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NK cells in pancreatic cancer demonstrate impaired cytotoxicity and a regulatory IL-10 phenotype

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ABSTRACT

Pancreatic ductal adenocarcinoma (PDAC) is one of the most common tumor subtypes and remains associated with very poor survival. T cell infiltration into tumor tissue is associated with improved clinical outcome but little is known regarding the potential role of NK cells in disease control. Here we analyze the phenotype and function of NK cells in the blood and tumor tissue from patients with PDAC. Peripheral NK cells are present in normal numbers but display a CD16^{hi}CD57^{hi} phenotype with marked downregulation of NKG2D. Importantly, these cells demonstrate reduced cytotoxic activity and low levels of IFN-γ expression but instead produce high levels of intracellular IL-10, an immunoregulatory cytokine found at increased levels in the blood of PDAC patients. In contrast, NK cells are largely excluded from tumor tissue where they display strong downregulation of both CD16 and CD57, a phenotype that was recapitulated in primary NK cells following co-culture with PDAC organoids. Moreover, expression of activatory proteins, including DNAM-1 and NKP30, was markedly suppressed and the DNAM-1 ligand PVR was strongly expressed on tumor cells. As such, in situ and peripheral NK cells display differential features in patients with PDAC and indicate local and systemic mechanisms by which the tumor can evade immune control. These findings offer a number of potential options for NK-based immunotherapy in the management of patients with PDAC.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) incidence is rising and remains associated with a 5 year survival rate of less than 5% with virtually no improvement in clinical outcome over the last 10 years.[1–4] Indeed, current predictions suggest that PDAC may become the second most common cause of cancer death by 2030.[5] Chemotherapy and radiotherapy-based regimens are currently limited and there is interest in developing novel forms of immunotherapy. Patients with PDAC display poor responses to checkpoint inhibition[6] and there is thus a clear need to better understand the mechanisms of immune evasion.

The microenvironment of pancreatic ductal adenocarcinoma is typically characterized by extreme hypoxia and a dense stromal 'desmoplastic' reaction (TME)[7] which together act to support tumor progression, metastasis, and chemoresistance.[8–10] Features of immune suppression, such as elevated serum and tissue levels of IL-10 and TGF-β, have been observed.[11–13] Despite this, an immune infiltrate is present within many tumors and has been associated with improved clinical outcome.[14–17] Indeed, although immune cells typically demonstrate an exhausted phenotype in situ[12] they can be expanded in vitro to recognize autologous tumor cells.[18]

In contrast, the phenotypic and functional features of Natural Killer (NK) cells within the PDAC microenvironment has been less intensively investigated although NK cells exhibit impaired killing of autologous PDAC cells due to NKG2D and DNAM-1 deficiency[19] and an increased number of peripheral NK cells may act as a prognostic marker for longer recurrence-free survival and overall survival.[20,21] NK cells play an important role in controlling the development of cancer[22–24] through loss of inhibitory signaling from cells with downregulation of HLA[25] and activatory engagement with 'stress' molecules such as NKG2D ligands.[26–28] The ultimate outcome of NK cell engagement with a target cell is mediated through the balance of inhibitory and activatory interactions and there is now considerable interest in understanding both how tumor cells evade NK cell recognition and if they may be exploited for immunotherapy.[29] Here we determined the phenotypic and functional profile of NK cells from patients undergoing surgical resection of PDAC tumors. We show that a range of mechanisms of immune evasion are present within NK cells and that these differ between the local and systemic environments.

Materials and methods

Participants

54 patients with PDAC were recruited from clinics at University Hospitals Birmingham NHS Foundation Trust,

Queen Elizabeth Hospital Birmingham over 3-years period (2016–2019) under appropriate ethical approval (REC no 16/WM/0214). Samples from 34 healthy age-matched donors were also obtained. Patients were selected who had localized disease, without local invasion or metastasis, and who were undergoing Whipple's resection with curative intent.

Immunophenotyping

25 ml of blood was collected prior to pancreaticoduodenectomy surgery and peripheral blood mononuclear cell (PBMC) were collected by density centrifugation. After defrosting, PBMCs were washed and re-suspended at 106cells/100 μl before staining with one of the following antibody panels. Panel 1; anti-DNAM-1 FITC (11A8 Biolegend) (5 μl); anti-PD-1 PE (EH12.2 H Biolegend) (3ul); anti-CD14/CD19 PE dazzle (HCD14/H1B19 Biolegend) (1.5 μl/2.5 μl); anti-NKp46 PerCP/Cy5.5 (Biolegend) (5 μl); anti-CD56 PEcy7(HCD56 Biolegend) (3 μl); anti-NKG2D APC (BD Biosciences) (5 μl); anti-CD3 AF700 (HIT3a Biolegend) (2.5 μl) and anti-CD16 Pacific Blue (3G8 Biolegend) (5 μl). Panel 2; anti-NKG2C FITC(11A8Milteny Biotec) (2μl); anti-CD94 PE (DX22 Biolegend)(1 μl); anti-CD14/CD19 PE-dazzle (HCD14/H1B19Biolegend) (1.5 μl/2.5 μl); anti-KIRS PerCP/Cy5.5 (HP-MA4 Biolegend)(5 μl); anti-CD56PECy7 (HCD56 Biolegend) (3 μl); anti-NKp30 APC (Biolegend)(5 μl); anti-CD3 AF700 (HIT3a Biolegend) (2.5 μl) and anti-CD57 Pacific Blue (HNK-1Biolegend) (3 μl).

Antibody stained samples were run on a BC Gallios flow cytometer within 1 h of staining. At least 100,000 events were acquired per sample using CytoSoftware, and analyzed offline using Kaluza v1.5 software.

Isolation of Tumor-infiltrating lymphocytes (TIL)

Tumor was cut into 1–2 mm pieces and incubated overnight in tissue media culture (RPMI 1640 + 10% FCS+1% Penicillin/Streptomicin+1% L-Glutamine) to allow TILs to migrate out of tissue. Media was spun at 1600 rpm for 5 min prior to flow cytometric analysis.

NK cell cytotoxicity assay

NK cells were enriched using EasySep™ Human NK cell enrichment kit (Stem Cell Technologies, Canada) and activated overnight at with IFNα (sigma, 10 ng/ml)., K562 cells were labeled with CFSE and co-cultured with the pre-activated NK cells at E:T ratio of 1:1. Cells were co-cultured for 16 hours prior to analysis using BD Accuri™ flow cytometer (BD Bioscience) and specific lysis calculated on live cell enumeration.[27] The equation for calculation is: $100 \times (1 - [(\text{experimental group cell count})/(\text{control cell count})])$.

Intracellular cytokine staining

PBMCs and K562 were co-cultured overnight with Brefeldin A (SIGMA ALDRICH) (1 μl/ml) at 1:1 ratio. Co-cultures were washed and stained with appropriate surface antibodies and PB live/dead dye (Invitrogen; Massachusetts, USA) before the fixation and permeabilization. Cells were then stained with antibodies against intracellular cytokines, washed, and analyzed using flow cytometry.

Quantitative reverse-transcription polymerase chain reaction (qRT-PCR)

Total RNA was isolated from normal pancreatic tissues and tumor tissues using QIAGEN RNeasy kit and treated with DNase I (Turbo DNA-free kit; Ambion). Q-RT-PCR assays were performed with TaqMan® Gene Expression Assays (Applied Biosystems), reference plasmids containing relevant target gene sequences were used to generate standard curves.

