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Supercharged NK cells, unlike primary activated NK cells, effectively target ovarian cancer cells irrespective of MHC-class I expression

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ABSTRACT

Objective To demonstrate the significance of supercharged natural killer (sNK) cells to target aggressive gynecological tumours.

Methods and analysis We used cell cultures of peripheral blood-derived mononuclear cells (PBMCs) and purified NK cells alone and in the presence of tumours. MHC-class gene expression assessments of ovarian tumours were performed using gene set enrichment analysis (GSEA). Secretion and expression levels of cytokines in PBMCs and NK cells were determined using ELISA and scRNA seq analysis, respectively. A flow cytometer was used for surface marker analysis. ^{51}Cr and eSight were used to determine the killing activity of NK cells.

Results We have observed a significant decrease in the numbers and functions of NK cells in patients with ovarian cancer. GSEA revealed differently expressed genes, decreased differentiation- and immune-related genes, and increased genes for cell cycle analysis in recurrent tumours compared with chemo-naive ovarian tumours. Increased gene expression as well as secretion of interferon-γ and tumour necrosis factor-α and increased avidity in binding to tumour cells by sNK cells was observed. Unlike primary interleukin (IL)-2-activated NK cells, sNK cells effectively lysed OVCAR8 ovarian poorly differentiated cancer stem-like cells (PDCSCs) and well-differentiated OVCAR4 tumours. Primary ovarian tumours with lower MHC-class I expression were highly susceptible to both primary IL-2-activated NK and sNK cells, whereas the well-differentiated tumours with high expression of MHC-class I were only susceptible to sNK cells.

Conclusion The use of sNK cells in immunotherapy emerges as a potentially effective strategy to target and eliminate the majority of ovarian tumour clones, thereby providing a potential therapeutic opportunity in preventing the recurrence of the disease.

WHAT IS ALREADY KNOWN ON THE TOPIC

⇒ Ovarian cancer, the most aggressive gynaecological cancer, is the sixth leading cause of cancer-related deaths among women in the USA, and once diagnosed, treatment options are often limited. Majority of ovarian cancer patients are post-menopausal, although it can occur in younger age women. Primary therapeutic approaches are surgery in combination with chemotherapy, radiation and, more recently, immunotherapy has been used in the setting of recurrent disease with limited efficacy.

WHAT THIS STUDY ADDS

⇒ Peripheral natural killer (NK) cells in patients with ovarian cancer have a significant deficiency in the numbers and functional ability to target aggressive recurrent tumours that lack MHC-class I surface expression. Supercharged natural killer (sNK) cells, generated in our laboratory, are unique populations of NK cells that exhibit increased avidity in binding to tumour cells, increased cytotoxicity and augmented interferon-γ and tumour necrosis factor-α secretion and expression. Recurrent ovarian tumours have increased cell cycle but decreased differentiation- and immune-related gene sets based on gene set enrichment analysis.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE, POLICY

⇒ Unlike conventional primary activated NK cells which mainly target poorly differentiated cancer stem-like cells (PDCSCs), sNK cells target both PDCSCs with lower expression of MHC-class I and well-differentiated tumours with higher expression of MHC-class I.

INTRODUCTION

The majority of ovarian cancer cases are diagnosed at stage III or IV, and 5-year survival rates[1–3] remain below 50%.[4] Ovarian tumours are thought to contain a subpopulation of tumour cells with cancer stem-like cell (CSC) properties or poorly differentiated cancer stem-like cells (PDCSCs), and these cells are thought to have self-renewal capacity and resist standard chemotherapy, resulting in

tumour progression and recurrence.[5–8] CSCs or PDCSCs are recognised to express lower surface expression levels of MHC-class I, which could be one reason for the limited effectiveness and success of T-cell-based immunotherapies in patients with cancer.[9–11] This, however, highlights the potential of MHC-independent approaches, such as natural killer (NK) cell-based therapies. NK cells are known to target cells with low MHC-class I surface expression.

To understand what types of tumours are targeted by the primary NK (pNK) cells, we needed a simple way to identify the differentiation status of the tumours with which NK cells interact. To accomplish this, we obtained pancreatic tumours at the poorly, moderately and well-differentiated stages based on the morphological and pathological grading. Based on these studies, activated pNK cells lysed CSCs/poorly differentiated significantly, whereas moderately differentiated tumours were killed less by NK cells and well-differentiated tumor had a very minimal NK cell-mediated killing.[12 13] The higher killing by NK cells was also correlated with the higher expression of CD44, and no or low expression of CD54, MHC-class I and PD-L1 on PDCSCs, whereas lower expression of CD44 and higher expressions of CD54, MHC-class I and PD-L1 was mainly seen in well-differentiated tumours.[12 13] Most tumours contain a heterogeneous population of cells, even when they are enriched with CSCs. Thus, they may contain a small subpopulation of intermixed moderately or well-differentiated cells. We refer to tumours that are enriched with populations harbouring the lowest MHC-class I expression and the greatest recognition and lysis by the primary interleukin (IL)-2-activated NK cells as PDCSCs.[14]

NK cells are known for their anticancer function, are identified by CD16 and CD56 surface receptors and are activated by several different cytokines.[15 16] We previously demonstrated that NK cells play a crucial role in limiting the survival and expansion of PDCSCs via direct killing or the induction of differentiation through their secreted interferon (IFN)-γ and tumour necrosis factor (TNF)-α.[11 17] However, for the NK cells to be able to differentiate tumours they need to shut down their killing ability and increase their cytokine secretion capability, a concept termed split anergy.[18] Indeed, when NK cells are treated with IL-2 and anti-CD16 mAbs, they exhibited decreased NK cell killing and increased IFN-γ and TNF-α secretion, exhibiting the classical split anergy.[18]

Differentiated tumours expressing higher levels of MHC-class I surface receptors are resistant to primary activated NK cell-mediated killing.[17] NK cells were shown to kill ovarian cancer cell lines expressing lower MHC-class I.[11] It is well known that pNK cells become activated by cells that have downmodulated or lost MHC-class I.[19–21] Studies have found diminished cytotoxic activity and other anticancer mechanisms in the peripheral blood-derived NK cells of patients with cancer.[22–25] To overcome this problem, our laboratory developed the methodology using osteoclasts (OCs) as feeder cells to generate large numbers of highly potent NK cells, named supercharged natural killer (sNK) cells, for adoptive NK cell-based therapeutics.[26] These NK cells were named sNK cells due to their superior expansion and significant anticancer activity against tumours[26 27] as well as many other tumour cells, including a great majority of haematological malignancies, breast, prostate and uterine tumours (manuscript in preparation and in press), as well as oral, pancreatic, hepatic and glioblastoma tumours.[12 13 28–30] sNK cells demonstrated higher expression of activating receptors[26] and downmodulation of inhibitory receptors (manuscript in review). In addition, they demonstrated increased secretion of IFN-γ and TNF-α.[13] The levels of Trail expression were also elevated in the sNK cells at the single-cell transcriptomic analysis (manuscript in review). sNK cells expressed higher levels of BCL2 and were able to resist the induction of cell death and loss of cytotoxicity within the tumour microenvironment (manuscript in review). Moreover, in contrast to primary activated NK cells, sNK cells were able to lyse both stem-like and differentiated tumours.[31] sNK cells were found to be superior to many other NK cell treatments, including IL-2, IL-2 and anti-CD16 mAbs, IL-2 and anti-CD16 mAbs, and sAJ2,[13 30] treatment with other NK-specific cytokines such as IL-12, IL-15 and IL-18, and oral squamous cancer stem-like cells (OSCSCs) or K562 expanded NK cells.[13 26] In addition, compared with cord blood-derived NK cells, iPSC-derived NK cells, NK92 and several other NK cells, sNK cells exhibited much higher levels of cytotoxicity and cytokine secretion.[31]

In this study, we tested the peripheral blood of patients with ovarian cancer and found decreased numbers of peripheral blood-derived mononuclear cells (PBMCs), decreased percentages of NK cells and suppressed function of immune cells compared with healthy individuals. We demonstrate the reduced antitumour activity of PBMCs of patients with cancer against established CSCs and autologous tumours. In addition, we previously reported the increased targeting of several ovarian tumours with decreased expression of MHC-class I by primary activated NK cells.[11] In this article, we observed increased sNK cell-mediated cytotoxicity compared with primary IL-2-activated NK cells against ovarian tumour cell lines and also primary patient high-grade serous ovarian tumours. In addition, sNK cells kill irrespective of MHC-class I expression on tumour cells, making them a unique NK cell-based treatment strategy in heterogeneous solid tumours such as ovarian cancers.

