently exploited as targets in immune therapy against cancer.(305, 306)

Tumour cells orchestrate the changes in the cellular and acellular component of the surrounding microenvironment. This requires a complex bi-directional network of signals which is not completely understood, and implies cytokines, chemokines, growth factors and remodelling enzymes.(307) Recently, new mediators have also been identified, such as cell-free DNA, apoptotic bodies and exosomes, as new potential horizontal mechanisms to transmit signals and information, inducing malignant transformation in normal cells or promoting metastases.(308) Unveiling this complex interaction would open new perspectives to develop effective therapeutic targets and diagnostic tools.

Cancer cells are characterised by a rapid and uncontrolled turnover, which requires an important supply of oxygen. The imbalance between supply and demand results in **hypoxia,** which is a typical characteristic of solid tumours. Hypoxia induces tumour cells to modify the surrounding landscape in order to develop an adequate

amount of blood vessels. However, tumour induced-de novo angiogenesis is ineffective: the new microvascular network is architecturally disorganised and hyper permeable, leading to vascular leakage and a greater interstitial pressure.(309) **HIF-1α**, expressed by tumour cells, is responsible of the hypoxia-driven modifications in the microenvironment. In particular, HIF-1α suppresses the expression of E-cadherin, promote EMT and induces VEGF, angiopietin-2 and angiopoietin-like 4 expressions, thus promoting invasion and metastasis.(310) The development of anti-VEGF treatments has revolutionised the outcome of metastatic colorectal cancer, renal cells carcinoma and hepatocarcinoma, among others.(311) Moreover, HIF-1α hypoxic stimuli shape the inflammatory response towards an immunosuppressive phenotype. In fact, they stimulate MDSCs differentiation, upregulate PD-L1 expression and impair the cytotoxic function and the proliferation of infiltrating T lymphocytes, creating an acidotic milieu.(312, 313)

Finally, the pressure exerted by hypoxia on tumour cells contributes to cell heterogeneity, promotes an aggressive and metastatic phenotype and concurs in developing drug resistance, as the current anti-tumour therapies are not able to reach poorly perfused regions.(314, 315)

1.4.3 Tumour microenvironment induces NK cells dysfunction to promote cancer growth: mechanisms of immune escape

Several studies have recently highlighted the active role of NK cells in detecting and killing circulating metastatic cells, either in mice or in humans.(316, 317) However, in primary solid tumours NK cells represent just a minor fraction of Infiltrating

Lymphocytes (TILs), these been mostly represented by T lymphocytes. For this reason, they have limited prognostic value compared to other T cells subpopulations, such as $CD8^*$ T cells or T_{reg} cells, and their role in the immune surveillance of primary solid tumour in humans is far to be clearly defined.(199) However, in some solid tumours such as colon,(318) breast,(319) stomach,(320) kidney(321) and lung,(322) the infiltration of NK cells has been associated with a better prognosis.

In solid cancers, tumour associated NK cells show an impaired cytotoxic activity: first, along with tumour progression, the percentage of infiltrating NK cells is drastically reduced; also, the downregulation of multiple molecules such as the activating receptors NKG2D, CD16, NCRs and CD226, but also INFγ, CD107a, FasL, TRAIL and perforin, demonstrates that the killing activity is compromised at different levels.(323, 324) Moreover, the NK cells' metabolism is affected, with a suppressed glycolysis which compromises viability and effector functions.(325) On the contrary, inhibitory receptors, in particular TIGIT, NKG2A and PD-1 have been reported to be upregulated in infiltrating NK cells, probably in response to the secretion of immune suppressive cytokines from the tumour microenvironment, such as IL-10 and TGF-β.(257, 326-328)

The microenvironment plays a pivotal role in inducing these modifications on NK cells, either directly or indirectly, by stimulating other immune suppressive cells like MDSC or T_{reg}.

Together with IL-10, whose role in suppressing NK cells has already been discussed in chapter 1.3.3.1, **TGF-β** is one of the most important cytokines inducing an immune suppressive microenvironment. The main sources of TGF-β in the TME are cancer cells, fibroblasts, TAMs, T_{reg} and platelets.(329) In homeostatic conditions, TGF-β counterbalances the activation of adaptive immunity by stimulating T_{reg}, and

inhibiting T_H1 response and APCs, such as dendritic cells. TGF-β affects NK cells' cytotoxicity directly downregulating the expression of NKG2D, NKp30 and DAP12.(330, 331) Also, TGF-β interferes with INFγ production, inhibiting T-bet transcription factor SMAD3 and prevents IL-15 to activate NK cells, by targeting mTOR metabolic pathway.(332, 333) NK cells rely on glycolysis as energy source to accomplish their functions. A recent study on infiltrating NK cells in lung cancer showed that TGF-β promotes gluconeogenesis instead, upregulating the enzyme fructose bisphosphatase 1 (FBP1), resulting in a reversible dysfunction. In fact, pharmacological inhibition of FBP1 is able to re-establish normal NK functions at least at an early stage of cancer promotion.(325) Finally, TGF-β is able to convert NK cells into ILC1, which lacks of cytotoxic activity.(334)

In TME, **prostaglandin E2 (PGE2**) is secreted by cancer cells, TAMs, MDSCs, DCs and fibroblasts.(335) PGE2, also known as dinoprostone, is a lipid molecule synthetized by COX enzymes, overexpressed in many cancers.(336) On NK cells, it binds the membrane receptors E-prostanoid 2 and 4 (EP2, EP4), downregulating the expression of important activating receptors, such as NKp30, NKp44, NKp46 and NKG2D.(337) Other than the cytotoxic activity, PGE2 is also able to interfere with NK maturation and recruitment in the TME.(338) Importantly, PGE2 inhibits the secretion of INFγ and TNFα, through EP4 binding, and increases the expression of the inhibitory complex NKG2A/CD94, reducing the cytolytic potential.(339, 340)

Another mechanism through which TME negatively interacts with NK cells is the production of **indolamine 2,3-dioxygenase (IDO),** an enzyme overexpressed in several cancers,(341) which converts tryptophan in different metabolites with immunosuppressive potential. Among those, L-Kynurenine has an important effect on

NK cells. In particular, this metabolite jeopardises the promoting effect of IL-2 on the expression of NKp46 and NKG2D, decreasing the ability to recognise those targets expressing NKp46 and NKG2D ligands. However, this effect is reversible and does not affect NKp30. The molecular mechanism underneath this process has not been clearly understood.(342)

An important role on the suppression of NK function is played by **hypoxia**, a pivotal characteristic of the TME, as already discussed in chapter 1.4.2.

Hypoxia is detrimental on NK cells cytotoxicity, as it directly induces downregulation of all NCRs, NKG2D, and inhibition of the secretion of perforins and granzyme B.(343) Also, the accumulation of lactate in response of hypoxia promotes apoptosis, as seen in infiltrating NK cells in colorectal liver metastasis, and downregulates the expression of NKp46.(344, 345) Moreover, hypoxia induces the synthesis of adenosine, which is a potent suppressor of NK activity through the A2a receptor (ADORA2A).(346) Interestingly, in prostate cancer hypoxia supports tumour escape by shedding MICA/B from the surface of cancer cells, through a mechanism that involves nitric oxide signalling.(347) This is also boosted by the secretion of metalloproteases in the TME, which facilitates the release of soluble ligands, such as MICA/B, which interact and saturate the activating receptors on NK cells.(199) Finally, hypoxia compromises the ability of NK cells to detect metastatic cells inducing the release of exomes containing TGF-β and miRNA targeting NKG2D receptor.(348)

1.4.4 Immune landscape in PDAC

The development of new biotechnologies has improved our understanding of the genomic and transcriptomic alterations in PDAC cancer. The transcriptional analysis of bulky tumour samples has helped to describe different subtypes of PDAC correlating with histopathological characteristics.(349-352) Moreover, different genomic phenotypes correlate with specific immune signature and therefore prognosis. For example, cancer cells with microsatellite instability evoke an important T cells infiltration and are more sensitive to immunotherapy.(353) All these studies, although offering different useful classifications, agree on the heterogeneity of the cellular component in PDAC. Therefore, the limitation of bulky tumour analysis. More recently, *Peng et al.,* using single-cell RNA-sequencing technology, demonstrated that the specific cancer cells component is characterised by two different type of duct cells, named type 1 and type 2. Type 1 duct cells still express genes related to normal pancreatic functions, such as digestion and bicarbonate transport, and are present both in cancer and normal tissues. Type 2 duct cells represent the proper malignant cell and express genes related to cell proliferation, migration and hypoxia. Moreover, a sub-analysis of T-cells, TAMs and fibroblasts confirmed previous data on the heterogeneity of the TME and identified different cluster of expression for each cell types. In particular, 5 subtypes of T cells expressing at different levels genes related to CD8, cytotoxicity, memory T-cells and T_{reg}, and 5 subtypes of TAMs, expressing genes involving in class II antigen presentation, remodelling of ECM, chemokines recruiting MDSC, such as CCL2, or cytotoxic T cells, such as CXCL9 and CXCL10. Therefore, based on this latter analysis on TAMs, the Authors suggested that the conventional M1 vs. M2 classification is too simplistic and does not reflect the variability showed by single cells analysis.(354)

PDAC is generally described as poorly immunogenic tumours, implying that, in the large majority of the cases, tumour cells show a low mutation rate and an immunosuppressive infiltrate. In fact, MDSCs and T_{reg} represent the two main lymphocytes populations and are found since the early PanIN stages, indicating their key role in immune escape.(355, 356) Also, TAMs express CCR2 which promotes tumour proliferation, angiogenesis and recruitment of other immune suppressive cells. The inhibitory receptor CD40 is also highly expressed in PDAC and correlates with staging and prognosis,(357) while EMT-promoting factors and overexpression of the checkpoint ligands PDL1 and PDL2 have also been reported and correlate with a scarce immune infiltrate.(355, 356) In particular, PDL1 is overexpressed in almost 40% of tumour cancer, and in DCs, macrophages and T_{reg}. (358) This “immune-evasive” phenotype is also supported by the secretion of high level of immune suppressive cytokines, such as IL-6, IL-10, TGF-β.

Opposite to this scenario, some PDAC could show a highly cytotoxic phenotype, characterised by a microenvironment rich in $CD4^+$ and $CD8^+$ T cells, peritumoral B cells, M1 macrophages and presence of tertiary lymphoid tissues (TLTs), which grant a strong anti-tumoral effect. As result, the numbers of T_{reg} and TAMs are extremely reduced. This phenotype holds the best prognosis and survival.

A last possibility is a mixed phenotype, also defined as “immune-exhausted”, in which PDL1 is extremely upregulated. This phenotype shows a rich immune infiltration, which probably exerts a high selective pressure leading to the development of an immune evasive mechanism based on the PD1-PDL1 axis. Tumours arising from IPMN display this phenotype and are characterised by microsatellite instability.(359)

Eventually, the biological behaviour of this phenotype becomes similar to classical “suppressive” one.(356)

Being TAMs and T cells the main infiltrative populations, less information on how PDAC escapes NK cells recognition is currently available. Several studies have compared the relative number of NK cells in PDAC versus healthy population, but the results are contradictory and based on small cohorts.(360, 361) Also, the majority of studies focus on circulating NK cells, demonstrating an impaired phenotype characterised by reduced cytotoxic activity: the production of granzyme B and perforin are impaired either at mRNA level or protein level;(324, 362) the expression of important activating receptors such as NKp46 and NKp30 is suppressed;(324) DNAM-1 and NKG2D are also suppressed, despite their ligands CD155 and MICA/B respectively are found on tumour cells and TME.(363) All these characteristics have been found to correlate with prognosis and survival.(324, 363)

Speculations on how PDAC is able to influence NK cells functions have taken into account several factors. Firstly, the degree of differentiation of pancreatic cancer cells: PDAC tends to express high level of MHC-I and ICAM-I, while NK cells preferably target cancer stem cells, as they express high level of MICA/B.(364, 365) Secondly, as discussed in chapter 1.4.2, PDAC microenvironment is extremely hypoxic and rich in lactic acid. This has a detrimental effect on NK metabolism, but also contribute to the shedding of MICA/B, significantly impairing the cytotoxic activation mediated by NKG2D. The proposed mechanisms have already discussed in chapter **Error! Reference source not found.**.(345)

Finally, the cellular component of pancreatic TME influences NK cells phenotype and functions. NK cells are attracted by CAFs rather than qPSC, being therefore segregated into the fibrotic component of the TME and not be able to reach in proximity of cancer cells.(366) Moreover, the suppressive influence of the infiltrating T_{reg} and TAMs has a detrimental effect on NK cells. In particular, these cells are a major source of TGF-β and IL-10, whose roles on suppressing NK cells have already been discussed in chapters 1.4.2 and **Error! Reference source not found.**. Finally, deficient NK cells are not able to recruit DCs from the bloodstream, as they are the main source in the TME of CXCL1 and CCL5, chemoattractant for DCs. Moreover, NK cells produce fms-like tyrosine kinase 3 ligand (FLT3L), a potent activator of DCs. Being this cross-talk impaired, the ability to process the tumoral antigens is also undermined.(367)

1.4.5 NK based immunotherapy in PDAC

Immunotherapy has recently been proved to be a formidable tool in the fight against cancer, either solid or haematologic tumours. Despite most of the therapies are now focusing on T cells, NK cells show some promising advantages, including the development of chimeric antigen receptor-NK cells (CAR-NK cells).(368, 369) CAR-NK cells are genetically engineered cells, “forced” to express particular antigen receptors. Preclinical studies with NK cells have been targeting CD19, CD20, CD224 and HER2.(370)

NK cells-based therapies can induce a potent cytotoxicity against cancer cells without antigen recognition or clonal differentiation once activated, as T cells.

Expressing CD16, NK cells can be used in combination with mAbs to boost ADCC response, like for example the anti-EGFR cetuximab in colo-rectal cancer or the anti-ErbB2/HER2 trastuzumab in breast cancer. Also, NK cells do not induce the “cytokine storm”, a typical side effect of T cells-based therapy, due to excessive release of pro-inflammatory cytokines. This could sometime be fatal. NK cells have a short half-life, therefore NK cells-based therapies are not expected to induce side effects in the long run, such as auto-immunity or secondary malignancies. Finally, no graft-versus-host disease has been reported.(371)

Despite these interesting features, NK cells-based therapy shows some limitations. Technically, NK cells are difficult to obtain and manipulate, even when viral vectors are used.(372) Most importantly, the infusion of NK cells or CAR-NK *in vivo* shows low persistence and low ability to infiltrate the tumour.

To overcome the limitations, some strategies have been adopted in pancreatic cancer: pre-activation with cytokines, such as IL-2, IL-15 or IL-18, in order to induce a memory-like phenotype;(373) the development of CAR-NK cells expressing IL-15 (ongoing trial, NCT03056339) or derived from a highly cytotoxic cell line, like NK92.(374)

NK cells activity against PDAC could also be boosted by the infusion of cytokines. The most tested cytokines is IL-2, but due its high toxicity and the contemporary stimulation of T_{reg}, its role is now restricted.(375) Other cytokines which do not stimulate T_{reg} have also been tested in PDAC, such as IL-15, IL-18 and IL-21, and they all show a good potential to promote NK cells infiltration and cytotoxicity.(376) IL-15 is a particularly interesting molecule, as it induces NK cells’ killing towards PSCs. Moreover, one of iIL-15 superagonist formulation (ALT-803) protects NK cells from

TGF-β suppression and promotes graft-versus-tumour effect in a murine module of B cell lymphoma.(376, 377)

Transfusion of *ex vivo* expanded NK cells have been tested either in mice and humans, with improved anti-tumour effect, reduction of metastasis and prolonged survival.(378, 379) Also, DCs based vaccines proved to have a beneficial effect on NK cells, and are currently tested in PDAC, either alone or in combination with chemotherapy.(376)

Finally, therapies blocking checkpoint inhibitors such as PD-1/PD-L1 and CTLA-4 have also been tested *in vivo* but showed limited improvement on NK cells activity against cancer cells.(380, 381) Conversely, the delivery of CD40L and 4-1BBL using a viral vector induced a strong NK activation and cytotoxic activity.(382)

In conclusion, NK cells-based therapies are promising weapons against cancer and pancreatic cancer in particular. Therefore, the urgent need to discover new targets, to make this approach more efficient and improve survival.

1.5 Hypothesis

Being an important cellular component of the innate immune system, NK cells have been demonstrated to efficiently protect not only against infections, but also in tumour surveillance. In particular, many studies confirm their key role in controlling blood cancers and circulating metastatic cells from solid tumours. However, their role in primary solid tumours is far to be demonstrated, partially because of the paucity of infiltrative cells, compared to other immune cells. For this reason, these cells could potentially be targeted to develop powerful immune strategies in oncology, and in particular in pancreatic cancer.

Pancreatic cancer is a major health problem: its incidence is increasing among Western populations; most patients have incurable disease at presentation; survival has not changed over the past 40 years and curative and non-curative treatments are potentially hazardous.(383) The need to improve the care, treatment and outcomes for patients with pancreatic cancer is therefore imperative.

In this view, the aim of this thesis is to elucidate the role of NK cells in pancreatic adenocarcinoma, in order to identify possible targets to be exploited in immunotherapy and thus improving overall survival in pancreatic cancer patients.

Chapter 2. Materials and Methods

2.1 Ethics and recruitment

This project is part of a larger study on pancreatic adenocarcinoma, titled “A comparative study of immunological and nutritional variable among patients with curable and incurable pancreatic cancer”. To independently collect the surgical samples, we first sought for ethical approval via IRAS platform. Once the protocol was granted the ethical acceptance (IRAS 199000), this was submitted to the Health Research Authority (HRA) and to UHB NHS Foundation Trust for local approval. After the reviews, the project was accepted and registered and subjected to the local authorities’ guidelines (REC 16/WM/0214, RRK5773 respectively). This study was also registered into the NIHR Portfolio (CRN007661, Study n. 31211). The entire process took almost nine months.

Patients were identified from the central specialist pancreatic MDT, to which all potential patients are referred to for clinical discussion. If surgery is the chosen option, the patient is asked to attend clinic, where the consent for surgery is taken. At this point, the HPB Consultant informs the patient on the possibility to take part into the research study and provide the Patient Information Sheet (PIS). The consent for taking part into the research was then signed on the day of the procedure on the ward, before being sent for surgery.

The blood samples were collected in the anaesthetic room, once general anaesthesia was induced and brought to the lab for being processed as soon as possible. The surgical specimens were sent fresh to the Pathology lab, where the tumour and normal tissue samples were collected by the HPB Pathology Consultant and brought to the lab to be processed and/or frozen down as soon as possible.

2.2 FACS analysis of PBMCs and TILS

2.2.1 PBMCs isolation from PDAC patients

Up to 25 ml of blood, distributed in 5 lithium heparin tubes (5x5ml), was collected from 54 patients by a Consultant Anaesthetist before surgery. Blood was then distributed in two 50 ml falcon tubes. An equal volume of RPMI-1640 was added and mixed. Blood was gently overlaid onto 15 ml of lymphoprep solution and was centrifuged at 2000 rpm for 30 minutes, with slow deceleration to prevent disruption of buffy layer. The interface layer was removed and transferred into a new 50 ml falcon tube. Fifteen millilitres of RPMI 1640 were added and then centrifuged again at 1500 rpm for 10 minutes to pellet PBMCs. Supernatant was then poured off and pellet resuspended in 20 ml of RPMI 1640. Cells were then counted on a haemocytometer. Between 0.5 and 1 x 10^6 cells were removed for flow cytometric staining. The remainder was centrifuged at 1200 rpm for 10 min to pellet PBMC, which were then cryopreserved in 90% FBS + 10% DMSO at approximately 5-10 x10^6 cells per vial.

2.2.2 PBMCs from healthy donors

Up to 25 ml of fresh blood were collected from 15 healthy donors and prepared according to the procedure described in chapter 2.2.1. Other 19 PBMCs were defrosted for 1-2 minutes in warm bath and wash with RPMI 1640, in 15 min Falcon tube. They were then gently centrifuged at 1000 rpm for 12 minutes. Approximately 5 x10^5 cells were transferred into FACS tube for staining.

2.2.3 Tumour Infiltrating Lymphocytes (TILs) collection and staining

The surgical specimen is sent fresh to Pathology, where a dedicated HPB Pathologist processes the specimen and provides the tumour tissue and the macroscopically uninvolved adjacent pancreatic tissue. The two tissues are then cut in 1-2 mm pieces, and either used to isolate immune and tumour cells, culture fibroblasts or cryopreserved at -80°C for future use.

The tumour pieces are placed in a 12 wells plate and incubate overnight in tissue media culture (RPMI 1640 + 10% FCS+1% Penicillin/Streptomicin + 1% L-Glutamine), in order to allow the TILs to spread out. The media is then collected the following day, and spin down at 1600 rpm for 5 minutes.

2.2.4 NK cells staining

Approximately 0.5-1x10^6 PBMCs and TILs cells were collected and transferred to FACS tubes. Two panels of antibodies were used. Details can be found in Table 2.1 were added. Cells were incubated for 30 minutes at 4°C, in the dark. Propidium Iodide (Thermo Fischer Scientific, USA; Catalogue number P1304) was used as viability dye. Tubes were washed with MACS buffer (PBS + 0.5% BSA + 2mM EDTA) and centrifuged at 1500 rpm for 5 min.

Table 2.1 Antibodies for FACS analysis

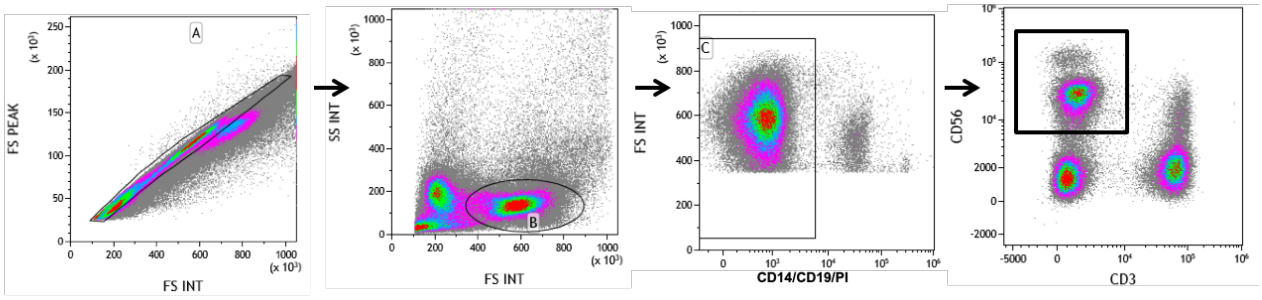
Panel 1	Clone	Company	Panel 2	Clone	Company	FC colour	Concentration
DNAM-1	11A8	BioLegend	**NKG2C**	11A8	Milteny Biotec	FITC	5 μl
PD-1	EH12.2H	BioLegend	**CD94**	DX22	BioLegend	PE	3 μl
CD14/CD19	HCD14/H1B19	BioLegend	**CD14/CD19**	HCD14/H1B19	BioLegend	PE dazzle	1.5/2.5 μl
NKp46		BioLegend	**KIRS CD16***	HP-MA4	BioLegend	PerCP/Cy5.5	5 μl
CD56	HCD56	BioLegend	**CD56**	HCD56	BioLegend	PECy7	3 μl
NKG2D	Not specified	BD Biosciences	**NKp30**		BioLegend	APC	5 μl
CD3	HIT3a	BioLegend	**CD3**	HIT3a	BioLegend	AF700	2.5 μl
CD16	3G8	BioLegend	**CD57**	HNK-1	BioLegend	Pacific Blue	5 μl

*7 PDAC and 5 HD

2.2.5 Flow Cytometer analysis

Antibody-stained samples were run on a Beckman Coulter Gallios flow cytometer within one hour from staining. At least 100,000 events were acquired per sample using CytoSoftware and analysed offline using Kaluza v1.5 software (Beckman Coulter Life Sciences, Indiana, USA). Figure 2-1 illustrates the gating strategies used to select circulating and infiltrative NK cells. Results are presented as percentage (%) of positive cells for the relative gate, and as MFI (Mean Fluorescence Intensity), which indicates the median level of expression of the antibody in the gated population. We decided to use the median, instead of the arithmetic mean, due to the relatively small number of circulating NK cells in the whole PBMCs, the small size of the two cohorts and because of the huge variation of the markers' expression among patients.

1. Gating strategy for PBMCs flow cytometry



2. Gating strategy for TILs flow cytometry

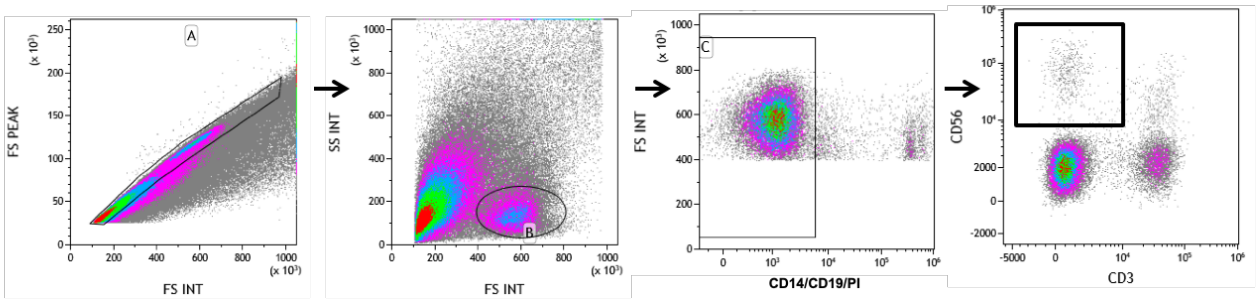


Figure 2-1. Gating strategies for flow cytometer analysis.

Gating strategies shown for the detection of $CD3^{neg}CD56^{pos}$. At first the singles were gated using gate A and subsequently lymphocytes were selected using gate B. Then, gate C marked the $CD3^{neg}CD56^{pos}$ NK cells, after the exclusion of the dead cells (PI^{+}), B cells ($CD19^{+}$) and monocytes ($CD14^{+}$).

2.2.6 Statistical analysis

Statistical analysis was performed with SPSS 25 (SPSS Inc., Illinois, USA) and Prism 8 (GraphPad, California USA), both for Macintosh. Each parameter was checked for normal distribution. The expression of the NK cells markers in the two groups (healthy donors vs. PDAC patients) was compared using a t-Student's test for parametric or Mann-Whitney U-test for non-parametric variables. To compare the expression in TILs vs. autologous circulating NK cells, a paired-t Student's test or the Related samples-Wilcoxon Signed-Rank test for non-parametric variables were used. A p value <0.05 was considered statistically significant.

2.3 NK cells functional assays

2.3.1 CD107a degranulation assay

2.3.1.1 Plate preparation

PBMCs from healthy donors and from PDAC patients were gently defrosted and resuspended at a concentration of $1x10^7$ ml with General Media (RPMI-1640 + 1% Penicillin/Streptomicin). The target cells (K562) were washed and resuspended at a concentration of $1x10^7$ ml. NK cells were isolated from PBMCs of healthy donors and PDAC patients using EasySep™ Human NK Cell Enrichment Kit (Stemcell Technologies, Canada; Catalogue number 19055).

In 96 wells round bottom plate, 100 µl of PBMCs are incubated with 100 µl of K562, at a concentration ratio of 1:5. The following scheme was used:

- 100 µl of NK cells + 100 µl of GM
- 100 µl of NK cells + 100 µl of GM + 3 µl of APC-conjugated CD107a antibody
- 100 µl of NK cells + 100 µl of K562 + 3 µl of APC-conjugated CD107a antibody

The plate is then incubated for 5 hours in 37ºC incubator. The media is then transferred into FACS tubes, topped with MACS buffer and wash at 1500 rpm for 5 minutes. Supernatant is the discharged, the pellet is resuspended in the residual volume and stained with CD3, CD56 and live dye. After 30 minutes of incubation at 4ºC and in the dark, cells are washed with MACS buffer and centrifuge at 1500 rpm for 5 minutes.

Flow cytometer analysis has been described in chapter 2.2

2.3.2 NK cytotoxic assay

At first, NK cells were enriched using EasySepTM™ Human NK cell enrichment kit (Stem Cell Technologies, Canada). Then, the NK cells were activated overnight with IFNα (Sigma Aldrich, USA; Catalogue number SRP-4596). Next day, K562 cells were first labelled with CFSE. Then $5x10^4$ K562 cells were co-cultured with the $5x10^4$ pre-activated NK cells at E:T ratio of 1:1 in 96-wells V-bottom plate, in 100µl volume. Every condition was triplicated. The cultures were incubated at 37°C for 16 hours before the analysis using BD Accuri™ flow cytometer (BD Bioscience, USA). PI was used as live dye to gate the live cells. The specific lysis was calculated on live cell absolute quantification.

2.3.2.1.1 Intracellular staining

PBMCs and K562 were co-cultured overnight with Brefeldin A (Sigma-Aldrich, USA; Catalogue number B7651) (1µl/ml) at 1:1 ratio. PBMCs/K562 culture were washed in MACS before been stained with appropriate surface antibodies and PB live/dead dye (Invitrogen, USA) for 40 minutes at 4°C. Then, the cells were washed in MACS buffer and fixed with 4% paraformaldehyde for 15 minutes at room temperature. The cells were permeabilised with saponin and stained with intracellular cytokines antibodies Table 2.2. The cells were washed again and finally analysed using flow cytometry, as described in chapter 2.2.

Table 2.2 Antibodies for intracellular staining

Antibody	Fluorochrome	Company	Clone	Concentration
TNFα	AF 488	BioLegend	Mab11	5 μl
IL-10	PE	BioLegend	JES3-19F1	5 μl
CD16	PE-Dazzle	BioLegend	3G8	5 μl
CD3	PerCP/Cy5.5	BioLegend	SK7	2.5 μl
CD56	PE/Cy7	BioLegend	5.1H11	3 μl
IL-5	APC	BioLegend	TRFK5	5 μl
INFγ	AF 700	BioLegend	B27	5 μl
IL-4	APC-Cy7	BioLegend	MP4-25D2	5 μl
CD19	PB	BioLegend	HIB19	2.5 μl
IL-2	BV 510	BioLegend	M-A251	5 μl

2.3.3 Statistical analysis

Statistical analysis was performed with SPSS 25 (SPSS Inc., Illinois, USA) and Prism 8 (GraphPad, California USA), both for Macintosh. Each parameter was checked for normal distribution. The expressions of CD107a and the cytokines in the two groups (healthy donors vs. PDAC patients) were compared using Mann-Whitney U-test for non-parametric variables. A p value <0.05 was considered statistically significant.

2.4 RT-qPCR

2.4.1 Tissue collection

The tissue collection was previously described in chapter 2.2.3. The frozen tissues, either tumour or normal pancreatic sample, were defrosted and immediately processed for RNA extraction.

2.4.2 Real Time–quantitative PCR

RNA was extracted from normal pancreatic tissue and tumour according to manufacturers' instruction (RNeasy Mini Kit (50), Quiagen, USA). Briefly, the tissue was disrupted and centrifuged for 3 minutes at maximum speed, with 350-600 μl of Buffer RNA*later*® (RLT), according to the amount of tissue available. 1x 70% of ethanol was added, then 700 μl of the samples was centrifuged again for 15 sec at >8000g. 700 μl of Buffer RW1 (included in the kit) was added, and the sample spin up again at >8000 x g. Finally, 500 μl of Buffer RPE was added and the sample centrifugated again at >8000 x g. This final step was repeated twice, first for 15 sec and last spin for 2 minutes.

RNA concentration was detected and quantified with Nanodrop Spectrophotometer (v1.8, ThermoFischer Scientific, USA) and recorded. Then, RNA was converted to cDNA: 1 μg RNA is mixed with 4 μl of iScript Reverse Transcriptase (BioRAD, Hercules, USA) and DNA free water, for a total of 20 μl. The reaction mix is incubated in Eppendorf® Mastercycler, according to the following protocol:

1. Priming, 5 minutes at 25°C
2. Reverse transcription, 20 minutes at 46°C
3. Reverse transcriptase inactivation, 1 minute at 95°C
4. Holding time, at 4°C

The reaction mix is then diluted to 200 µl to reach a concentration of 5µg/µl with DNA free water.

In a 90 wells plate for qPCR, 5 µl of cDNA is added to 20µl of cocktail solution (1.2 µl of primer + 12.5 µl of TaqMan Universal Mastermix + 6.25 µl of DNA free water). The primers tested are listed in Table 2.3. Finally, the plate is run in RT-qPCR reader for 50 cycles (7500 System SDS software, v1.5.1, Applied Biosystems, Invitrogen, USA).

Table 2.3 List of primers for RT-qPCR

Primer	Company	Assay ID	Reporter	Quencher	Concentration
MIC-A	Thermo Fisher	Hs00741286_m1	FAM	none	1.2 µL
MIC-B	Thermo Fisher	Hs04187752_m1	FAM	none	1.2 µL
ULBP 1	Thermo Fisher	Hs00360941_m1	FAM	none	1.2 µL
ULBP 2	Thermo Fisher	Hs00607609_mH	FAM	none	1.2 µL
ULBP 3	Thermo Fisher	Hs00906262_m1	FAM	none	1.2 µL
ULBP 4	Thermo Fisher	Hs01026642_m1	FAM	none	1.2 µL
ULBP 5	Thermo Fisher	Hs01584111_mH	FAM	none	1.2 µL
ULBP 6	Thermo Fisher	Hs04194671_s1	FAM	none	1.2 µL
HLA-A	Thermo Fisher	Hs01058806_m1	FAM	none	1.2 µL
HLA-DRA	Thermo Fisher	Hs00219575_m1	FAM	none	1.2 µL
HLA-E	Thermo Fisher	Hs03045171_m1	FAM	none	1.2 µL
HLA-C	Thermo Fisher	Hs00740298_g1	FAM	none	1.2 µL
PVR	Thermo Fisher	Hs00197846_m1	FAM	none	1.2 µL
PVRL2	Thermo Fisher	Hs00607609_m1	FAM	none	1.2 µL
PDL-1	Thermo Fisher	Hs00204257_m1	FAM	none	1.2 µL
PDL-2	Thermo Fisher	Hs00228839_m1	FAM	none	1.2 µL
GADPH	Applied Biosystems	4310884E	VIC	TAMRA	0.5 µL
18S	Thermo Fisher	Hs03003631_g1	FAM	none	1 µL
B2M	Thermo Fisher	Hs00187842_m1	FAM	none	1 µL
TBP	Thermo Fisher	Hs00427620_m1	FAM	none	1 µL

The housekeeping genes GADPH, 18S, B2M and TBP were tested in normal and tumour tissue, and 18S was chosen for its stability. The primers listed in Table 2.3 were compared against 18S in term of number of cycles. For each primer, a standard curve was created, to convert the number of cycles into copy number, expressed as logarithmic value (Figure 2-2).