Immunohistochemistry

10 cases of paraffin wax embedded PDAC Tissue sections were de waxed and re-hydrated. Endogenous peroxidase activity was blocked using 0.3% H2O2 and the citrate buffer antigen retrieval method was performed. Before application of primary antibodies, slides were blocked in 5x casein (Vector Laboratories Ltd.). Primary antibodies Anti-ULBP2 (ab88645; Abcam) and Anti-PVR (HPA012568; Atlas Antibodies) were diluted in PBS to desired concentration. For visualization of staining DAB substrate was added (Vector Laboratories Ltd). Stained tissue was analyzed under the Eclipse E400 microscope (Nikon) and images were taken with 40x magnification.

Co-culture of organoid and autologous PBMCs

Tumor was diced into approximately 2 mm sections and crushed with the butt of a syringe. Homogenate was poured into basal culture media containing Type II Collagenase (Life Technologies; [2.5 mg/mL]) and shaken at 37°C for 60 min prior to dilution with basal culture medium and filtration through a 70uM filter (Falcon Catalog number 352350). The cell suspension was pelleted and washed prior to incubation with growth factor-reduced Matrigel (Corning Catalog number CB-40230 C), with final concentration at 75%. The suspension was then rapidly plated into a 24-well plate with 50uL of suspension per well. 20 min later, 600uL of general culture medium was added to each well of the solidified Matrigel. General culture medium was composed of Advanced DMEM/F12, supplemented with 1% penicillin streptomycin, 1X Glutamax, 1% HEPES, 100 ng/mL R-spondin 1 (Peprotech catalog number 120–38), 100 ng/mL Noggin (Peprotech catalog number 120–10 C), 50 ng/mL EGF (Peprotech catalog number 100–15), 10 ng/mL FGF-10 (Peprotech catalog number 100–26), 10 ng/mL FGF2 (Peprotech catalog number 100–18B), 1x B27 (Life Technologies Catalog number 17504044), 10 mM Nicotinamide (Sigma Aldrich Catalog Number N0636), 1.25 mM N-acetylcysteine (Sigma Aldrich catalog number A9165), 1uM Prostaglandin E2 (Catalog number R&D

Systems Catalog number 2296), 10uM SB202190 (Sigma Aldrich Catalog number S7076), and 500 nm A83-01 (Sigma Aldrich catalog number SML0788). Medium was replaced every 3–4 days and matured organoids passaged 1:3 every 14 days. Autologous PBMC were defrosted and cultured *in vitro*, with or without organoid, with IL-2 added after 3 days at 200IU/ml. Co-cultures were maintained for 7–14 days prior to immuno-phenotyping.

Statistical analysis

Statistical analysis was performed with SPSS 24 and Prism 8. Each parameter was checked for normal distribution. A paired-t or the related samples-Wilcoxon Signed Rank test for non-parametric variables were used accordingly. A *p* value <.05 was considered statistical significant. The clinical correlation was carried out using Spearman's Rank correlation test. Also the effect of NK cell on overall survival, disease-free survival, and recurrence were plotted using the Kaplan–Meier method and assessed with the log-rank (Mantel-Cox) method.

Results

Patients with PDAC have normal numbers of NK cells within blood but expression of CD16 is increased on the CD56^{dim} subset

The overall percentage of NK cells within the blood of patients with PDAC was not reduced and comprised 18% of PBMC compared to 16% in healthy donors ($p = .52$) (Figure 1b). The relative distribution of NK cells within major NK subsets was then examined by analysis of CD16 and CD56 expression. These four major subsets comprise CD56^{dim}CD16^{bright}, CD56^{bright}CD16^{negative}, CD56^{bright}CD16^{bright} and CD56^{dim}CD16^{negative} subgroups[30] (Figure 1a). As anticipated, CD56^{dim}CD16^{bright} cells were the dominant population in healthy donors, representing 79% of NK cells, with values of 6.4, 2.2, and 12% for CD56^{bright}CD16^{negative}, CD56^{bright}CD16^{bright} and CD56^{dim}CD16^{negative} cells, respectively (Figure 1c+1d). Interestingly, the CD56^{dim}CD16^{bright} population was further increased in the blood of PDAC patients where it comprised 87% of total cells ($p = .001$). The CD56^{bright}CD16^{negative}, CD56^{bright}CD16^{bright} and CD56^{dim}CD16^{negative} subsets were measured at 4.2, 1.9, and
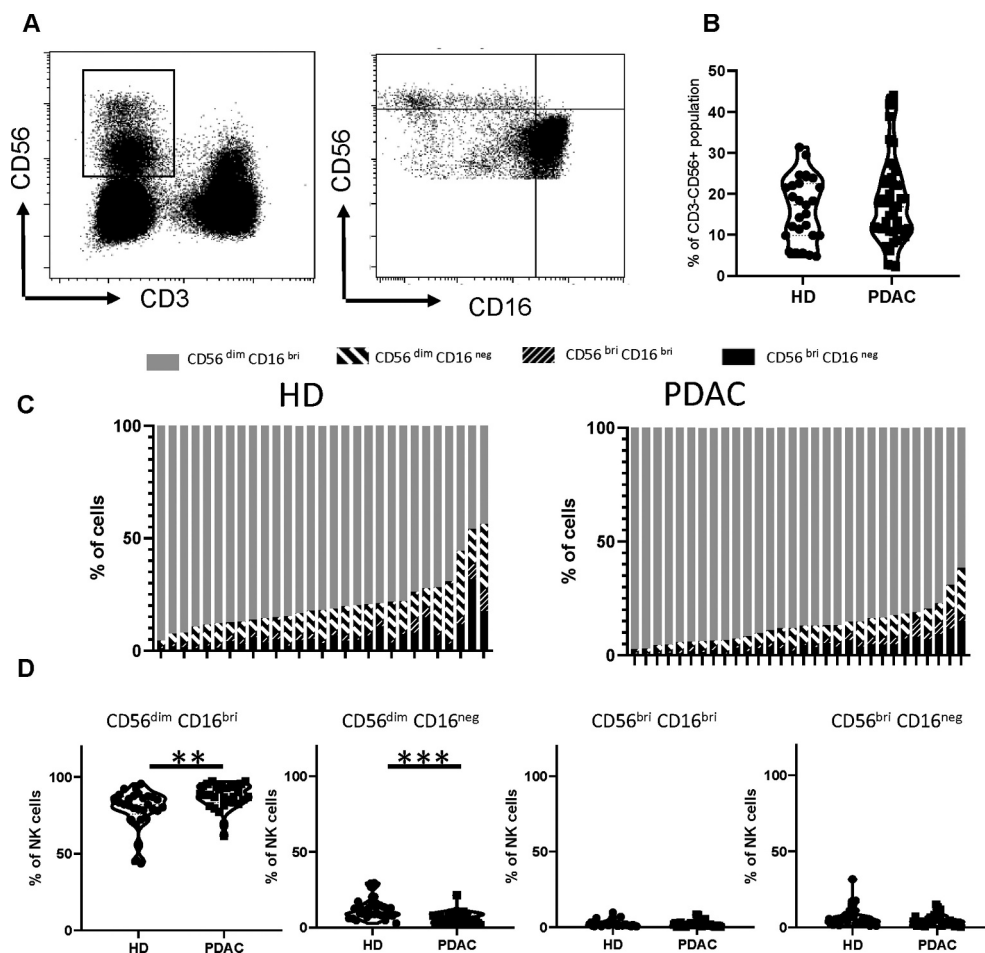
Figure 1. Patients with PDAC have normal numbers of NK cells within blood but expression of CD16 is increased on the CD56dim subset. (A): Left panel: An example of dot plot from flow cytometry staining to gate the NK cells using CD3- CD56+ as the makers. Right panel: An example of dot plot to gate the four NK subsets according to CD56 and CD16 expression, CD56^{dim}CD16^{bright}, CD56^{bright} CD16^{bright}, CD56^{dim}CD16^{negative} and CD56^{bright}CD16^{negative} NK cells. (B): The percentage of CD3- CD56+ NK cells out of PBMCs were compared between PDAC patients and healthy donors using violin plot with each point represent a donor. (C): The composition of these four NK subsets in the bloods from PDAC patients and HDs were compared in bar chart. (D): Percentage of four NK subsets in the bloods from PDAC patients and HDs were compared individually using violin plot with each point represent a donor with significance was determined using Mann-Whitney testing, *p*< .01(**) and *p*<.0001(***).