RESULTS

Decreased PBMC numbers and decreased percentages of NK cells in the peripheral blood of patients with ovarian cancer

The number of PBMCs per 1 mL of peripheral blood was found to be lower in patients with ovarian cancer compared with healthy individuals (figure 1A). Significantly lower percentages of CD16+CD56+ NK cells, and a moderate decrease in CD14+ monocytes were seen in

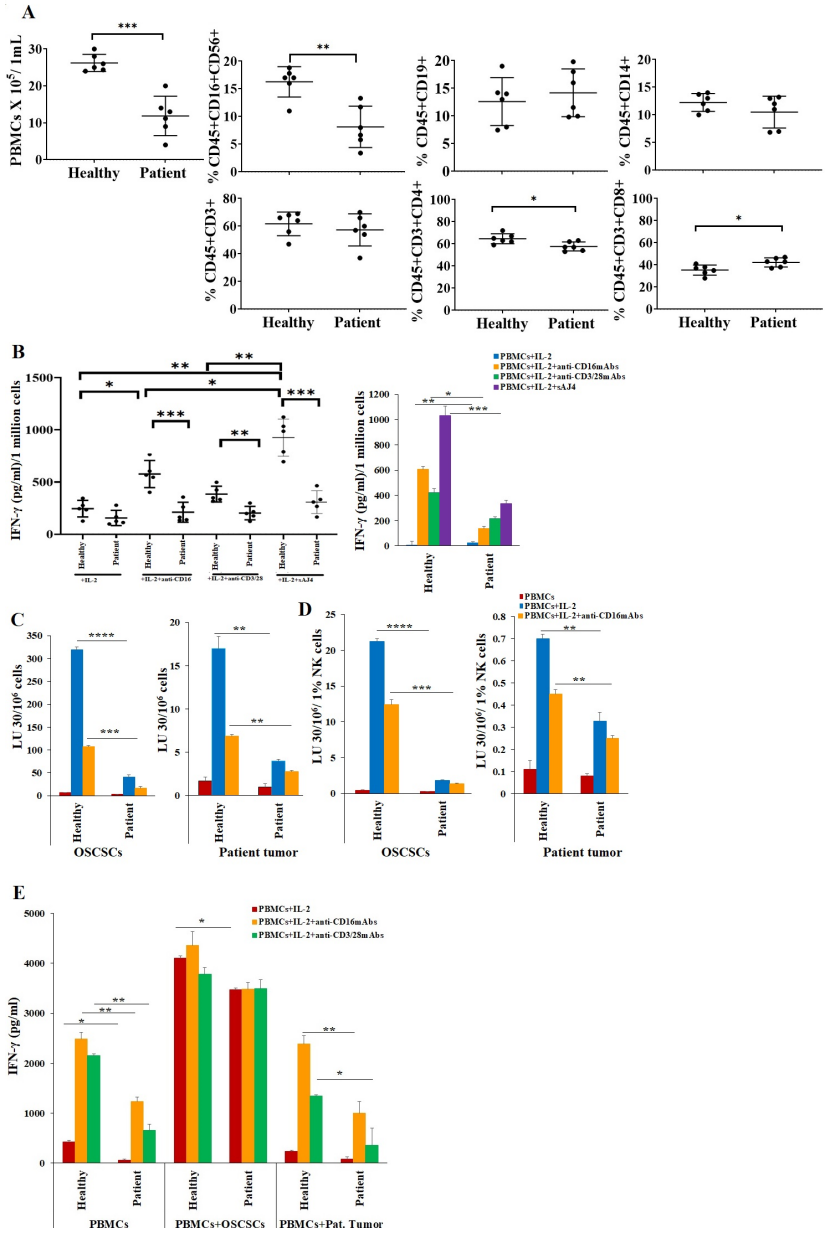


Figure 1 Decreased number of peripheral blood-derived mononuclear cells (PBMCs), percentages of natural killer (NK) cells, interferon (IFN)-γ secretions and cytotoxicity in peripheral blood-derived immune cells of patients with cancer. PBMCs of age- and sex-matched healthy individuals and patients with cancer (patient characterisation is given in online supplemental table S2) were isolated from 1 mL of peripheral blood, and the number of cells was counted using microscopy. PBMCs (2×10^5 cells) were used to determine the percentages of CD16+CD56+, CD19+, CD14+, CD3+, CD3+CD4+ and CD3+CD8+ subsets within CD45+ immune cells using flow cytometric analysis (n=5; one patient blood collected in two different disease time points) (A). PBMCs of healthy individuals and patients with cancer were treated with interleukin (IL)-2 (5000 U/mL) or with a combination of IL-2 (5000 U/mL) and anti-CD16 mAbs (3 µg/mL) or with a combination of IL-2 (5000 U/mL) and anti-CD3/28 antibody (25 µL/mL) or with a combination of IL-2 (5000 U/mL) and sAJ4 (PBMC:sAJ4, 1:20) for 18 hours before the supernatants were harvested to determine IFN-γ secretion using single ELISA (n=5; one patient blood collected in two different disease time points). A representative experiment is shown as a bar graph, and data are presented as mean±SD (B). PBMCs of healthy individuals and patients with cancer left untreated or were treated with IL-2 (5000 U/mL) or with a combination of IL-2 (5000 U/mL) and anti-CD16 mAbs (3 µg/mL) for 18 hours before they were used as effectors in standard 4-hour ^{51}Cr release assay against oral squamous cancer stem-like cells (OSCSCs) or autologous primary patient high-grade serous ovarian cancer (C, D). The lytic units $30/10^6$ cells were determined using the inverse number of PBMCs required to lyse 30% of tumours × 100 (C). Lytic units per 1% NK cells were determined using CD16+CD56+ % obtained from flow cytometric analysis (D). PBMCs of healthy individuals and patients with cancer were treated with IL-2 (5000 U/mL) or with a combination of IL-2 (5000 U/mL) and anti-CD16 mAbs (3 µg/mL) or with a combination of IL-2 (5000 U/mL) and anti-CD3/28 antibody (25 µL/mL) for 18 hours before they were co-cultured with either OSCSCs or autologous primary patient high-grade serous cancer (PBMCs: tumours; 41:1). After overnight co-culture, the supernatants were harvested to determine IFN-γ secretion using single ELISA (E). ****p<0.0001, ***p<0.001, **p=0.001–0.01, *p=0.01–0.05.