2.4.3 Statistical analysis

Statistical analysis was performed with SPSS 25 (SPSS Inc., Illinois, USA) and Prism 8 (GraphPad, California USA), both for Macintosh. All variables were checked for normal distribution. The Related samples-Wilcoxon Signed Rank test for paired non-parametric values was used to compare the log of copy numbers in normal and tumour tissues. A p value <0.05 was considered statistically significant.

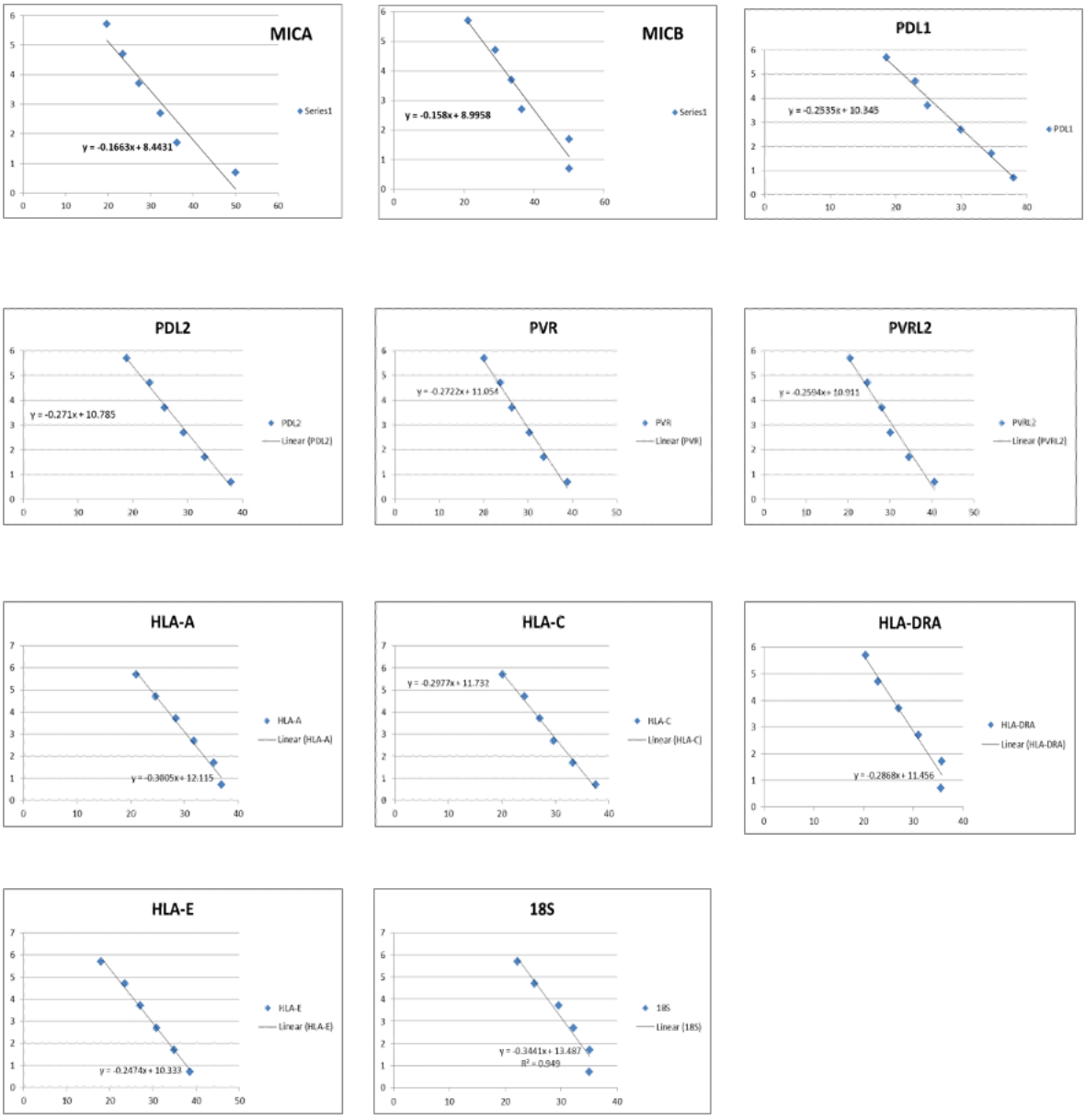


Figure 2-2. Standard curves

The housekeeping genes GADPH, 18S, B2M and TBP were tested in normal and tumour tissue, and 18S was chosen for its stability. The primers were compared against 18S in term of number of cycles. For each primer, a standard curve was

created, to convert the number of cycles into copy number, expressed as logarithmic value. Statistical analysis was finally carried out.

2.5 Sandwich ELISA

2.5.1 Sera collection and preparation

One 5 ml blood sample (no-anticoagulant-red lid) was collected from patients by the Anaesthetist in the anaesthetic room, prior to surgery. This was centrifuged at 2200 rpm for 5 min (brake on). Around 2 ml of serum was removed and cryopreserved at -80°C.

Sera from healthy donors and from patients with pathological proven PDAC were defrosted. The following soluble NK ligands were tested: ULBP 1, ULBP 2, ULBP 3, MICA, MICB (all DuoSet® ELISA, R&D Systems, USA) and PVR/CD155 (LifeSpan BioSciences Inc., USA).

2.5.2 ELISA

All samples were run in duplicate in a 90 wells plate, according to manufactory's instruction. Briefly, each plate was coated with capture antibody over night at room temperature and then washed three to four times with wash buffer (PBS + 1% Tween 20). A 50 µl sample per well and an 8-fold standard solution are added and incubated at room temperature for two hours. After a carefully wash, 100 µl of secondary detection antibody is added and incubated at room temperature for 1 hour. 100 µl of Streptavidin-HRP complex and, after 20 minutes of incubation, 100 µl of TMB Substrate Solution are added. At this point, positive wells acquired a blue colour,

according to the concentration of the target ligand. Finally, 100 µl of Stop Solution (1M HCl) is added and the blue positive colour turns into a bright yellow.

The ELISA experiment for PVR/CD155 differed from the others, as the plate was sold pre-coated. Main difference was the incubation at 37ºC for 1 hour.

The Optical Density (OD) of each well is then immediately determined using a microplate reader set to 450 nm with a wavelength correction set to 650 nm, to correct the optical imperfection in the plate.

2.5.3 Statistical analysis

Statistical analysis was performed with SPSS 25 (SPSS Inc., Illinois, USA) and Prism 8 (GraphPad, California USA), both for Macintosh. All variables were checked for normal distribution. Concentrations in the two groups were compared using Mann-Whitney U test. A p value<0.05 was considered statistically significant.

2.6 Co-culture of organoid and autologous PBMCs

2.6.1 Development of Organoids

According to *Hill et al.*, the tumour was diced into approximately 2 mm sections and then crushed with the butt of a syringe. This homogenate was then poured into basal culture media containing Type II Collagenase (Life Technologies, USA; Catalogue number 17101015) at a final concentration of 2.5mg/ml. The homogenate was shaken at 37°C for 60 minutes and then diluted 1:1 with basal culture media and filtered through a 70uM filter (Falcon Catalog number 352350). The cell suspension was then spun at 1000RPM to create a cell pellet. The pellet was washed with red blood cell lysis buffer two to three times, and then washed once with basal culture media.

For general culture, once a pellet of either liquid or solid tumour cells was obtained, the cells were mixed with growth factor reduced Matrigel® (Corning, USA; Catalog number CB-40230C), with the final concentration of Matrigel at 75%, The suspension was then rapidly plated into a 24-well plate with 50uL of suspension per well. Once the Matrigel was solidified, 600uL of general culture medium was added to each well.

General culture medium was composed of Advanced DMEM/F12, supplemented with 1% penicillin streptomycin, 1% Glutamax, 1% HEPES, 100 ng/ml R-spondin 1 (Peprotech, UK; Catalogue number 120-38), 100 ng/ml Noggin (Peprotech, UK; Catalogue number 120-10C), 50 ng/ml EGF (Peprotech, UK; Catalogue number 100-15), 10 ng/ml FGF-10 (Peprotech, UK; Catalogue number 100-26), 10 ng/mL FGF2 (Peprotech, UK; Catalogue number 100-18B), 1x B27 (Life

Technologies, Catalog number 17504044), 10 mM Nicotinamide (Sigma-Aldrich, USA; Catalog Number N0636), 1.25mM N-acetylcysteine (Sigma-Aldrich, USA; Catalog number A9165), 1uM Prostaglandin-E2 (R&D Systems, USA; Catalog number 2296), 10uM SB202190 (Sigma-Aldrich, USA; Catalog number S7076), and 500ng A83-01 (Sigma Aldrich, USA; Catalog number SML0788).(384)

The medium was replaced every 3-4 days, and the matured organoids will be passaged 1:3 every 14 days. The basic phenotype was checked using flow cytometry staining with anti-EpCAM.

2.6.2 Co-culture of Organoids with autologous PBMC

Autologous PBMCs were defrosted. Then $1x10^6$ PBMCs with $2x10^5$ Organoids were mixed with Matrigel before they were plated on a 24-well plate. After the Matrigel was set, 600 μl Organoids medium was added. The PBMCs-only control was also setup with the same medium and condition. 200IU/ml IL-2 were added 3 days after the co-culture was setup. Co-cultures were harvest at day7 and day14, and the phenotype of NK cells were studied using the two flow panels described in chapter 2.2 and listed in Table 2.1. Due to the big variation of different donors, the data were shown as the relative level of the expression compared with the control medium only PBMCs.

2.7 Immunohistochemistry

Formalin-fixed paraffin-embedded slides from ten patients diagnosed with pancreatic cancer were selected and cut by Pathology Consultant with a specific interest in HPB disease. The slides included PDAC and sections of normal pancreatic tissue.

2.7.1 Antigen retrieval

The slides were firstly dewaxed in Histoclear for 10 minutes, then placed in IMS for 5 minutes. While the slides were rinsing in tap water, a Citrate buffer was prepared (1 litre distilled water + 1.26 gr Sodium Citrate + 0.25 gr Citric Acid). The buffer was brought to boiling temperature with microwave, and the slides immerse and boiled for 10 minutes at medium power and 10 minutes at low power. Then, the slides were left cooling down in cold room (4°C) for 15-20 minutes and then washed in running water for other 5 minutes. Subsequently, the slides were conditioned with TBS and blocked with 100μl/slides of 5x casein for 10 minutes. 100μl of primary antibody was therefore applied to each slide and incubated overnight in cold room.

2.7.2 Staining

The slides were thoroughly washed with TBS. 100 μl/slides of the secondary antibodies listed in Table 2.5 were applied for 30 minutes, followed by 1-2 drops of Vector Real EnVision HRP Rabbit. They were then washed with TBS and DAB substrate was applied (1 ml buffer + 1 drop DAB). The slides were rinsed in tap water for 5 minutes,

stained in Meyers Haematoxylin for 2-5 minutes and then rinsed again in warm tap water until blue colour developed. They were left at increasing concentration of IMS for a total of 10 minutes and eventually placed in Histoclear for 10 minutes. The cover slip was mounted with Omnimount 1-2 drops and left to dry for one hour.

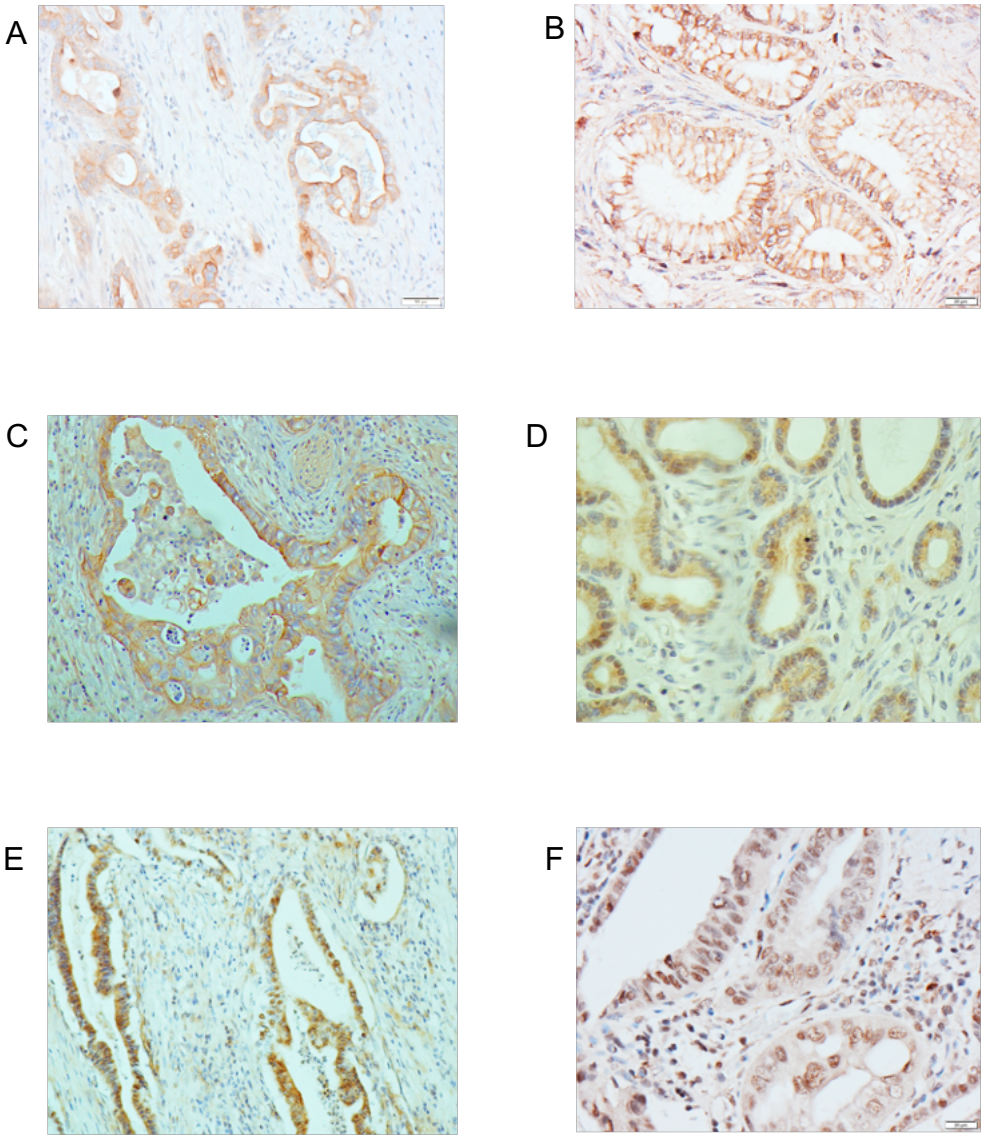


Figure 2-3 Immunohistochemistry on historical slides.

Examples of IHC performed on historical slides to confirm expression of NK cells activating ligands by PDAC. The historical slides were selected and cut by a Pathology Consultant with a specific interest in HPB disease. All pictures were taken at x20 magnification. A. MICA/B; B. PVR; C. PVRL2; D. PDL1; E. PDL2; F. ULBP2.

Table 2.4 Lists of Reagents

Reagents	Company Name	Catalogue no
RPMI-1640	Sigma-Aldrich	R0883
Lympholyte	Cederlane	CL5020
Fetal Bovine Serum (FBS)	Life Technologies, Gibco	2614007
DMSO	Sigma-Aldrich	D2650
BSA	Sigma-Aldrich	
Propidium Iodide	BioLegend	B283509

Table 2.5 List of secondary antibodies for IHC

	Company	Catalogue n.o	Isotype	Concentration
MICA/B	R&D System	MAB13001-100	monoclonal IgG2A	1:200
CD56	Atlas Antibodies	HPA039835	Polyclonal IgG	1:200
PDL1	BioLegend	329710	monoclonal IgG2B	1:200
PDL2	BioLegend	345404	monoclonal IgG1	1:200
PVR	Atlas Antibodies	HPA012568	polyclonal IgG	1:200
PVRL2	Atlas Antibodies	HPA012759	polyclonal IgG	1:200
ULBP2	ThermoFisher Scientific	MA5-29636	monoclonal IgG	1:200

Chapter 3. Phenotypic differences in circulating NK cells between PDAC patients and Healthy Donors

3.1 Introduction

NK cells are an important component of the innate immune system and are crucial for controlling cancer's growth. Most of the studies has been focused on the circulating NK cells, due to the limited number of tissues infiltrating NK cells.

In this study, 54 PBMCs from patients undergoing surgery for PDAC were analysed by flow cytometry and compared to 38 PBMCs collected from healthy donors (HD). These are young people who have previously donated their blood for clinical purpose and for research. We do not have much clinical information on these donors, apart from the age, which unfortunately could not be matched with our cohort of patients. The PBMCs were stained with two 10-colors flowcytometry panels and the composition and phenotype of NKs were compared between PDAC patients and age-matched HD. As the two subsets of NK cells $CD56^{bright}$ and $CD56^{dim}$ are supposed to have different roles, the first be regulatory and the latter the true cytotoxic population, the composition of NK cells was analysed and compared based on CD56 and CD16 expression. Finally, the expression of main activating receptors and inhibiting receptors were analysed in patients with PDAC and compared with healthy donors.

3.2 There are no differences in total $CD3^{-}CD56^{+}$ NK cells percentage

The PBMCs from patients with PDAC and HD were stained with panel 1 and panel 2 antibodies listed in Table 2.1. At first, the singlets were gated through Forward Scatter Height vs. Forward Scatter Area. Then the forward and side scatter density plots were used to identify lymphocytes and exclude debris. Subsequently, the live dye, CD14 and CD16 antibodies were used to exclude dead cells and monocyte and B cells. At last, the whole NK population was gated according to CD3 and CD56 expression. The $CD3^{neg}CD56^{pos}$ gate defines the NK cells, and clearly it is separated in two main clusters, which according to CD56 intensity are defined $CD56^{dim}$ and $CD56^{bright}$ (Figure 2-1).

After the NK cells were identified, the percentage of NK cells from whole PBMC population was studied. The percentage of NK cells varies hugely among donors: in our HD cohort, the percentage of NK cells varies from 4.78% to 59%, while for the PDAC group, the range was from 2.2% to 43.6%. Despite this difference, representative individual dot graphs indicate that, overall, the total percentage of NK cells in PDAC did not differ from HD (21% vs. 16%, p=0.12, respectively).

However, the subclassification according to the intensity of the expression of CD56 indicates that the $CD56^{bright}$ population is reduced in the PBMCs of PDAC patients (0.7% vs. 1.35%, p=0.0026), while the $CD56^{dim}$ population remains stable, despite a slight reduction, which does not reach the statistical significance (16% vs. 20%, p=0.3).

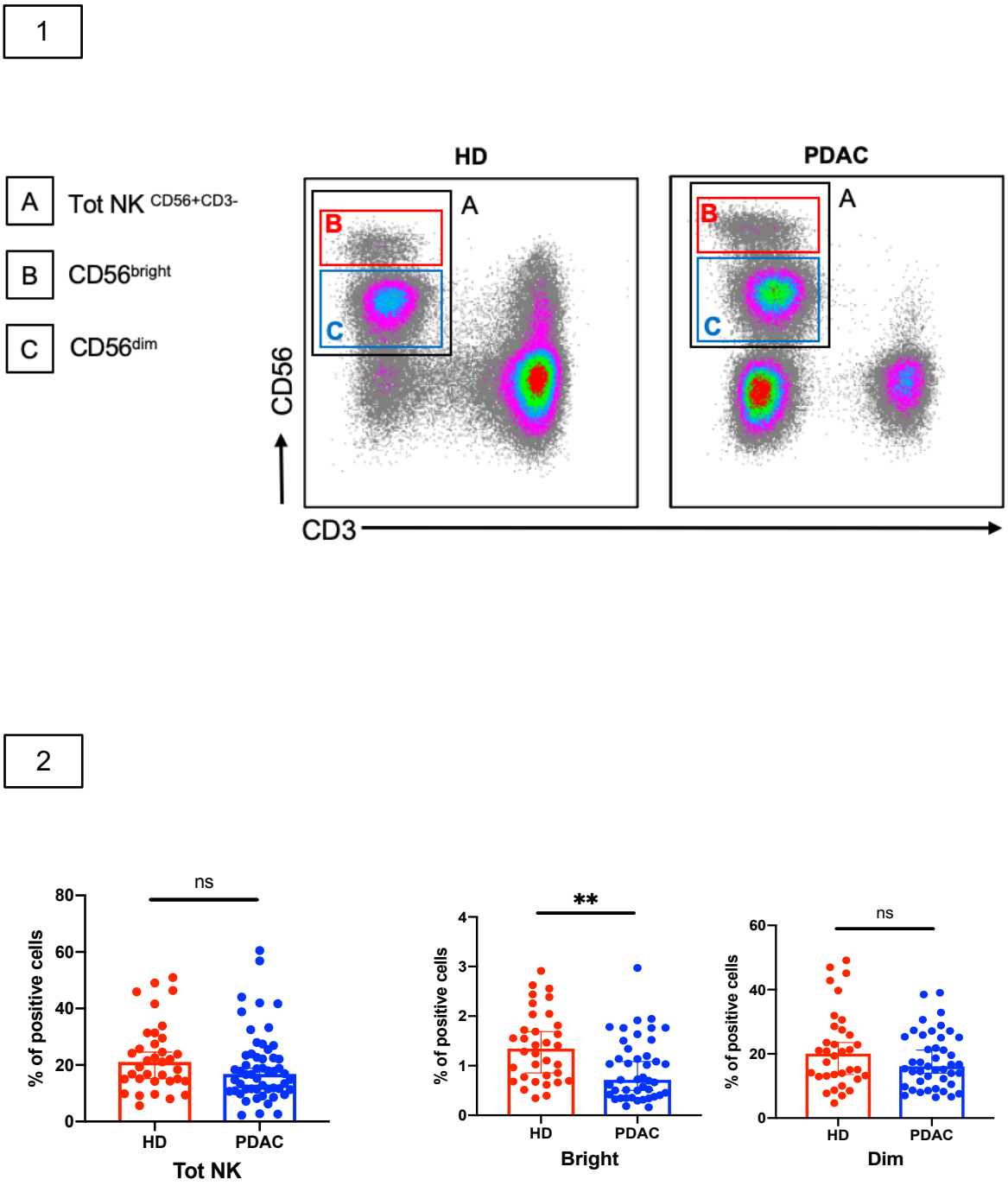


Figure 3-1. Total relative number of circulating NK cells is similar in HD vs. PDAC

1. An example of dot plot from flow cytometry staining, to gate the NK cells using $CD3^{neg}CD56^{pos}$ as makers; **2.** Percentage of the total NK cells and the two subsets in

the bloods from PDAC patients and HDs were compared individually, using scatter plot with bars, with each point represent a donor. Significance was determined using Mann-Whitney U-testing, $p < 0.01$(**).

3.3 Activating receptors are downregulated on NK cells from PDAC patients

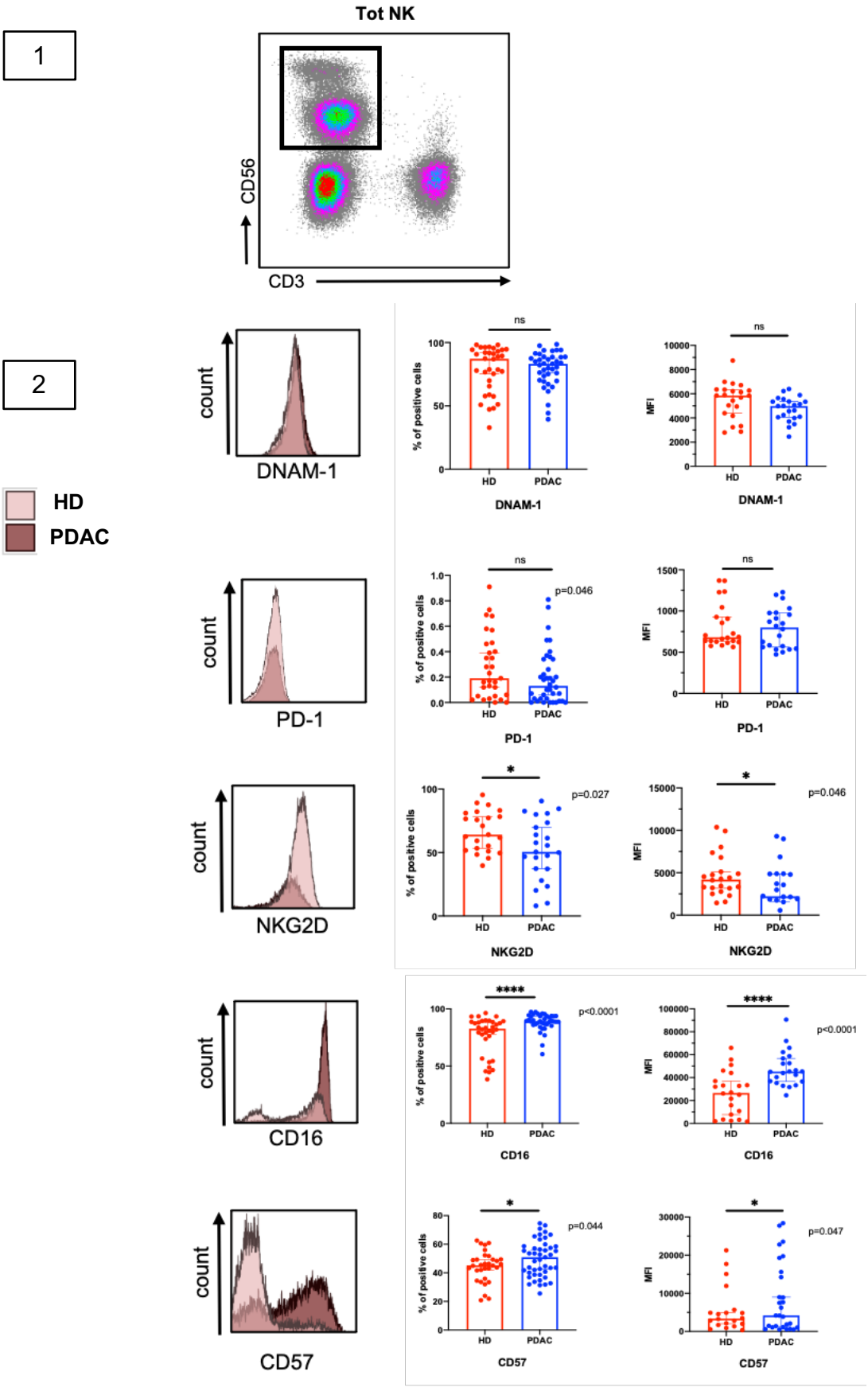
After the percentage of NK population was compared, the phenotype of NK cells was compared between PDAC and HD as well. In the two panels, activating receptors, including NKp30, NKp46, NKG2D, DNAM-1, CD57 and inhibiting receptors, including KIRs, NKG2C were studied and compared. The overall data revealed that circulating NK cells in PDAC patients show a functionally impaired phenotype compared to HD, with a lower expression of important activating receptors. Here, we show in detail the level of expression of each receptor, either in the whole NK cells or in the two subpopulations, $CD56^{dim}$ and $CD56^{birght}$. Figure 3-2, Figure 3-3 and Figure 3-4 summarise our results.

While the down regulation of NKp30 involves the entire NK population (57% vs. 44%, p=0.017; MFI: 9298 vs. 8423, p=0.9) and the two subsets of $CD56^{bright}$ (78% vs. 57%, p=0.03; MFI: 9295 vs. 10588, p=0.6) and $CD56^{dim}$ (64% vs. 47%, p=0.023; MFI: 9341 vs. 8477, p=0.9), the DNAM-1 expression is markedly impaired in the $CD56^{bright}$ (99% vs. 90%, p<0.0001; MFI: 7526 vs. 7060, p=0.7) and $CD56^{dim}$ (97% vs. 82%, p<0.000; MFI: 2693 vs. 5628, p=0.5), but not in NK cells as a whole (87% vs. 83%, p=0.5; MFI 6348 vs. 5648, p=0.5). On the other hand, NKG2D is downregulated in the total circulating NK cells (63% vs. 50%, p=0.27; MFI: 4193 vs. 2214, p=0.04), but just the $CD56^{dim}$ shows a significant decrease in terms of MFI (4103 vs. 1962, p=0.04; 30% vs. 47.9%, p=0.7), as in the $CD56^{brigth}$ we did not find any differences in the level of expression (85% vs. 88%, p=0.3; MFI: 6587 vs. 7842, p=0.2). Also, the CD16 expression is markedly increased in the cytotoxic $CD56^{bright}$ population in terms of MFI

(1440 vs. 5370, p<0.0001) but not in positive numbers of cells (27% vs. 28%, p=0.9). Furthermore, CD16 expression shows a marked increasing in the whole population (83% vs. 89%, p<0.0001; MFI: 38054 vs 45245, p=0.0001), and in the $CD56^{dim}$ (87.5% vs. 94 %, p<0.0001; MFI: 39774 vs. 55261, p<0.0001).

The total NK cells and the $CD56^{dim}$ population are characterised by a significant increase of the expression of the maturation marker CD57 (44% vs. 50%, p=0.04, MFI: 5794 vs. 11401, p=0.047; 45% vs. 52%, p=0.17, MFI: 5196 vs. 7084, p=0.3, respectively), while the $CD56^{bright}$ population shows no differences (1.75% vs. 2.3%, p=0.6; MFI: 148 vs. 150, p=0.8).

We did not identify any significant differences in the expression of NKp46, NKG2C, CD94 or KIRS. These data are summarised in Table 3.1.


1
Tot NK
CD56
CD3
2
HD
PDAC
count
DNAM-1
% of positive cells
MFI
ns
HD
PDAC
DNAM-1
PD-1
p=0.046
NKG2D
p=0.027
p=0.046
CD16
****
p<0.0001
CD57
p=0.044
p=0.047

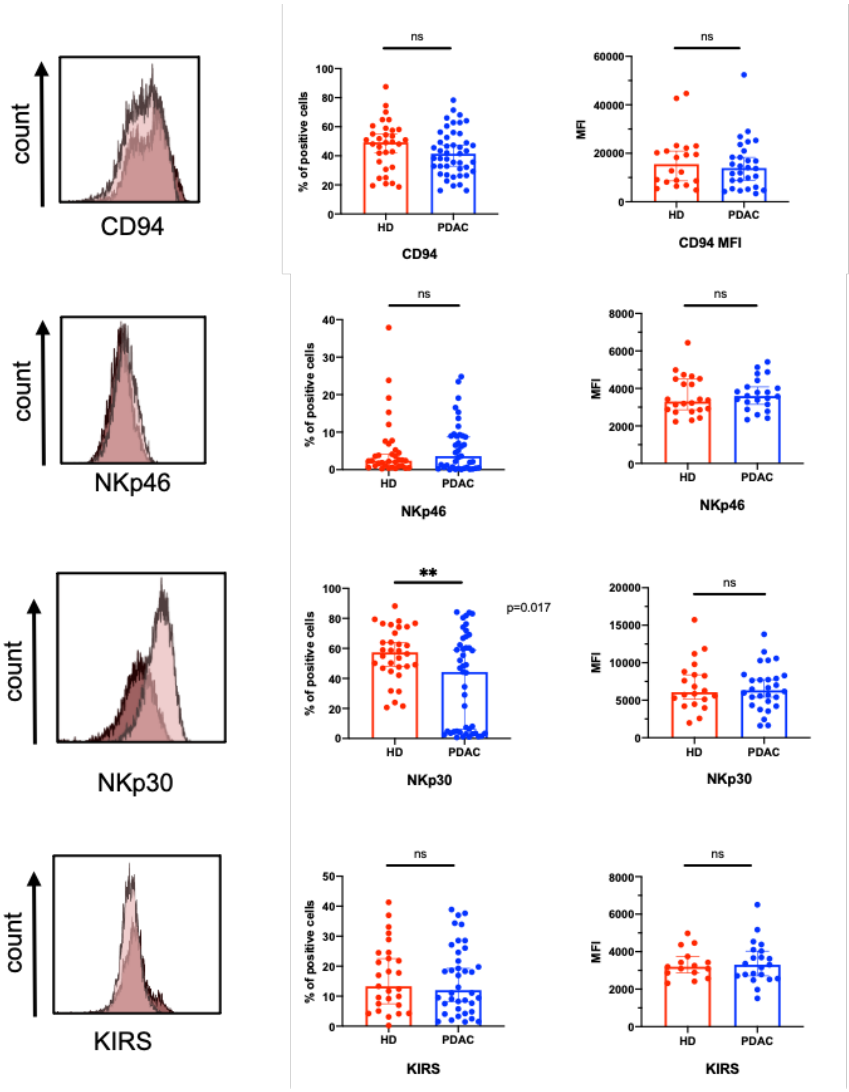
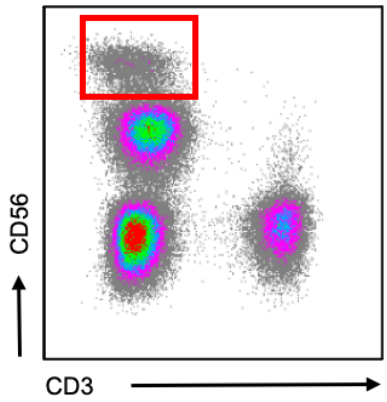


Figure 3-2. Total Circulating NK cells in PDAC patients show a highly differentiated phenotype and reduced expression of activating receptors.