6.7%, respectively (Figure 1c right panel and 1D), representing a 44% reduction of CD56^{dim}CD16^{negative} cells within the patient group ($p = .0001$).

Peripheral NK cells from PDAC patients express reduced levels of the activating receptors NKG2D and NKp30

We next went on to assess the expression of markers associated with NK cell differentiation and activation on peripheral NK cells. CD57 expression is expressed on late differentiated cells and associated with high cytotoxic activity.[31] Of note, CD57 was expressed on 50% of cells in the patient group compared to only 44% within healthy donors ($p = .04$) (Figure 2a). The major activatory protein CD16 was also increased in patients, expressed on 88% compared to 77% of controls ($p = .0002$) (Figure 2a). A trend toward increased expression of NKG2C (Figure 2a) was also seen in the patient group (NKG2C: 4.8% vs 5.2% for HD vs PDAC, $p = .68$). Overall this profile reveals
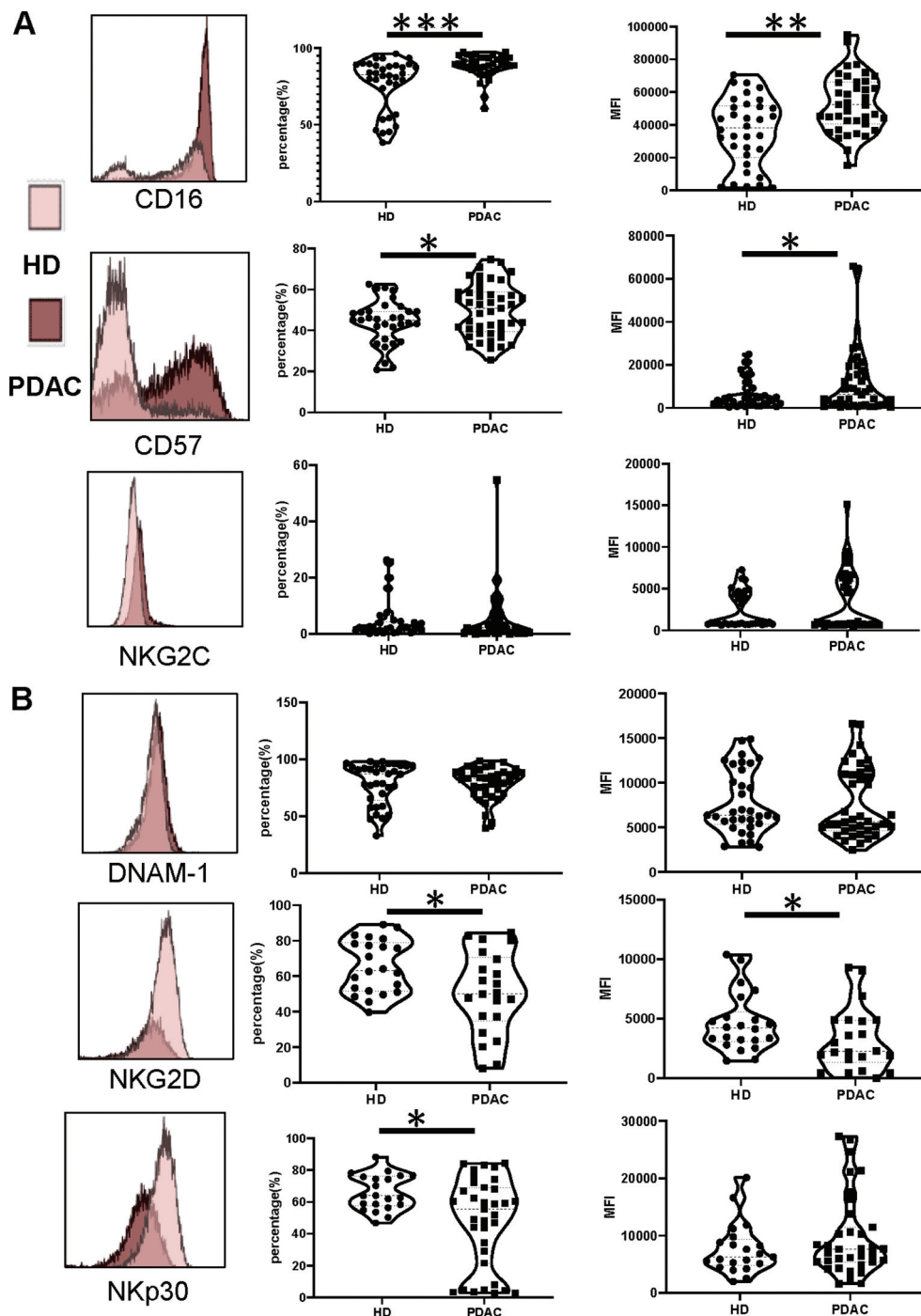
Figure 2. Peripheral NK cells from PDAC patients express reduced levels of the activating receptors NKG2D and NKp30. The expression of differentiation markers including CD16, CD57, NKG2C(A) and activatory makers including DNAM-1. NKG2D, NKp30 (B) were compared between PDAC patients and HDs. The left panel of each marker is the histogram of representative examples to show the expression of each marker in PDAC patients and HDs. The comparison was carried out with percentage (middle panel) and mean florescence intensity (MFI) (right panel).The significance was determined using Mann-Whitney testing, $p< .05$(*), $p< .01$(**) and $p< .001$(***).

a somewhat more differentiated profile of peripheral NK cells within the patient group.

Activating receptors are critical regulators of NK cell activity and are frequently downregulated in the setting of cancer. We next defined the expression of the major activatory receptors NKG2D, DNAM-1 and NKp30. NKG2D expression was downregulated in the patient group, present on 51% of cells compared to 66% of healthy donors ($p = .027$) (Figure 2b). The mean florescence intensity (MFI) of expression was also lower at 3200 ± 564 and 4620 ± 525, respectively ($p= .047$). NKp30 expression was also reduced, present on 47% and 66% of cells in PDAC patients and controls, respectively ($p= .023$) (Figure 2b). In contrast, expression of DNAM-1 did not differ between the two groups, present on 79% of cells in both cohorts (Figure 2b).

NK cells within the blood of PDAC patients have reduced cytotoxic function and express high levels of IL-10

The functional capacity of NK populations was next assessed by assessment of relative cytotoxic activity and the pattern of cytokine production. Cytokine production was assessed by incubation of PBMC with K562 prior to assessment of cytokine production using intracellular staining. NK cells normally produce substantial amounts of proinflammatory cytokine such as IFN-γ and TNF-α and this pattern was observed in healthy donors. However, IFN-γ production was substantially reduced in NK cells from PDAC patients (1.9% vs. 5.1% in controls; $p= .003$) (Figure 3a) and atrend toward reduced levels of TNF-α was also seen (2.4% vs 3.9%, $p= .12$) (Figure 3b left panel). IL-2 production was also lower in the patient group, present within only 0.7% of cells compared to 2.4% from healthy donors ($p= .009$) (Figure 3a). 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 ($p= .001$) (Figure 3a). Expression of IL-4 and IL-5 was comparable between donors (Figure 3b middle and right panel). Lytic activity of NK cells was analyzed on K562 target cells and cytotoxicity was markedly reduced compared to healthy donors (42% vs. 70%, respectively, at E:T ratio 1:1 ($p= .026$) (Figure 3c).