PBMCs of patients with cancer (figure 1A). PBMCs of both patients with cancer and healthy individuals exhibited very similar percentages of CD3+ T cells, although there was a slight decrease in CD3+CD4+ T cells and a slight increase in CD3+CD8+ T cells in PBMCs of patients with cancer (figure 1A). No significant differences in the percentage of CD19+B cells were seen in patients with ovarian cancer compared with healthy individuals (figure 1A).

Suppressed IFN-γ secretion and cytotoxicity against oral cancer stem cells as well as autologous tumours by PBMCs of patients with ovarian cancer

PBMCs of healthy individuals and patients with ovarian cancer were treated with IL-2, or a combination of IL-2 and anti-CD16 mAbs, or a combination of IL-2 and anti-CD3/28 mAbs, and a combination of IL-2 and probiotic bacteria sAJ4 before IFN-γ secretion levels were determined using ELISA. PBMCs of patients with cancer secreted significantly lower amounts of IFN-γ compared with healthy individuals (figure 1B). In healthy individuals but not in patients with cancer, increased IFN-γ secretion was observed when PBMCs were treated with IL-2 + anti-CD16 mAbs, IL-2 + anti-CD3/28 mAbs and IL-2 + sAJ4 compared with IL-2 alone-treated PBMCs (figure 1B). We next used untreated, IL-2 treated and IL-2 + anti-CD16 mAbs-treated PBMCs in a 4-hour chromium release assay to determine their cytotoxicity against OSCSCs, and also against autologous isolated ovarian cancer cells (figure 1C,D). PBMCs of patients with cancer mediated significantly lower cytotoxicity against both OSCSCs and autologous tumours (figure 1C). The level of NK cell-mediated cytotoxicity by 1% NK cells in PBMCs was determined using CD16+CD56+ percentages in PBMCs and was found to be lower in PBMCs of patients with cancer against both OSCSCs and autologous tumours (figure 1D).

We co-cultured IL-2, or IL-2 + anti-CD16 mAbs, and IL-2 + anti-CD3/28 mAbs-treated PBMCs with OSCSCs, and also with autologous ovarian cancer cells to determine the levels of IFN-γ secretion using ELISA (figure 1E). PBMCs of patients with cancer secreted significantly lower amounts of IFN-γ with or without tumour co-cultures compared with healthy individuals' PBMCs (figure 1E). A higher decrease in IFN-γ secretion was seen when patients' PBMCs were co-cultured with autologous tumors (figure 1E).

Recurrent ovarian tumours exhibit increased cell cycle activity alongside decreased levels of differentiation, immune cell infiltration and MHC-class I and II expression

To better understand the differences between paired chemo-naive and recurrent ovarian tumours, we applied gene set enrichment analysis (GSEA)—an approach that elucidates patterns within differentially expressed genes. Our study incorporated a data set of 18 longitudinal, paired samples from patients with chemo-naive and recurrent ovarian cancer.[32] The GSEA based on bulk RNA-seq data revealed decreased expression of differentiation- and immune-related gene sets, and increased expression of cell cycle and proliferation-related gene sets in recurrent tumours (figure 2A). In addition, GSEA demonstrated the significant downregulation of MHC-class I pathway genes in post-therapy recurrent ovarian cancer samples compared with their longitudinally paired chemo-naive counterparts (figure 2C, E, online supplementary figure S2). A similar significant pattern of downregulation was also observed in MHC-class II pathway genes (figure 2C,E and online supplemental figure S2). The enrichment levels of all listed gene set categories were statistically significant as shown in figure 2. GSEA waterfall and mountain plots further highlighted the downregulated genes from representative MHC-class I (figure 2B–D) and antigen processing gene sets (figure 2C,D and online supplemental figure S2).

Increased IFN-γ and TNF-α expression levels in sNK compared with pNK cells

Our results demonstrate that NK cells in patients with ovarian cancer are defective in numbers and function, providing a therapeutic opportunity to use off-the-shelf NK cell-based immunotherapeutic strategies. We previously demonstrated the crucial role of OCs and probiotic bacteria sAJ2 contributing to the generation of sNK cells and discussed sNK cells' characteristics.[26 27] In this study, we have further characterised the sNK cells at a single-cell level to understand their antitumour activity. Using single-cell RNA sequencing data, we demonstrate increased IFN-γ and TNF-α transcript levels in sNK cells compared with untreated and IL-2-treated pNK cells (figure 3A,B). We previously showed that despite not being completely identical in their Uniform Manifold Approximation and Projection localisation, the clusters of untreated pNK cells, IL-2-treated pNK cells and sNK cells were homogeneous, with a similar percentage of each cluster. We also found a significant increase in the secretion of IFN-γ and TNF-α from sNK cells compared with IL-2-treated pNK cells (see [13] and online supplemental figure S3). Thus, sNK cells can produce cytokines that differentiate tumour cells, resulting in the curtailment of tumour growth and an increase in MHC-class I.[17]

When the avidity of binding was determined between the NK cells and tumour cells over the force ramp from 0 to 1000 pN using z-Movi, sNK cells yielded stronger interactions with the cancer cells, thereby exhibiting much higher avidity. The per cent bound at 1000 pN is higher for sNK cells due to a greater portion of sNK cells having tight, force-resistant associations with the cancer cells than the primary IL-2-activated NK cells (figure 3C,D). We previously observed increased expression levels of activating receptors in sNK cells compared with pNK cells. In addition, increased expression of adhesion factor CD54 and decreased expression level of inhibitory receptor NKG2A were seen in sNK cells compared with