1. Gating strategy for circulating Total NK cells (black rectangle); **2.** The expression of differentiation markers (CD16, CD57, NKG2C, CD94, PD-1) and activating or inhibitory makers (DNAM-1, NKG2D, NKp30, Nkp46, KIRs) was compared between PDAC patients and HD. On the left, the representative histogram of each marker; on the right,

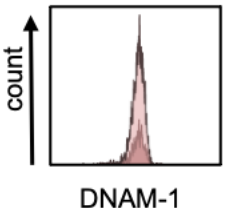
the scatter plots with bars: the comparison was carried out with percentage (middle) and mean florescence intensity (MFI) (right). Significance was calculated with t-Student's test or Mann-Whitney U-testing according to normal distribution; $p<0.05$ (*), $p<0.01$(**), $p<0.001$ (***), $p<0.0001$ (****)

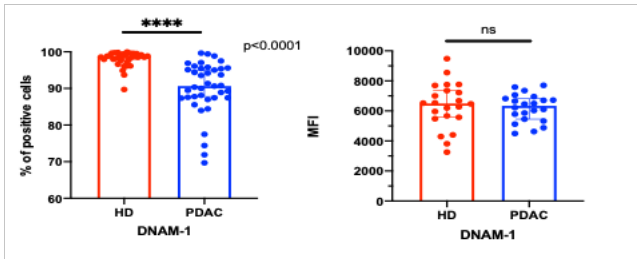
1

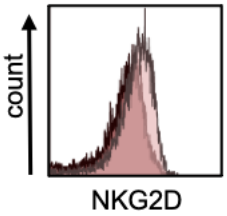

CD56
CD3

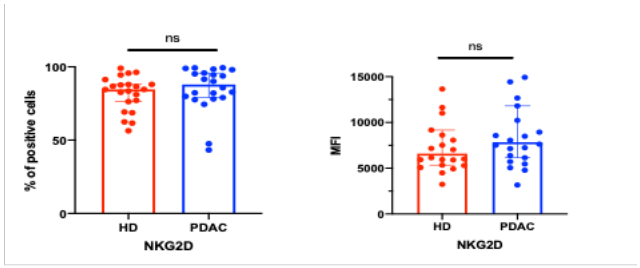
2

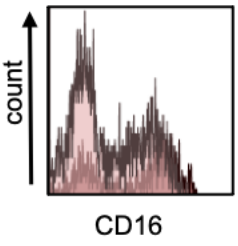
HD
PDAC

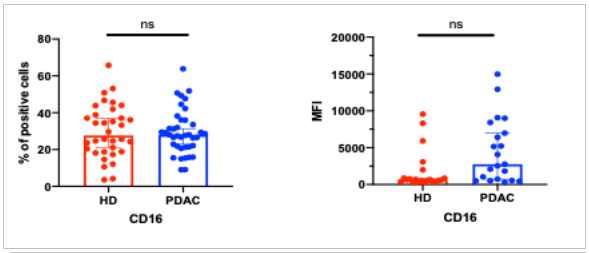

count
DNAM-1

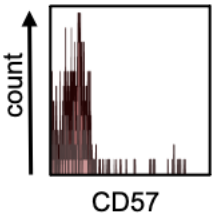

****
p<0.0001
% of positive cells
HD
PDAC
DNAM-1
ns
MFI
HD
PDAC
DNAM-1

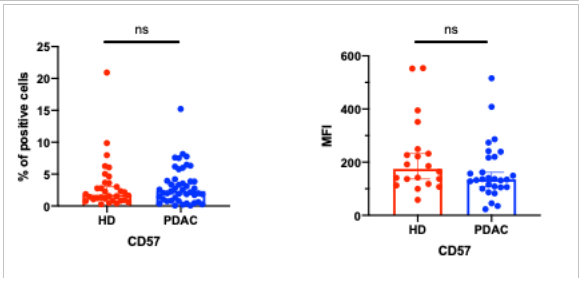

count
NKG2D


ns
% of positive cells
HD
PDAC
NKG2D
ns
MFI
HD
PDAC
NKG2D


count
CD16


ns
% of positive cells
HD
PDAC
CD16
ns
MFI
HD
PDAC
CD16


count
CD57


ns
% of positive cells
HD
PDAC
CD57
ns
MFI
HD
PDAC
CD57

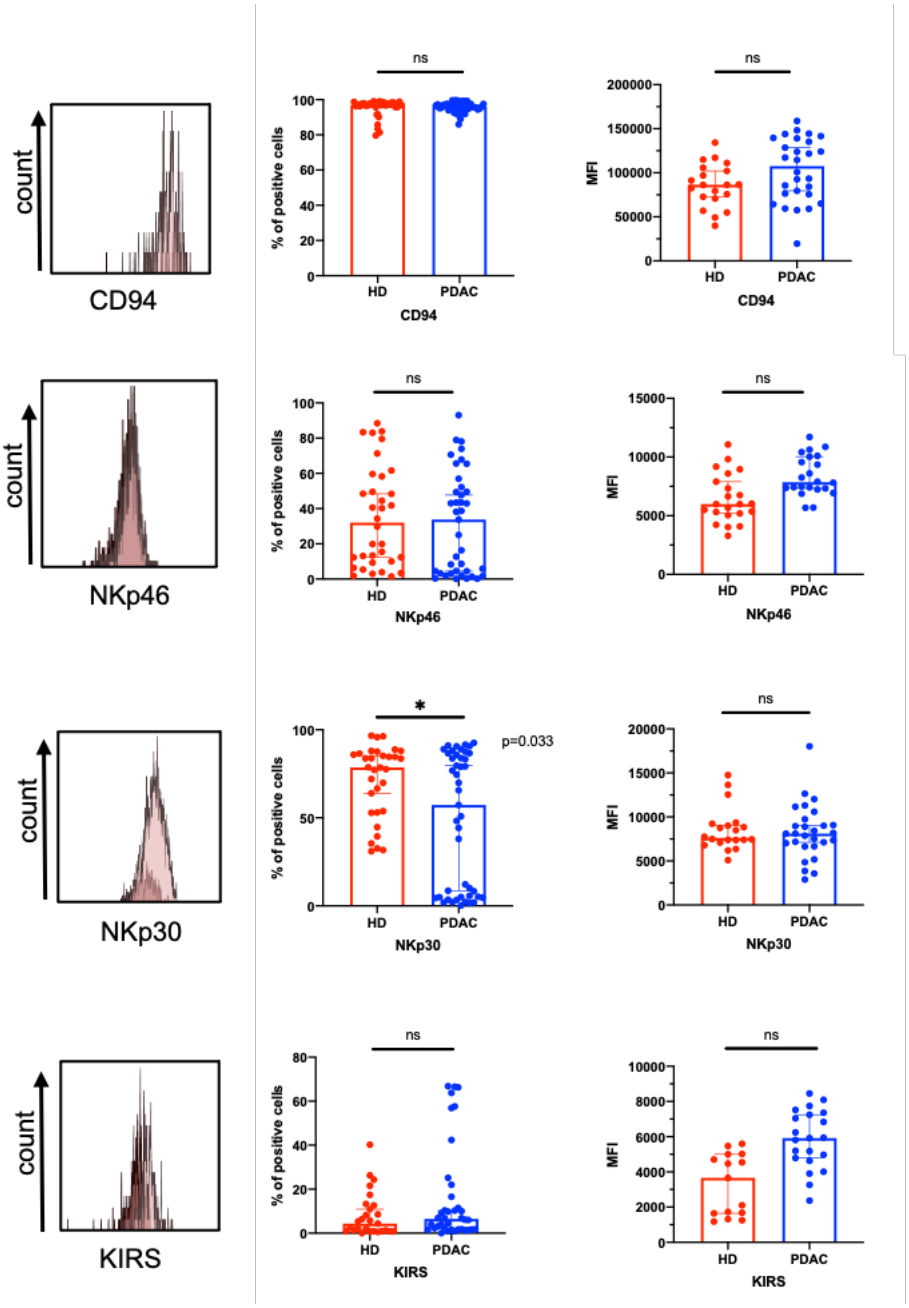
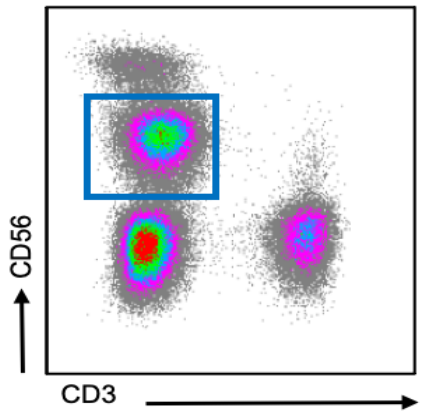


Figure 3-3. Circulating CD56^{bright} subset demonstrates reduced expression of activating receptors.

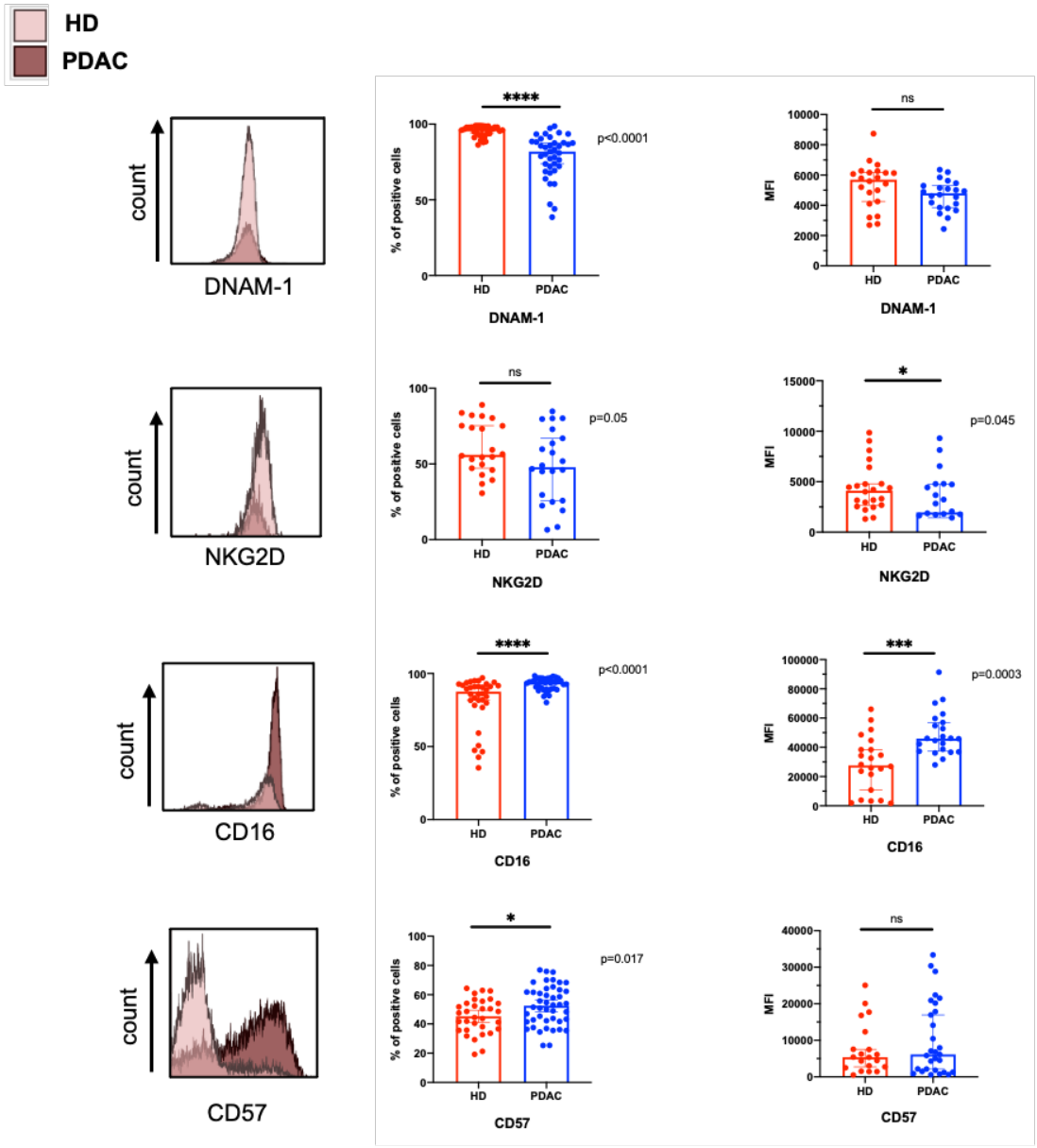
1. Gating strategy for CD56^{bright} (red rectangle); 2. The expression of differentiation markers (CD16, CD57, NKG2C, CD94) and activating or inhibitory makers (DNAM-1, NKG2D, NKp30, Nkp46, KIRs) was compared between PDAC patients and HD. On

the left, the representative histogram of each marker; on the right, the scatter plots with bars: the comparison was carried out with percentage (middle) and mean florescence intensity (MFI) (right). Significance was calculated with t-Student's test or Mann-Whitney U-testing, according to normal distribution; $p<0.05$ (*), $p<0.01$(**), $p<0.001$ (***), $p<0.0001$ (****)

1


CD56
CD3

2


HD
PDAC
count
DNAM-1
NKG2D
CD16
CD57
% of positive cells
MFI
HD
PDAC
****
p<0.0001
ns
p=0.05
*
p=0.045
***
p=0.0003
p=0.017

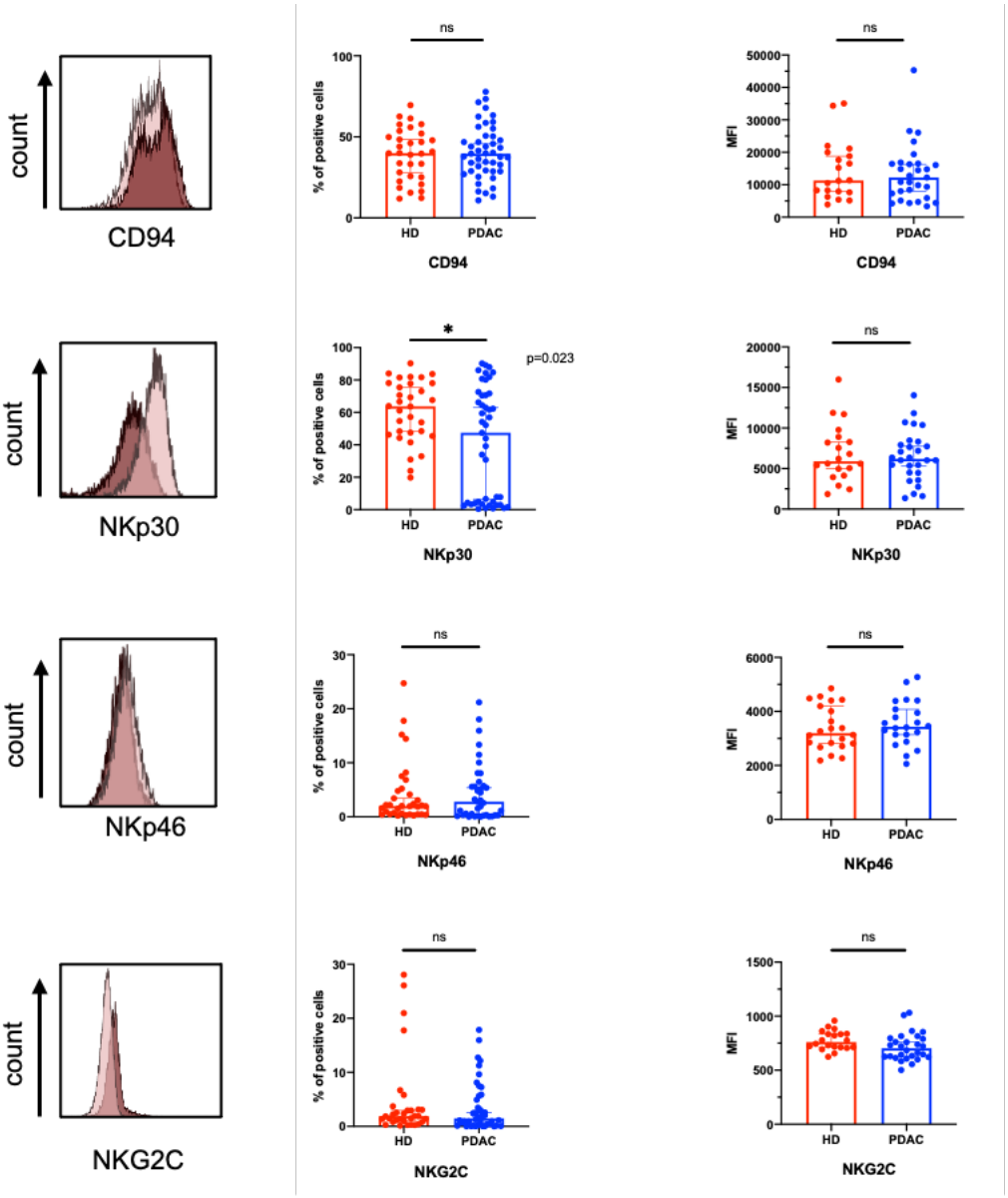


Figure 3-4. Circulating CD56^{dim} NK cells in PDAC patients show a highly differentiated phenotype and reduced expression of activating receptors.

1. Gating strategy for CD56^{dim} (blue rectangle); **2.** The expression of differentiation markers (CD16, CD57, NKG2C, CD94) and activating or inhibitory makers (DNAM-1, NKG2D, NKp30, Nkp46, KIRs) was compared between PDAC patients and HD. On the left, the representative histogram of each marker; on the right, the scatter plots with

bars: the comparison was carried out with percentage (middle) and mean florescence intensity (MFI) (right). Significance was calculated with t-Student's test or Mann-Whitney U-testing, according to normal distribution; $p<0.05$ (*), $p<0.01$(**), $p<0.001$ (***), $p<0.0001$ (****).

Table 3.1 FACS analysis in circulating PBMCs: HD vs. PDAC

	$CD3^{neg}CD56^{pos}$					
	HD (%)	PDAC (%)	p	HD (MFI)	PDAC (MFI)	p
NKp46	32	34	0.5	8250	10107	0.3
NKG2C	3	3.4	0.4	1088	1035	0.8
CD94	97	96	0.6	88988	92629	0.1
KIRs	4.3	6.4	0.11	3200	3306	0.9
	$CD56^{dim}$					
	HD (%)	PDAC (%)	p	HD (MFI)	PDAC (MFI)	p
NKp46	2	2.7	0.9	4299	4414	0.29
NKG2C	1.8	1.7	0.2	849	815	0.7
CD94	40	39.6	0.8	17553	14766	0.4
KIRs	18	12	0.4	4392	4363	0.9
	$CD56^{bright}$					
	HD (%)	PDAC (%)	p	HD (MFI)	PDAC (MFI)	p
NKp46	2.4	3.6	0.8	4511	4791	0.3
NKG2C	2.1	2.2	0.7	858	820	0.7
CD94	49	41	0.1	19404	16887	0.5
KIRs	13	12	0.7	4461	4457	0.9

3.4 Difference composition of NK cells subpopulations classified according to the simultaneous expression of CD56 and CD16.

NK cells are usually divided into two subpopulations, according to the intensity of CD56 expression. However, a more thorough investigation must take into account the simultaneous expression of CD56 and CD16, as other four NK cells subpopulations could be identified, each of them showing a potential different role, as described in chapter 1.3.1. These four subpopulations studied in this project are $CD56^{dim}CD16^{neg}$, $CD56^{bright}CD16^{neg}$, $CD56^{bright}CD16^{bright}$ $CD56^{dim}CD16^{neg}$ as shown in Figure 3-5.

Our analysis indicates that the overall $CD16^{neg}$ population is significantly reduced in the PBMCs of patients, in particular the $CD56^{dim}CD16^{neg}$ subpopulation (6% vs. 12%, $p<0.0001$), while the $CD56^{bright}CD16^{neg}$ is slightly but significantly reduced (4.7% vs. 3.3%, $p=0.03$). This is accompanied by the increase of the $CD56^{dim}CD16^{pos}$ subpopulation (87% vs. 80%, $p=0.0003$), while the $CD56^{bright}CD16^{pos}$ subpopulation remains stable, both in PDAC and HD (1.3% vs. 1.5%, $p=0.5$).

The analysis of NK cells receptors listed in panel 1 (DNAM-1, NKG2Dand NKp46) according to the four subpopulations demonstrates a different pattern of expression as well. The cytotoxic $CD56^{dim}CD16^{pos}$ subpopulation is characterised by a reduced expression in PDAC patients of DNAM-1 in terms of MFI (6605 vs. 4851, $p=0.02$) but not in the relative number of positive cells (86% vs. 85%, $p=0.6$), and a reduced expression of NKG2D and PD-1 (54% vs. 33%, $p=0.03$, MFI 3756 vs. 1931, $p=0.09$; 3.5% vs. 0.1%, $p<0.0001$, MFI 674 vs. 792, $p=0.6$, respectively). No

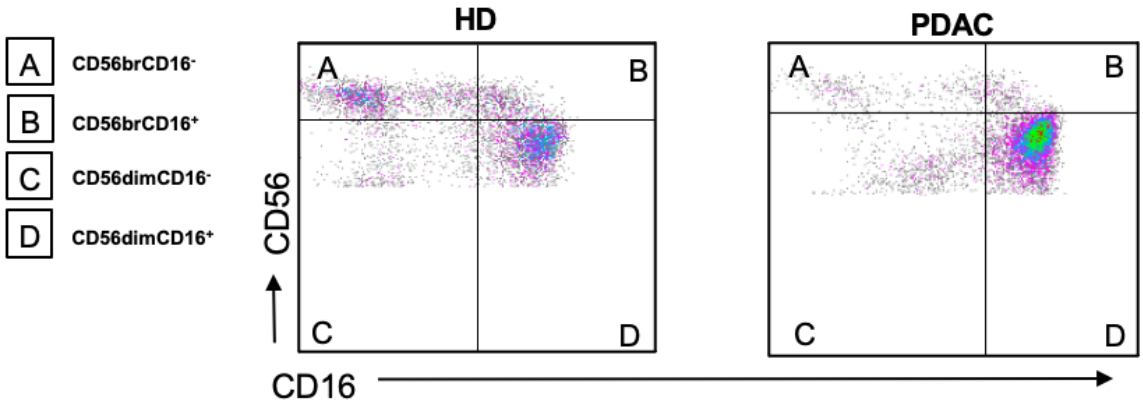
differences were found in the expression of NKp46 (3% vs. 3.9%, p=0.07, MFI 3319 vs. 3565, p=0.5).

Interestingly, the regulatory $CD56^{bright}CD16^{neg}$ shows an increased level of the activating receptor NKp46 compared to HD in terms of MFI (30% vs 31%, p=0.5, MFI 5976 vs. 8315, p=0.001). The expressions of DNAM-1 and NKG2D are similar in both cohorts [*DNAM-1:* 91% vs. 92%, p=0.2, MFI 6231 vs. 6185 p=0.7; *NKG2D*: 80% vs. 81%, p=0.9, MFI 8070 vs. 7151, p=0.1].

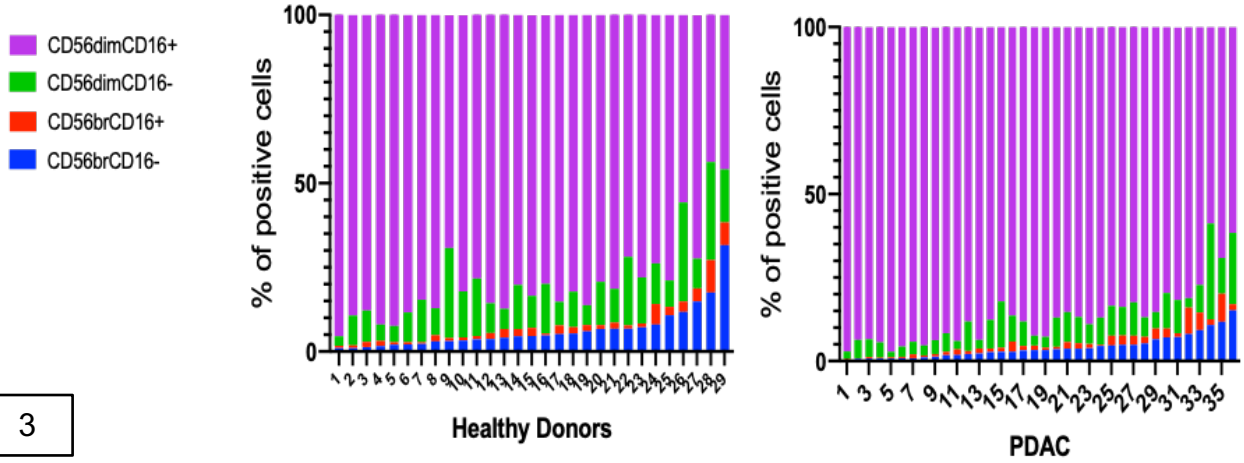
The $CD56^{dim}CD16^{neg}$ and $CD56^{bright}CD16^{pos}$ probably represent intermediate stages of maturation. In both these populations, the expression of NKG2D and PD-1 are markedly impaired, compared to HD. In the $CD56^{dim}CD16^{neg}$ subpopulation, we demonstrate a reduced expression of NKG2D compared to HD (70% vs. 56%, p=0.02, MFI 4717 vs. 3984, p=0.4). In the $CD56^{bright}CD16^{pos}$ subpopulation, NKG2D expression is decreased but does not reach the statistical significance (91% vs. 88%, MFI 7900 vs. 9600, p=0.05). The expression of DNAM-1 and NKp46 is not affected in either subpopulations [$CD56^{dim}CD16^{neg}$: DNAM-1, 71% vs. 66%, p=0.7, MFI 443 vs. 3866, p=0.3; NKp46, 11% vs. 12%, p=0.2, MFI 3081 vs. 3973, p=0.2. $CD56^{bright}CD16^{pos}$: DNAM-1, 94% vs. 91%, p=0.7, MFI 7030 vs. 6491, p=0.1; NKp46, 36% vs. 23%, p=0.1, MFI 6601 vs. 7743, p=0.6].

Figure 3-6, Figure 3-7, Figure 3-8 and Figure 3-9 summarise my results.

1


A CD56brCD16-
B CD56brCD16+
C CD56dimCD16-
D CD56dimCD16+
HD
PDAC
A
B
C
D
CD56
CD16

2


CD56dimCD16+
CD56dimCD16-
CD56brCD16+
CD56brCD16-
% of positive cells
Healthy Donors
PDAC

3

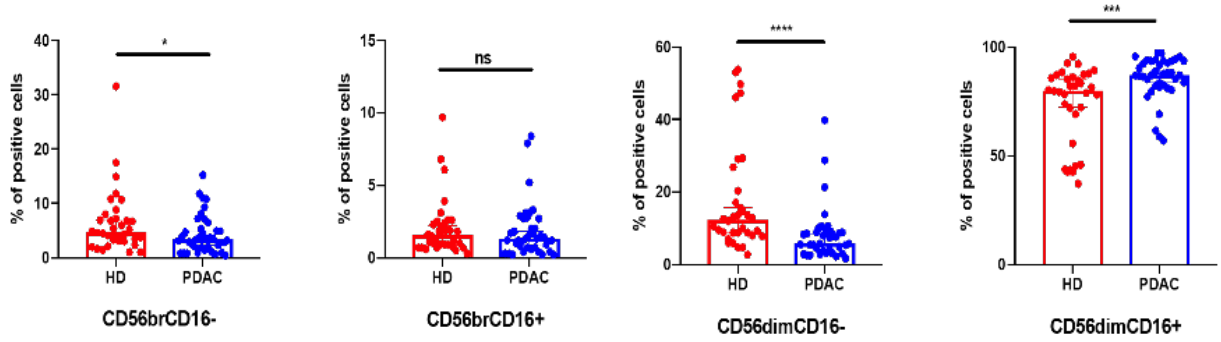

% of positive cells
HD
PDAC
CD56brCD16-
CD56brCD16+
CD56dimCD16-
CD56dimCD16+
ns

Figure 3-5. The distribution of circulating NK cells subsets is different in PDAC patients compared to HDs.

1. An example of dot plot from flow cytometry staining to gate the four NK subsets according to CD56 and CD16 expression. **2.** The composition of the four NK subsets in the bloods from PDAC patients and HDs were compared in bar charts; **3.** Percentage of four NK subsets in the bloods from PDAC patients and HDs were compared individually using scatter plots with bars. Significance was calculated with t-Student's test or Mann-Whitney U-testing, according to normal distribution; $p<0.05$ (*), $p<0.01$(**), $p<0.001$ (***), $p<0.0001$ (****).

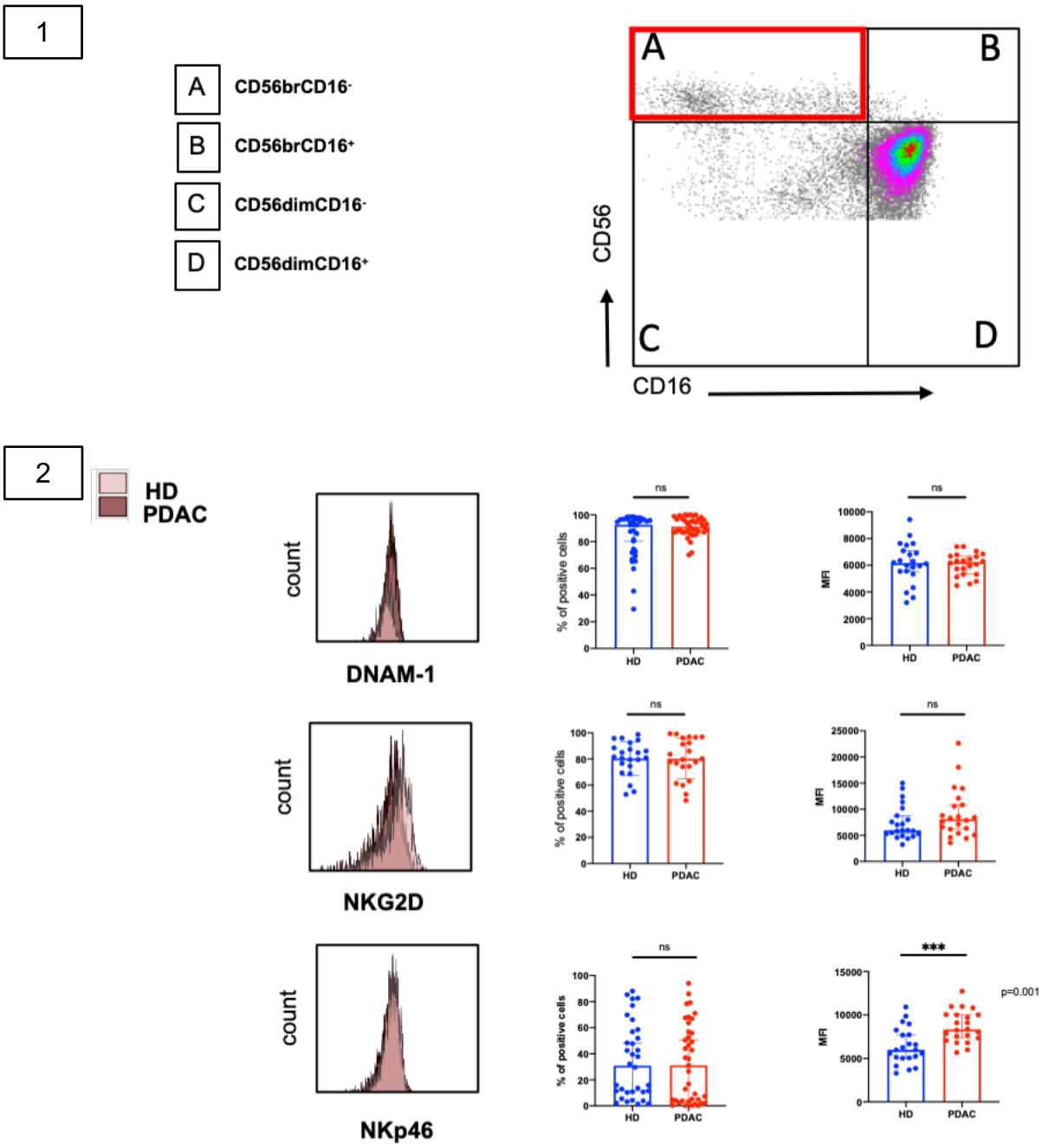


Figure 3-6. NKp46 is upregulated in the circulating CD56^{br}CD16^{neg} of PDAC patients compared to HDs.

1. An example of dot plot to gate the CD56^{br}CD16^{neg} NK subset according to CD56 and CD16 expression; **2.** The expression of the activating makers DNAM-1, NKG2D and NKp46 was compared between PDAC patients and HD. On the left, the

representative histogram of each marker; on the right, the scatter plots with bars: the comparison was carried out with percentage (middle) and mean florescence intensity (MFI) (right). Significance was calculated with t-Student's test or Mann-Whitney U-testing, according to normal distribution; $p<0.05$ (*), $p<0.01$(**), $p<0.001$ (***), $p<0.0001$ (****).