NK cells are a minority population within PDAC tumors and show strong downregulation of CD16

Having observed substantial differences in the phenotype and function of NK cells in the blood of PDAC patients we next went on to assess the phenotype of NK cells isolated from the tumor microenvironment using 9 paired samples of blood (PBMC-NK) and tumor-infiltrating lymphocytes (TIL-NK).

The percentage of NK cells was markedly suppressed within TIL populations, representing only 3.5% of cells compared to 17% within PBMC ($p= .005$) (Figure 4a and 4b). The relative
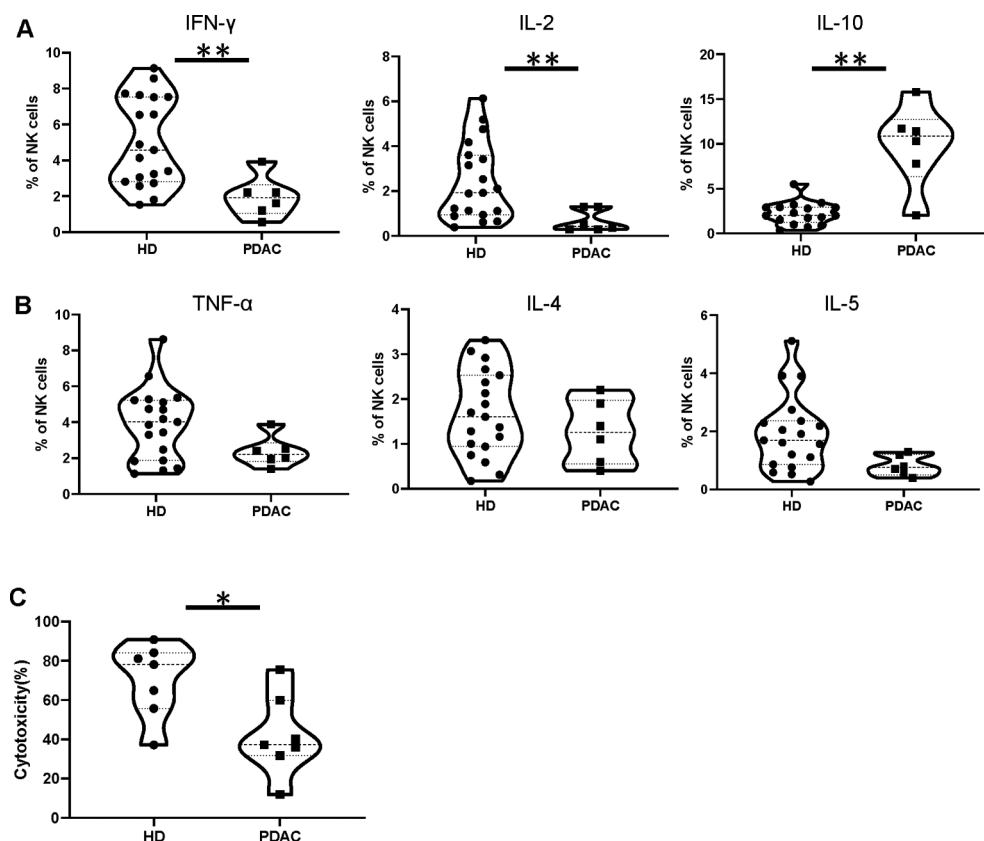
Figure 3. NK cells within the blood of PDAC patients have reduced cytotoxic function and express high levels of IL-10. (A and B): Whole PBMCs from PDAC patients and HDs were co-cultured with K562 cells at 1:1 ratio in the presence of Brefeldin A for overnight before the cells were stained for surface receptors, fixed permeabilised and stained with IFN-γ, IL-2, IL-10, TNF-α, IL-4 and IL-5 antibodies. The percentage of NK cells that produce each cytokine was compared between PDAC patients and HDs. Each plot represents one cytokine. (C) NK cells were enriched and co-cultured with CFSE labeled K562 cells for 16hrs. The cytotoxicity was calculated according to the relative cell count of the live populations of target cells using flow cytometry. Data shown are as violin plots with each dot represents adonor. The significance was determined using Mann-Whitney testing, $p< .05(*)$ and $p< .01(**)$.
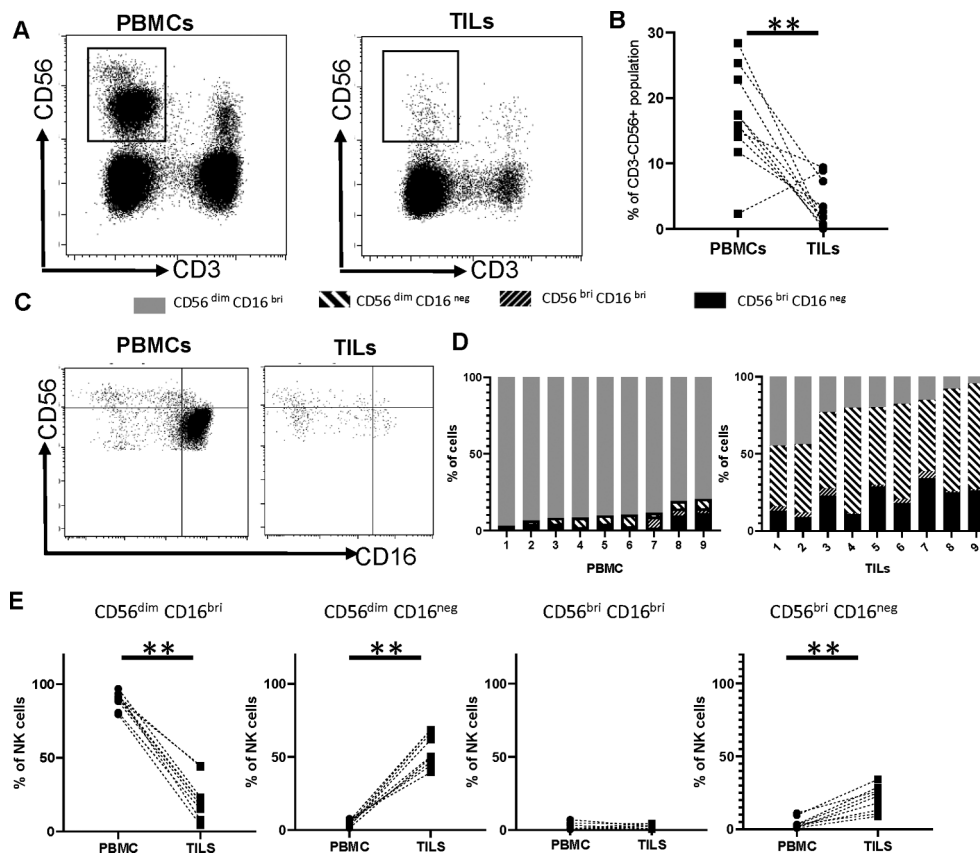
Figure 4. NK cells are a minority population within PDAC tumors and show strong downregulation of CD16. (A): Examples of dot plot from flow cytometry staining to gate the NK cells using CD3- CD56+ as the makers with left panel represents PBMCs and right panel represents TILs from PDAC patient. (B) The percentage of CD3- CD56 + NK cells were compared between PBMCs and TILs from PDAC patients with each point represent a donor. (C) Examples of dot plots to gate the four NK subsets according to CD56 and CD16 expression, CD56^{dim}CD16^{bright}, CD56^{bright} CD16^{bright}, CD56^{dim}CD16^{negative} and CD56^{bright}CD16^{negative} NK cells. The left panel represents PBMCs and right panel represents TILs from PDAC patient. (D): The composition of these four NK subsets in PBMC and TILs from PDAC patients were compared in bar chart. (D): Percentage of four NK subsets in the PBMCs and TILs from PDAC patients were compared individually with each point represent a donor with significance was determined using Wilcoxon matched-pairs signed rank test, $P < .01(**)$.