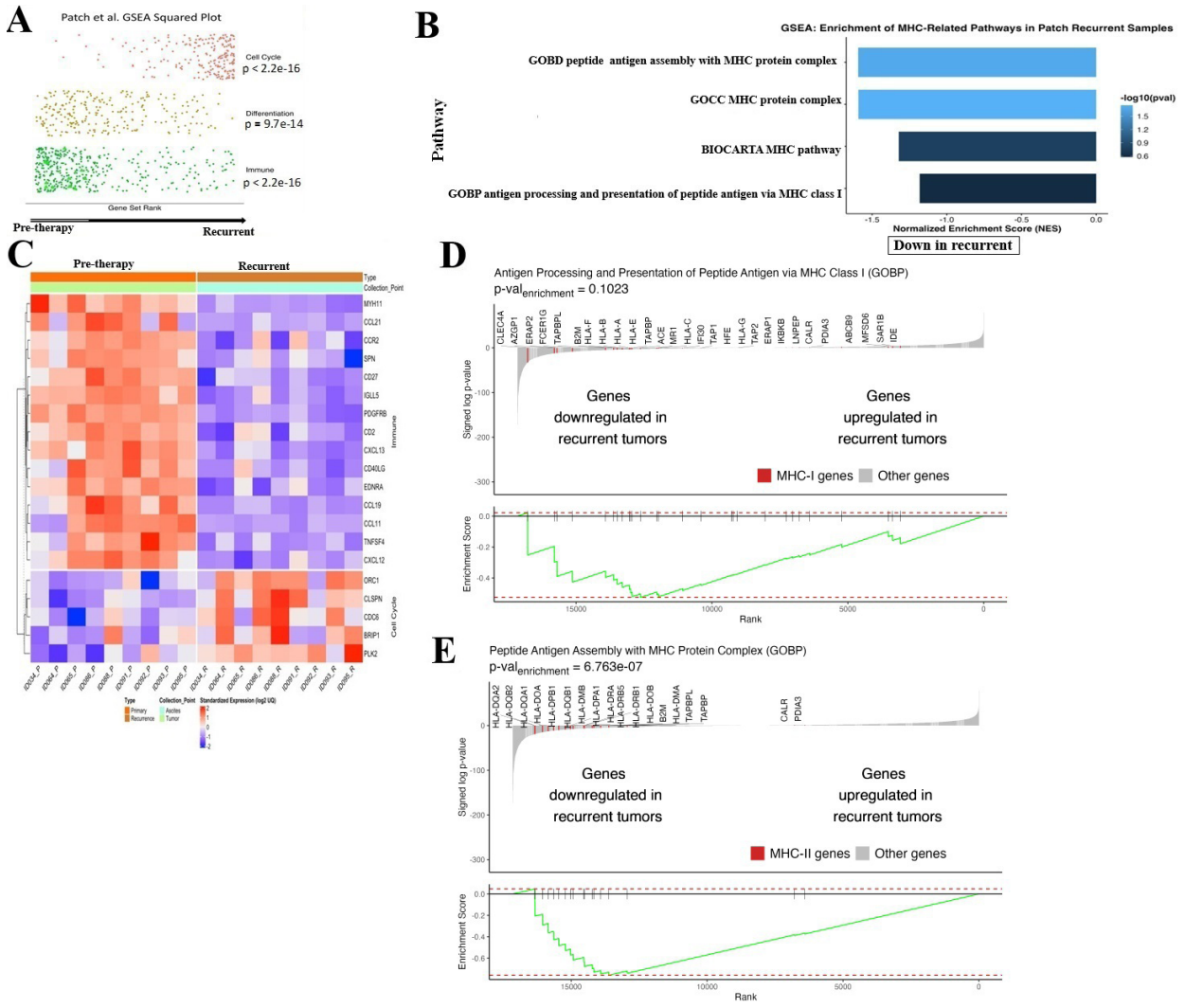


Figure 2 Differentially expressed gene sets between chemo-naïve and recurrent ovarian tumour samples. Gene set enrichment analysis (GSEA) based on differentially expressed genes between chemo-naive and recurrent ovarian tumour samples demonstrates that differentiation- and immune-related gene sets are significantly de-enriched in recurrent tumours (bright green triangles mark antigen processing gene sets from MSigDB, Kolmogorov-Smirnov (KS) enrichment p=0.014). A total of 18 longitudinal, paired chemo-naïve and samples of patients with recurrent ovarian cancer were used in this analysis.[32] Enrichments of all listed categories are significant (p<0.001) by KS test (A). GSEA enrichment of MHC and other related genes pathways in recurrent samples, coloured by log-transformed p-values (B). Hierarchically clustered heatmap showing the top 20 differentially expressed genes in recurrent versus primary samples, based on selected biological categories (C). GSEA demonstrates the downregulation of MHC-class I and MHC-class II and other related gene pathways in post-therapy recurrent ovarian cancer samples compared with their longitudinally paired chemo-naive samples. The GSEA waterfall plot (top panel) and GSEA mountain plot (lower plot) show the downregulated genes from a representative MHC-class I and MHC-class II and other related gene set. Enrichment p-value was determined from KS statistics (D, E).

pNK cells (manuscript in press). A summary of our previous studies[12 29–31 33–36] highlighting the enhanced characteristics of sNK cells and illustrating how these advanced traits make them more effective in targeting and eliminating tumor cells compared with pNK cells is shown in figure 3E.

sNK cells mediate significantly higher cytotoxicity against ovarian cancer cell lines OVCAR4 and OVCAR8 compared with primary IL-2-activated NK cells

Our previous studies demonstrated that CSCs or PDCSCs exhibit lower MHC-class I and are excellent targets for NK cell-mediated cytotoxicity, whereas well-differentiated tumours exhibit increased MHC-class I surface expression levels and are resistant to pNK cell-mediated cytotoxicity.[12 14 17 18 25 37] We found higher surface expression of MHC-class I in ovarian cancer cell lines OVCAR4 compared with OVCAR8, as reported in our previous manuscript[11] (online supplemental figure S4). This result suggested that OVCAR4 exhibits differentiated tumour phenotype, whereas OVCAR8 exhibits CSCs or PDCSC phenotype based on our previously established criteria.[11 25] We then used IL-2 treated pNK cells (NK+IL-2) and sNK cells as effectors against OVCAR4 and OVCAR8 in a