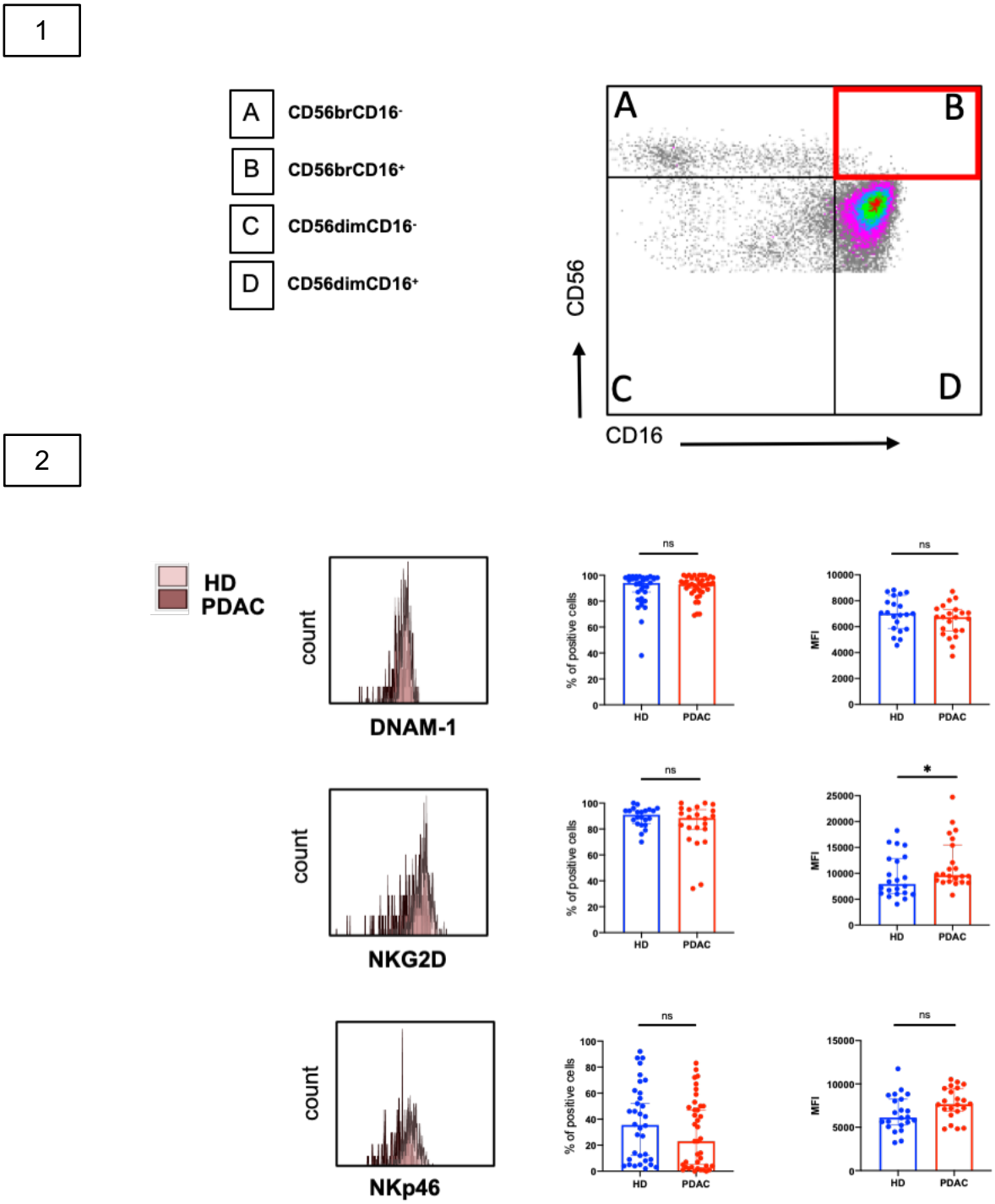


Figure 3-7. There is no difference in the expression of activating markers in the circulating CD56^{br}CD16^{pos} subset of PDAC patients, compared to HDs.

1. An example of dot plot to gate the CD56^{br}CD16^{pos} NK subset according to CD56 and CD16 expression; **2.** The expression of the activating makers DNAM-1, NKG2D

and NKp46 was compared between PDAC patients and HD. On the left, the representative histogram of each marker; on the right, the scatter plots with bars: the comparison was carried out with percentage (middle) and mean florescence intensity (MFI) (right). Significance was calculated with t-Student's test or Mann-Whitney U-testing, according to normal distribution; $p<0.05$ (*), $p<0.01$(**), $p<0.001$ (***), $p<0.0001$ (****)

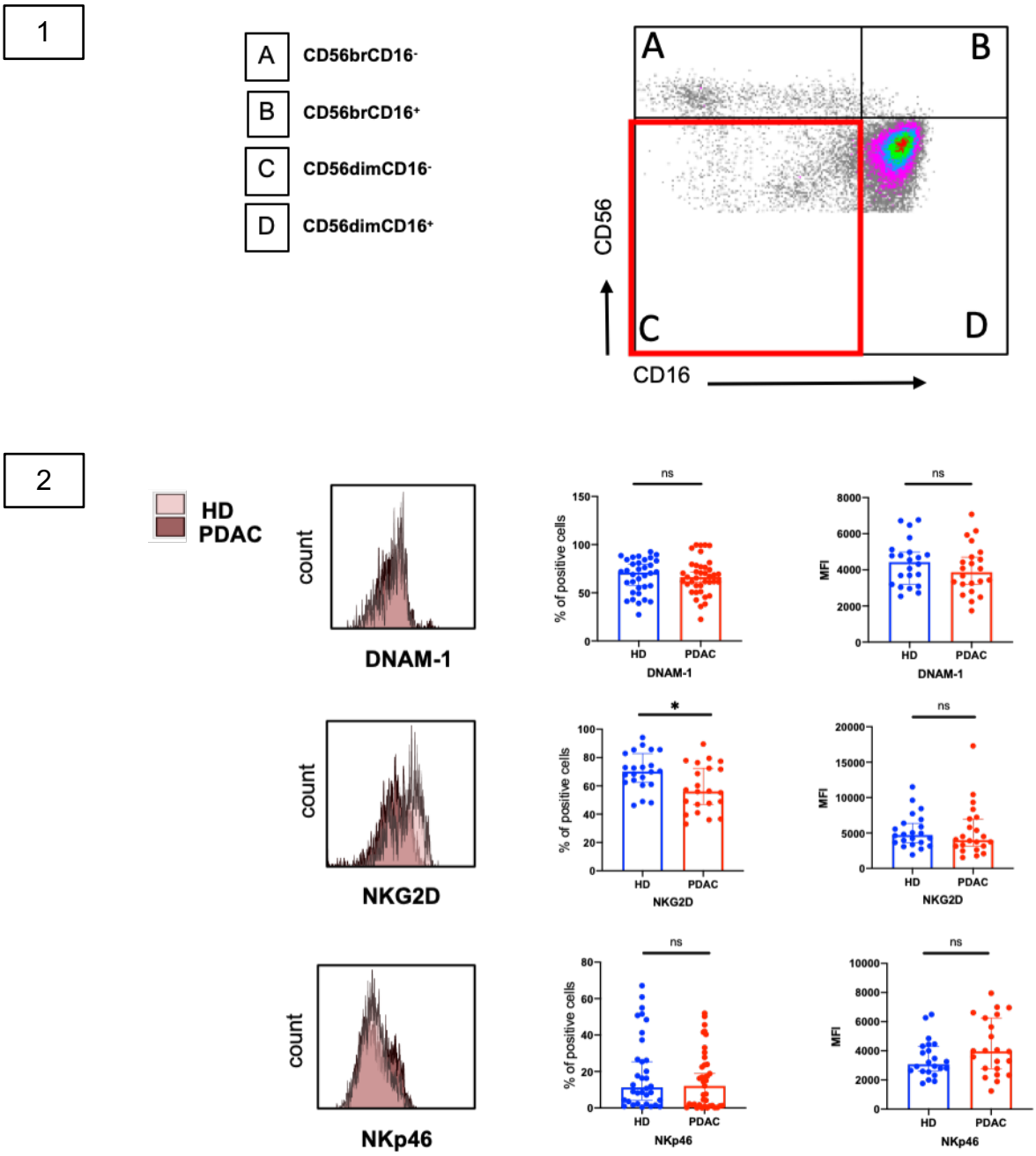


Figure 3-8. NKG2D is downregulated in the circulating CD56^{dim}CD16^{neg} of PDAC patients, compared to HDs.

1. An example of dot plot to gate the CD56^{dim}CD16^{neg} NK subset according to CD56 and CD16 expression; **2.** The expression of the activating makers DNAM-1, NKG2D

and NKp46 was compared between PDAC patients and HD. On the left, the representative histogram of each marker; on the right, the scatter plots with bars: the comparison was carried out with percentage (middle) and mean florescence intensity (MFI) (right). Significance was calculated with t-Student's test or Mann-Whitney U-testing, according to normal distribution; $p<0.05$ (*), $p<0.01$(**), $p<0.001$ (***), $p<0.0001$ (****).

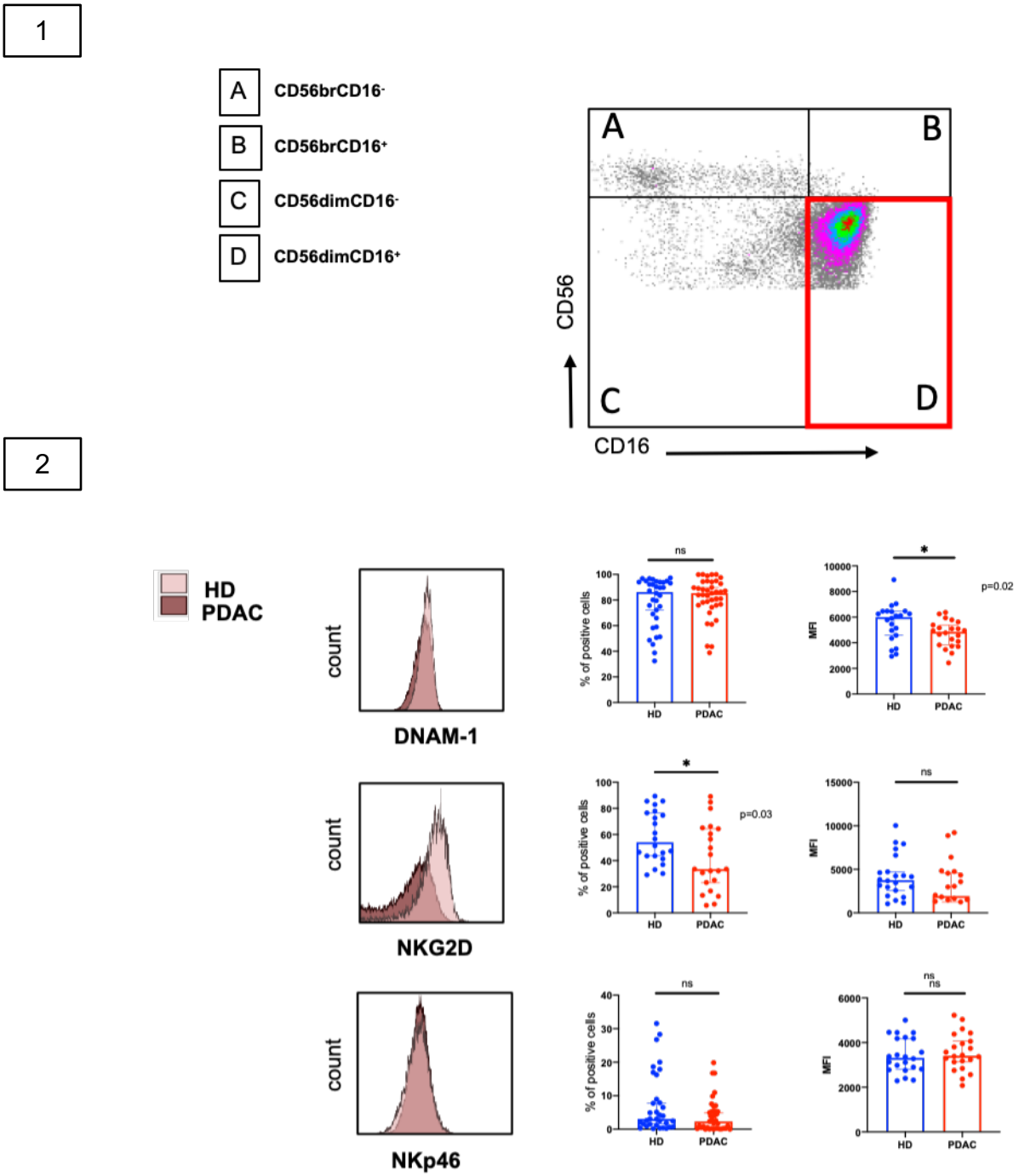


Figure 3-9. The cytotoxic CD56^{dim}CD16^{bright} shows an impaired phenotype, in peripheral blood of patients affected by PDAC, compared to HDs.

1. An example of dot plot to gate the CD56^{dim}CD16^{pos} NK subset according to CD56 and CD16 expression; **2.** The expression of the activating makers DNAM-1, NKG2D

and NKp46 was compared between PDAC patients and HD. On the left, the representative histogram of each marker; on the right, the scatter plots with bars: the comparison was carried out with percentage (middle) and mean florescence intensity (MFI) (right). Significance was calculated with t-Student's test or Mann-Whitney U-testing, according to normal distribution; $p<0.05$ (*), $p<0.01$(**), $p<0.001$ (***), $p<0.0001$ (****).

3.5 Discussion

NK cells are a class of the innate immune system, playing a pivotal role in defending the organism against damage, infections and tumours. In particular, their importance in protecting against tumour development, has been demonstrated by the observation of increased tumours incidence in mice in which NK cells were either depleted or functionally impaired.(385) In humans, the role of NK cells in oncology has been addressed just recently, as most of the studies have been focusing on T-cells. However, many Authors now agree that NK cells are crucial in the fight against cancers, not only because they can be used as a prognostic tool, but also because they can be exploited either as a target or as a weapon in immunotherapy.

3.5.1 The percentage of NK cells in PBMCs in PDAC patients

In this chapter, firstly we showed that the number of circulating NK cells does not differ in patients affected by pancreatic cancer, compared to healthy donors. This observation is confirmed by similar studies comparing the percentage of circulating NK cells in pancreatic cancer and healthy volunteers. In *Yamamoto et al.,* NK cells from 100 PDAC patients undergoing surgery where compared to 20 healthy donors and no significant difference was found between the two cohorts (8.1% vs. 6.5%, p=0.489, respectively).(386) Similarly, *Bang et al.* compared the leukocytic population of the peripheral bloods from 13 patients with advanced pancreatic cancer with 7 sex and age-matched healthy donors. In the first group, NK cells represented 17% of the whole leukocytes, while in HD NK cells where 15% (p value not showed).(387) However,

whether these data reflect a real alteration in the number of circulating NK cells in PDAC or this is the result of interindividual variation, is still a matter of debate, as other studies have demonstrated opposite results. For example, *Xu et al.,* in their analysis of 160 PDAC patients vs. 25 HD, showed that circulating NK cells represented almost 22% of the entire leukocytes, compared to 15% in HD (p=0.001). Similarly, *Ullenhag et al* demonstrated the increase of NK population in peripheral bloods' of PDAC patient, compared to healthy group, 12% vs. 6%, $p<0.05$. Another recent study compared the distribution of NK cells in peripheral blood of PDAC patients and to healthy donors and confirmed the increase, with NK cells representing 28% of the circulating leukocytes in patients and 16% in the normal cohort ($p<0.001$).(388) Due to the huge variation between patients, the study cohort needs to be expanded and also to include multiple clinical centres, which will enable us to make a more solid conclusion. Also, the percentage cannot represent actual number of NK cells, we plan to use counting bead in the future study to obtain the absolute count of NK population, thus avoiding biases related to the differences in the whole PBMCs number among donors.

3.5.2 NK cell subset population

NK cells are typically identified as $CD3^{neg}CD56^{pos}$, but they can also be subdivided according to the intensity of the expression of CD56. Therefore, two groups are recognised, $CD56^{dim}$ and $CD56^{bright}$. $CD56^{dim}$ represent almost 90% of the circulating NK cells and have the highest cytotoxic potential. Instead, $CD56^{bright}$ are efficient cytokines producers and are typically localised in lymph nodes.(143) Therefore, in the peripheral bloods of healthy donors, $CD56^{bright}$ represents a minor

fraction of the circulating leukocytes [8.65%±6.9% (range, 0.63-38%)], as demonstrated by *Angelo et al*. in their study aiming to establish the normal range of NK cell subsets in 40 adult healthy volunteers.(389)

We found no difference in the relative percentage of total peripheral NK cells, but the distribution of $CD56^{dim}$ and $CD56^{bright}$ is modified, with a reduction of the latter in PDAC patients, compared to HD, while no difference was found in $CD56^{dim}$. The same trend is confirmed by other studies investigating the proportion of NK cells in different malignancies, such as breast cancer and head and neck cancer.(390, 391) However, this is not confirmed in other solid tumours such as ovarian cancer (392) or oesophageal squamous cell carcinoma (393). Moreover, contradictory results in breast cancer shows that the relative proportion of $CD56^{bright}$ is actually increased and correlates to the stage of the disease.(148)

Several studies have investigated the origin of $CD56^{bright}$. According to the most endorsed theory, this subset represents an immature stage of the cytotoxic $CD56^{dim}$, of which they are therefore precursors.(157, 394) However, other Authors speculate an independent origin of the two subsets, based on the observation that patients with mutation of GATA2 present $CD56^{dim}$ but have no $CD56^{bright}$.(373) Interestingly, aging is known to induce an increase of the absolute number of circulating NK cells,(395-397) but also the internal distribution of the two subsets is altered, with $CD56^{dim}$ prevailing on $CD56^{bright}$. This is supposed to depend on a deficient output of immature $CD56^{bright}$ from the bone marrow.(398)

Therefore, we can speculate that the decrease in the percentage of the $CD56^{bright}$ observed in our cohort of patients, could be related either to a high turnover of cytotoxic $CD56^{dim}$ engaged in their response against the tumour, or to a direct

suppressive role of the tumour itself on the bone marrow, which is not able to overcome the request of output of immature $CD56^{bright}$ NK cells in the blood stream. A third intriguing hypothesis could be the specific recruitment of $CD56^{bright}$ by the tumour itself. Probably, a combination of the three mechanisms could explain our findings in the peripheral blood of PDAC patients.

3.5.3 The phenotype of NK cells in PDAC patients

NK cells are the first line against transformed cells. Their activation relies on a delicate balance between inhibitory and activating receptors. To escape T cells recognition, malignant cells lose the expression of MHC class I molecules, which are potent deactivators of NK cells cytotoxicity, through KIRs-transmitted signals and NKG2A/CD94 complex. In addition, malignant cells expressed stress-related molecules (e.g., MICA/B and ULBP1-6), which are recognised by the activating receptors expressed on the surface of NK cells. This activating signal is able to overcome the inhibition provided by MHC class I molecules. However, according to the immune editing theory, cancer cells "learn" to evade NK cell recognition through different mechanisms. Cancer cells release soluble NK cells' ligands in the microenvironment, which, once bound to the activating receptor, induce its blockage and internalisation, and ultimately the downregulation of the receptor itself. Another mechanism is the secretion of cytokines, such as TGF-β, IL-10, PGE_2 or IDO, all able to raise a potent and direct suppression of the expression of NK cells' activating receptors.(200, 331, 339, 399, 400) Finally, cancer cells are able to interfere with the transcription of mRNA, through the release of microRNA. All these mechanisms

participate to the immune evasion of tumour cells, impairing NK cells' cytotoxic function and their ability to recognise the targets, and are associated with tumour progression in PDAC.(400-403).

NKG2D, DNAM-1 and NCRs (NKp30, NKp44 and NKp46) are the main activating receptors expressed by NK cells, and among them, NKG2D is the best characterised. **NKG2D** is a potent NK cells activator and in humans, it is also expressed on $CD8^+$ T-cells, $\gamma\delta$-T cells, NKT cells and T_{reg}. It recognises MICA/B and ULBP1-6, which are usually absent on the surface of healthy cells but are typically induced by carcinogenesis. Here, we report the downregulation of NKG2D in circulating NK cells of patients affected by PDAC. This is particularly evident in the $CD56^{dim}CD16^{pos}$ cells, and marginally involves the $CD56^{dim}CD16^{neg}$ subgroup. Conversely, NKG2D expression in the $CD56^{bright}$ and subgroups, is the same between patients and healthy donors. Other than the intensity of CD56 expression, NK cells can be subclassified according to the simultaneous expression of CD56 and CD16. Therefore, four groups are recognised: the $CD56^{dim}CD16^{pos}$ displays the highest cytotoxic activity and are the more abundant subgroup among circulating NK cells; the $CD56^{bright}CD16^{neg}$ corresponds to the "regulatory" NK cells, located primary in the lymph nodes; the other two subgroups $CD56^{bright}CD16^{pos}$ and $CD56^{dim}CD16^{neg}$ represent a minor fraction and probably intermediate stages of maturation of the other main two subgroups. Their roles still need further investigations to be defined. In our analysis, NKG2D was found to be downregulated particularly in the $CD56^{dim}CD16^{pos}$ fraction. The downregulation of NKG2D is a well-known mechanism of tumour evasion, reported also in other tumours, PDAC included.(220, 324, 404, 405) In particular, *Jun et al.* demonstrated that NKG2D expression in PDAC is downregulated since early

stages of the disease, but there is no difference in the level of expression between healthy donors at later stages, i.e. stage III and IV. Besides, NKG2D expression on circulating NK cells does not correlate with a reduced cytotoxic activity, expressed as percentage of CD107a positive or INFγ positive cells, after stimulation with the target cell line, implying that also other mechanisms impairs the degranulation activity of NK cells.(405) Moreover, the decrease of the expression of NKG2D seems to significantly correlate with lymph node metastasis in PDAC, gastric cancer and colorectal cancer, as demonstrated by *Peng et al*.(324) Being the $CD56^{dim}CD16^{pos}$ the most active cytotoxic NK cells subgroup, the decrease of NKG2D confirms the important role of NK cells' dysfunction in immune escape, in PDAC. In fact, NKG2D is a pivotal trigger of the perforin-dependant cytotoxicity.(406) The implication of the decrease of NKG2D expression in the $CD56^{dim}CD16^{neg}$ is more dubious, as the role of this subset is far to be defined. The gating strategy plays a fundamental role in discriminating $CD56^{dim}CD16^{neg/dim}$, as according to *Amand et al*. $CD56^{dim}CD16^{dim}$ could represent a specific NK cells subset.(407) Recent data suggest that $CD56^{dim}CD16^{neg}$ is a heterogeneous population, with both high and poor cytotoxic function. They could also represent cytotoxic $CD56^{dim}CD16^{pos}$ cells already undergone degranulation, as CD107a expression inversely correlate with CD16 shedding. The downregulation of NKG2D receptor seem to correlate with high concentration of the immune suppressive cytokine TGF-β, as high levels of this cytokine are often found in the sera of PDAC patients.(405) Also, high secretions of the NKG2D ligands MICA/B and ULBP1-6 are known to negatively affect the expression of this activating receptors. A combination of all these mechanisms is present in PDAC patients. This last topic will be further discussed in chapter Chapter 7.

DNAM-1 is another important receptor involved in NK cells-mediated killing of PDAC tumours.(388, 400) DNAM-1 recognises CD112 and CD155, two stress-induced proteins present on the surface of tumour cells. In our cohort of patients, even if we did not find the significant difference of DANM-1 for whole NK population, we found that DNAM-1 is significant reduced in patients compared to healthy donors in NK cells subsets. In fact, the decrease involved either the $CD56^{bright}$ or $CD56^{dim}$ subpopulations, but we did not find any differences when performing the analysis according to CD56/CD16 phenotypes, in peripheral bloods. The importance of DNAM-1 in immunosurveillance has been highlighted by the development of a murine model, in which DNAM-1-deficient mice exposed to chemical carcinogens, showed a significant higher incidence of fibrosarcoma and papilloma compared to the wild-type.(225) In PDAC, contradictory results have been reported in literature on the expression of DNAM-1 in peripheral bloods. While *Peng et al.* demonstrated a significant reduction of the percentage of circulating DNAM-1-positive NK cells compared of healthy donors, *Jun et al.* showed no differences in the expression between the two groups and no relation to the stage of disease. (324, 405) However, DNAM-1 was found to be important in NK cells recognition of several tumours, such as neuroblastoma, myeloma, Ewing's sarcoma and melanoma.(408, 409) Moreover, in breast cancer DNAM-1 is confirmed to be downregulated in patients compared to healthy volunteers and this correlates with the stage disease, being more pronounced in the metastatic patients. Interestingly, the expression of DNAM-1 in breast cancer patients is not affected by the supernatant, suggesting that soluble factors may not be implied in DNAM-1 downregulation.(319) This result was confirmed in patients affected by ovarian cancer, in which NK cells isolated from peripheral bloods showed to lose

DNAM-1 receptor within hours from exposure to CD155-positive target cells.(408) Altogether, these data suggest that DNAM-1 is more susceptible to physical contact with cancer cells rather than being affected by cytokines or other soluble factors, as conversely NKG2D is. This may also explain why we found than DNAM-1 is markedly more reduced in tumour infiltrating NK cells rather than circulating cells, as we will show in the next chapter.

In this chapter, we also analysed the expression of the NCRs, NKp30 and NKp46. While we did not see any differences between healthy and patients' groups in the level of expression of NKp46, the percentage of NKp30-positive NK cells was markedly suppressed in PDAC patients, suggesting an important role of this receptor in PDAC immune escape. Similar results were reported in *Jun et al.*, who found no difference in the expression of NKp46 between control and malignant patients, but a severe decrease of NKp30. Moreover, while NKp46 expression did not correlate with tumour stage, NKp30 was suppressed in the early but not in the late stages of the disease, similarly to what was reported in the same study for NKG2D. Also, NKp30 MFI significantly correlated with the presence of INFγ-positive NK cells in the patients group.(405) Other studies confirmed that the suppression of NKp30 in circulating NK cells is a common mechanism of tumour escape in other tumours, such as gastric and colorectal cancers,(402) cervical cancer (410), breast (319) and melanoma (411).

NCRs were the first identified activating receptors in NK cells.(409) They recognise many different ligands involved in tumour surveillance, and their engagement triggers NK cytotoxicity and cytokine production.(230) NKp30 is expressed on mature and activated NK cells, and it recognises, among others, B7-H6

and BAT3, also known as Bcl2-associated athanogene 6, both found to be expressed in cancers.(412, 413) While B7-H6 is expressed on the cell surface of cancer cells, BAT3 is a nuclear protein which controls different cell processes, from gene regulation to protein degradation.(230) BAT3 was found to be released in an exosomal form by tumour cells and this form is able to bind NKp30 and to evoke the activation.(413, 414) In Chronic Lymphocytic Leukaemia patients, the release of a soluble BAT3 competes with the exosomal form and suppress NK cells activation.(415) B7-H6 is the main NK cells activator expressed on K562 cells, a cell line highly susceptible to NK cells lysis, and it is driven by the proto-oncogen *Myc*.(416) High concentration of B7-H6 was found in the sera of patients affected by hepatocellular carcinoma, GIST, neuroblastoma and in ovarian cancer.(230) Importantly, B7-H6 is also highly expressed in pancreatic cancer.(417) The shedding of this ligand by metalloproteases is thought to be one of the main mechanisms to escape NKp30 recognition, and it is correlated with poor survival.(230) Moreover, the secretion of TGF-β1 by cancer cells could excruciate NKp30 suppression, as recently demonstrated in neuroblastoma.(418) Finally, it was reported that NKp30 could undergo different post-transcriptional splicing, which generates three isoforms, namely NKp30a/b/c. NKp30c evokes a suppressive phenotype, with IL-10 secretion, impaired degranulation and defective secretion of INFγ and TNFα, in GIST and neuroblastoma.(235) It is unknown if a similar mechanism is present in pancreatic cancer.

We found that CD16 and CD57 are both increased in circulating NK cells of patients affected by PDAC cancer, compared to healthy donors. The increase involves particularly the $CD56^{dim}$ fraction, while no differences are reported in the $CD56^{bright}$.

CD16 is the main receptor involved in antibody-dependant cellular cytotoxicity in humans: infected or transformed cells are coated with IgG antibodies, whose Fc domain is recognised by Fcγ receptors expressed on NK cells and monocytes. This binding ignites the release of cytotoxic granules or the upregulation of death receptors and lead to the destruction of the target cells. In humans, there are two main Fcγ receptors, FcγRIIa and FcγRIII, the latter also known as CD16. These are characterised by a high polymorphism, which affects their affinity for the IgG. CD16 in particular recognises the Fc portion of IgG1 and IgG3.(419) ADCC seems to be the main mechanism by which antibodies mediate their anti-tumour effect *in vivo*, the others being the direct interference with tumour cell growth and the activation of complement-dependent cytotoxicity (CDC).(420) NK cells represent the main effectors of ADCC, and therefore it is not surprising that CD16 expression on NK cells correlates with better prognosis in cancers, chronic viral infection and some autoimmune disease.(421) Particularly in the oncological setting, therapeutic mAbs recognise cancer cells and make them susceptible to NK-mediated-ADCC, binding CD16, such as trastuzumab for breast cancer and cetuximab for colorectal cancer.(422, 423) For example, in colorectal cancer, patients presenting with more than 12% of circulating $CD16^{pos}$NK cells showed a better survival, after FOLFOX chemotherapy.(424) Moreover, the expression of CD16 is found to be pivotal in the internalisation and killing of HPV infected cells, while in chronic HIV infected patients this mechanism is deficient due to the downregulation of CD16.(421, 425) Discordant results are reported on the level of expression of CD16 in circulating NK cells, in different cancers: in breast cancer, CD16 is downregulated in parallel with the progression of the disease, being the lowest in metastatic patients, while in ovarian cancer it is overexpressed, but it is

not found on NK cells isolated from ascitic fluids.(220, 319) Experiments with pancreatic cell lines demonstrated the induction of CD16 expression on NK cells,(400) but *Jun et al.* did not find any differences between healthy donors and PDAC patients in peripheral bloods.(405) Conversely, our data show that peripheral NK cells in PDAC are highly activated and potentially able to induce ADCC against cancer cells. However, in the next chapter we will demonstrated that this mechanism is lost in infiltrating NK cells.

CD57 is a glycan carbohydrate expressed on T cells and NK cells, and it is typical marker of cellular senescence, chronic immune activation, particularly in viral infection, and aging.(426) Typically, T cells expressing CD57 are still able to produce INFγ, but are less prone to proliferation, as demonstrated by the presence of shorter telomerases. Also, $CD57^{+}CD8^{+}$ T cells are expanded in many tumours and this has been associated with poor prognosis.(427) In contrast with these evidences, the expansion of circulating $CD57^{+}$ NK cells has been associated with a better prognosis, and observed in many different tumours, for instance colorectal, breast, gastric, head and neck, oesophageal and pulmonary cancer.(427) CD57 is usually expressed by $CD56^{dim}CD16^{pos}$ NK cells, while it is almost absent in the $CD56^{bright}$ fraction. Interestingly, its expression directly correlates with age, being completely absent in new-borns and reaching more than 60% of the circulating NK cells in European adults.(428) This is probably due to the exposition to viral infections during the course of life, as CD57 is typically overexpressed in hCMV-infected adults.(429) CD57 is acquired during late stages of NK cells maturation: these cells show a potent cytolytic effect through CD16 signalling, but are less prone to proliferate in response to IL-12 and IL-18. Moreover, the production of INFγ after CD16 activation is enhanced

compared the $CD56^{dim}CD16^{pos}CD57^{neg}$ counterpart.(426) Our data are consistent with the current literature: CD57 is expressed by 1%-2% of $CD56^{bright}$ circulating NK cell and they are not amplified in patients. On the contrary, CD57 is significally increased in the $CD56^{dim}$ subgroup of patients, compared to healthy donors. This demonstrates the presence of mature NK cell in the bloods of patients affected by PDAC cancers, potentially able to trigger a potent cytolytic affect against cancer cells. However, this phenotype could also be acquired after viral infections, in particular hCMV infections. Unfortunately, patients' viral status was not available. However, we did not find any differences in the expression of the NKG2C/CD94 complex. hCMV infection is known to induces the preferential expansion of NK cells co-expressing CD57 and NKG2C/CD94, the main receptor for the recognition of hCMV. Therefore, we can speculate that the overexpression of CD57 in the $CD56^{dim}$ subset could be genuinely related to the chronic release of PDAC-related antigens in the bloodstream.

Finally, we analysed the expression of KIRs. To this aim, we used anti-CD158 antibodies, which was a cocktail of antibodies against different KIRs either inhibitory or activating receptors: KIR2DL1 (CD158a) is described as inhibitor of NK cells function, while KIR2S1(CD158h) and KIR2S3/S5 (CD158g) are activating receptors. We did not find any differences in the expression of these receptors in PDAC patients. KIR2DL1 recognises HLA-C and induces the suppression of NK cells' lytic activity. It is found to be significantly expressed in breast cancer and in non-small cell lung cancer patients.(430, 431) However, limited data are available on the role of KIRs in solid tumours, other than skin cancers and cholangiocarcinoma.(432, 433) On the contrary, KIRs have been driven attention for their potential role in immunotherapy against

haematological malignancies.(260) For example, Lirilumab, a mAb targeting KIR2DL1-2-3, improves NK cells cytotoxic activity against acute leukaemia cells either in vitro or *in vivo*. Moreover, *Ewen et al.* have demonstrated that *in vitro* stimulation with IL-12, IL-15 and IL18 are able to reduce the expression of KIR2DLs on circulating NK cells, leading to an improved CD16-mediated cytotoxic activity against tumour cells.(434) These results could be exploited in future as potential treatments in patients affected by solid tumours.