distribution of TIL-NK and PBMC-NK within the four major NK subsets was then defined by CD16 and CD56 expression. Of note, the dominant phenotype of TIL-NK cells was the CD56^{dim}CD16^{negative} population which comprised 55% of the total pool. CD56^{dim}CD16^{bright}, CD56^{bright}CD16^{negative} and CD56^{bright} CD16^{bright} subgroups represented 22, 21, and 2.2%, respectively, of the remaining population (Figure 4d). This was a substantially different profile from that seen within PBMC-NK where, as expected, 89% of cells expressed aCD56^{dim}CD16^{bright} phenotype. CD56^{bright}CD16^{negative}, CD56^{bright} CD16^{bright} and CD56^{dim}CD16^{negative} cells also comprised 4%, 2.2% and 4.6%, respectively (Figure 4d). As such these findings show a marked downregulation of CD16 within the tumor microenvironment, which was present on only 27% of total TIL-NK compared to 92% of PBMC-NK ($p < .0001$) (Figure 5a).

Expression of activatory receptors is reduced substantially on NK cells within the PDAC tumor

The extended phenotype of TIL-NK was then assessed using flow cytometry. CD57 expression was seen to be very low, present on 11% of cells compared to 44% in blood ($p = .0001$; Figure 5a) . This data from CD16 and CD57 suggests aless differentiated phenotype for TIL-NK

populations. But interestingly, NKG2C expression was marginally increased at 9.2% compared to 6.1% on PBMC-NK ($p = .04$).

Strikingly, downregulation of NK activatory receptors was profoundly suppressed on TIL-NK cells, further extending the profile that had been observed in PBMC-TIL populations. This was seen most clearly in both the percentage and median fluorescent intensity (MFI) of DNAM-1 and NKp30 expression. DNAM-1 was present on only 32% of TIL-NK cells compared to 83% of PBMC-NK ($p < .0001$) (Figure 5b) with NKp30 expression of 26% and 60%, respectively ($p = .007$) (Figure 5b). Relative MFI values were 1430 ± 214 and 4860 ± 294 for DNAM-1 and 2860 ± 475 and 7120 ± 1308 for NKp30, respectively (Figure 5b).

Although NKG2D expression had been seen to be reduced on peripheral NK cells from PDAC patients, this remained stable within the TIL populations with no further reduction (48% versus 58% in TIL-NK and PBMC-NK, respectively; $p = .15$) (Figure 5b).

The PDAC tumor microenvironment expresses high levels of NK cell ligands and tumors induce downregulation of CD16 and CD57 on NK cells

In order to interrogate mechanisms that might lead to the characteristic phenotype of NK cells within PDAC tumors
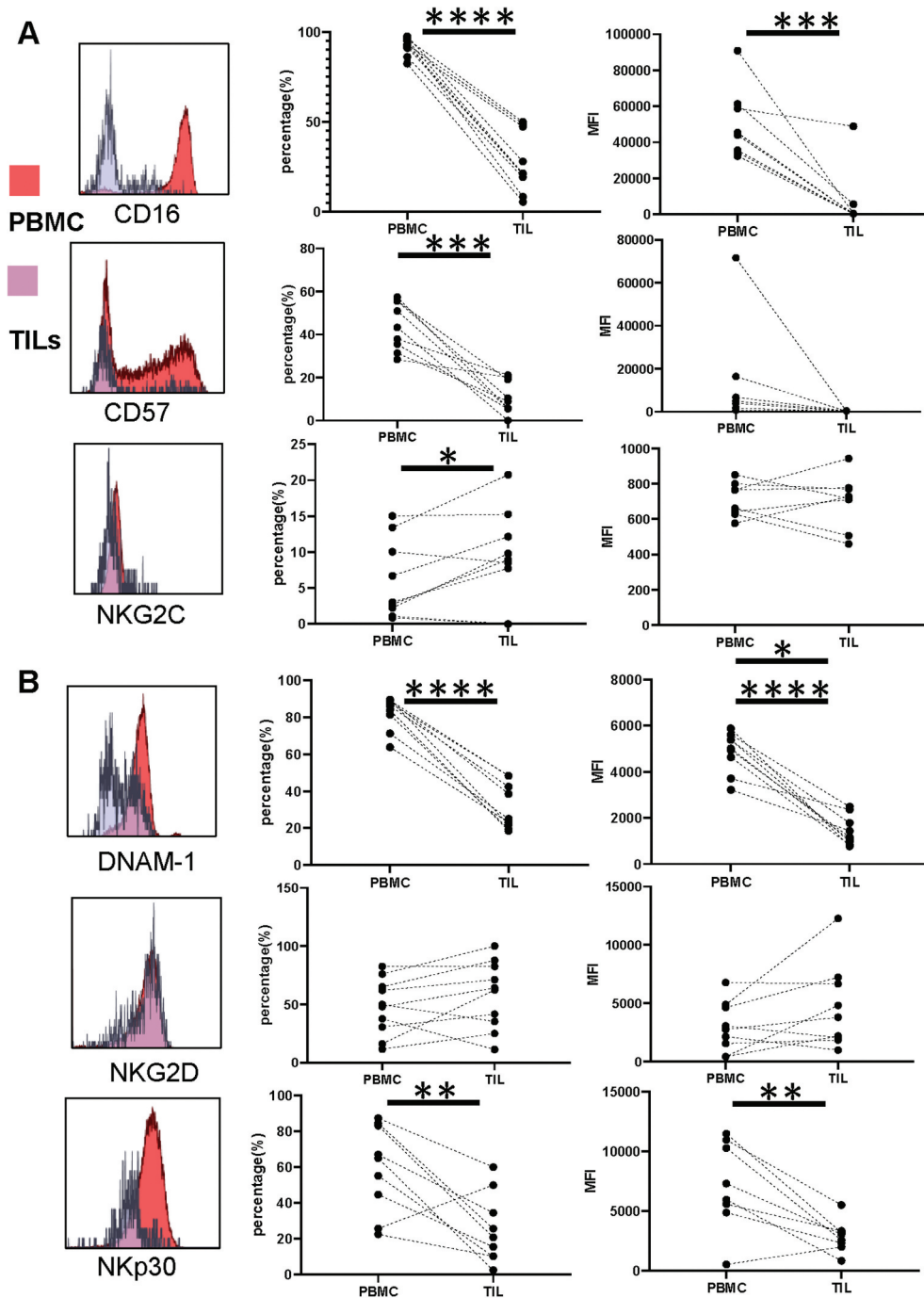
Figure 5. Expression of activatory receptors is reduced substantially on NK cells within the PDAC tumor. The expression of differentiation markers including CD16, CD57, NKG2C(A) and activatory makers including DNAM-1. NKG2D, NKp30 (B) were compared between PBMCs and TILs from PDAC patients. The left panel of each marker is the histogram of representative examples to show the expression of each marker in PBMCs and TILs from PDAC patients. The comparison was carried out with percentage (middle panel) and mean florescence intensity (MFI) (right panel).The significance was determined using Wilcoxon matched-pairs signed rank test, $p < .05(*)$, $P < .01(**)$, $p < .001(***)$ and $p < .0001(****)$.