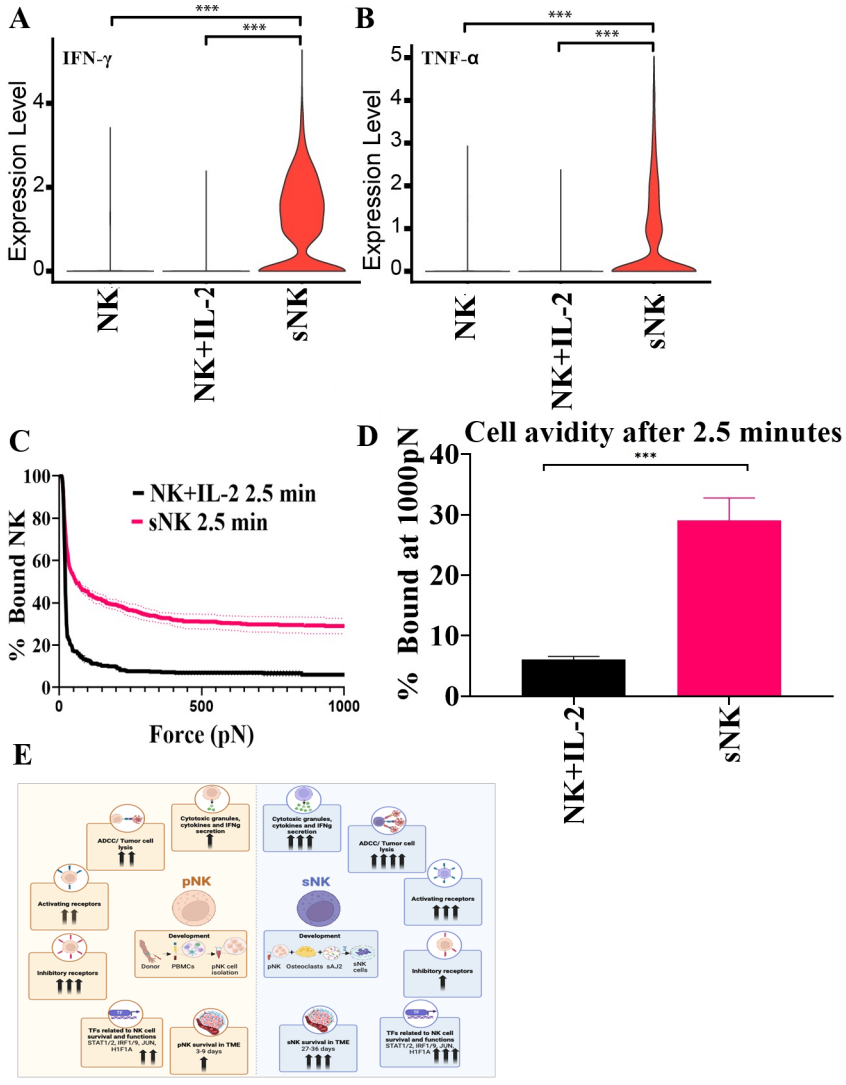


Figure 3 Osteoclast (OC)-expanded supercharged natural killer (sNK) cells exhibit higher cytokine expression levels compared with primary NK cells. OCs were generated as described in the 'Materials and methods' section. NK cells (1×10^6 cells/mL) from healthy individuals were treated with a combination of interleukin IL-2 (5000 U/mL) and anti-CD16 mAbs (3 µg/mL) overnight before they were cultured with OCs and probiotic bacteria sAJ2 at a ratio of 1:2:4 (OCs:NK:sAJ2). Primary NK cells were treated overnight with IL-2 (5000 U/mL). The expression level of interferon (IFN)-γ was determined in IL-2-treated primary NK cells and sNK cells at scRNA seq analysis (A). The expression level of tumour necrosis factor (TNF)-α was determined in IL-2-treated primary NK cells and sNK cells at scRNA seq analysis (B). The per cent of the indicated effector cell bound over the force ramp from 0 to 1000 pN is plotted in this graph (C). sNK cells had much higher binding to target cells in 500 and 1000 pN force (n=2). Therefore, exhibiting much higher avidity compared with those activated with IL-2 (D). The bar chart represents the per cent bound at a single point along the curve using the end point of the force curve, 1000 pN. The per cent bound at 1000 pN is higher for sNK cells due to a greater portion of sNK cells having tight, force-resistant associations with the cancer cells than the primary IL-2-activated NK cells (n=3). ***p<0.0001–0.001. The illustration highlights the features of sNK cells that contribute to their enhanced tumour-targeting capabilities compared with primary NK cells (E). ADCC, antibody-dependent cellular cytotoxicity; sAJ2, a combination of seven probiotic bacteria used for sNK proliferation and expansion; TF, transcription factor; TME, tumour microenvironment.

4-hour chromium release assay. IL-2-treated pNK cells mediated much lower cytotoxicity against OVCAR4 compared with OVCAR8 (online supplemental figure S4B,C), and fold increase of sNK cells versus IL-2 treated pNK cells was higher for both OVCAR4 and OVCAR8 (online supplemental figures S4D and S5).

In addition to the chromium release assay, which is a short-term killing assay (4 hours), we also used a long-term killing assay using eSight to determine sNK cells' cytotoxicity against OVCAR4 and OVCAR8. Our positive control cell lines were OSCSCs and well-differentiated oral squamous carcinoma cells (OSCCs) oral tumours were[14 29] previously been characterized and are used to determine the differentiated status of ovarian tumours in this paper (online supplemental figures S6 and S7A). OSCSCs and OSCCs (online supplemental figures S6 and S7A) and OVCAR8 and OVCAR4 (figure 4 and online supplemental figure

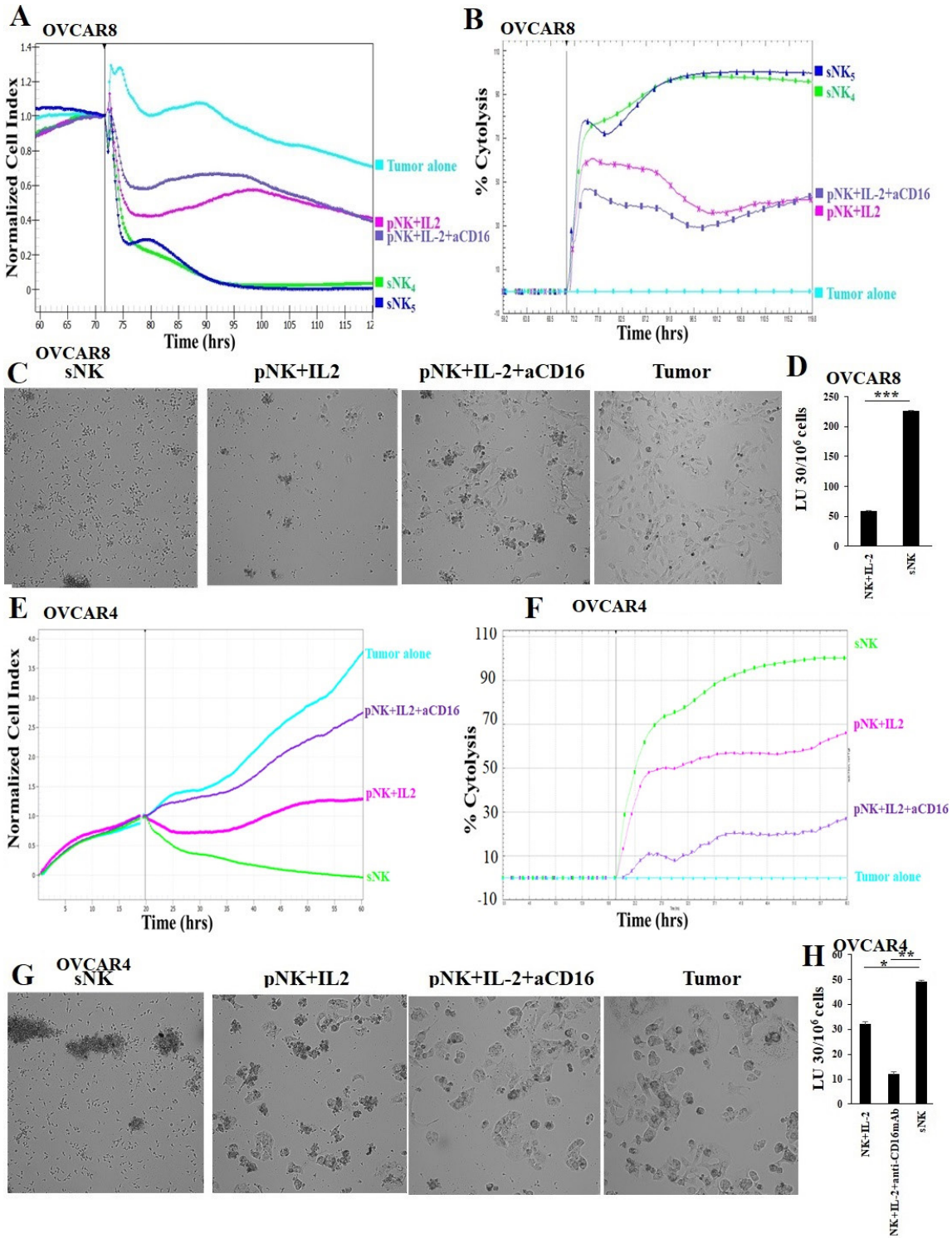


Figure 4 Unlike primary activated natural killer (NK) cells, supercharged NK (sNK) cells greatly lyse both OVCAR8, the poorly differentiated ovarian tumours, and well-differentiated OVCAR4 when assessed in a long-term killing assay using eSight OVCAR4 (A–C) and OVCAR8 (E–G) were cultured on eSight plates for 20–24 hours before the sNKs, primary NK cells activated with interleukin (IL)-2 (5000 U/mL) or with a combination of IL-2 (5000 U/mL) and anti-CD16 mAbs (3 µg/mL) were added at 2.5:1 E:T ratio, and co-cultures were continued to 55–65 hours (A, E). The graphs for normalised cell index and %cytolysis were assessed using the eSight RTCA and RTCA pro software (B, F). Microscopic images of tumour and NK cell interactions as shown in the figures were captured with eSight after 24 hours of incubation (scale: 0–200 µm) (C, G). NK cells are significantly smaller compared with tumour cells (C, G). OVCAR4 (D) and OVCAR8 (H) were treated as above, and ^{51}Cr release assay was conducted for 4 hours at different E:T ratios. The lytic units 30/10^6 cells were determined using the inverse number of peripheral blood-derived mononuclear cells (PBMCs) required to lyse 30% of tumours × 100. ***p<0.001, **p=0.001–0.01, *p=0.01–0.05.