In conclusion, in this chapter we have demonstrated that circulating NK cells of patients affected by PDAC showed an impaired phenotype, characterised by the downregulation of pivotal receptors, such as NKG2D, DNAM-1 and NKp30, that ignite the cytotoxic response against the tumour. This is probably the result of multiple mechanisms of evasion, for instance the chronic release of activating ligands and the production of immunosuppressive cytokines by tumour cells and/or the microenvironment. However, circulating NK cells show also a mature phenotype characterised by the expression of CD16 and CD57, possibly in response to chronic stimulation by epitopes released by cancer cells or a direct stimulation by cancer cells. This makes these cells potentially capable of mounting an effective ADCC against cancer cells. If these activated NK cells are eventually able to access the tumour site and carry out their potential will be the subject of the next chapters.

Chapter 4. Clinical and pathological characteristics: correlations with circulating NK cells receptors

4.1 Introduction

In the United Kingdom (UK), PDAC is the 12th most common tumour in males and the 9th in females, but its incidence has been steadily increasing since the early 1990s. The mortality rate is very close to the incidence rate and thus, for the vast majority of patients a diagnosis of PDAC is fatal. Therefore, the need to improve the care, treatment and outcomes for patients with pancreatic cancer is pivotal. Being an important cellular component of the innate immune system, NK cells have been demonstrated to be efficient in tumour surveillance. In this chapter, we wanted to investigate any relations between the level of expression of NK cell's activating receptors and the clinical and pathological characteristics of patients undergoing pancreatic resection, with curative intent. This could potentially delineate new prognostic markers that could help the selection and treatment of patients affected by pancreatic cancer.

4.2 Material and methods

Complete clinic information was collected from 52 patients over 54 patients and summarised in Table 4.1. Two patients were excluded because of missing data.

Statistical analysis was performed with SPSS 25 (SPSS, Inc., Chicago, Illinois). All variables were checked for normal distribution. Comparisons between two groups were performed with-t student's test for parametric variables, or Mann-Whitney U test for non-parametric variables. Correlations were determined with Spearman's rho test or linear regression analysis for categorical or continuous variables, respectively. Kaplan-Mayer was used to calculate survival analysis and log rank (Mantel Cox) test was used to estimate the significance. A p value <0.05 was considered statistically significant.

4.3 Results

The female population compared to males, demonstrates a significant higher expression of NKp46 (6053 vs. 4012, p=0.046), CD16 (62330 vs. 45059, p=0.007) and CD94 (19789 vs. 13829, p=0.02) in terms of MFI, while the percentage of positive cells remains the same in the two cohorts (6.3% vs. 5.9%, p=0.8; 86% vs. 90%, p=; 43% vs. 40%, p=0.5, respectively). The same pattern is found in the $CD56^{dim}$, with NKp46 (5992 vs. 3945, p=0.04), CD16 (62751 vs. 45881, p=0.002) and CD94 (16671 vs. 12226, p=0.02) are more expressed in the female population rather than in males. Again, there is no difference in the relative number of positive cells remain in the two populations (4.5% vs. 4.6, p=0.8; 91% vs. 93%, p=0.2; 42% vs. 39%, p=0.4, respectively).

The total number of NK correlates with the size of the tumour (r=0.360, p=0.02, n=39) but it is not predictive (r^2= 0.0178, p=0.4). The lymph node ratio (LN ratio), calculated as the number of lymph nodes positive for metastasis over the total number of lymph nodes harvested, is significantly associated with the expression of DNAM-1,

NKp46 and KIRS either considering the total $CD56^{pos}$ NK cell population (*DNAM-1:* r=-0.503, p=0.005, n=30; *NKp46:* r= -0.413, p=0.023, n=30; *KIRS:* r= -0.380, p=0.027, n=34), and the two main subgroups, either the $CD56^{dim}$population (*DNAM-1:* r= -0.49, p=0.006, n=30; *NKp46:* r= -0.427, p=0.019, n=30; *KIRS:* r= -0.370, p=0.029, n=35) or the $CD56^{bright}$, (*DNAM-1:* r=-0.438, p=0.017, n=29; *NKp46:* r= -0.493, p=0.007, n=29; *NKG2C*: r=0.313, p=0.05, n=40, *NKp30:* r= -0.377, p=0.017, n=40; *KIRS*: r= -0.418, p=0.012, n=35). Interestingly, the expression of these receptors can also predict the degree of lymph node involvement. Figure 4-1 summarises these results.

The expression of NKp30 and NKp46 were found significantly reduced in advanced disease, in terms of T and N, as illustrated in Figure 4-2. We found that PDAC characterised by perineural invasion demonstrate a significant suppression of NKp46 in the whole NK population (2.1% vs. 13.6% p=0.001) and subgroups ($CD56^{dim}$: 1.7% vs. 11.5%, p=0.002; $CD56^{bright}$: 15% vs. 57%, p=0.03), compared to those adenocarcinomas without perineural invasion. Moreover, perivascular invasion is characterised by a reduction in the number of $CD56^{bright}$ subpopulation (0.3% vs. 1.3%, p=0.004) and a reduced expression in the same subpopulation of the NKG2D (82% vs. 97%, p=0.02) and CD94 (95% vs. 98%, p=0.04).

Finally, mortality is characterised by a lower expression of NKp46 in the total NK population (1.2% vs. 8,8%, p=0.013) and in $CD56^{dim}$ (0.8% vs. 5.3%, p=0.039). Of notice, in the $CD56^{bright}$, NKp46 is also markedly reduced but this does not reach the statistical significance (8.5% vs. 43%, p=0.1). Mortality is also characterised by a higher presence of NKG2C in terms of MFI, in all the groups analysed (Total NK: 962 vs. 660, p=0.04; $CD56^{dim}$: 947 vs. 647, p=0.04; $CD56^{bright}$: 1304 vs. 953, p=0.04, respectively). However, there are no differences in terms of percentage of positive

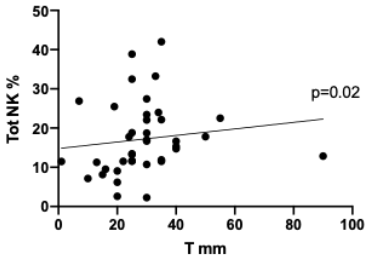
cells. The level of expression of none of the receptors analysed, has an impact on the incidence of recurrence.

Table 4.1 Patients' characteristics

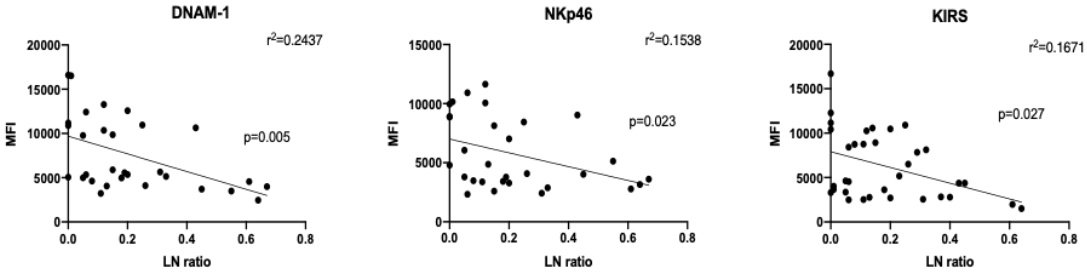
		Patients n=52
Age		67.5 (34-85)
Gender (M/F)		25/27 (48%/52%)
Resected (y/n)		44/8 (85%/15%)
Pathology		
T (mm)		30 (1-90)
T	*x*	2 (4%)
	1	2 (4%)
	2	16 (36%)
	3	23 (52%)
	4	2 (4%)
N	*0*	10 (23%)
	1	22 (50%)
	2	12 (27%)
LN ratio		0.15 (0-67)
Differentiation	*Well*	2 (5%)
	Moderate	38 (93%)
	Poor	(2%)
Perivascular Invasion (Y/N)		31/13 (70%/30%)
Perineural Invasion (Y/N)		32/12 (73%/27%)
R1		12 (27%)
Neoadjuvant Chemo		5 (9%)
Adjuvant Chemo		33 (63%)
Death		
<90 days		6 (12%)
>90 days		46 (88%)
Recurrences		25 (57%)
OS (days)		354 (9-1050)
DFS (days)		334 (10-952)
Pre-operative Bilirubin (Umol/L)		173 (3-499)
Pre-operative Ca 19-9		256 (6-7150)

*Continuous data are shown as median with min and max intervals

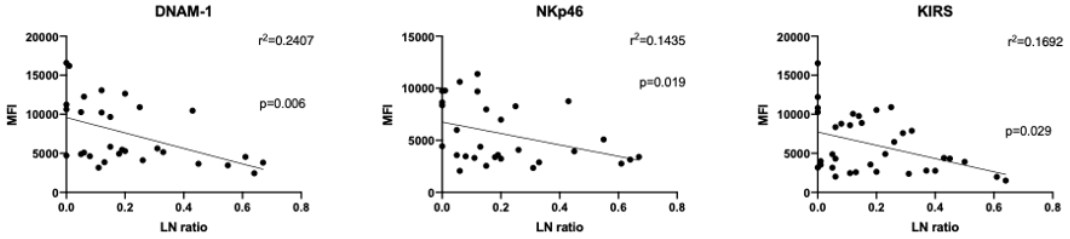
A


Tot NK %
T mm
p=0.02

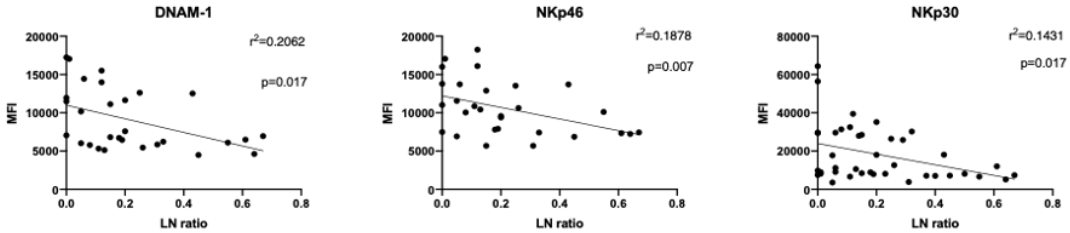
B


DNAM-1
r²=0.2437
p=0.005
NKp46
r²=0.1538
p=0.023
KIRS
r²=0.1671
p=0.027
MFI
LN ratio

C


DNAM-1
r²=0.2407
p=0.006
NKp46
r²=0.1435
p=0.019
KIRS
r²=0.1692
p=0.029
MFI
LN ratio

D


DNAM-1
r²=0.2062
p=0.017
NKp46
r²=0.1878
p=0.007
NKp30
r²=0.1431
p=0.017
MFI
LN ratio

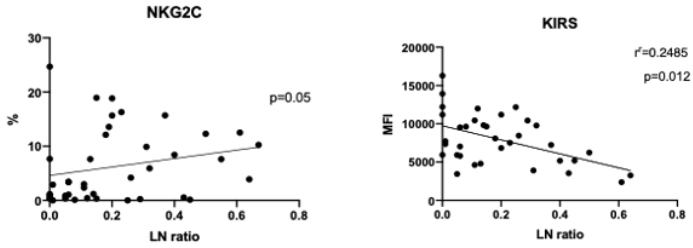

NKG2C
%
p=0.05
KIRS
r²=0.2485
p=0.012
MFI
LN ratio

Figure 4-1. The phenotype of circulating NK population affects local growth and lymph node metastasis

A. The percentage of total NK population in blood from PDAC patients correlate with tumour size. The expression of activating markers DNAM-1, NKp46 and KIRS inversely correlates with lymph node metastasis in the total NK population **(B)**, in the $CD56^{dim}$ **(C)** and in the $CD56^{bright}$ **(D)** subsets**.** NKp30 correlate with LN ratio only in the $CD56^{bright}$ subset (D). The line in the figure represents the linear regression analysis. Spearman's rho test was used to calculate significance.

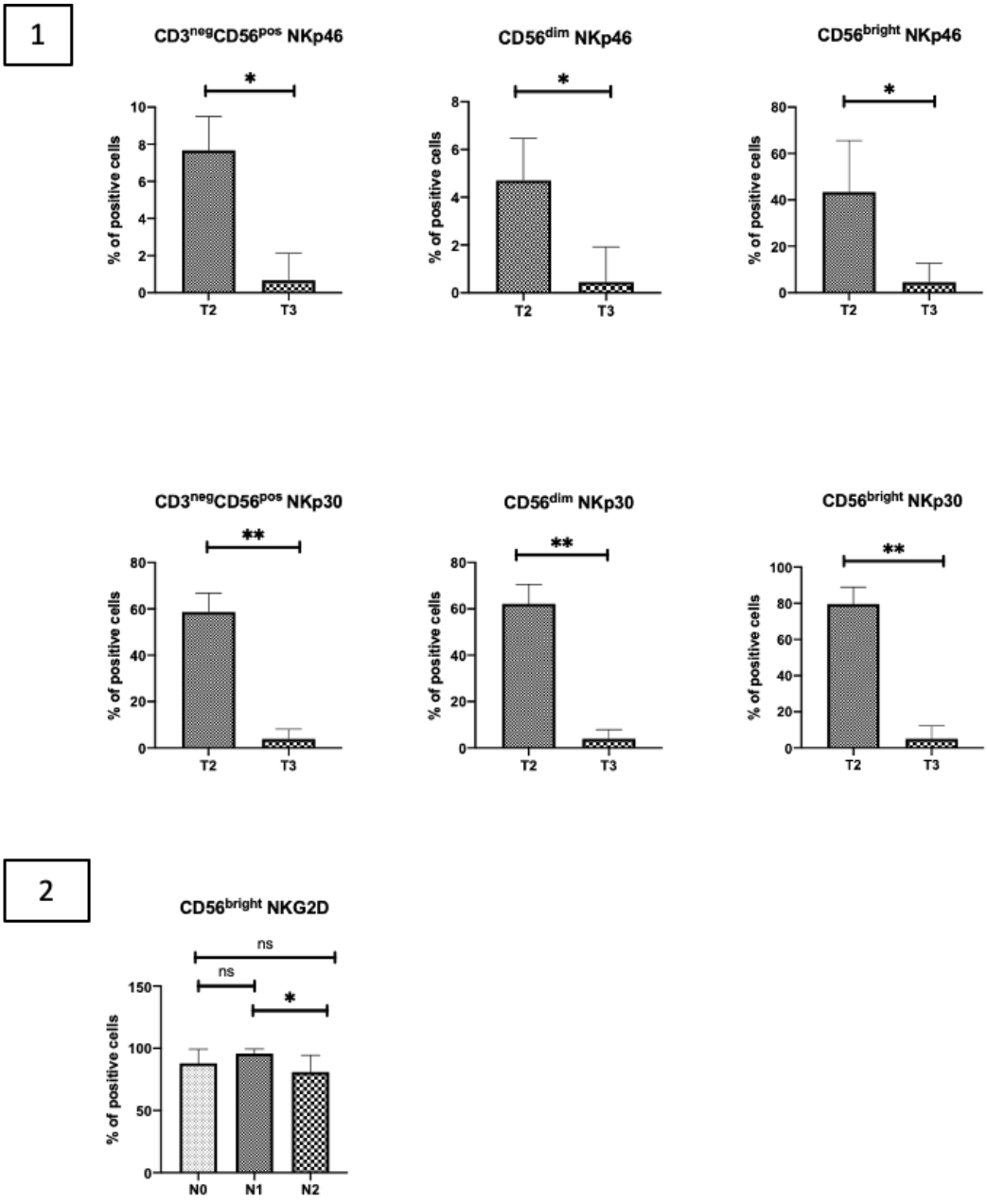


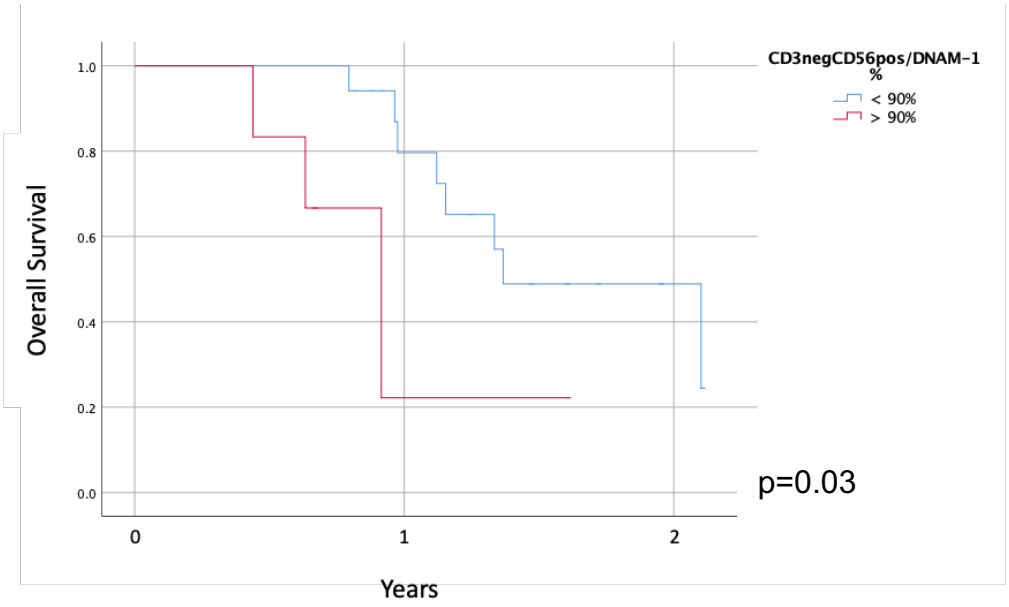
Figure 4-2. Phenotypic impairment in circulating NK cells is a characteristic of advanced stages of PDAC.

1. The expression of the activating receptors NKp46 and NKp30 in blood of PDAC patients, is significantly downregulated in advanced stages of disease. **2.** The

expression of NKG2D in blood of PDAC patients is significantly downregulated in advanced lymph nodes stage (N2: metastasis in >4 regional LN).

Then, we performed survival analysis on patient underdone pancreatic resection with curative intent and who had adjuvant chemotherapy, excluding those who died within the first 90 days after surgery. This to avoid bias related to death for surgical complications after pancreatic resection.

The cut off for all receptors was set up at 75° percentile. The Kaplan-Meier estimates indicate that the overall survival (OS) rate is improved when DNAM-1 expression in the whole and in the $CD56^{dim}$ subgroup is below the 75° percentile, as illustrated in Figure 4-3. No difference in OS was found for the other receptor markers. No difference in DFS was found for any receptors or relative numbers of NK cells.



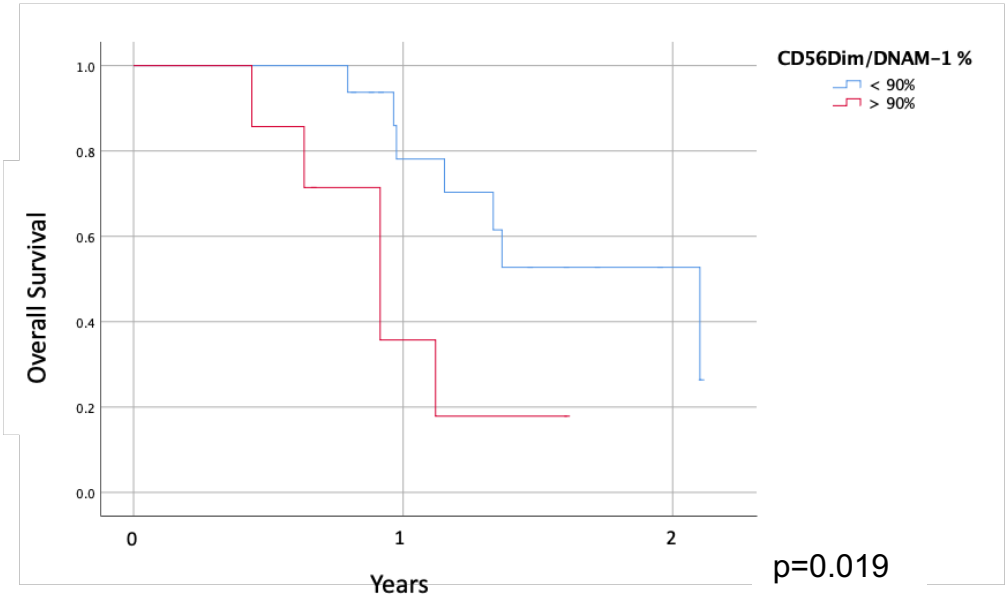


Figure 4-3. Overall Survival according to DNAM-1 expression in the blood of PDAC patients.

Overall Survival (OS) is improved when the expression of the activating marker DNAM-1 represents less than 90% (75° percentile) of the expression of total NK cell (upper figure) or $CD56^{dim}$ subset, in the blood of patients affected by PDAC.

4.4 Discussion

In this chapter, we wanted to investigate whether the level of expression of activating receptors in circulating NK cells have any impact on pathological and clinical characteristics of patients affected by pancreatic cancer.

Interestingly, the relative number of positive NK cells in the blood inversely correlate with dimension of the tumour, suggesting that NK cells are able to stem local tumour growth. Also, the presence of higher level of important activating receptors such as DNAM-1 and NKp46 seems to protect against lymph node spread: LN ratio is calculated as the number of positive lymph nodes over the total of lymph nodes harvested within the surgical specimen. LN ratio is an important prognostic marker in many gastro-intestinal, ovarian and head and neck tumours, also able to better stratifying tumour stages.(435-438) However, having the denominator as the total number of lymph nodes harvested, it is biased by the extension of the surgical lymph nodes dissection, the experience of the surgeon and pathologist, and the local extension of the tumour. Therefore, in pancreatic cancer, a minimum of twelve lymph nodes is required by the Royal College of Pathologists to define an oncological adequate surgical resection, fifteen for the AJCC.(439, 440) A recent systemic review confirmed a LN ratio over 20% as a worse prognostic factor for the OS of patients affected by pancreatic cancer, similar to other gastro-intestinal malignancies. Also, it is confirmed to be superior to the total number of positive lymph nodes alone.(441) In our study, LN ratio inversely correlates with the expression of DNAM-1, NKp30, NKp46 in terms of MFI, and directly correlate with the percentage of relative NKG2C positive NK cells. Therefore, we can speculate that the level of NK cells activation is pivotal in

controlling lymph node metastasis in pancreatic cancer, which is also confirmed by the direct correlation of the lymphatic spread with a suppressive receptor such as NKG2C. Finally, of notice, NKp46 and NKG2D expressions are protective against perineural and perivascular invasion, signs of aggressive tumour biology and negative prognostic factors.(442, 443) To the best of our knowledge, this is the first study showing such correlations in pancreatic cancer.

The expression of the activating receptors is dramatically reduced in advanced T3 stage, compared T2. According to the AJCC, T describes the size in mm of the tumour, being T2 a tumour between 2-4 mm and T3 greater than 4 mm, but still confined within the organ.(444) Moreover, the percentage of NKG2D-positive cells is reduced in advanced lymph nodes metastasis, defined as N2 according to the AJCC.(444) These findings confirm that later stages of pancreatic disease are characterised by NK cells impairment, with reduced expression of the main activating receptors NKp30, NKp46 and NKG2D. Similarly, as discussed in the previous chapter, *Jun et al.* investigated the differences of NKp30 and NKp46 in different stages of disease. However, differently from our findings, they did not see any differences in the expression of NKp46 and no differences between early and late stages in NKp30 expression.(405) These differences could be due to intrinsic differences within the population, the sample size or the gating strategies.

Finally, we performed survival analyses according to the level of the expression of the receptors. We decided to use as cut off the 75° percentile as the ROC curves were not accurate enough to set up a threshold, due to the limited size of pour population.

In our analysis, only DNAM-1 expression showed a better survival for expression less than 90% in the whole NK cells population or in the $CD56^{dim}$ subpopulation. We can speculate that higher expression of DNAM-1 could represent later stages of disease and therefore worse prognosis. However, this is difficult to demonstrate in our cohort due to limited numbers and a follow up <5 years.

In conclusion, in this chapter we demonstrate that NK cells activation could have an impact on tumour size and on the lymphatic spread of pancreatic cancer cells, confirming their pivotal role in controlling tumour growth and an anti-metastatic activity. In particular, NKp30 and NKp46 expression seems to correlate with T stage and LN ratio and could be of use to better identify subclasses of patients that could benefit of immune therapy.

Chapter 5. Phenotypic differences between PBMC and TILs

5.1 Introduction

In the previous chapter, we have demonstrated that NK cells show a functionally impaired phenotype and are correlated with clinical characteristics, which confirms their importance in controlling tumorigenesis. We therefore wanted to confirm the presence of NK cells within pancreatic cancer and investigate any differences compared to circulating NK cells.

Infiltrating NK cells is a rare population among tumour infiltrating lymphocytes, nonetheless their presence correlates with a better prognosis in many solid cancers, including colorectal, breast, gastric and lung adenocarcinoma.(445, 446) Moreover, they play a pivotal role in the control of haematological malignancies, such as chronic myeloid leukaemia and B cell lymphoma.(199) Despite increasing data suggesting an active role in the control of distal metastasis from solid tumours, a local influence on tumour growth is far to be demonstrated, compared to other infiltrating cells, such as $CD8^+$ T cells or T_{reg}. Also, infiltrating NK cells. appears to have a different phenotype compared to the circulating counterpart, being the $CD56^{bright}CD16^{neg}$ subset the most represented NK cells. As demonstrated above, this subset does not show cytolytic activity, but it is an important source of cytokines, among which INFγ is the most important.

5.2 Material and methods

PBMCs (n=10) collected from patients undergoing pancreas resection for PDAC, were compared to autologous TILs (n=10), isolated from fresh tissue, according to the technique described in Chapter 2.2.3. The PBMCs were stained with two 10-colors flowcytometry panels and the composition and phenotype of NKs were compared between the two groups.

5.3 Differences in the distribution of total NK cells and in the two subpopulations, $CD56^{bright}$ and $CD56^{dim}$

We have previously demonstrated that the total relative number of circulating NK cells do not differ between PDAC patients and the healthy population. Conversely, when we compared the presence of NK cells in 10 TILs and their corresponding PBMCs, we found a dramatic drop in the percentage of NK cells infiltrating the tumour (16% vs. 2%, p=0.005). Also, differently to circulating PBMCs, TILs show a severe decrease in the cytotoxic $CD56^{dim}$ population (15% vs. 2%, p=0.002), while $CD56^{bright}$ does not show any significant changes in the relative number (0.6% vs. 0.3% p=0.9) (Figure 5-1).

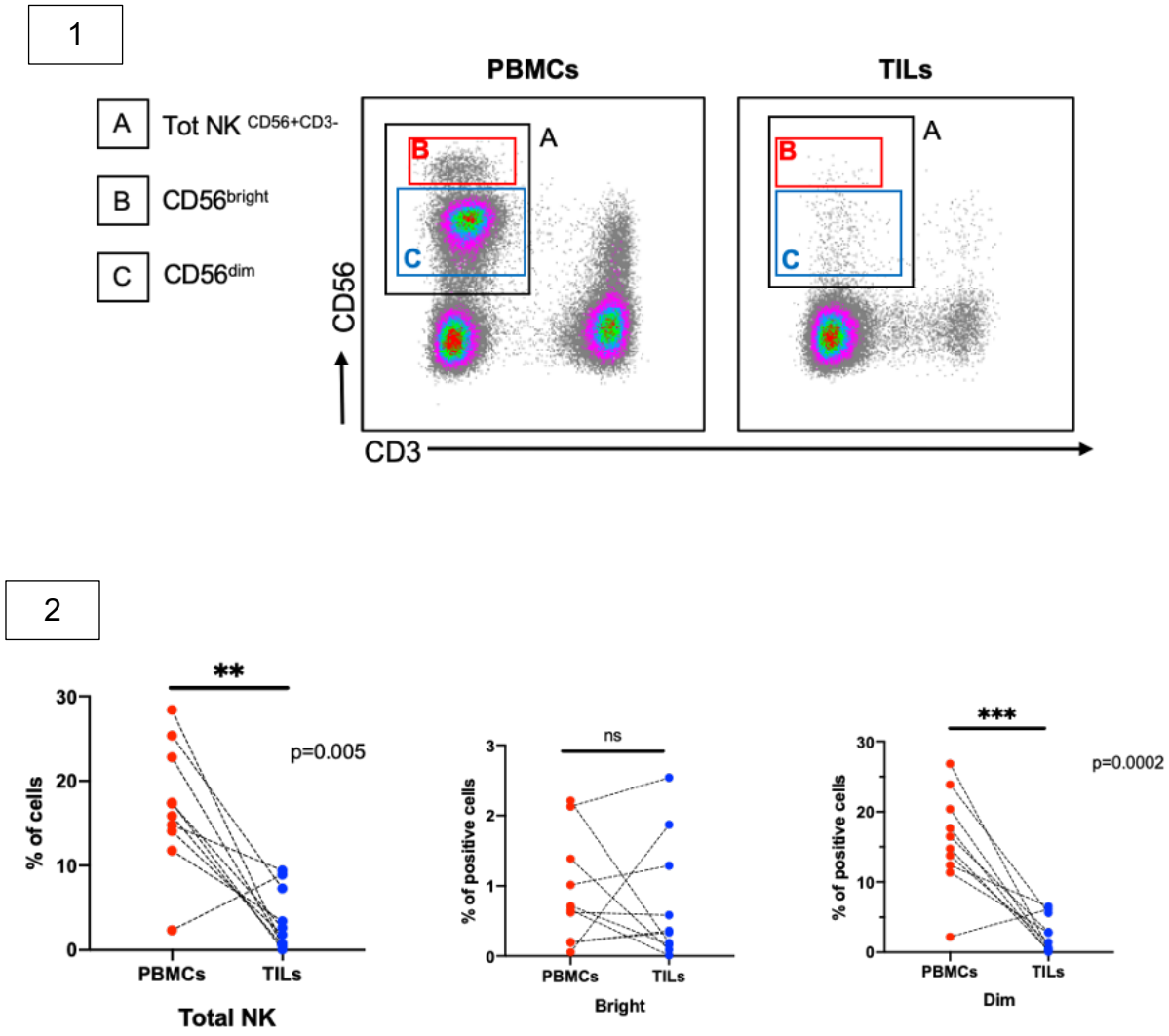


Figure 5-1. NK cells are a minority population within PDAC

1. An example of dot plot from flow cytometry staining, to gate the NK cells using $CD3^{neg}CD56^{pos}$ as makers; **2.** Percentage of the total NK cells and the two subsets in in PDAC patients and in autologous TILs were compared individually, using scatter plots, with each point represent a donor. Significance was calculated with paired-t Student's test or the Related samples-Wilcoxon Signed-Rank test, according to normal distribution; $p<0.05$ (*), $p<0.01$(**), $p<0.001$ (***), $p<0.0001$ (****).

A further analysis of the distribution of circulating NK cells and infiltrating NK cells according to CD16 expression, shows an opposite pattern compared to what was found in PBMCs vs. HD.

Infiltrating NK cells show a dramatic loss of CD16 expression: while in peripheral bloods of patients $CD56^{bright}CD16^{neg}$ represents not more than 3% of NK cells, the percentage of this subset in infiltrating NK cells is 10-times increased to almost 21% [(2.5% vs. 20.5%, p=0.007)]. Moreover, the $CD56^{dim}CD16^{neg}$, which in peripheral bloods are less than 5%, reaches almost 50% of the NK cells within the tumour (4% vs. 55%, p<0.0001). On the other hand, the $CD56^{bright}CD16^{pos}$ demonstrates a down trend in the percentage of positive cells, but this does not reach the significance, (2.6% vs. 1%, p=0.8), while the $CD56^{dim}CD16^{pos}$ is dramatically reduced in infiltrating NK cells (90% vs. 22%, p<0.0001). Results are summarised in Figure 5-2.

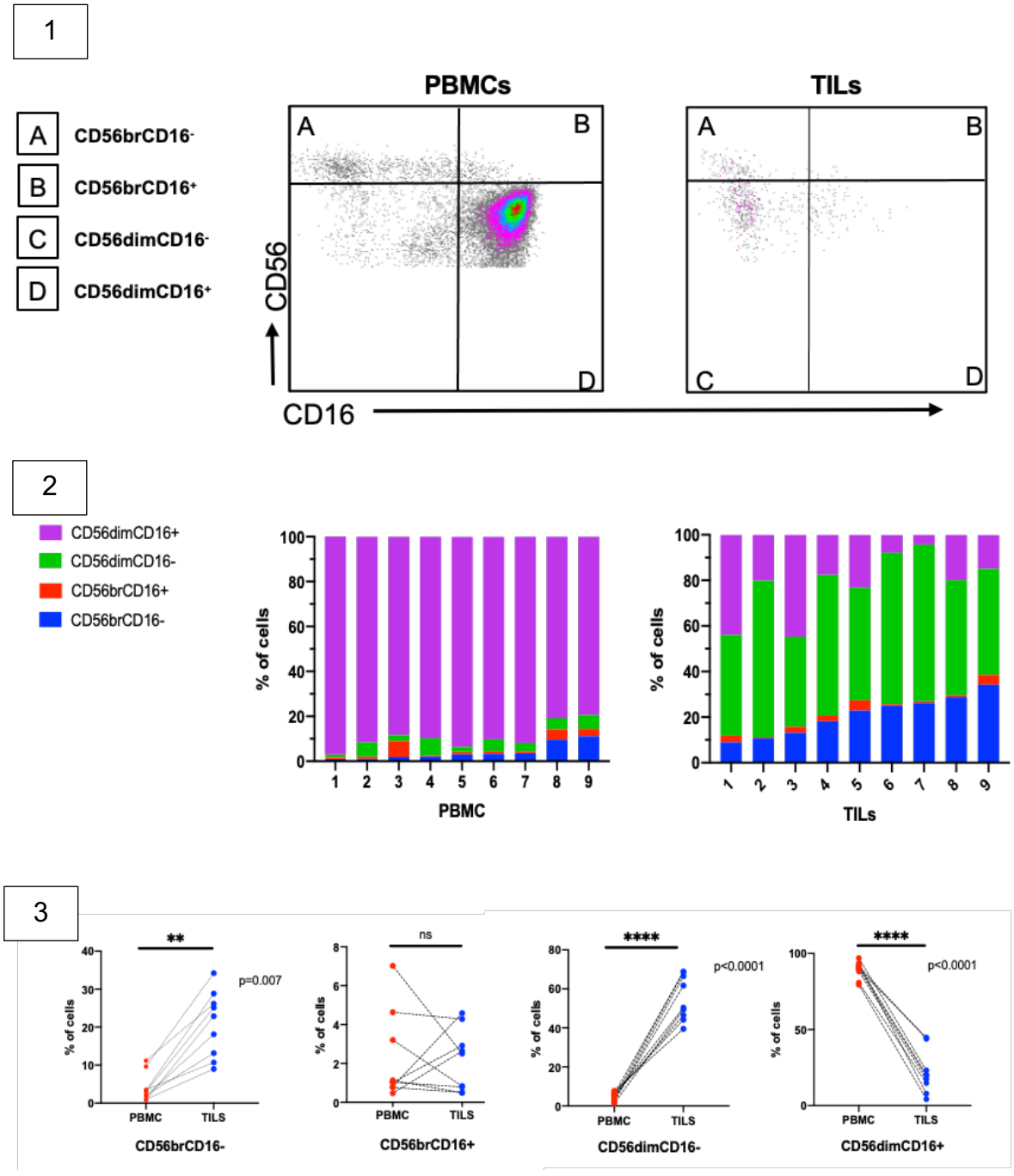


Figure 5-2. Infiltrative NK cells show an opposite distribution in the four subgroups and a drastic reduction of CD16, compared to corresponding PBMCs.