we next examined the expression of ligands for NK activatory receptors within the tumor microenvironment. qPCR was used to assess transcription of the NKG2D ligands and PVR within tumor tissue and adjacent uninvolved margin. Interestingly, expression of the NKG2D ligands ULBP2 and MICA was increased by 6.2 and 2.7 fold, respectively, within tumor tissue (ULBP2: 1000 & 162 for PDAC vs margin, $p= .0059$; MICA: 1510 vs 567 ($p= .01$) (Figure 6a)). PVR is aligand for DNAM and was markedly increased by 18-fold

in the tumor microenvironment (4370 vs 245 ($p= .0024$) (Figure 6a)). Immunohistochemical analysis confirmed strong PVR, MICA, and ULBP2 expression on PDAC tumor tissue (Figure 6a).

To further understand how PDAC tumor cells might act to directly influence NK phenotype we next co-cultured primary PDAC cells with autologous PBMC-NK cells *invitro*. PDAC tumor organoids were generated and after 2–3 week maturation were co-cultured with the PBMC for 14 days. NK cell
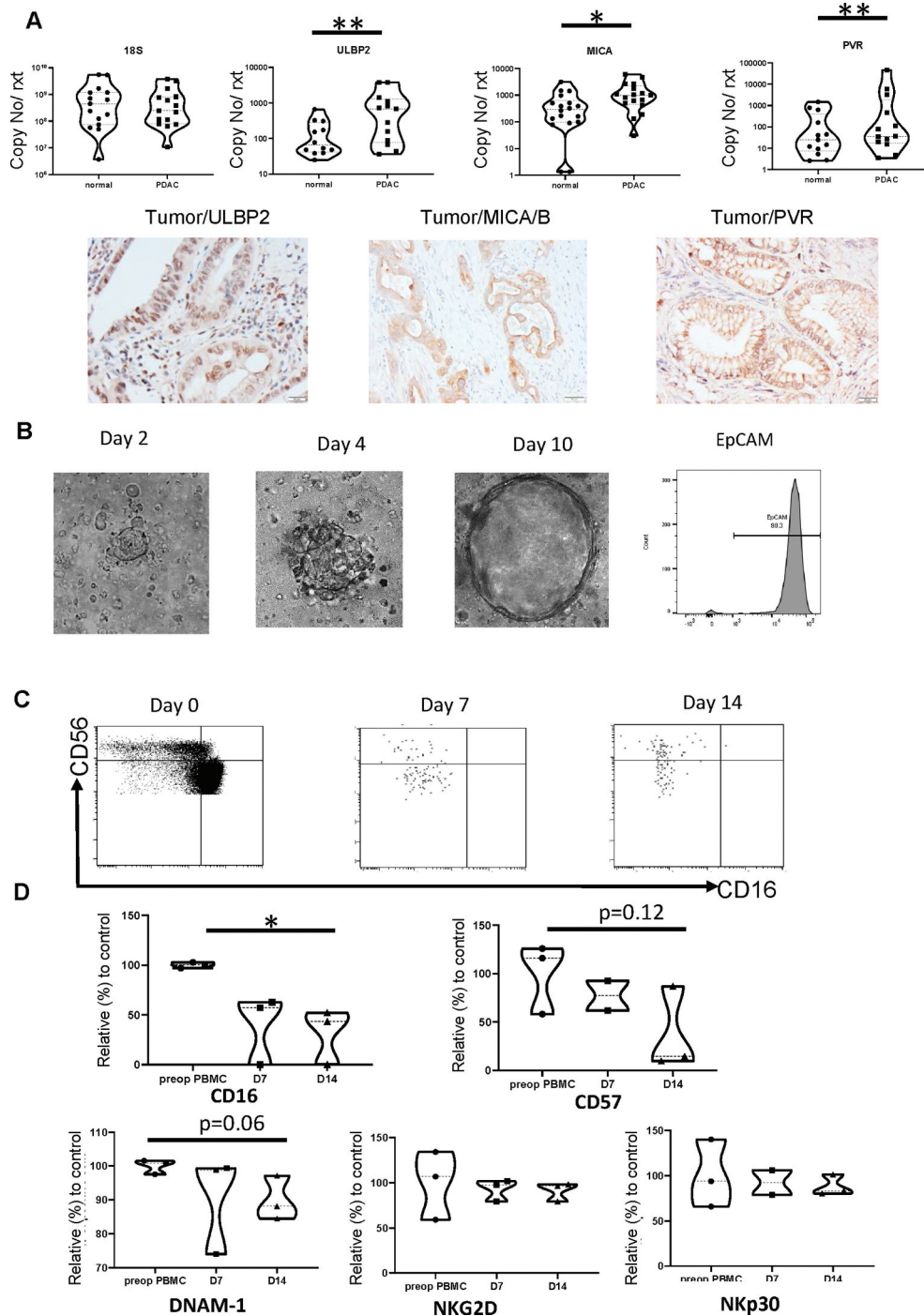
Figure 6. The PDAC tumor microenvironment expresses high levels of NK cell ligands and tumors induce downregulation of CD16 and CD57 on NK cells. (A): Total RNA from tumor tissues and normal margins were extracted and and reverse-transcripted to cDNA before the Q-PCR was performed to calculate absolute quantity of copy numbers using standard curve. The copy numbers per reaction were identified for 18s, ULBP2, MICA and PVR. The expression of PVR, MICA/B and ULBP2 in PDAC was demonstrated using the immunohistochemistry staining contrasting to normal adjacent pancreatic tissue. (B): The PDAC tumor organoids were generated through 3D culture. These pictures are representative organoids culture from day2, day4 and day 10 after the setup of the organoids. Right panel is the histogram of the EpCAM staining with the organoids culture after they were matured. (C): After the organoids culture was matured, the co-culture with the autologous PBMCs were set up. The phenotype of NK cells from the co-culture were monitored at day 7 and These flow dot plots represent the expression pattern of CD16 and CD56 of NK cells (CD3- and CD56+) from one experiment(left panel: before the co-culture, middle panel: day 7 post co-culture and right panel: day14 post co-culture). (D) The percentage of CD16, CD57, DNAM-1, NKG2D and NKp30 on NK population was compared before the co-culture, D7 and D14 post co-culture. Expression of each marker (D7 andD14) is shown as relative percentage on NK cells cultured with organoids compared with NK cells under control medium. The data was shown as using violin plot with each point represent a donor with significance was determined using Mann-Whitney testing, $p < .05(*)$.

phenotype was determined prior to incubation and then atday 7 andday 14 following co-culture, the data were shown as relative percentage of surface expression compared to PBMC cultures without organoid (n = 3). CD16 and CD57 expression in the control group remained stable during culture but, interestingly, CD16 expression was profoundly downregulated

during organoid co-culture, falling to 32% of the control group at day 14 (p= .01). As such the majority of NK cells came to develop aCD56^{dim}CD16^{negative} phenotype typical of those found in primary PDAC tumors (Figure 6c right panel). CD57 expression was also reduced during co-culture, falling to 37% of control values atday 14 (p= .12) (Figure 6d). This phenotypic change was not observed with organoid-conditioned medium. Expression of the activatory receptors DNAM-1, NKG2D and NKp30 expression was broadly stable during culture. As such tumor cells can rapidly directly down-regulate CD16 and CD57 expression but do not modulate expression of activity ligands during short-term culture.