S7B) were cultured on eSight plates for 20–24 hours before the sNKs or pNK cells activated with IL-2 or IL-2+anti-CD16 mAbs were added to the tumours and co-cultures were continued to 55–65 hours (Figure 4 and online supplemental figures S6 and S7). sNK cells cultured with tumour cells had the lowest cell index with the highest percentages of cytolysis of both PDCSC tumours OSCSCs (online supplemental file 3) and OVCAR8 (figure 4A,B and online supplemental figure S7B) compared with either IL-2-activated or both IL-2 and anti-CD16 mAbs-activated pNK cells (see online supplemental file 1). Microscopic images of tumour and NK cell interactions, as shown in online supplemental figures S6C and 4C, were captured with eSight after 48 hours of incubation. As seen in the microscopic analysis, sNK cells lysed all of the tumours; therefore, no residual tumour cells could be visualised (online supplemental figures S6C and 4C). Tumour alone had the highest seeding on the plates (online supplemental figures S6C and 4C), followed by the treatment of pNK cells with IL-2 and anti-CD16 mAbs which exhibited relatively higher survival of tumour cells compared with primary IL-2-activated NK cells which still had residual tumours, although the levels were less than either tumour alone or NK treated with IL-2 and anti-CD16 mAbs (online supplemental figures S6C and 4C). Similar findings were observed between the data obtained with eSight and ^{51}Cr release assay (online supplemental figures S6D and 4D). On the other hand, sNK cells were the only NK cells that effectively lysed the well-differentiated OSCCs (online supplemental figure S6E,F) and OVCAR4 (figure 4E,F) as evidenced in both normalised cell index (online supplemental figures S6E and 4E) or at the levels of cytolysis (online supplemental figures S6F and 4F), whereas IL-2-activated NK cells in comparison were much less capable of lysing these tumours. Therefore, either the tumours started growing in the presence of IL-2-activated NK cells or more tumours were visible in the co-cultures of IL-2-activated NK cells with differentiated tumours (online supplemental figures S6G and 4G). In all cases, sNK cells greatly lysed the tumour cells and no visible tumour could be visualised (online supplemental figures S6G and 4G). Finally, there was a clear correlation between the data obtained with eSight and ^{51}Cr release assay (online supplemental figures S4-7 and 4).

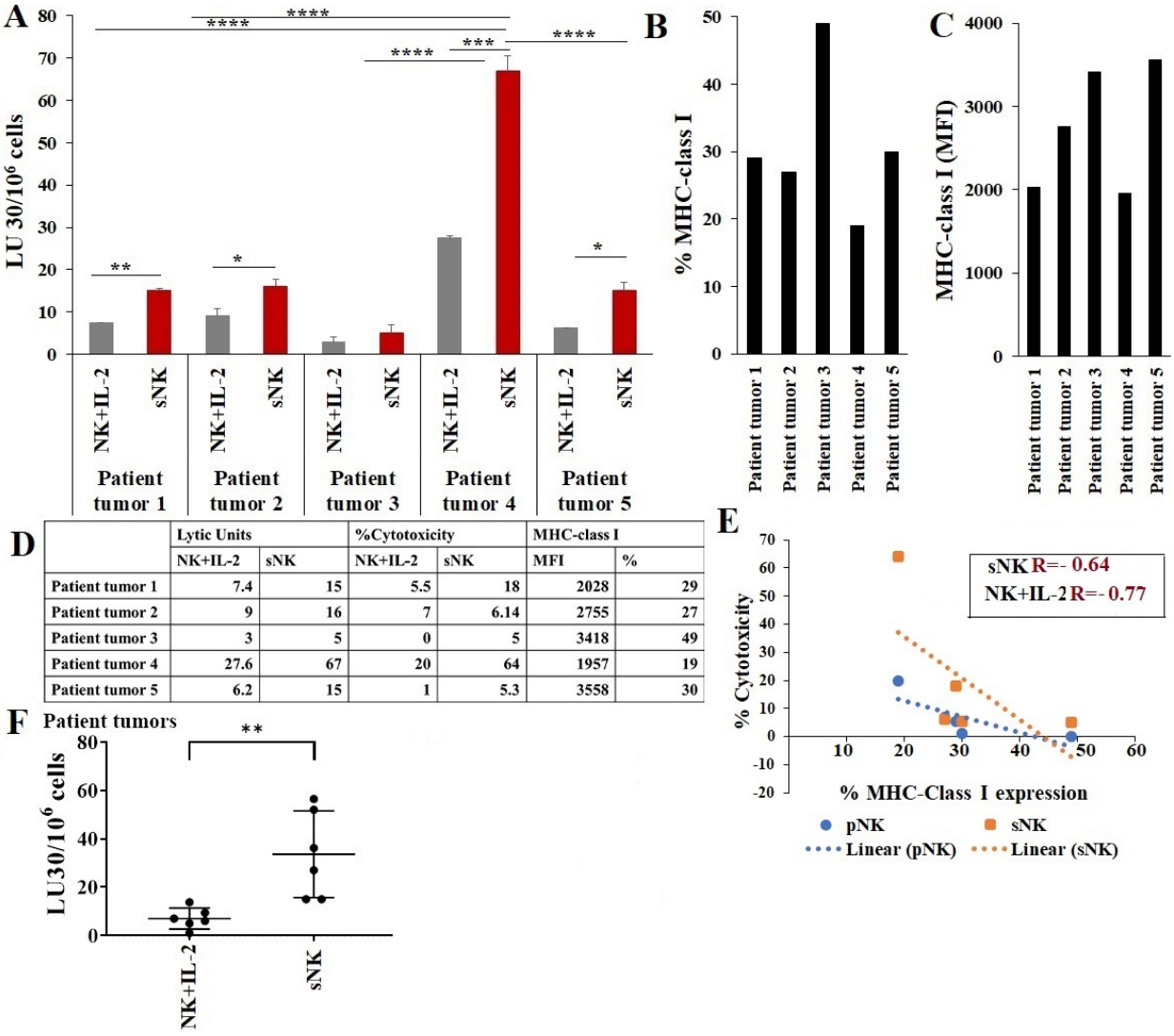
Characterisation of primary ovarian cancer tumour cells based on NK cell-mediated cytotoxicity and MHC-class I surface expression

We used primary IL-2-treated NK (pNK + IL-2) and sNK cells as effector cells in a 4-hour ^{51}Cr release assay against five different primary patient high-grade serous ovarian tumours and observed higher sNK cell-mediated cytotoxicity compared with IL-2-treated pNK cells (figure 5A,D,E,F and online supplemental figure S8). We observed a correlation between NK cell-mediated lysis and surface expression levels of MHC-class I on primary patient high-grade serous ovarian tumours (R=−0.77 (NK+IL-2); R=−0.64 (sNK)) (figure 5A–C,E). Tumours with lower surface expression levels of MHC-class I were excellent targets of both primary IL-2-treated NK cells and sNK cell-mediated cytotoxicity; however, sNKs induced higher tumour lysis compared with primary IL-2 NK cells (figure 5A–E and online supplemental figure S9).