1. An example of dot plot from flow cytometry staining to gate the four NK subsets according to CD56 and CD16 expression. **2.** The composition of the four NK subsets

in PBMCs and in TILs was compared in bar charts; **3.** The percentages of four NK subsets in PBMCs and TILs were compared individually, using scatter plots. Significance was calculated with paired-t Student's test or the Related samples-Wilcoxon Signed-Rank test, according to normal distribution; $p<0.05$ (*), $p<0.01$(**), $p<0.001$ (***), $p<0.0001$ (****).

5.4 TILs expressed markedly reduced level of activating receptors

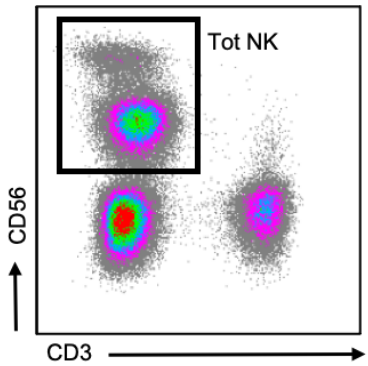
The comparative analysis of the expressions of NK cells receptors in the two groups, PBMCs and TILs, shows two different phenotypic patterns of expression.

Compared to PBMCs, TILs show a dramatic reduction of the expression of activating receptors and maturation markers, such as DNAM-1, NKp30, CD57 and KIRs. This is showed at all levels, either considering the entire NK population (*DNAM-1*: 84% vs. 38%, p<0.0001, MFI 5185 vs. 1304 p=0.005; *NKp30*: 60% vs. 25%, p=0.007, MFI 7315 vs. 3064, p=0.007; *CD57*: 43% vs. 8%, p=0.004, MFI 4000 vs. 320, p=0.004; *KIRS*: 15% vs. 3% p=, MFI 1204 vs. 1830, p= 0.06), and the two main subgroups $CD56^{bright}$ (*DNAM-1*: 98% vs. 80%, p=0.003, MFI 6588 vs. 2797 p=0.005; *NKp30*: 70% vs. 31%, p=0.03, MFI 13339 vs. 6800, p=0.008; *CD57*: 3% vs. 0%, p=0.004, MFI 4000 vs. 320, p=0.004; *KIRS*: 15% vs. 3% p=0.04, MFI 295 vs. 249, p= 0.12) and $CD56^{dim}$ (*DNAM-1*: 95% vs. 50%, p<0.0001, MFI 4984 vs. 1002 p=0.005; *NKp30*: 61% vs. 28.6%, p=0.001, MFI 7308 vs. 2965, p=0.008; *CD57*: 51% vs. 14%, p<0.0001, MFI 4927 vs. 335, p=0.02; *KIRS*: 17% vs. % p=0.011, MFI 1870 vs. 1456, p= 0.06). The expression of CD16 is markedly suppressed both in the total NK cell population (92% vs. 21%, p<0.0001; MFI 45,294 vs. 421, p= 0.002) and in $CD56^{dim}$ (96.5% vs. 26%, p<0.0001; MFI 46251 vs. 468, p=0.035), while the $CD56^{bright}$ shows a similar trend, which however does not reach the statistically significant (55% vs. 17.5%, p=0.14, MFI 3607 vs. 268, p=0.07). The activating receptor NKp46 is reduced is the $CD56^{bright}$ subpopulation (65% vs. 41%, p=0.011, MFI 8000 vs. 4800, p=0.007), while the $CD56^{dim}$ and the whole NK cell population do not seem affected. Interestingly, NKG2C is significantly upregulated in the whole infiltrating NK cell population (6% vs.

9%, p=0.04) and in $CD56^{dim}$ (2.7% vs. 8.8%, p=0.021) compared to circulating NK cells, but this is not demonstrated in the $CD56^{bright}$ (15% vs. 11%, p=0.1). Finally, NKG2D shows is suppressed in the $CD56^{bright}$ (75% vs. 49%, p=0.003, MFI 9784 vs. 4679, p= 0.003), but upregulated in the cytotoxic $CD56^{dim}$ (41% vs. 53%, p=0.07, MFI 4000 vs. 6500, p=0.035).

The analysis of expression of the other receptors does not show any significant difference between circulating and infiltrating NK cells. Figure 5-3, Figure 5-4 and Figure 5-5 summarise our results.

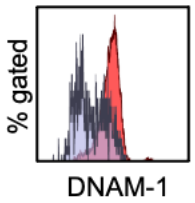
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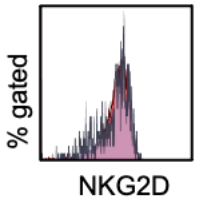

Tot NK
CD56
CD3

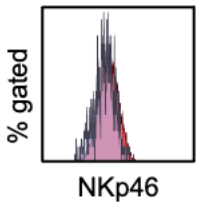
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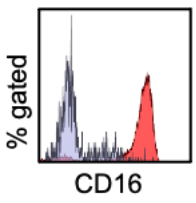
PBMC

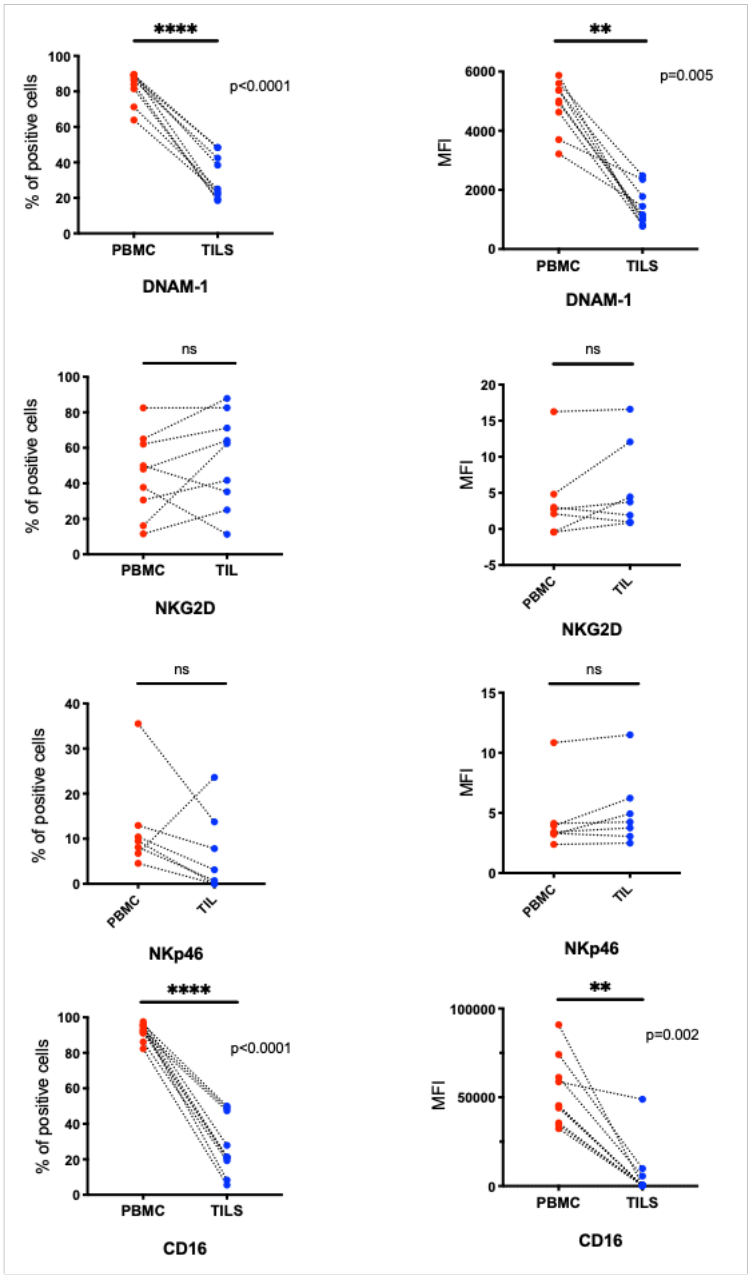
TILs


% gated
DNAM-1


% gated
NKG2D


% gated
NKp46


% gated
CD16


****
p<0.0001
% of positive cells
PBMC
TILS
DNAM-1
**
p=0.005
MFI
PBMC
TILS
DNAM-1
ns
% of positive cells
PBMC
TIL
NKG2D
ns
MFI
PBMC
TIL
NKG2D
ns
% of positive cells
PBMC
TIL
NKp46
ns
MFI
PBMC
TIL
NKp46
****
p<0.0001
% of positive cells
PBMC
TILS
CD16
**
p=0.002
MFI
PBMC
TILS
CD16

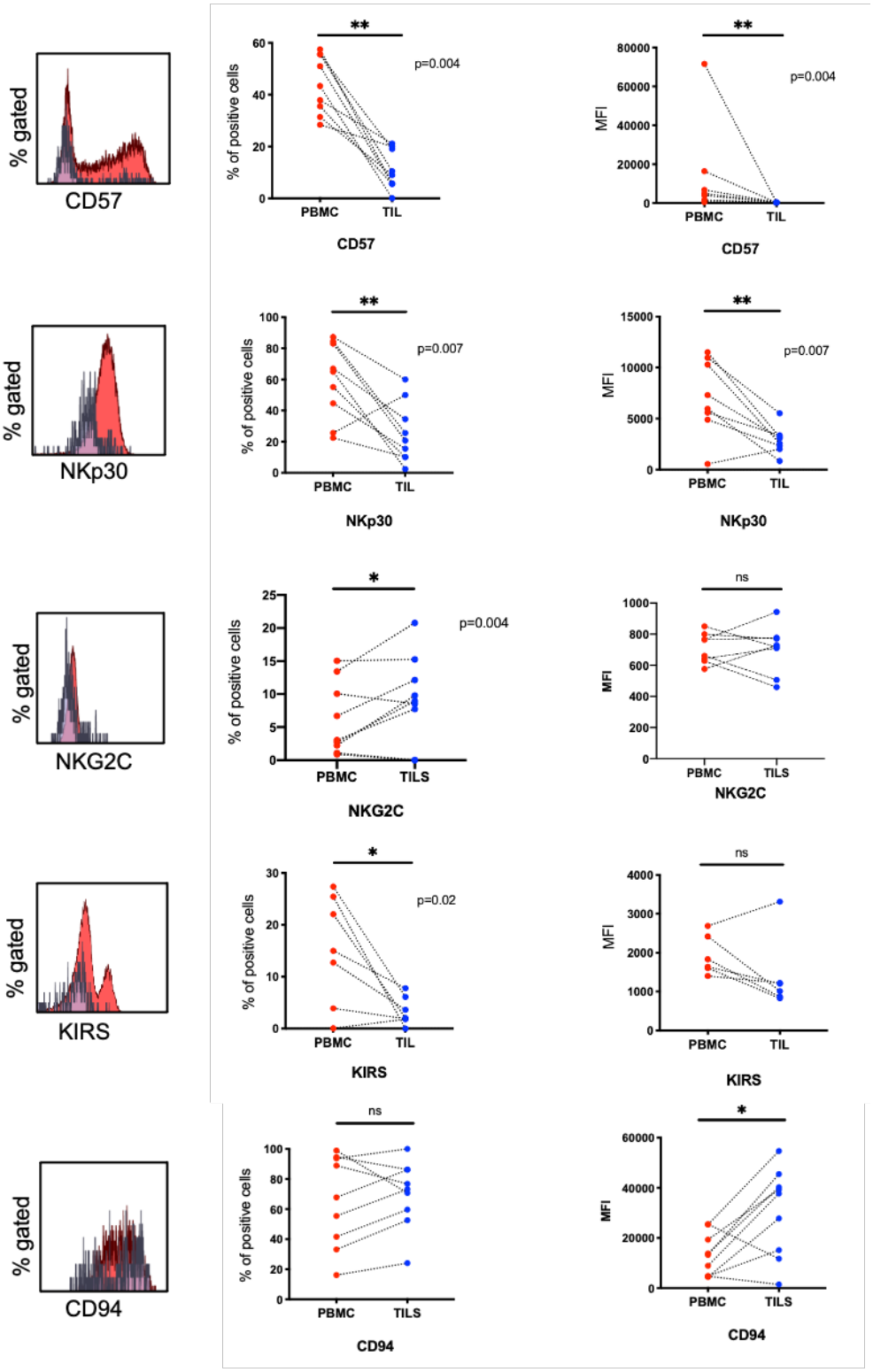
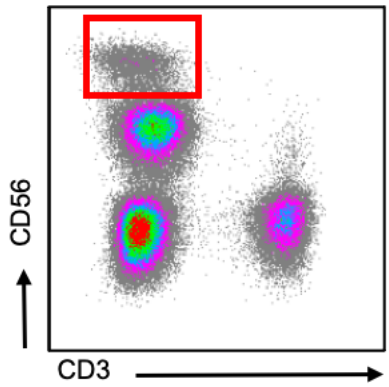

% gated
CD57
% of positive cells
**
p=0.004
PBMC
TIL
CD57
MFI
**
p=0.004
PBMC
TIL
CD57
% gated
NKp30
% of positive cells
**
p=0.007
PBMC
TIL
NKp30
MFI
**
p=0.007
PBMC
TIL
NKp30
% gated
NKG2C
% of positive cells
*
p=0.004
PBMC
TILS
NKG2C
MFI
ns
PBMC
TILS
NKG2C
% gated
KIRS
% of positive cells
*
p=0.02
PBMC
TIL
KIRS
MFI
ns
PBMC
TIL
KIRS
% gated
CD94
% of positive cells
ns
PBMC
TILS
CD94
MFI
*
PBMC
TILS
CD94

Figure 5-3. Total NK cells within the tumour show a substantial reduction of expression of activating receptors, compared autologous PBMCs.

1. Gating strategy for total $CD3^{neg}CD56^{pos}$ (black rectangle); **2.** The expression of differentiation markers (CD16, CD57, NKG2C, CD94) and activating or inhibitory makers (DNAM-1, NKG2D, NKp30, Nkp46, KIRs) was compared between PBMCs and autologous TILs. On the left, the representative histogram of each marker; on the right, the scatter plots: the comparison was carried out with percentage (middle) and mean florescence intensity (MFI) (right). Significance was calculated with paired-t Student's test or the Related samples-Wilcoxon Signed-Rank test, according to normal distribution; $p<0.05$ (*), $p<0.01$(**), $p<0.001$ (***), $p<0.0001$ (****).

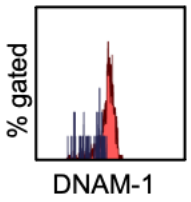
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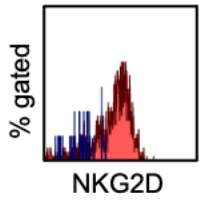

CD56
CD3

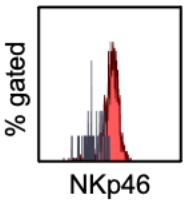
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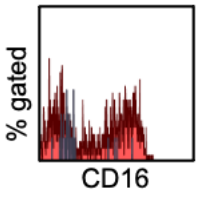
PBMC

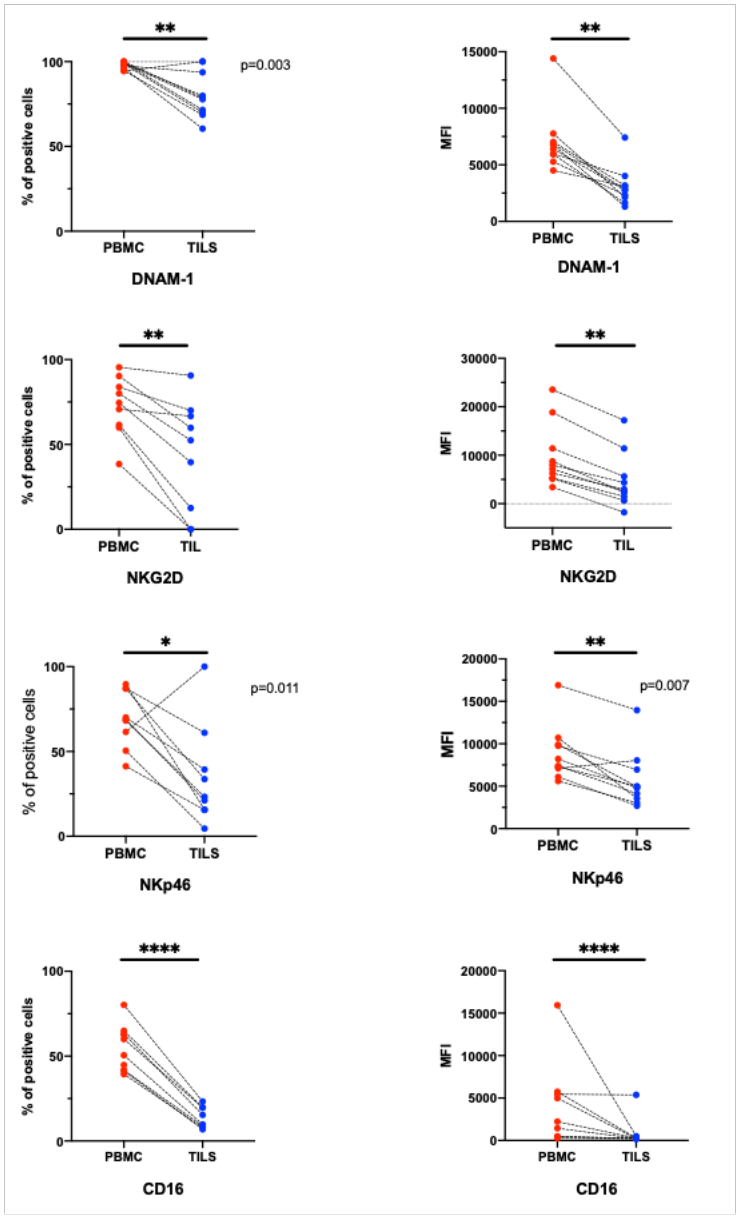
TILs


% gated
DNAM-1


% gated
NKG2D


% gated
NKp46


% gated
CD16


**
p=0.003
% of positive cells
PBMC
TILS
DNAM-1
**
MFI
PBMC
TILS
DNAM-1
**
% of positive cells
PBMC
TIL
NKG2D
**
MFI
PBMC
TIL
NKG2D
*
p=0.011
% of positive cells
PBMC
TILS
NKp46
**
p=0.007
MFI
PBMC
TILS
NKp46
****
% of positive cells
PBMC
TILS
CD16
****
MFI
PBMC
TILS
CD16

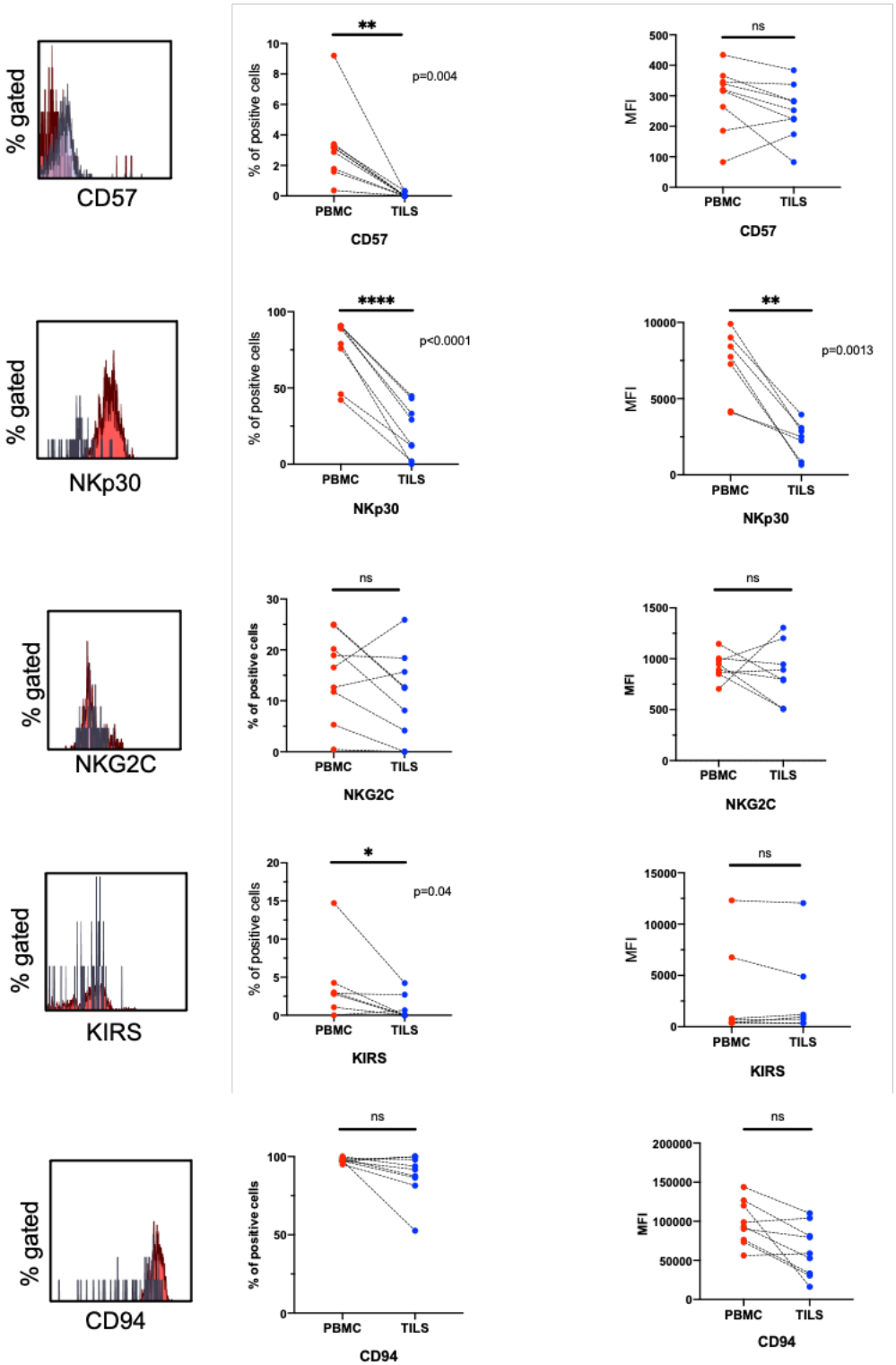
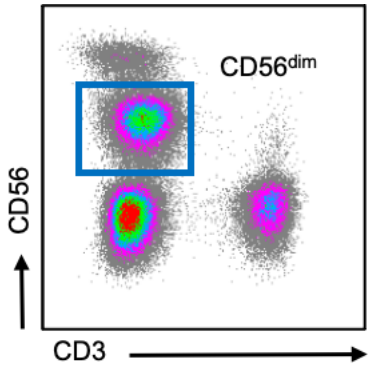

% gated
CD57
**
p=0.004
% of positive cells
PBMC
TILS
CD57
ns
MFI
PBMC
TILS
CD57
% gated
NKp30
****
p<0.0001
% of positive cells
PBMC
TILS
NKp30
**
p=0.0013
MFI
PBMC
TILS
NKp30
% gated
NKG2C
ns
% of positive cells
PBMC
TILS
NKG2C
ns
MFI
PBMC
TILS
NKG2C
% gated
KIRS
*
p=0.04
% of positive cells
PBMC
TILS
KIRS
ns
MFI
PBMC
TILS
KIRS
% gated
CD94
ns
% of positive cells
PBMC
TILS
CD94
ns
MFI
PBMC
TILS
CD94

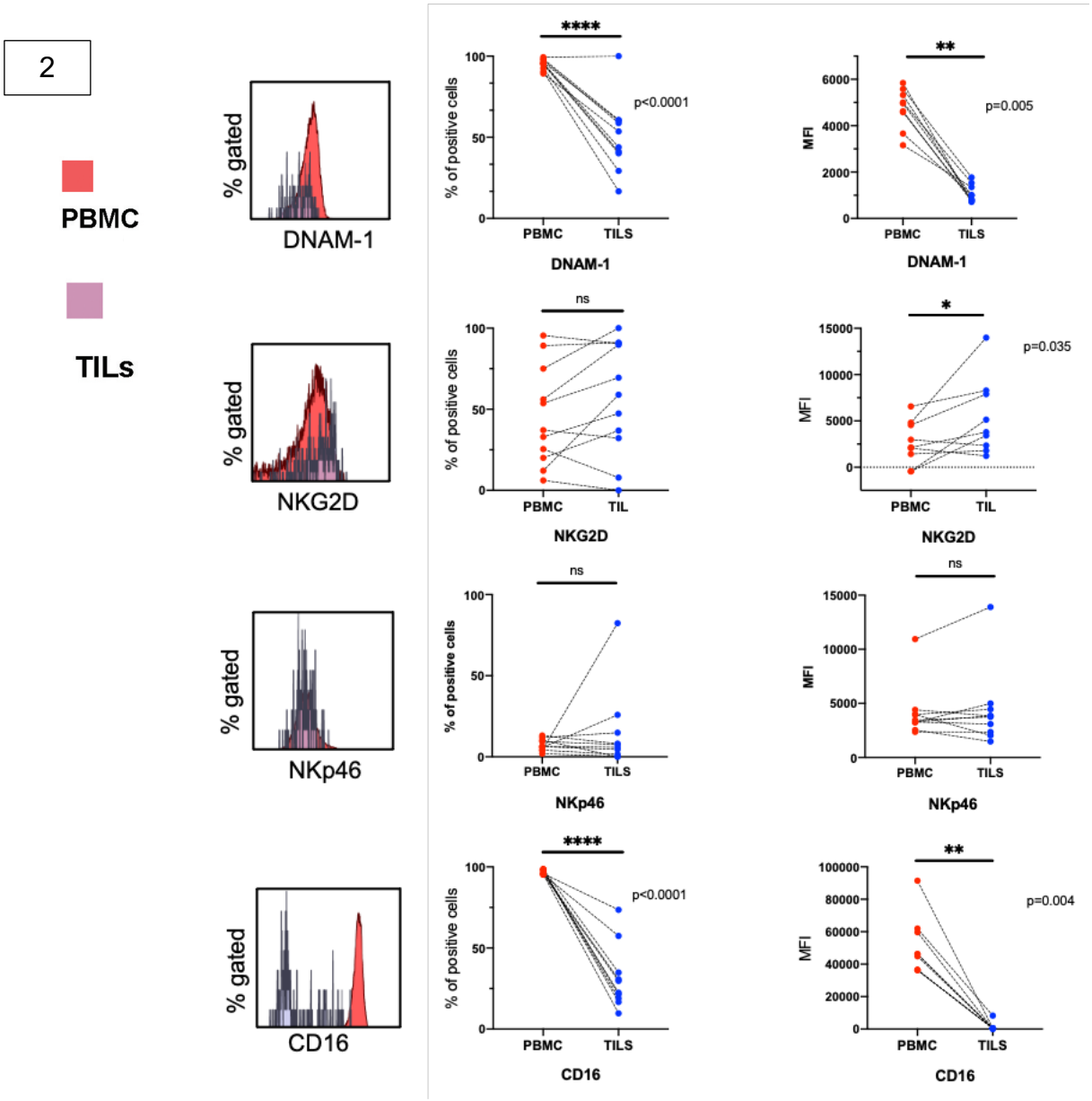
Figure 5-4. $CD56^{dim}$ NK cells within the tumour show a markedly suppressed cytotoxic function and an immature phenotype compared to autologous PBMCs

1. Gating strategy for total $CD56^{bright}$ (red rectangle); **2.** The expression of differentiation markers (CD16, CD57, NKG2C, CD94) and activating or inhibitory makers (DNAM-1, NKG2D, NKp30, Nkp46, KIRs) was compared between PBMCs and autologous TILs. On the left, the representative histogram of each marker; on the right, the scatter plots: the comparison was carried out with percentage (middle) and mean florescence intensity (MFI) (right). Significance was calculated with paired-t Student's test or the Related samples-Wilcoxon Signed-Rank test, according to normal distribution; $p<0.05$ (*), $p<0.01$(**), $p<0.001$ (***), $p<0.0001$ (****).

1


CD56dim
CD56
CD3

2


PBMC
TILs
% gated
DNAM-1
NKG2D
NKp46
CD16
% of positive cells
MFI
PBMC
TILS
TIL
****
**
*
ns
p<0.0001
p=0.005
p=0.035
p=0.004

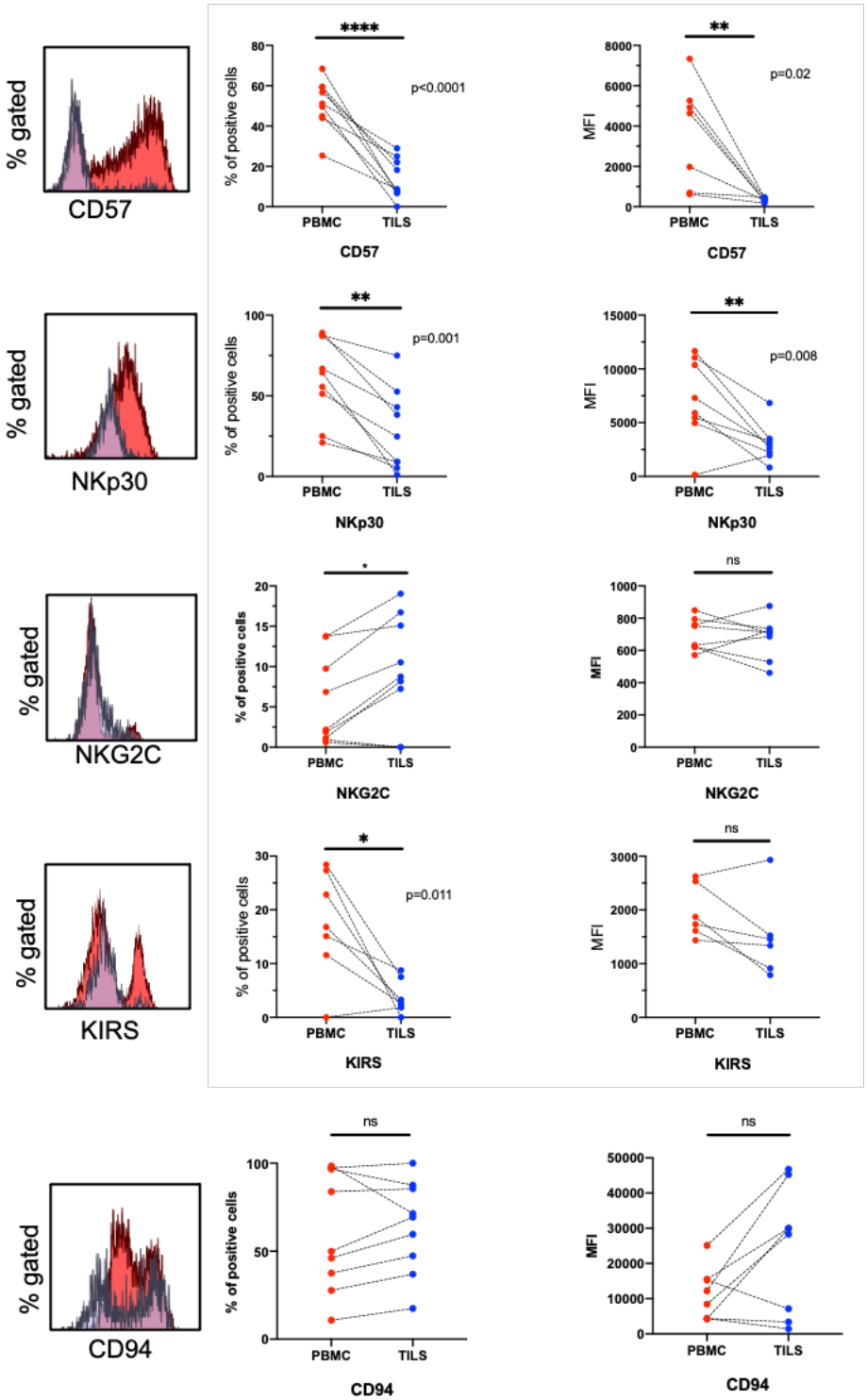

% gated
CD57
****
p<0.0001
% of positive cells
PBMC
TILS
CD57
**
p=0.02
MFI
PBMC
TILS
CD57
% gated
NKp30
**
p=0.001
% of positive cells
PBMC
TILS
NKp30
**
p=0.008
MFI
PBMC
TILS
NKp30
% gated
NKG2C
*
% of positive cells
PBMC
TILS
NKG2C
ns
MFI
PBMC
TILS
NKG2C
% gated
KIRS
*
p=0.011
% of positive cells
PBMC
TILS
KIRS
ns
MFI
PBMC
TILS
KIRS
% gated
CD94
ns
% of positive cells
PBMC
TILS
CD94
ns
MFI
PBMC
TILS
CD94

Figure 5-5. Cytotoxic CD56^{dim}NK cells within the tumour show a markedly suppressed cytotoxic function and an immature phenotype compared to autologous PBMCs

1. Gating strategy for total CD56^{bright} (red rectangle); **2.** The expression of differentiation markers (CD16, CD57, NKG2C, CD94) and activating or inhibitory makers (DNAM-1, NKG2D, NKp30, Nkp46, KIRs) was compared between PBMCs and autologous TILs. On the left, the representative histogram of each marker; on the right, the scatter plots: the comparison was carried out with percentage (middle) and mean florescence intensity (MFI) (right). Significance was calculated with paired-t Student's test or the Related samples-Wilcoxon Signed-Rank test, according to normal distribution; $p<0.05$ (*), $p<0.01$(**), $p<0.001$ (***), $p<0.0001$ (****).