The CD56^{dim} CD16^{neg} NK population within blood does not affect overall survival but is positively correlated with tumor recurrence

Finally, we next assessed how the size and phenotype of the NK pool correlated with clinical outcome. Interestingly, the proportion of total NK cells within blood correlated directly with the size of the tumor

(R = 0.38, P= .02). A trend toward reduced survival was also observed (Figure 7a).

Given our earlier observations on differential populations of CD56^{dim}CD16^{neg} NK within patients, and expansion after incubation with tumor organoids, we considered that these cells may be tumor-reactive and of particular interest. The cohort of PDAC patients undergoing potentially curative surgery was divided into two groups on the basis of the median percentage of CD56^{dim}CD16^{neg} NK cell populations (<1% and ≥ 1%) and correlated with pathologic and clinical outcome (Table 1). Patients with ≥ 1% CD56^{dim}CD16^{neg} NK cells had higher CA19-9 levels than those with <1% CD56^{dim}CD16^{neg} NK cells. The overall survival and disease-free survival curves were plotted using the Kaplan–Meier method and the significance was assessed using log-rank (Mantel-Cox) method. Patients with a CD56^{dim}CD16^{neg} NK population higher than 1% of PBMCs demonstrated significantly reduced tumor recurrence (Figure 7b and Table 2) with amedian of disease-free survival of 451 days (164–737) compared with 185 days (17–352) with those in the <1% group. These data indicate that CD56^{dim}CD16^{neg} NK populations may play acrucial role in containing recurrence of PDAC tumor after surgery.
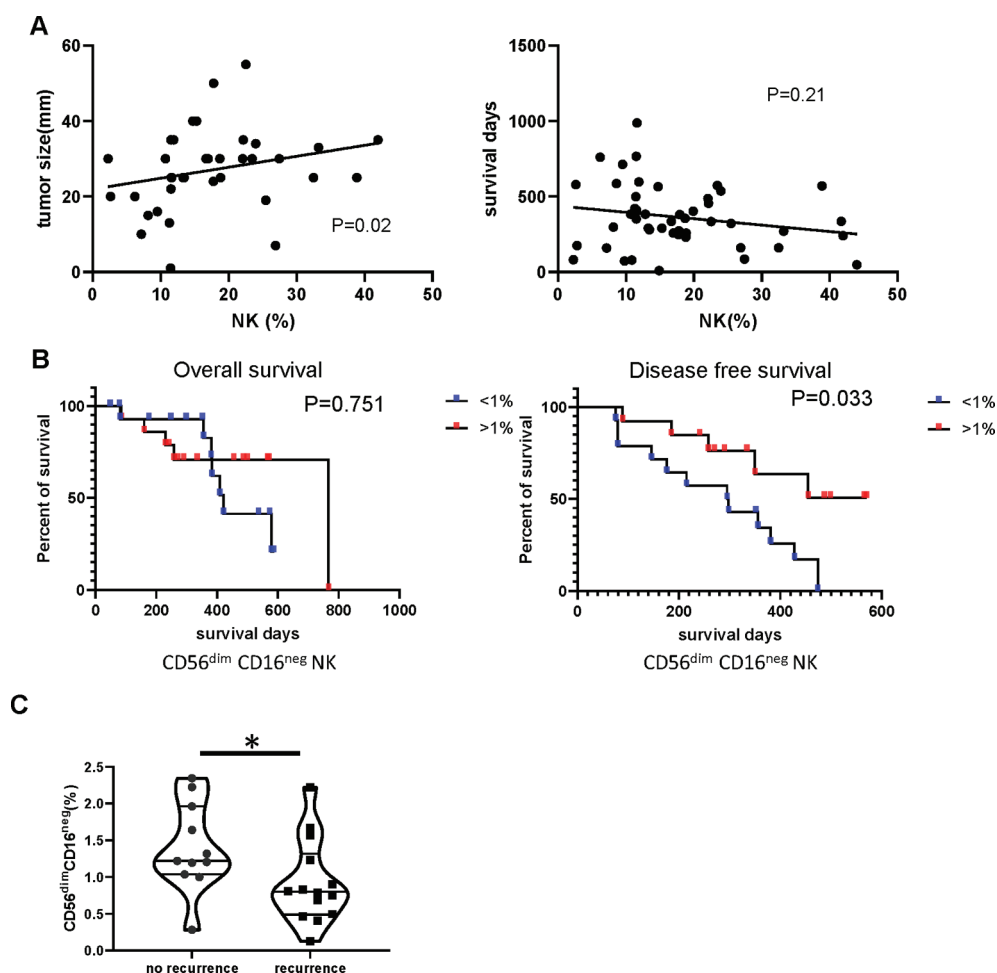
Figure 7. The CD56^{dim} CD16^{neg} NK population within blood does not affect overall survival but is positively correlated with tumor recurrence. (A) Correlation between the percentage of total NK population in blood from PDAC patients with overall survival and tumor size was assessed. The line in the figure represents the linear regression line. In addition, the Spearman's Rank correlation was used to test the significance and P value is indicated for each panel. (B) Kaplan-Meier curve (log-rank test) to compare the overall survival and disease free survival between two groups with different percentage of CD56^{dim} CD16^{neg} NK (cutoff point as median value 1%) from whole PBMCs. (C) Violin plot (Mann-Whitney test) to compare the percentage of CD56^{dim} CD16^{neg} NK cells in the patients who developed recurrent and who did not recurrent with significance was determined using Mann-Whitney testing, p < .05(*).

Table 1. Demographic, diagnostic and therapeutic characteristics of two groups of PDAC patients with defined on the basis of CD56^{dim} CD16^{neg} NK cell population (<1% and ≥ 1%).

CD56^{dim} CD16^{neg} NK cells	NK low (<1) n = 16	NK high (1+) n = 14	p
Age	67 (61–75)	75 (65–79)	0.525
BMI	27.3 (25.1–29)	26.9 (23.6 – 28.8)	0.856
Gender (male)	10 (63%)	6 (43%)	0.272
Smoker (yes)	3 (19%)	3 (21%)	1
Preoperative biliary drainage (Yes)	4 (25%)	2 (14%)	0.651
Jaundice at presentation (yes)	15 (94%)	13 (93%)	1
Vit D	25 (11.5–27.25)	31 (26–39)	0.9
CA19-9	109 (11–459)	625 (156–3036)	0.041
Neoadjuvant therapy (yes)	1 (6%)	1 (7%)	1
Resected at surgery (yes)	13 (81%)	13 (93%)	0.602
T stage 1 2 3	3 10 3	2 10 1	0.701
N Stage 0/1/2	2 7 5	3 7 3	0.775
Positive resection margin (yes)	7 (44%)	7 (50%)	1
Tumor size (mm)	25 (22.75–28.75)	25 (25–35)	0.223
Lymph node ratio	0.23 (0.13–0.53)	0.09 (0.04–0.43)	0.395
Comprehensive complication index	4.5 (0–17.9)	21 (0–26)	0.553
Adjuvant therapy (yes)	10 (62.5%)	12 (86%)	0.093
Palliative therapy (yes)	8 (50%)	5 (35%)	0.464

Table 2. Clinical survival of two groups of PDAC patients with defined on the basis of CD56^{dim} CD16^{neg} NK cell population (<1% and ≥ 1%).