DISCUSSION

We previously showed that NK cells are defective in patients with[38] cancer.[12 26 39 40] Similarly, patients with ovarian cancer have a significant deficiency in the number of PBMCs, in particular NK cells. Both the numbers and functions of NK cells are compromised in these patients. Due to this deficiency, any arising PDCSCs that have lost or downmodulated MHC-class I and/or II may not be eliminated since primary activated NK cells are well known to target aggressive PDCSC tumours,[11 41] and thus, the patient will be predisposed to recurrence. Our global analysis of genes points to a decrease in MHC-class I and II genes with recurrent disease, further supporting the abovementioned hypothesis (figure 2). Moreover, recurrent ovarian tumours are shown to demonstrate increased cell cycle markers, decreased differentiation and decreased immune cell infiltration (figure 2A). Therefore, due to a severe decrease in NK cells in patients, such aggressive tumours will not be controlled, and thus will be susceptible to recurrence. Therefore, based on the data presented in this article, cell therapy using sNK cells should be able to control disease progression of ovarian tumours. SNK cells are equipped to not only lyse PDCSCs significantly but are also capable of targeting differentiated tumours with the high expression of MHC-class I, a promising and unique attribute of sNK cells which were not observed in primary activated NK cells normally inhibited via MHC-class I expression on target cells. Compared with many other NK cell treatments, such as IL-2, IL-2 and anti-CD16, IL-2 and anti-CD16 and sAJ2,[13] or OSCSC and K562 expanded NK cells were not able to survive and expand, although they were functionally activated for a short time.[13 26] Similarly, activation with a combination of NK-activating cytokines was not able to expand NK cells.[13] More recently, NK cells stimulated with IL-12, IL-15 and IL-18, termed cytokine-induced memory-like NK cells, were shown to have promising results in clinical trials.[42 43] However, we have not observed a significant expansion of NK cells using such a combination of cytokines. Expansion of NK cells is important for clinical application since adequate number of NK cells are necessary to lyse all the tumours.

There are many advantages to using off-the-shelf sNK cells for tumour immunotherapy. First, unlike CAR-T therapy, they will be readily available to use in therapy and one does not have to wait to generate them from the patient's cells. Second, there have not been any reports of NK cell therapy causing graft versus host disease



	Lytic Units		%Cytotoxicity		MHC-class I	
	NK+IL-2	sNK	NK+IL-2	sNK	MFI	%
Patient tumor 1	7.4	15	5.5	18	2028	29
Patient tumor 2	9	16	7	6.14	2755	27
Patient tumor 3	3	5	0	5	3418	49
Patient tumor 4	27.6	67	20	64	1957	19
Patient tumor 5	6.2	15	1	5.3	3558	30

Figure 5 Characterisation of primary patient high-grade serous ovarian cancer cells based on natural killer (NK) cell-mediated cytotoxicity and MHC-class I surface expression. Primary overnight interleukin IL-2 (5000 U/mL)-treated NK cells and day 15 supercharged NK (sNK) cells of healthy individuals were used as effectors in a standard 4-hour ^{51}Cr release assay against primary patient high-grade serous ovarian cancer (A, D, E, F (n=6)). The lytic units 30/10^6 cells were determined using the inverse number of NK cells required to lyse 30% of tumours × 100 (A, D, F (n=6)), and percentage cytotoxicity at 2.5:1; effector:target (E:T) is shown in figures (D, E). The surface expression of MHC-class I on primary patient high-grade serous ovarian cancer was determined using flow cytometry. IgG isotype control was used to assess non-specific binding (B, C, E). ****p<0.0001, ***p<0.001, **p=0.001–0.01, *p=0.01–0.05.

and/or cytokine storm syndrome.[44 45] These important advantages make sNK cells an ideal cell to use in tumour immunotherapy.

We previously showed that primary activated NK cells were responsible for lysing and differentiating PDCSCs, whereas well-differentiated tumours were not or minimally lysed by pNK cells.[14 37] By using four specific biomarkers of CD44, MHC-class I, CD54 and PD-L1 we were able to distinguish between PDCSCs and well-differentiated tumours in several tumour models, including pancreatic, oral, ovarian and hepatic tumours, and study their interaction with the primary activated NK cells and compare with sNK cell function[11–14 26 29 31] (manuscript in preparation).There was a correlation between the differentiation state of ovarian tumours and their susceptibility to pNK cell-mediated cytotoxicity.[11] In addition, using primary activated NK cells against several ovarian tumours at different stages of differentiation, we could distinguish between PDCSCs and well-differentiated tumours.[11] Thus, based on the susceptibility to pNK cell-mediated cytotoxicity and the four biomarkers, several ovarian tumour cells mentioned above,[11] we selected two ovarian tumour lines of OVCAR8 with PDCSC phenotype and OVCAR4 for the well-differentiated phenotype to further understand their dynamic differences and susceptibility to primary activated NK cells and sNK cells. Our results indicated that, similar to other tumour models, pNK cells were able to lyse OVCAR8 PDCSCs and much less OVCAR4 or NK-supernatant differentiated OVCAR4, whereas sNK cells not only had much higher levels of

cytotoxicity than pNK cells against OVCAR8 PDCSCs, but they were also able to target OVCAR4 or NK-supernatant differentiated OVCAR4 much more than their primary activated NK cells (figure 4 and online supplemental figures S4 and S5).

In this article, we present the significant attributes of sNK cells armed with the ability to kill ovarian tumours irrespective of the levels of MHC-class I expression or the stage of differentiation of the tumours. These attributes of sNK cells are very different from the conventional primary activated NK cells as increased MHC-class I on differentiated tumours deliver an inhibitory signal to pNK cells, resulting in suppressed NK cell function against tumours with increased MHC-class I expression.[11] Since sNK cells are laboratory-generated, at the moment, it is not clear whether these cells are laboratory-created or such a subpopulation of NK cells exists either in the blood or tissues of humans. Indeed, we have not observed such cell populations either in patients or obtained with other activators of NK cells in the absence of OCs as feeder cells. Other NK cell activation methods tested, such as the use of tumour cells as feeder cells, treatment with the combination of multiple cytokines or the use of engineered tumours to express 4IBBL and IL-21, do not increase cytotoxicity of primary activated NK cells against differentiated tumours.[46] Considering that OCs are usually found in the bone microenvironment, it is possible that these cells are not in the periphery and thus we may not have such subpopulation of NK cells in the periphery. Alternatively, there may be such subpopulation of NK cells in patients with cancer that have not yet been identified or characterised. In addition, in the bone marrow microenvironment, the majority of NK cells are in an immature state, and, therefore, may not have the capability to give rise to sNK cells until they mature and exist towards the periphery. sNK cells, therefore, have the characteristics of both classical pNK cells and T cells since they can effectively target cells that have lost MHC-class I and can also target tumours with high MHC-class I expression levels, respectively.

In the sNK engineering process, before exposure to OCs, the NK cells are treated with IL-2 and anti-CD16 mAbs in which NK cells cease to kill tumours but they are programmed to secrete cytokines, a concept coined as split anergy.[18 21 26] Indeed, these are the cells that give rise to sNK cells after culture with OCs. In addition, IL-2+ anti-CD16 mAbs-activated NK cells also express higher levels of 4IBB and 4IBBL; therefore, it is possible that in the presence of OCs in which higher expressions of NKG2DL and cytokines such as IL-15, IL-12 and IL-18 are present, NK cells greatly expand due to the increase in survival, activation and secretion of key cytokines such as IFN-γ and TNF-α which are known to expand NK cells.[13 26] Future work should focus on identifying endogenous sNK-like cells in humans if such cells exist.