5.5 Discussion

In 2001, Shankaran et al. demonstrated that $Rag2^{-/-}$ mice, lacking B and T cells but not NK cells, were more susceptible to chemical-induced carcinogenesis than the immunocompetent mice.(447) This first study initiated the era of immune oncology, as demonstrated that cancers can be immunogenic and potentially be controlled by host immune system. The immune editing theory gave more insights on this matter, adding the concept that immune infiltrate is directly involved not only in detecting the cancer cells, but also in later stages in shaping cancer growth and ability to escape. This was already discussed in chapter 1.4.1. Moreover, a large amount of data is continuously demonstrating that the stromal component of the tumour actively participates either in the immune response or immune escape, and it has become a key target in developing therapeutical strategies.

The phenotype of this infiltrate dictates the effectiveness of the immune response and the prognosis of the host. For instance, an immune infiltrate represented by $CD8^+$ T cell, T_H1 cells, M1 macrophages, type 1 dendritic cells (DC) and NK cells is considered as a strong effective immune response against the cancer cells and holds the best prognosis. At the opposite pole, an immunosuppressive phenotype is characterised by the presence of T_H2 cells, M2 macrophages, type 2 DC, MDCSs, T_{reg} cells and absence of NK cells. However, realistically the immune infiltrate presents characteristic in between of these two opposite scenarios. The clinical outcome and prognosis are thus the result of this balance.

As lymphocytes are the main white cell population infiltrating the tumours, the experimental research has been focused on T cells, producing a gigantic amount of

strong data which have led to the publication of metanalysis and systematic reviews.(448-451) On the contrary, infiltrating NK cells (iNK cells) represent approximately 3-10% of the TILs, but a large amount of data have now demonstrated that their presence correlates with a good prognosis in solid tumours.(445, 446, 452, 453) However, discrepant results have also been reported and need to be mentioned.(454, 455) In pancreatic cancer, iNK cells are even a rarer population compared to other solid tumours such as colorectal, breast, liver or lung.(388) In fact, their frequency is less than 0.5%, and this makes these cells difficult to analyse. This could be due to many factors: (1) the important desmoplastic reaction that characterised pancreatic cancer, which plays as a physical barrier; (2) the release of cytokines that exclude NK cells from the tumoral milieu or (3) The hypoxic and acidotic microenvironment; (4) a combination of the above.(456)

In this chapter, we analysed the iNK cells isolated from ten fresh pancreatic tissues, and we compared their phenotype with corresponding circulating NK cells. While total $CD3^{neg}CD56^{pos}$ NK cells represent approximately 20% of the circulating lymphocytes, their frequency within the tumour tissue varies from complete absence to not more than 9%. Also, the $CD56^{bright}CD16^{neg}$ becomes the main infiltrating NK subpopulation, while the cytotoxic $CD56^{dim}CD16^{pos}$ is completely absent or very rare in the infiltrate. This is an opposite scenario compared to bloodstream. Moreover, the number of $CD56^{dim}CD16^{neg}$, which does not reach more than 5% of the NK cells in the blood, represents approximately 50% of the iNK cells.

Other Investigators have reported the $CD56^{bright}CD16^{neg}$ as the most prevalent iNK cells in many solid tumours, included, lung, breast, colorectal and bladder cancers.(445, 457, 458) These cells show low cytotoxic activity due to the

downregulation of activating receptors (DNAM-1, NKG2D, NKp30, NKp46 and CD16), lack of perforins and granzymes. Interestingly, although rare, they are mostly localized within the stromal tissue. An important characteristic of these cells is their pro-angiogenic phenotype, represented by their ability to express VEGF, which makes them functionally similar to decidual NK cells (dNK cells). dNKs represent the large majority of the lymphocytes infiltrating the maternal-foetal interface. The expression of pro-angiogenic factors, such VEGF and PLGF, together with their immunosuppressive activity, is critical to allow the implantation of the trophoblast, and regulates the structural changes of the arterial vascularisation in the decidua.(459) In fact, a lack of dNK is associated with a higher risk of miscarriage.(460) dNK are probably recruited from the peripheral bloods, as they express receptors for chemotactic cytokines, as CXCR4 and CXC3. Interestingly, also tumour infiltrating $CD56^{bright}$ are reported to express high level of CXC3 and CD9, therefore we can speculate on a peripheral origin rather than their differentiation from tissue resident NK cells. Moreover, the expression of NKG2C also characterised dNK cells, and interestingly we found that NKG2C is also upregulated in our samples. These findings suggest a possible dual role of iNK, as potentially they could facilitate cancer development, rather to represent an effective immune response against the tumour. The secretion of TGF-β by the TME is probably responsible for this dysfunctional maturation. In fact, it was demonstrated that TGF-β is able to convert peripheral $CD56^{dim}CD16^{pos}$ into $CD56^{bright}CD16^{neg}$, which are similar to dNK.(319, 461, 462) Hypoxia could also be a key player in the polarisation of iNKs towards a proangiogenic phenotype, as recently demonstrated by *Xi et al.* The single-cell RNA sequencing of iNK cells shows the upregulation of HIF-α, a transcription factor which activates angiogenesis and cell proliferation (see chapter 1.4.2). The

inhibition of HIF-α enhanced NK cells antitumour activity and control tumour progression.(463)

Our analysis demonstrates that expression DNAM-1 is dramatically reduced, while NKG2D is downregulated only in the $CD56^{bright}$ subpopulation.

In the previous chapter, we demonstrated a similar trend in the expression of DNAM-1 in the peripheral bloods of patients, compared to healthy donors. The expression of DNAM-1 has been found to be more susceptible to direct contact with cancer cells, rather than the release of soluble ligands, as for instance NKG2D is. In fact, *Sanchez-Correa* et al. analysed the level of expression of DNAM-1 on NK cells of patients affected by acute myeloid leukaemia. They found an inverse correlation between DNAM-1 expression on NK cells and the expression of CD112 on leukemic blasts. Moreover, *in vitro* co-culture of HDs' NK cells and leukemic blasts expressing CD112, induced the downregulation of DNAM-1 expression on NK cells.(464) Similar results have been demonstrated also in solid tumours: the incubation of NK cells with ovarian cancer cells expressing CD155 (PVR), leads to the downregulation of the DNAM-1 receptor.(408) These findings support the hypothesis that the expression of a receptor could be negatively influenced by the chronic exposure to its ligands. This could in part explain the drastic reduction of DNAM-1 in iNK cells of our patients, compared to circulating NK cells. However, soluble ligands as well could potentially be involved in some extent to NK cells cytotoxic dysfunction. In fact, it has been demonstrated in a mouse model that soluble CD155 (sCD155), is able to negatively interfere with the DNAM-1-mediated degranulation process.(319, 465) Immunohistochemistry analysis on 92 samples of pancreatic cancer confirmed the

downregulation of DNAM-1 expression, and the correlation with the stage of the disease.(466)

The downregulation of NKG2D expression on iNK cells was demonstrated by previous studies on different solid tumours, and here confirmed.(467, 468) Interestingly, we also noticed that the reduction of NKG2D expression involves in particular the $CD56^{bright}$ subpopulation, which is involved in the production of cytokines, such as INFγ, implying a possible impairment in the regulation of the NK-driven inflammatory response. However, the $CD56^{dim}$ iNK cells showed a slight increase in the MFI expression of NKG2D, suggesting that a certain level of degranulation activity is still present, although probably impaired, as other activating receptors are downregulated. As seen in DNAM-1, TGF-β released by the TME seems to impact negatively the expression of NKG2D, even in the presence of the ligands MICA/MICB (see chapter Chapter 6).

The impairment of the anti-tumour toxicity is also supported by the decrease the activating receptors NKp30 and NKp46, similar to what we observed in the peripheral blood. In particular, NKp30 is downregulated in both main subgroups $CD56^{dim}$ and $CD56^{bright}$, while NKp46 is downregulated only in the $CD56^{bright}$. The suppression of NCRs is a typical mechanism of immune escape, as this has been observed in other solid tumours. For example, *Rocca et al.* analysed the phenotype of infiltrating NK cells in fresh tissues of colorectal cancer and found a drastic decrease in the level of NKp30 and NKp46, along with NKG2D, DNAM-1 and CD16.(468) Similar observations were reported also in lung and breast cancers.(319, 469, 470) Moreover, a metanalysis by *Zhang et al.* showed that the expression of NKp46 correlates with a better OS and RFS and, interestingly, NKp46 is also an independent prognostic factor for survival. Due to

the lack of survival analyses, the same conclusion could not be reached for NKp30.(446) Interestingly, the study by *Rocca et al.* also confirmed the downregulation of KIRs, as observed in our samples. According to the Authors, the downmodulation of KIRs could represent a state of “hypo-responsiveness”, as previous studies have also described.(471)

Finally, the marker of maturation CD57 is extremely reduced in all infiltrating NK cells, particularly in the $CD56^{dim}$, while it is almost absent in the $CD56^{bright}$. This is an opposite result compared to the peripheral bloods, in which CD57 is upregulated, particularly in the cytotoxic $CD56^{dim}$ fraction. We have already seen that $CD57^{pos}$ NK cells show a robust cytotoxic activity, and in fact the infiltration $CD57^{pos}$ NK cells of in tumours has been associated with better clinical outcomes, stages and prognosis, in different solid tumours and haematological malignancies.(427, 446) Interestingly, $CD57^{pos}$ NK cells were also found in brain metastases from various cancers, included lung, breast and renal, but the significance of such phenomenon is unclear, as this did not correlate with an enhanced activity against the metastatic cells.(472) Therefore, the absence of $CD57^{pos}$ iNK cells could represent a further indication of cytotoxic dysfunction of iNK cells. However, $CD57^{neg}$ NK cells are usually the main source for INFγ in response to cytokines, and in fact usually the regulatory $CD56^{bright}$ NK cells express very low level of CD57. If $CD57^{neg}$ NK cells are preferentially recruited by the tumour or CD57 is lost as a consequence of the interaction with tumour cells or the TME is difficult to establish, but it would be interesting to investigate this topic in future.

We could speculate that this phenotypic impairment could be the result of several mechanisms of immune escape: a direct downregulation of the receptors induced by secretion of TGF-β, PGE2 or IDO by the hypoxic TME; a prolonged cell-to-

cell contact with tumour cells, which leads to “cellular exhaustion”; the secretion of soluble ligands that shed the receptors from proper activation; finally, the induction of a different developmental program of differentiation leading to different stage of maturation.

In conclusion, tumour infiltrating NK cells show a profoundly different phenotype compared to their circulating counterpart, characterised by the prevalence of $CD56^{dim/bright}CD16^{neg}$ cells. All the important activating receptors are downregulated, implying a deficient cytotoxic activity in terms of degranulation and INFγ production, in line with other Authors have demonstrated. This peculiar phenotype resembles decidual NK cells, a specific subtype of NK cells infiltrating the maternal-foetal interface during the very early stage of pregnancy. These cells stimulated angiogenesis and allow the correct implantation of the trophoblast in the decidua. The phenotypic changes found in our iNK cells corroborate the hypothesis that iNK, at a certain stage of tumour development, could stimulate tumour growth rather than contain it, thus playing a dual role in tumorigenesis.

Chapter 6. Functional impairment of circulating NK cells

6.1 Introduction

Circulating NK cells in patient affected by PDAC displayed an altered phenotype, characterised by the downregulation of important activating receptors, such as DNAM-1, NKG2D and NKp30. To exert effective activity against cancer cells, NK cells require synergic stimulation with different activating receptors.(405) Therefore, in this chapter, we wanted to investigate if these changes have any effect on peripheral NK cells ability to mount an effective response against pancreatic tumour, as previous studies have showed functional impairment in solid cancers such as breast and colorectal.(319, 468, 473) Thus, we investigated circulating NK cells functionality through their ability to degranulate, expressed as percentage of CD107a positive NK cells, their ability to kill target cells (cytotoxic activity against K562) and the production of cytokines, such as INFγ, TNFα, IL-2, , IL-4, IL-5 and IL-10. Ideally, the same analysis would have been planned for iNKs, but due to the paucity of iNK cells, this was not eventually attempted.

6.2 Material and methods

The lytic activity was tested by measuring the level of expression of the degranulation marker CD107a on NK cells (HD, n=5 vs. PDAC, n=5), against K562 target cells. Also, NKs' lytic activity was assessed by the relative cytotoxic activity (HD, n=7 vs. PDAC,

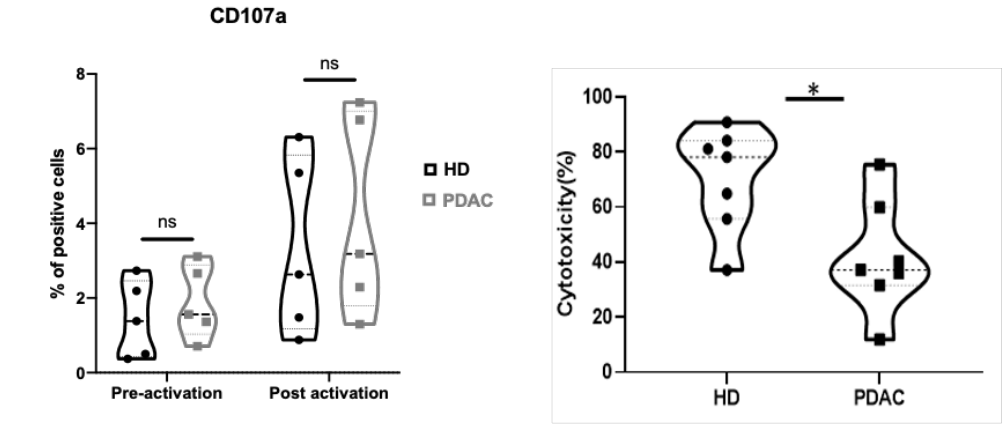
n=7). The cytokine production was assessed by intracellular staining, after incubation of PBMCs with K562 (HD, n=19; PDAC n=6).

6.3 Results

At first, the CD107a expression was investigated after K562 cell stimulation: there was no difference in CD107a expression between patients and healthy donors after the culture with target cells at 1:5 ratio (3.3% vs. 4.2%, p=0.6; delta increment, 150% vs. 137%, p=0.7). Afterwards, the relative lytic activity was measured by the absolute count of target cells K562 before and after co-culture with NK cells. The cytotoxicity of NK cells was markedly reduced in PDAC patients, with values of 42% and 70% in patients and donors respectively, at an E:T ratio of 1:1 (p=0.026).

Finally, the NK cells cytokines profile was studied after K562 stimulation. NK cells normally produce substantial amounts of proinflammatory cytokine such as IFNγ and TNFα and this pattern was observed in healthy donors. However, IFNγ production was substantially reduced in NK cells from PDAC patients (1.9% vs. 5.1%, p=0.003), and a trend towards reduced levels of TNFα was also seen (2.4% vs 3.9%, p=0.12). IL-2 production was also lower in the patient group, present within only 0.7% of cells compared to 2.4% from healthy donors (p=0.009). Strikingly, IL-10 production was markedly increased in NK cells from PDAC patients, present in 9.8% of cells compared to only 2.2% in healthy donors (p=0.001). Expression of IL-4 and IL-5 was comparable between donors and patients.

1



2

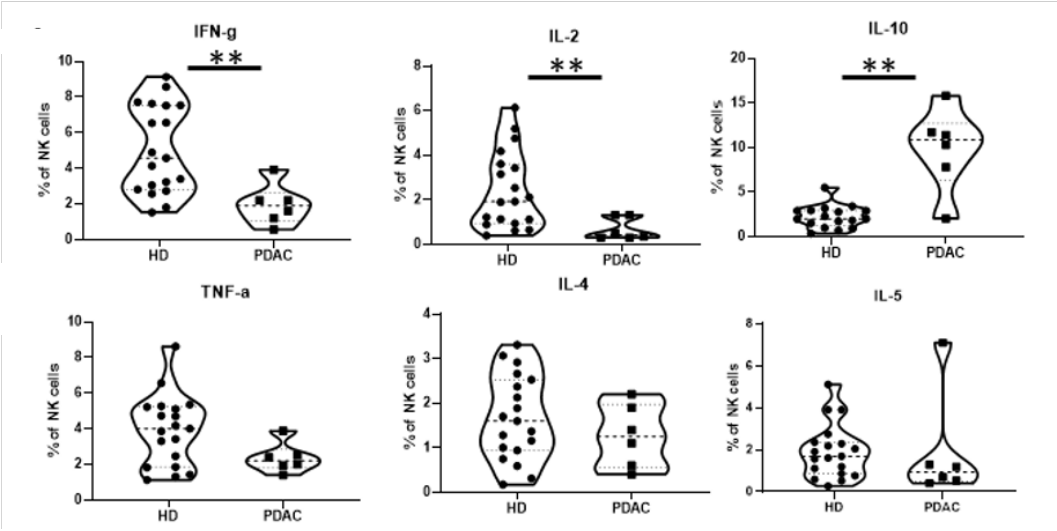


Figure 6-1. NK cells within the blood of PDAC patients show impaired cytotoxic function and suppressive phenotype.

1. NK cells lytic activity was investigated firstly measuring the expression of degranulation markers CD107a after co-culture with K562 target cells, at a 1:5 ratio in PDAC patients and HDs (on the left). Subsequently, the relative lytic activity was measured as the absolute count of CFSE-labelled K562 cells, before and after co-culture with NK cells, at an E:T ratio of 1:1 (on the right). **2.** Whole PBMCs from PDAC

patients and HDs were overnight co-cultured with K562 at 1:1 ratio, in the presence of Brefeldin A. Then, the cells were stained for surface receptors, fixed, permeabilised and stained with INFγ, IL-2, IL-10 TNFα, IL-4 and IL-5 antibodies. The percentage of NK cells producing each cytokine was compared between PDACs and HDs. Data are shown using violin plots, with each point representing a donor. Significance was determined using Mann-Whitney U-testing, $p<0.5$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$ (****)

6.4 Discussion

In this chapter, the functional profile of peripheral NK cells was assessed by flow-cytometry through cytotoxicity assay and cytokine profile, after stimulation. We could not perform the same analysis on iNK cells, due to the paucity of available cells.

We here noticed that NK cells from PDAC patients have a markedly altered profile of cytokine production, characterised by reduced levels of IFNγ and IL-2 and a substantially increased production of IL-10. As such, they express a 'regulatory' phenotype, a concept that is emerging within NK biology.(474) However, we did not find any differences between healthy and PDAC patients in the degranulation potential, defined as CD107a expression level. Conversely, the relative cytotoxic activity against target cells was significally impaired.

$CD56^{bright}$ are the main source of IL-10, and the secretion is modulated by the intensity and the duration of IL-12 released by DCs.(474) Therefore, the appearance of IL-10 secreting NK cells could reflect a chronic stimulation, as in tumours or viral infection like hCMV.(475) Also, IL-10 producing NK cells have been demonstrated after stem cell transplantation (476) and parasitic infestation.(477) Systemic concentration of IL-10 levels are 3-5 fold higher in the blood of patients with PDAC, where they strongly correlate with impaired T cell function (478-480), and our data indicates that NK cells are likely to be a major source of IL-10 production within the periphery. Importantly, all patients studied had undergone operative resection of PDAC with curative intent and had no evidence of metastatic disease at that time. Despite this, our findings reveal that the peripheral immune system has already been significantly

modulated by the disease process and it is likely that the microenvironment plays an important part in tumour spread.

INFγ is known to boost cancer immunosurveillance either in mice models or in humans.(189, 481) The co-engagement of the activating receptors NKG2D, B24 and LFA-1 is required to induce INFγ and TNFα production and secretion.(141) In our samples, INFγ secretion appears deficient, while no difference was noted for TNFα. This goes in parallel with the NKG2D downmodulation found in circulating NK cells. However, as the activation of CD16 alone is adequate to stimulate INFγ and TNFα secretion, other unknown mechanisms must be involved. In fact, CD16 is upregulated in peripheral NK cells and hypothetically could overcome NKG2D deficiency. This demonstrates that the expression and secretion of INFγ is more strictly regulated and requires a more complex engagement of different activator receptors, with probably different thresholds.(141) Interestingly, our results are in contrast with other Authors. In fact, *Jun et al.* found that the percentage of $INF\gamma^{pos}$ NK cells did not correlate with NKG2D or NKp30 expression. However, of notice, INFγ reduction was present in early stages of disease and did not correlate with tumour progression or prognosis.(405) Contradictory results were reported also in breast cancer: INFγ was reduced in peripheral NK cells compared healthy donors, but more interestingly the reduction run in parallel with the disease progression, being at its lowest level in metastatic cancer.(319) Soluble factors released in the bloodstream by TME seem to play a significant role in inducing these changes. In particular, TGF-β secreted by cancer cells, fibroblast and T_{reg} is able to profoundly inhibit INFγ secretion, as demonstrated by the restoration of NK cells functions after TGF-β-blocking antibodies *in vitro.*(319)

During the degranulation process, NK cells express lysosomal-associated membrane protein-1 (LAMP-1, also known as CD107a), whose level of expression correlates with granule exocytosis and the release of perforin and granzyme B.(482) Therefore, the level of CD107a has been used to indirectly assess cytotoxicity in NK cells. In healthy population, it was reported that only 1-2% of circulating NK cells should demonstrate expression of CD107a.(228) In our cohort of patients, the level of CD107a expression does not differ from healthy donors, either at rest or after co-culture with target cells K562. These results differ from previous studies in breast, colorectal and pancreatic cancer, in which CD107a was found reduced in peripheral NK cells of patients after incubation with target cells.(319, 405, 468) Moreover, in pancreatic cancer, CD107a was found to correlate with poor prognosis, in terms of survival and disease recurrence. Also, CD107a expression clearly correlates with the expression of NKG2D and NKp30.(405) These contradictory results could be explained by the low number of samples available in our experiments and also by different methods used to isolate peripheral NK cells from blood. Strangely, the CD107a level of expression did not correlate with the direct assessment of cytotoxicity against target cells, which is conversely very much impaired. Probably, this reflects the impairment of alternative mechanisms of cytotoxicity, other than the release of perforin and granzyme B, as for example Fas-FasL and/or TRAIL pathways. NKG2D, DNAM-1 and KIRs mismatch represent pivotal triggers of these Fas-FasL and TRAIL killing pathways in haematological malignancies, such as acute lymphatic leukaemia and multiple myeloma, but also in many solid tumours (neuroblastoma, ovarian carcinoma, gastric, colon and renal cancer).(228) These receptors are downregulated in peripheral NK cells of our patients as demonstrated in the previous chapter. We could therefore

speculate that the downregulation of these receptors profoundly affects NK cells cytotoxic activity and potentially represent a mechanism of immune escape.

In conclusion, we have demonstrated that peripheral NK cells of patients affected by pancreatic cancer are functionally impaired at multiple levels. Firstly, they lack ability to target cancer cells, as reflected by the reduced cytotoxicity. This seems dependent to downregulation of important activating receptors, such as DNAM-1, NKG2D and KIRs, which triggers the caspase 8 dependant killing pathways Fas-FasL and TRAIL. On the contrary, the perforin and granzymes mediated cytotoxicity pathway appear to be intact in our patients. Secondly, the secretion of cytokines is imbalance towards an immunosuppressive pattern, with reduced pro-inflammatory INFγ and IL-2 and markedly increased of the suppressive IL-10. We can speculate that these changes in the cytokines production is the result of the release by TME of suppressive modulators, in particular TGF-β, PGE_2 and IDO have a strong negative effect on INFγ and TNFα release.

Chapter 7. The tumour microenvironment rapidly induces downregulation of CD16 and CD57 expression on NK cells and undermines cytotoxic function

7.1 Introduction

The analysis of the expression profile on tumour-infiltrating NK cells shows a peculiar phenotype, previously illustrated in Chapter Chapter 5. In order to understand the mechanism that might induce these changes, we investigated the amount of expression of the ligands for NK activating receptors through RT-qPCR. This was tested within the tumour microenvironment and compared to the adjacent uninvolved tissue. The expression was confirmed by immunohistochemistry on historical slides.

Also, the release of soluble ligands in the microenvironment is a well-known mechanism of immune escape, already described in chapter 1.3.4. In order to confirm this mechanism in pancreatic cancer, we measured the concentration of NKG2D and DNAM-1 ligands in the sera of our cohort of patients and we compared to the healthy population.

Finally, we generated three organoids from primary PDAC cells, and then we co-cultured them with autologous PBMCs-NK cells *in vitro.*

7.2 Material and methods

For the RT-qPCR, twenty patients with histologically proven PDAC were selected. Four patients were excluded from the analysis because of instability of 18S expression or scarcity of RNA/cDNA. The total RNA was extracted from tumour site and surrounding heathy tissue before they were reverse transcribed into cDNA. Then the quantitative PCR was carried out with different primers and probes. The copy number was calculated using standard curve. Confirmation of the ligands' expression was carried out by immunohistochemistry on historical slides, according to the protocol described in chapter 2.7.

Sera from PDAC patients and healthy donors were defrosted (*MICA*: PDAC n=62, HD n=40; *MICB*: PDAC n=40, HD n=40; *ULBP-1:* PDAC n=40, HD n=40; *ULBP-2* PDAC =64, HD n=40; *ULBP-3*: PDAC n=40, HD n=40; *PVR*: PDAC n=20, HD n=20). The presence and concentration of target ligands were established by sandwich ELISA, according to the protocol described in chapter 2.5.

Three PDAC tumour organoids were generated, and after 2-3 weeks maturation, they were co-cultured with the PBMC for 14 days. NK cell phenotype was assessed prior to incubation and then at day 7 and day 14, following co-culture.

7.3 Results

7.3.1 RT-qPCR and ELISA

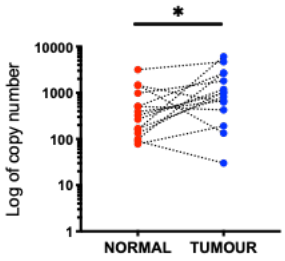
Our analysis demonstrated that, compared to the normal adjacent pancreatic tissue, the tumour microenvironment significantly overexpressed both NKG2D ligands [MICA (295 vs. 904, p=0.02), MICB (12.4 vs. 1636.5, p=0.001), ULBP-2 (50.6 vs. 219, p=0.01), ULBP-3 (27.5 vs. 151, p=0.04) and ULBP-6 (311 vs. 1702, p=0.04)] and the DNAM-1 ligands PVR (13.4 vs. 31, p=0.039), as well as PVRL2 (783 vs. 2918, p=0.01). On the other hand, MHC class I molecules were all downregulated in the tumour, but the statistical significance was not reached. On the contrary, the expression of the immune check-point PD ligands, PDL1 and PDL2, were both upregulated, but again not significantly. Table 7.1 and Figure 7-1 summarizes these results.

Surprisingly, we found that sera of PDAC patients showed a lower concentration of NKG2D and DNAM-1 ligands, compared to healthy population: *MICA*, 468 pg/ml vs. 78.3 pg/ml, p=0.002; MICB 2437 pg/ml vs. 895 pg/ml, p=0.002; ULBP-1 2243 pg/ml vs. 1017 pg/ml, p=0.8; ULBP-2 504 pg/ml vs 0 pg/ml, p=0.04; ULBP-3 533 pg/ml vs 0 pg/ml, p<0.0001; PVR 666 pg/ml vs. 36 pg/ml, p<0.0001. Results are summarized in Figure 7-2.

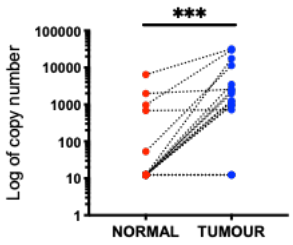
Table 7.1 Results: Genes' log of copy number

	Normal tissue[1]	Tumour core[1]	p value
MIC A	295.47 (1.34-3163.48)	904.07 (30.14-6105.23)	0.02
MIC B	12.47 (12.47-6476.58)	1636.56 (12.47-32033.03)	0.001
ULBP 1	11.89 (10.56-23.88)	19.08 (8.56-148.05)	0.72
ULBP 2	50.64 (17.33-660.27)	219.11 (5.04-3744.98)	0.01
ULBP 3	27.56 (13.39-88.48)	151.34 (9.99-2416.12)	0.04
ULBP 4	12.33 (2.13-61.76)	32.59 (9.51-253.83)	0.08
ULBP 5	62.2 (17.27-128.39)	95.32 (81.44-544.17)	0.47
ULBP 6	311.39 (78.03-2627.33)	1701.96 (357.55-4076.6)	0.04
HLA-A	$4.37x10^{20}$($5.5x10^{18}$-$1.38x10^{27}$)	$7.75x10^{19}$($1.38x10^{27}$-$2.54x10^{22}$)	0.605
HLA-C	379.23 (0-27969.56)	212.83 (0-34853.57)	0.477
HLA-E	919.36 (7.05-7695.75)	1630.16 (12.5-28756.39)	0.214
HLA-DRA	1204.39 (0-24994.93)	1570.16 (116.46-11226.66)	0.134
PDL1	6.27 (0.45-37.69)	11.39 (0.92-83.41)	0.278
PDL2	4.22 (0-62.94)	10.41 (0.82-34.57)	0.278
PVR (CD155)	13.35 (0-1468)	31.24 (0-46842.26)	0.039
PVRL2 (CD122)	783.24 (0.03-12156.15)	2918.02 (48.58-24235.89)	0.01

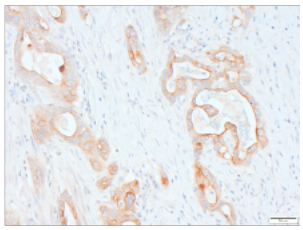
[1] data are shown as median and minimum and maximum values

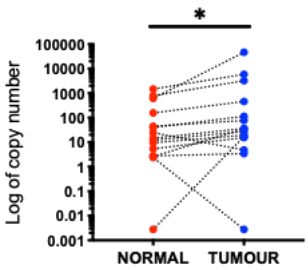


MIC-A

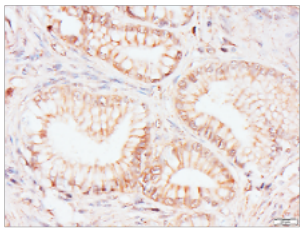


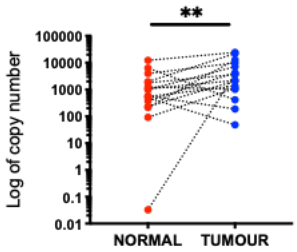
MIC-B



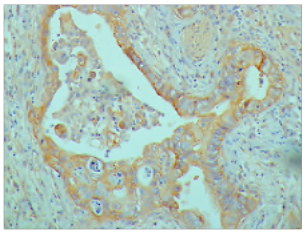


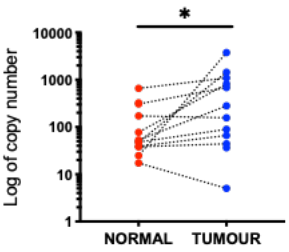
PVR



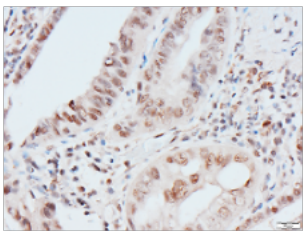


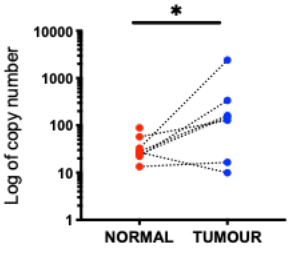
PVRL2



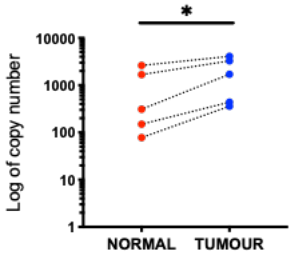


ULBP-2





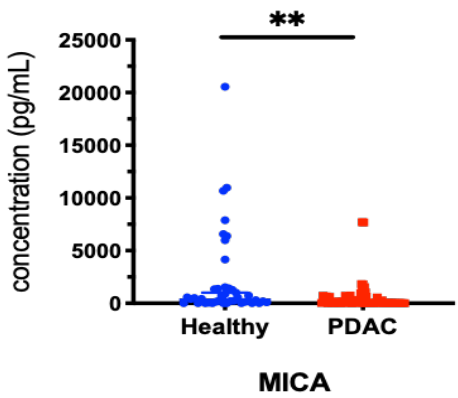
ULBP-3

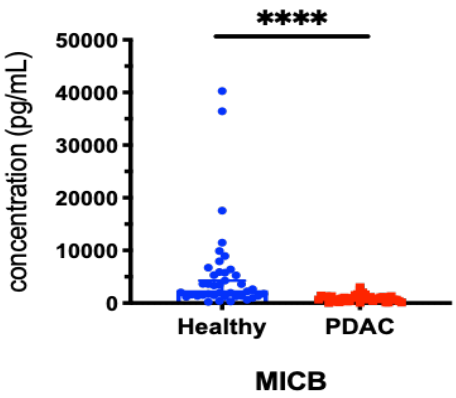


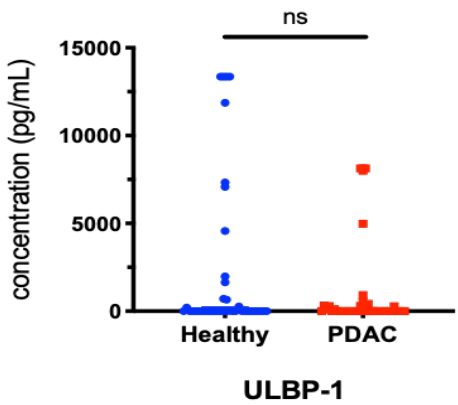
ULBP-6

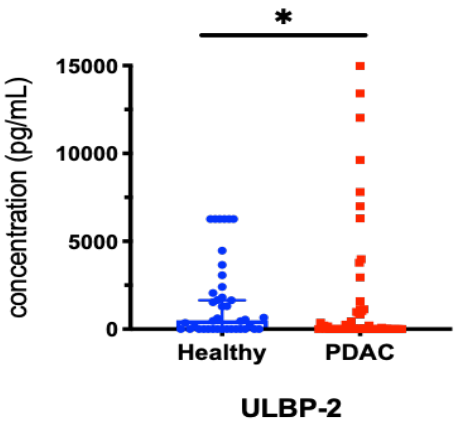
Figure 7-1. The PDAC microenvironment expresses high levels of NK cell ligands.