CD56^{dim} CD16^{neg} NK cells	NK low (<1) n = 16	NK high (1+) n = 14	p
OS median (95% CI)	409 (383–434)	499 (276–718)	0.751
DSS median (95% CI)	405 (360–449)	487 (173–800)	0.853
DFS median (95% CI)	185 (17–352)	451 (164–737)	0.033

Discussion

Most studies on the mechanisms of immune evasion in PDAC patients have focussed on T cell immune responses against tumor tissue but here we investigated NK cell phenotype and function. Differential features of NK cell immune evasion are observed within the tumor microenvironment and peripheral blood. As local invasion and metastasis ultimately develop in the majority of patients with PDAC, these findings indicate that immunotherapy protocols might seek to reverse these features in both settings.

A clear difference was apparent in the number of NK cells within the blood and tumor compartments. Lim et al have previously reported a higher percentage of NK cells in blood from PDAC patients compared to HD[19] and a similar trend was seen in our study but did not reach statistical significance (Figure 1b). NK proportions were markedly lower within tumor tissue, representing only 3.5% of the TIL population. As such migration of NK cells into the tumor site is likely suppressed and lack of CXCR2 expression on NK cells has been shown to be one critical determinant.[19] However, it should also be noted that NK cells numbers are frequently excluded from normal tissue and future work should address the profile of NK cells in control pancreatic tissue. The expression of CD16 and CD57 also differed between compartments. This is likely to partially reflect differential NK phenotype within normal tissue and peripheral blood as NK cells within secondary lymphoid tissue express a CD56^{bright}CD16^{neg} phenotype[30,32] whilst the cytotoxic CD56^{dim}CD16^{pos} subgroup predominates in

blood.[33,34] However, at least four NK subsets can be defined according to CD56 and CD16 expression[30] and we show that NK cells with a CD56^{dim}CD16^{neg} phenotype are enriched within tumor but depleted within peripheral blood. This subset expresses a broadly immature phenotype with high expression of NKG2A+ and reduced CD57,[35] findings that concur with our observations within NK-TIL. In contrast, NK cells in the blood of PDAC patients demonstrate a differentiated phenotype, with high levels of expression of CD16, CD57 expression. Organoid cultures provide an opportunity to interrogate interactions between primary tumor and immune effector cells *in vitro* and it was noteworthy that the features of NK cell phenotype within the tumor microenvironment were largely recapitulated following culture with PDAC organoids, with substantial downregulation of CD16 and CD57.

Downregulation of activating receptor expression on NK cells and impaired NK function is a common mechanism of immune evasion in cancer, and has been associated with tumor progression in PDAC.[36–39] We also observed this profile but it was noteworthy that a different pattern was observed on cells from blood or tumor microenvironment. NKG2D expression is often reduced on NK cells in cancer[26,27] and although modest downregulation was observed in PDAC patients this pattern remained stable within blood and TIL populations. In contrast, although NKp30 was only partially downregulated within blood, a profound reduction in expression was observed within tumor tissue. DNAM-1 expression was also selectively and comprehensively downregulated within the tumor microenvironment. Interestingly, expression of the inhibitory TIGIT receptor,[40] which binds to the same ligands as DNAM-1, is significantly higher on NK cells from blood in PDAC patients compared with HD (Supplementary Figure 3) and reinforces an important role for this pathway. Another mechanism for impairment of NK cell function within the tumor microenvironment may be the conversion of NK cells into innate lymphoid cell due to high concentration of TGF-β.[41]

The expression of ligands for NK activatory receptors was examined by PCR and immunohistochemistry. NKG2D ligand expression was increased in tumors, most notably for ULBP2 and MICA which were increased by 2–6 fold. Interestingly, the concentration of serum NKG2D ligands is an independent marker of survival in PDAC[42–45] and it most strongly associated with ULBP2.[43] The expression of PVR, a major ligand for DNAM, was markedly increased on tumor cells (Figure 6a) and is likely to explain the profound downregulation of DNAM in this setting. This is particularly noteworthy as DNAM-mediated cytotoxicity represents a major mechanism of lysis of PDAC cells.[19]

The functional profile of peripheral NK cells was assessed using cytotoxicity assay and cytokine profile after stimulation although too few cells were available within TIL for this analysis. One of the most striking observations was that NK cells from PDAC patients have a markedly altered profile of cytokine production, with reduced levels of IFN-γ and IL-2 but substantially increased production of IL-10. As such they express a 'regulatory' phenotype, a concept that is emerging within NK biology. IL-10 producing NK cells have been demonstrated after stem cell transplantation[46] and viral infection[47] and likely mediate a regulatory role, promoted by

sustained proliferation after chronic stimulation.[47,48] CD56[bri] NK cells are generally regarded as the major subset of cytokine-producing cells[49] but CD56^{dim} cells may also display rapid cytokine production after stimulation,[50] particularly from the CD16^{neg} subset.[35]

These findings raise questions as to how IL-10 production might act to suppress tumor-specific immune responses. Systemic concentrations of IL-10 levels are 3–5 fold higher in the blood of patients with PDAC, where they are strongly correlated with impaired T cell function,[11–13] and our data indicate that NK cells are likely to be a major source of IL-10 production within the periphery. IL-10 might also have a direct impact on NK cell function and has been shown to inhibit NK-92 killing of PDAC cells *in vitro*. Importantly, this effect was restored by anti-IL-10 receptor blockade, indicating a potential novel therapeutic intervention.[51] Of note, all patients studied had undergone operative resection of PDAC with curative intent and had no evidence of metastatic disease at this time. Despite this, our findings reveal that the peripheral immune system is already significantly modulated by the disease process and it is likely that this immunoregulatory environment may play an important part in tumor spread. Indeed the number of peripheral NK cells has been identified as an independent marker of increased survival.[20]

Interestingly, a positive correlation between the percentage of peripheral NK cells and the size of the PDAC tumor. The reasons underlying this are not clear but may include direct tumor-mediated stimulation of NK proliferation or increased partitioning of the NK pool from the tumor and into the peripheral circulation. The CD56^{dim}CD16^{neg} NK cell subpopulation was of particular note as it was reduced within blood but enriched in TILs. Interestingly, patients with peripheral CD56^{dim}CD16^{neg} NK populations above the median level (>1%) suffered less disease recurrence than patients with <1%. Our observation that this subset can be expanded by co-culture with autologous organoids reveals a potential mechanism for therapeutic intervention.

Our findings indicate that a range of mechanisms of immune evasion are apparent within NK cells in patients with PDAC. These include exclusion from the tumor site, downregulation of activatory receptors with impaired differentiation in the tumor microenvironment, and promotion of an immunoregulatory IL-10 phenotype within the periphery. This suggests that primary NK cells might have potent activity to suppress PDAC development and that these evasion mechanisms are a necessary component of tumor evolution. As such, approaches that overcome these mechanisms could have considerable potential in a disease with few therapeutic options. Approaches such as IL-10 blockade, mechanisms to support activatory receptor expression[52], or NK cellular therapy all represent options for future investigation.

Acknowledgments

Authors' contributions

JZ, GM, KR and PM designed the study. FM, JZ, HP. SN, SMD and MF performed the experiments in the research lab. FM, BM, and RB collected the patient samples. JZ, FM, KR and PM wrote the manuscript. All authors read and approved the final manuscript.

Availability of data and material

All data supporting the conclusion of this study have been included within the article. They can be requested through corresponding authors.

Consent for publication

This is not applicable for this study.

Competing interests

The authors declare that they have no competing interests.

Ethics approval and consent to participate

This study was approved by the Birmingham Local Research Ethics Committee REC 16/WM/0214. Written informed consent was obtained from all of the patients.

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