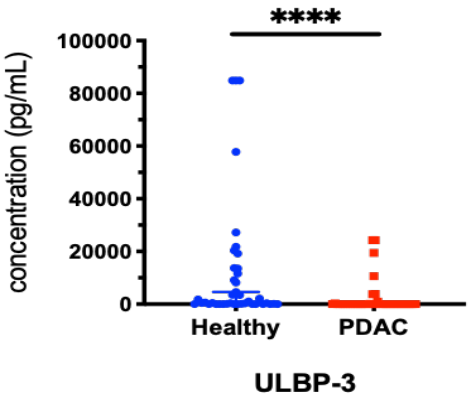
Total RNA from tumour tissue and normal margins was extracted and reverse-transcribed to cDNA, before the RT-qPCR was performed to calculate absolute quantity of copy numbers, using standard curves. The expression of MICA/B, PVR, PVRL2 and ULBP-2 was confirmed using immunohistochemistry staining, contrasting to normal adjacent pancreatic tissue. Data are shown using scatter plots, each point representing a donor. Significance was calculated with paired-t Student's test or the Related samples-Wilcoxon Signed-Rank test, according to normal distribution; $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$ (****).


**
25000
20000
15000
10000
5000
0
concentration (pg/mL)
Healthy
PDAC
MICA


****
50000
40000
30000
20000
10000
0
concentration (pg/mL)
Healthy
PDAC
MICB


ns
15000
10000
5000
0
concentration (pg/mL)
Healthy
PDAC
ULBP-1


*
15000
10000
5000
0
concentration (pg/mL)
Healthy
PDAC
ULBP-2


****
100000
80000
60000
40000
20000
0
concentration (pg/mL)
Healthy
PDAC
ULBP-3

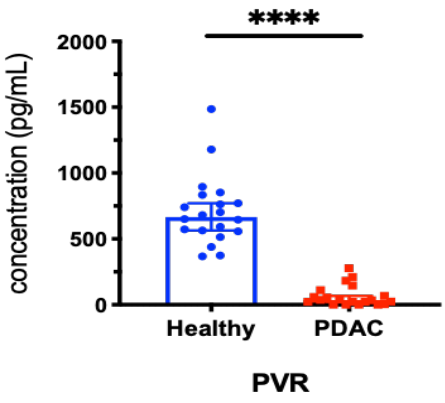
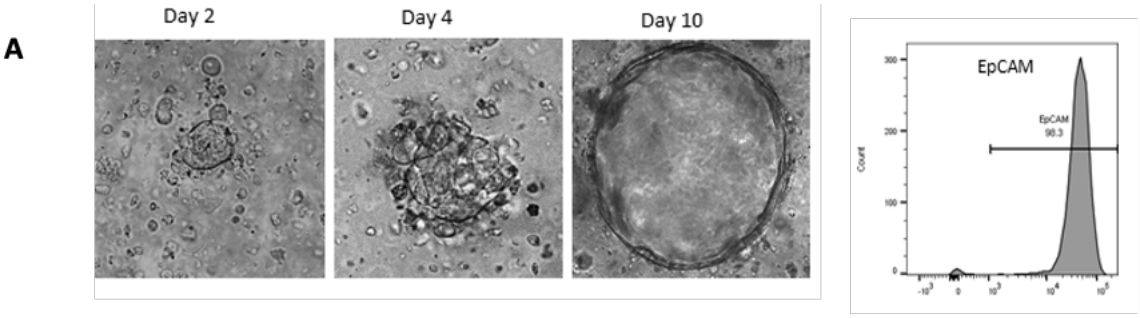

****
2000
1500
1000
500
0
concentration (pg/mL)
Healthy
PDAC
PVR

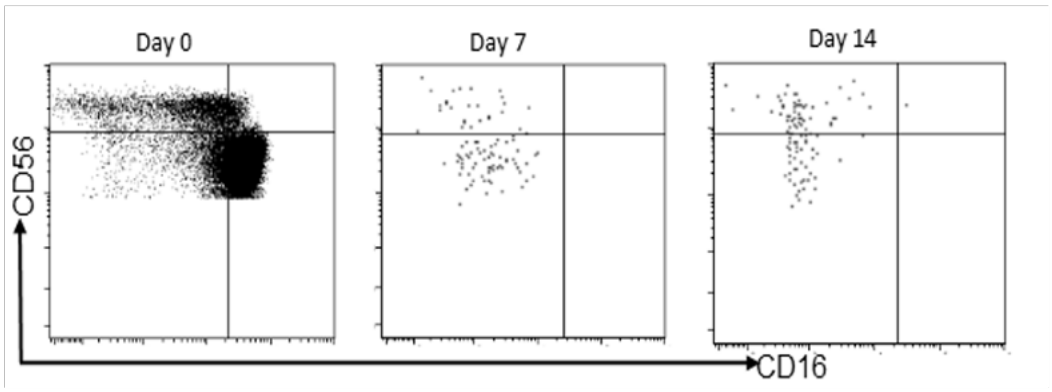
Figure 7-2. Sera of PDAC patients show a decreased level of soluble NK cell ligands.

Sera from PDAC patients and healthy donors were defrosted and the concentration of soluble ligands was measured by sandwich-ELISA. Optical Density of each well was immediately determined using a microplate reader set to 450 nm, with a wavelength correction set to 650 nm. Data are shown using scatter plots with bar, each point representing a donor. Significance was determined using Mann-Whitney U-test; $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$ (****).

7.3.2 Organoids co-culture

Finally, three PDAC tumour organoids were generated and co-cultured with autologous PBMC for 14 days. The NK cell phenotype was assessed prior to incubation, at day 7 and at day 14, following co-culture. The data were shown as relative percentage of surface expression compared to PBMC cultures without organoid (n=3). The surface markers studied remain stable in the control PBMCs culture without organoids. Interestingly, CD16 expression was profoundly downregulated during co-culture, falling from 98% to 32% at day 14 ($p=0.01$). As such, the majority of NK cells came to develop a $CD56^{dim}CD16^{negative}$ phenotype typical of those found in primary PDAC tumours. CD57 expression was also reduced during co-culture, falling from 95% to 37% at day 14 ($p=0.12$). The expression of the activating receptors DNAM-1, NKG2D and NKp30 was broadly stable during culture. However, tumour cells can rapidly and directly downregulate CD16 and CD57 expression, but do not modulate expression of activating ligands during short term culture. This implies that other unknown mechanisms of immune escape must be in place.


A
Day 2
Day 4
Day 10
EpCAM
EpCAM
90.3
Count


B
Day 0
Day 7
Day 14
CD56
CD16

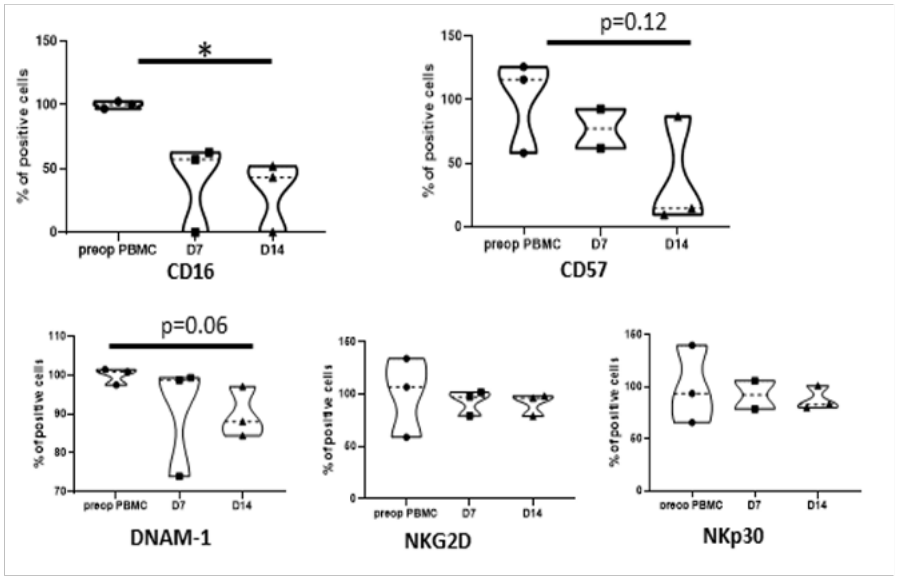

C
% of positive cells
preop PBMC
D7
D14
CD16
*
CD57
p=0.12
DNAM-1
p=0.06
NKG2D
NKp30

Figure 7-3. Pancreatic cancer induces downregulation of CD16 and CD57 on NK cells.

A) Organoids from PDACs were generated through 3D culture. The pictures represent organoids culture at day 2, day 4 and day 10, after the setup of the organoids. The right panel is the histogram of the EpCAM staining of the organoids once matured. **B)** Co-culture of organoids with autologous PBMCs. The phenotype of NK cells in culture were monitored at day7 and day14. The flow dot plots represent an example of the expression pattern of NK cells according to CD56 and CD16 expression. **C)** The expression of maturation markers (CD16 and CD57) and activating receptors (DNAM-1, NKG2D and NKp30) was compared before the culturing, at day7 and day14. The expression of each marker (day7 and day14) is shown as relative percentage on NK cells cultured with organoids, compared with NK cells in controlled medium. Data are shown using violin plot, each point represents a donor. Significance was determined using Mann-Whitney U-test, $p<0.05$ (*).

7.4 Discussion

The ability of NK cells to recognise the target cells relies on two mechanisms: the loss of inhibitory signals from the recognition of HLA class I ligands, and/or the expression of stress molecules which bind activating receptors.

7.4.1 The expression of NK cells ligand in PDAC tumour site

In this chapter, we firstly wanted to investigate the expression of NK cells ligands within the tumour and compare it with normal adjacent tissue. Subsequently, we determined the concentration of the soluble forms of the ligands in the plasma. Our results indicate that pancreatic tumour express NK cells activating ligands, and therefore is susceptible to lysis by NK cells. However, the expression is not followed by a significant increase in the sera of patients of the soluble form of the ligands, ULBP-2 excluded. This last information must be cautiously interpreted as the majority of the sera came from fast-tracked patients, biased by high concentration of bilirubin, which could have interfered with the analyses.

MICA and MICB are the two-best characterised NKG2D ligands, and their expression is documented in many solid and haematological tumours in humans, including breast, lung, liver, colon, renal, prostate and pancreatic cancer.(218) In pancreatic cancer, MICA polymorphism has been associated with an increased risk of developing the disease and higher serum concentration of the ligand. In particular the MICA A5.1 was also associated with cervical, hepatocellular and head and neck carcinoma, but not in colon, stomach or melanoma.(483) Disentegrins and the

metalloproteases ADAM 10 and 17 were demonstrated to lyse MICA and MICB from the tumoral cell surface, therefore allowing their soluble forms to be released in the TME and/or the bloodstream. Soluble forms of MICA and MICB retain the ability to bind NKG2D, however this is not followed by the initiation of the activating signal leading to cytolytic effect. On the contrary, NKG2D is downregulated by endocytosis and degraded, resulting in severe impairment of NK cells functions. Moreover, the same mechanism has an impact of $CD8^+$ T cells functionality, as they could express NKG2D.(216) In our samples, we confirmed that pancreatic tumour expresses MICA and MICB, and a similar mechanism of shedding could explained the downregulation of NKG2D noticed either in peripheral and infiltrating NK cells in our cohort of patients. However, we could not confirm an increase concentration of soluble forms of MICA/MICB in the sera of our patients, probably due to technical difficulties related to the presence of high level of bilirubin, which could have masked the ligands. Despite this, other Authors confirmed the shedding of s-MICA/MICB as prognostic factors in several solid and haematological cancers.(484, 485) Moreover, sMICA/MICB could also suppress the immune response inducing the recruitment and expansion of MDSCs and promoting the development of TAMs in the TME.(486) Thus, if during the early phase of tumour development the expression of NKG2DL could lead to an effective NK cells response and killing, at later stages their shedding probably promotes tumour growth and stimulate immune suppression and tumour escape.

We also analysed the presence of ULBPs in the tumour and in the sera of patients affected by PADC. The expression of ULBP2-3-6 is significantly increased in the tumour compared to adjacent normal tissue, while the concentration of the soluble ligands is either null or reduced. As seen for MICA/MICB, this last result is probably

due to technical issues due to the presence of high bilirubin in our samples. The best characterised ULB protein is ULBP2, whose expression has been demonstrated in solid and haematological malignancies. The expression of membrane ULBPs (m-ULBPs) induces a strong activating signal on NK cells, able to overcome the inhibitory signal from MHC class I molecules. Therefore, tumours expressing m-ULBPs are actively recognised by NK cells. However, the apoptosis induced by NK cells together with the presence of metalloproteases and disentegrins, determines the release of soluble forms of ULBPs (s-ULBPs). These have a dual effect: they induce the release of INFγ, TNFα, GM-CSF and promote the recruitment of macrophages,(219) but also have an important immune suppressive effect, characterised by the reduction and degradation of NKG2D. As on $CD8^+$ T cells NKG2D acts a co-stimulator of the TCR similar to CD28, this property affects also the adaptive cellular mediated immune response.(487) In fact, high concentration of s-ULBPs correlates with worse prognosis in several solid tumours, like s-ULBP-1 in cervical cancer or s-ULBP-2 in ovarian and pancreatic cancer and melanoma.(488) Here, we confirm that pancreatic cancer expresses high level of ULBP2-3-6, therefore it is potentially susceptible to NK cells cytolysis mediated by NKG2D. However, the reduction of NKG2D seen in the peripheral blood and in the TILs, makes us speculate about a possible mechanism of immune escape mediated by the release of soluble forms of ULBPs, as seen for MICA/MICB.

Our analysis on the expression of activating NK cells ligands in our cohort of patients demonstrated the induction of DNAM-1 ligands CD155 (PVR) and CD112 (Nectine-2) on pancreatic cancer cells. CD155 and CD112 are two adhesion molecules, upregulated in solid and haematological cancers, in response to FGF

receptor stimulation and/or *ras* mutation.(489) In particular, CD155 has been demonstrated to promote proliferation and migration in colon cancer and the adhesion of myeloma cancer cells to stroma in the bone marrow. Also, CD155 has been found in advanced stage of solid tumours, such as melanoma, glioblastoma, pancreas and lung cancers.(490) Nevertheless, the expression of high level of CD155 makes the tumour more susceptible to NK and T cell recognition, as DNAM-1 is expressed also on $CD8^+$ T cells.(491) For example, the elimination of circulating cancer cells in myeloid and lymphoid leukaemia is predominantly dependant on the CD155/DNAM-1 system, and the addition of an antibody blocking CD155 severely impaired the elimination of leukemic cells by NK cells.(492) Additionally, in human neuroblastoma, the level of expression of CD155 correlates with cellular sensitivity to NK cells-mediated killing.(491) However, the engagement of DNAM-1 is not sufficient to trigger an adequate killing response, and other receptors need to be involved, at least in solid tumours.(226) Still, the role of CD155 in cancer is dual. The chronic membrane expression of CD155 downregulates the expression of DNAM-1 on NK cells, as demonstrated in ovarian cancer and acute myeloid leukaemia.(464, 493) This could partially explain the dramatic reduction demonstrated in peripheral and infiltrating NK cells in our patients. Moreover, soluble forms of CD155 and CD112 are shed by cancer cells and compete for DNAM-1 binding, interfering with the proper CD155/DNAM-1 signalling. Several human solid cancers are characterised by high plasma level of CD155, which correlates with the stage of the disease.(494) Again, the plasma samples collected from our patients were characterised by high concentration of bilirubin, which could have jeopardised our analyses. Hence, we cannot confirm this information in our patients, but we could speculate that a similar mechanism is also in

place. Furthermore, CD155 is also able to bind two suppressive receptors, CD96 and TIGIT, both present on NK cells and cytotoxic T cells. While a suppressive role of CD96 in humans is still under debate, TIGIT is a well-known NK cells' inhibitory receptor, either in humans and in mice, and as such, it is one of the markers of cellular exhaustion. It binds CD155 with a greater affinity than DNAM-1. Tumour progression is therefore the result of an impair balance between the activating receptor DNAM-1 and the suppression mediated by TIGIT/CD96 pathways. Moreover, DCs expressing TIGIT could interact with CD155 and induce IL-10 release.(495)

Finally, the analysis of NK ligands demonstrates that pancreatic cancer maintains the ability of expressing HLA class I, although we found a slight downtrend which does not reach the statistical significance. HLA class I molecules are strong inhibitory signals for NK cells, and their loss is one of the main mechanisms to induce activation on NK cells, according to the "missing self" theory.(237) HLA class I molecules are recognised by KIRs receptors on NK cells, which we have previously demonstrated to be downregulated in infiltrating NK cells. This could represent another mechanism of immune escape.

7.4.2 The co-culture of organoids with PBMCs

Organoids are three-dimensional cellular culture able to mimic cancer structure and cell-to-cell contacts, and to reliably represent tumour heterogeneity and genetic expression. The use of patient derived organoids has been introduced only recently, to overcome the difficulties faced with other complex cell cultures, such as patient-derived mouse xenograft (PDX). In fact, PDX are expensive and time-consuming as

the passage to mouse can require months. On the contrary, patient-derived organoids show many advantages: they can be obtained from biopsies; they can be stored and genetically manipulated; moreover, they can also reproduce the stage of the disease and the pathological architecture.(496, 497) Organoids models are obtained from different cancer types, including pancreatic cancer. For example, Tsai et al. have cultured a pancreatic cancer-derived organoid and put in co-culture with autologous T cells. They observed the ability of T cells to infiltrate the organoids, probably due to the release of the chemotactic chemokine CXCL12, and demonstrated the impairment of T cells grown in the organoid grown media (OGM).(498)

To better understand the interaction that leads to the phenotypic impairment of infiltrating NK cells, we developed a pancreatic cancer organoid and we put it in co-culture with autologous circulating NK cells. We observed a sudden and dramatic decrease in the expression of two particular receptors, CD16 and CD57, while other activating receptor such as DNAM-1, NKG2D and NKp30 remained stable during the culturing. However, of notice, DNAM-1 showed clearly a sharper downtrend compared the others. This could be explained by the susceptibility of DNAM-1 of chronic cell to cell interaction, while the downregulation of NKG2D and NKp30 is influenced by the release of cytokines and/or their soluble ligands, as previously noticed. However, the co-culture was maintained for 15 days, we could also hypothesise that these receptors need a longer exposure to the organoids before a significant downtrend in their expression is recorded.

The loss of CD16 and CD57 reflects the same phenomenon seen in tumour infiltrating NK cells. Therefore, we could speculate that such an important change happens quite rapidly after NK cells contact with cancer cells. This could be explained

by different mechanisms: NK cells could lose CD16 expression after degranulation or can be clavated by metalloproteases secreted by cancer cells itself or the TME; NK cells are induced by cytokines or other factors to shift their phenotype in order to produce INFγ more efficiently, as we already saw that $CD56^{bright}CD16^{neg}CD57^{neg}$ NK cells are the main source of INFγ. Therefore, these changes could also represent a shift towards a "regulatory" phenotype at the expense of the cytotoxic effect, and possibly a mechanism of immune escape induced by the tumour since early exposure to NK cells.

Being a quite recent technique, not many data are currently available in the literature on co-culturing pancreatic cancer-derived organoids and immune cells. However, some data are emerging on other type of cancers, but the majority focuses on the ability of immune cells, NK cells included, to infiltrate and kill the organoid spheroids, while a deepen phenotype characterization is still missing.(499-503) For example, *Courau et al.* investigated the ability of T and NK cells to infiltrate colon cancer-derived spheroids, and demonstrated the killing effect of NK cells thought the NKG2D/MICA-B pathways and INFγ production. They also demonstrated the upregulation of the suppressing NK cells receptor NKG2A and the expression of its ligand HLA-E in the spheroids.(504)

Clearly, organoid models are a promising tool that could shed highlights in the in vivo interactions between cancer cells, surrounding immune infiltrates and TME. Hopefully, this could lead in the future to the development of new immunotherapeutic approaches.

In summary, in this chapter we confirmed that, potentially, PDAC could be a target for NK cells killing, as it expresses the corresponding activating ligands. However, the release of soluble ligands in the TME and/or the chronic cell-to-cell interaction could have the opposite effect to downregulate the activating receptor on the NK cells surface, resulting in deficient cytotoxic activity. Finally, the development of an PDAC-derived organoid in culture with autologous NK cells, demonstrated the rapid loss of ADCC ability and a quite significant and rapid downregulation of DNAM-1 receptor. This could represent a shift towards a more “regulatory” phenotype at the expense of the killing activity, and therefore a further mechanism of immune escape.

Chapter 8. Conclusions

Our study demonstrates that circulating NK cells of patients affected by PDAC show an impaired phenotype, characterised by the downregulation of important activating receptors, particularly NKG2D, DNAM-1 and NKp30. At the same time, the circulating cytotoxic $CD56^{dim}$ NK cells have undergone a process of activation and maturation, highlighted by the overexpression of CD16 and CD57. This could be due either to the chronic stimulation from cytokines produced by the tumour itself or the release of cancer-related epitopes, able to induce a chronic inflammatory response. The expression of CD16 makes NK cells potentially capable of mounting an effective ADCC response against the tumour. However, NK cells are confirmed to be a rare population within PDAC, and those cells eventually reaching the tumour microenvironment, are very different compared to their circulating counterpart. In fact, pancreatic cancer microenvironment is characterised by the prevalence of $CD56^{dim/bright}CD16^{neg}$ NK cells and the downregulation of all the most important activating receptors. These phenotypic modifications lead to a defective killing ability and a limited cytokines production. Interestingly, this phenotype is similar to a particular subset of NK cells, usually found during the very early stage of pregnancy, in the decidua, which represents the maternal-foetal interface. Their primary role involves the remodelling of the decidua, to allow the proper implantation of the trophoblast. Any functional defects of these important cells could lead to an early termination of the pregnancy. These changes support the hypothesis that NK cells, at a certain stage of tumour development, are induced to overcome their protective role against cancer cells and

stimulate tumour growth rather than containing it, thus playing a dual role in tumorigenesis. This phenomenon is not unusual in tumour development, as it has been demonstrated for other immune cells such as T cells, macrophages or neutrophils, and probably represents one of the main mechanisms of tumour escape. We have also demonstrated that PDAC expresses the ligands to stimulate NK cells activation, and therefore, it is potentially a target for cytotoxicity. However, the release into the microenvironment of the soluble forms or the chronic cell-to-cell stimulation could partially explain the phenotypic impairment of the few infiltrating NK cells. Definitely, the tumour itself has a great importance in shaping the NK cells response, as demonstrated by the rapid downregulation of DNAM-1, CD16 and CD57 seen when culturing the PDAC-derived organoid with autologous NK cells. This could represent a shift towards a more “regulatory” phenotype at the expense of the killing activity, and therefore a further mechanism of immune escape. However, the mechanism in place leading the phenotypic changes on NK cells, is far to be understood and deserve more experiments.

Finally, we found important correlations between NK cells and histopathological characteristics, in particular between the relative number of circulating NK cells correlate and the size of the tumour, and between the expression of activating receptors, such as NKp30 and NKp46, with the lymph node ratio, an important prognostic tool in solid tumours. Moreover, their expression is inversely correlated to pathological signs of aggressive biological behaviour, such as perineural and perivascular invasion. In addition, NKp30 and NKp46 are found to be more expressed in earlier T stages. These finding suggest that NK cells activity has an impact on tumour

growth locally and lymphatic metastasis. Due to the limited size of our cohorts, it was difficult to determine if NK cells activating receptors could be used as prognostic markers helping stratifying patients, that could benefit from a more aggressive chemotherapy or could respond to immune therapy.

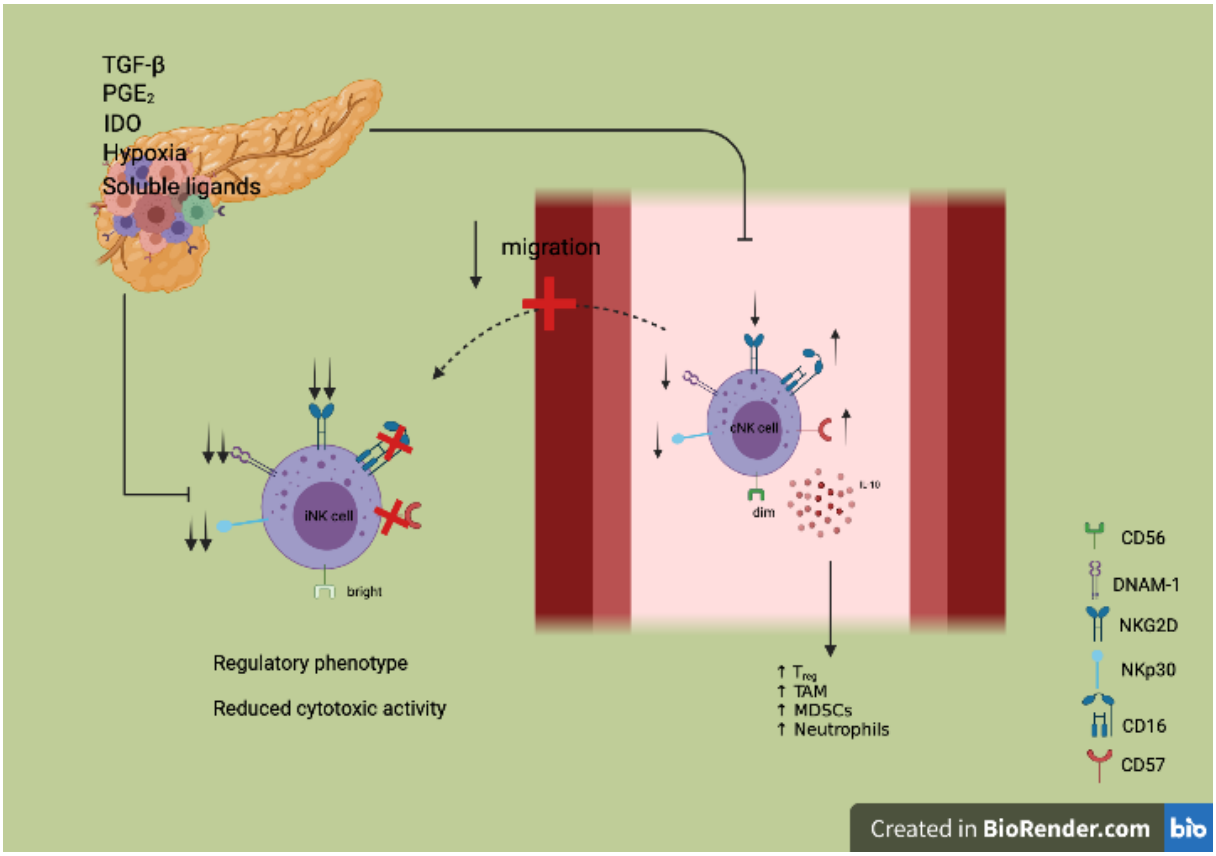


Figure 8-1. Possible mechanisms of immune escape induced by pancreatic adenocarcinoma.

The release of immune suppressive cytokines, like TGF-β and/or the secretion of soluble ligands in the blood stream by pancreatic cancer, induces the downregulation of the main NK activating receptors. This phenomenon affects either the circulating or the infiltrating NK cells, where however this is even more pronounced, with a drastic reduction of activating markers and the loss of CD16 and CD57. Moreover, circulating NK cells acquire the ability to secrete IL-10, a cytokine able to recruit other immune suppressive cells, like TAMs, MDSCs and N1-neutrophils, which promote tumour growth. Furthermore, the tumour site is precluded from the NK cells recognition, by a drastic reduction in their migration, due either to the mechanical inability to reach the

site induced by the desmoplastic reaction, or to an impaired kinetic, induced by hypoxia and/or hypoxia-related molecules, such as IDO or PGE_2.

8.1 Limitations

Our study has several limitations. Firstly, the limited size of the two cohorts, which could not cover the interindividual variations in the number of NK and in the expression of activating receptors. Then, the healthy donors used to perform the comparison were previously collected and frozen down. We do not have much clinical information on these donors, apart from the age, which unfortunately could not be matched with the median age of our patients. This because healthy donors are usually younger people who voluntarily donate their blood for either clinical use or for research. Therefore, it is quite challenging to find healthy volunteers of the same age.

The analysis of the expression profile of circulating and infiltrating NK cells was performed by Beckman Coulter Gallios flow cytometry, which could analyse up to ten colours per panel. The advent of CyTOF (Cytometry by the Time Of Flight) based on mass spectrometry, has revolutionised cytometry analysis, as it allows to perform more accurate analysis on complex cellular population, using up to 45 colours per panel. Also, it requires a fewer number of cells, which is an important advantage in rare population such as tumour infiltrating NK cells. Unfortunately, it is a quite expensive technique and requires a specific expertise in building the panels. Moreover, the flow rate is quite low, compared to flow cytometry. Not ultimately, the logistic access to this technology is challenging, with a long waiting list that is not suitable for such samples that cannot be scheduled with short notice.

The immunohistochemistry was performed according to a standard protocol, which was adequate for the purpose of this study. However, a more modern approach would have sought the use of multiplex immunofluorescence, which allows the

identification of multiple targets on the same section. This technique could give more insights on the spatial correlation between the cellular components of the tumour and the immune infiltrate.

Another limitation is the quantification of the NK cells' soluble ligands by ELISA, whose results cannot be realistic. As most of our patients were jaundiced at the time of the surgery, the high bilirubin in their sera could have interfered with the analysis. In this case, Western blot should have been tested as alternative technique to quantify the soluble ligands.

Lastly, we used organoid-based co-culture as surrogate of the interaction between cancer cells and infiltrating NK cells. Despite the many advantages already discussed in chapter 7.4.2, organoids still cannot replace complex pre-clinical animal models, such as patient-derived mouse xenograft (PDX). Therefore, our data will need to be confirmed on mouse model in the future.

8.2 Future works

Our findings indicate that PDAC growth develops as the results of different mechanism of immune evasion. These include the downregulation of activating receptors in the circulating compartment, the exclusion from the tumour site along with an impaired differentiation, and promotion of an immunoregulatory IL-10 phenotype within the periphery. Therefore, the development of therapeutic approaches that overcome these mechanisms could bring considerable benefits in a disease which nowadays holds few therapeutic options.

To overcome the limitations of our study and to have a better understanding of the mechanisms underlying the phenotypic changes we have highlighted, further studies need to be planned.

Firstly, the phenotypic changes in circulating NK cells and autologous infiltrative NK cells need to be confirmed in larger, prospectively recruited cohorts. Also, the flow cytometry analysis could be integrated with single-cells RNA sequencing technique on different populations: circulating and infiltrative NK cells, cancer cells and TME. If possible, CyTOF will be the preferable approach.

The implementation of the organoid co-culturing with autologous NK cells, along with the analysis of the related media culture, would bring more insights on the soluble molecules released by the cancer cells, and their role in inducing the phenotypic changes on NK cells. In particular, we will drive our attention on the identification and quantification of IL-2, IL-10, INFγ and TNFα, to confirm our results either in the sera of our patients or in the media culture. This will be performed with ELISA or Western blot analysis, but ELISPOT assays would be considered too. Moreover, we will develop a patient-derived mouse model to confirm the results.

Immunohistochemistry of historical slides and of the organoids will be integrated with multiplex immunofluorescence, for spatial characterisation of the immune infiltrate.

We hope that these investigations would help developing patient-specific immune approaches, such as cytokine blockage, mechanisms to enhance activating receptor expression, or the development of NK cellular therapies, such as CAR-NK cells.

Appendix I

Publications resulting from this work:

1. Francesca Marcon, Jianmin Zuo, Hayden Pearce, Samantha Nicol, Sandra Margielewska-Davies, Mustafa Farhat, Brinder Mahon, Gary Middleton, Rachel Brown, Keith J. Roberts & Paul Moss (2020). NK cells in pancreatic cancer demonstrate impaired cytotoxicity and a regulatory IL-10 phenotype, OncoImmunology, 9:1, 1845424, DOI: 10.1080/2162402X.2020.1845424

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