When the effect of sNK was determined in a long-term assay using eSight, important differences between primary activated NK cells and those of sNK cells were observed. In all different assessments, sNK cells had a profound ability to lyse and prevent tumour growth (figure 4, online supplemental figure S6 and online supplemental videos). Particularly when sNK cells were cultured in the presence of well-differentiated oral OSCCs and ovarian OVCAR4 tumours, the ability of sNK cells to lyse such tumours was superior to primary activated NK cells (figure 4, online supplemental figures S6 and S7 and online supplemental videos). Indeed, sNK cells lysed these tumours and were responsible for the growth suppression of these tumours in a long-term assessment. However, primary activated NK cells, even though they initially had some suppressive activity, were not able to maintain suppression of tumours, and the tumour growth started picking up after the initial decrease (figure 4, online supplemental figures S6 and S7 and online supplemental videos). Unlike IL-2-activated pNK cells, the addition of sNK cells to the tumour cells induced rapid lysis of tumour cells and subsequent aggregation of the dead tumours at the end of the incubation period (online supplemental videos).

Our recent findings have demonstrated that sNK cells persists and retained their function in the tumor microenvironment, unlike IL-2-activated pNK cells (manuscript in press).[11 31] In addition, in our transcriptomic and single-cell RNAseq analysis, sNK cells demonstrated decreased gene expression of NKG2A and increased expression of activating receptors (manuscript in press), likely providing the rationale for the decreased inhibition through the inhibitory MHC-class I ligands. Therefore, sNK cells were uniquely poised and equipped to lyse the PDCSCs as well as well-differentiated tumours at a much higher rate than primary activated NK cells, and the extent of differences in killing well-differentiated tumours was much higher than IL-2-activated NK cells[31] (figure 4 and online supplemental videos).

We showed previously and, in this article, that sNK cells secrete higher levels of IFN-γ and TNF-α, and the combination of these two cytokines drives differentiation of tumour cells, rendering them more resistant to primary activated NK cells, but not to sNK cells[13] (figure 3). Addition of antibodies to both IFN-γ and TNF-α completely abrogated NK supernatant-mediated differentiation of tumour cells, and tumours remained susceptible to pNK cell-mediated cytotoxicity.[11 17] Similarly, the differentiation of many ovarian tumours, with the exception of OVCAR8 either by NK cells' supernatants or recombinant IFN-γ and TNF-α, rendered them resistant to pNK cell-mediated cytotoxicity but not to sNK cells (data not shown). Indeed, when cytotoxicity was assessed by long-term eSight assay (ie, 40 hours), pNK cells were able to initially lyse OVCAR4 tumours, but the suppression of growth did not last since the tumour continued to grow in the presence of activated pNK cells, whereas sNK cells completely eliminated these tumours and suppression continued until the assay was terminated (figure 4). These differences were not seen in OVCAR8 since primary IL-2-activated NK cells eliminated these tumours, although sNK cells were still more potent compared with primary

activated NK cells in the killing of tumour cells (figure 4). This is due to the resistance of sNK cells to tumour-mediated cell death since sNK cells were found to exhibit the least amount or no cell death due to increased anti-apoptotic genes, whereas almost half of the primary activated NK cells had undergone cell death in the presence of the tumours in our preliminary studies. In accordance, more viable tumours were seen in the presence of IL-2-activated NK cells in OVCAR4, whereas more OVCAR8 tumours were eliminated by primary IL-2-activated NK cells, although sNK cells still mediated the highest lysis of OVCAR8 tumour cells. Indeed, in all tumour models tested sNK cells had a much higher ability to lyse tumour cells than the primary IL-2-activated NK cells. sNK cells exhibited higher binding avidity as determined by z-Movi compared with IL-2-activated pNK cells, indicating their increased potential to form conjugates with the tumour cells. Increased avidity of immune cells has been correlated with increased effectiveness of NK and T cells in lysing tumours in vivo.[47]

Next, we planned in vivo studies to test the ability of sNK cells to target and eradicate ovarian tumours in hu-BLT mice. Based on our previous in vivo studies with pancreatic,[12 36] oral[29 36] and melanoma[36] (manuscript in preparation), we believe sNK cells will function similarly in targeting ovarian tumours in vivo.

There is a significant correlation between the ability of NK cells to lyse aggressive primary patient high-grade serous tumours and the MHC-class I expression. The lower the levels of MHC-class I on ovarian tumours, the better targets they are for NK cells (figure 5). These tumours will not be targeted by CD8+T cells since they do not express adequate levels of MHC-class I and/or MHC-class II. In addition, they are highly resistant to chemotherapeutic and radiotherapeutic strategies.[13 37] Differentiation of such aggressive tumours with NK cell supernatants is capable of inducing increased tumour susceptibility to chemotherapeutic and radiotherapeutic strategies.[37] Moreover, an increase in MHC-class I will activate CD8+T cells; thus, a combination of sNK cells with anti-PD1 antibody treatment should exhibit a synergistic effect in augmentation of immune activation as previously reported in hu-BLT mice.[13] Interestingly, OVCAR8 PDCSCs have lost the ability to upregulate MHC-class I when differentiated by NK supernatants. This is opposite to what was found with several other tumours, in which differentiation by NK supernatants, in general, was able to increase MHC-class I.[17 37]

Based on the data obtained in our studies, in recurrent ovarian tumours, a subpopulation of tumours with loss of MHC-class I may survive, potentially due to the defective nature of NK cells in cancer patients, and the use of sNK cells in immunotherapy should provide an effective strategy in targeting such tumours to prevent recurrences.

Limitations of the study

Although we have used more than 400 hu-BLT mice to study the function of sNK cells in vivo in other tumour models,[12 13 29 33] we have yet to perform such studies using ovarian cancer. However, our preliminary in vivo experiments using OVCAR8 as a tumour model indicated that the delivery of sNK cells to the hu-BLT mice increased NK cell- and T cell-mediated secretion of IFN-γ in PBMCs and splenocytes (data not shown). We are in the process of completing these experiments.

We are also in the process of studying the underlying mechanisms of sNK cell-mediated lysis of differentiated tumours, focusing on the levels and functions of inhibitory receptors.

We believe that the immune cell activation initially seen in mice models and humans is due to sNK cell function, but later is because of restoration of autologous NK cell function, and therefore, we are planning further experiments to demonstrate this hypothesis. Indeed, a single infusion of sNK cells in a chronic lymphocytic leukemia (CLL) and a patient with glioma, and in an individual suffering from deep vein thrombosis, augmented percentages of NK cells and increased NK cell function up until 120 days of testing (manuscript in review), and therefore, such an increase was found to be due to the expansion and activation of autologous NK cells after sNK therapy. Indeed, sNK cells at the best in the in vitro culture conditions could only survive up until 30–60 days.

Finally, even though MHC-class I is a well-known modulator of the NK cell function, it is possible that other receptors such as MHC-class II and IB receptors which are also downmodulated in ovarian tumours (figure 2) could influence the function of NK cells. Overall, even though our studies have addressed many important questions in the field of NK cells, there are many more questions to explore in the future in order to understand the physiological role of these cells in humans. Indeed, killing is not their only function.

MATERIALS AND METHODS

Cell lines, reagents and antibodies

OSCSCs were isolated from patients with tongue tumours at UCLA.[14 17 18 48] OSCSCs were grown in RPMI 1640 medium (Gibco, Thermo Fisher, California, USA) with 10% FBS (Gemini Bio-Products, California, USA), 2% antibiotic/antimycotic solution (Cytiva, Massachusetts, USA), 1.4% sodium pyruvate (Gibco, California, USA), 1.4% MEM non-essential amino acids (Gibco, California, USA) and 0.15% sodium bicarbonate. OVCAR8 and OVCAR4 cells were obtained from the National Institute of Health (NIH) Division of Cancer Treatment and Diagnosis Tumor Repository. Human PBMCs were maintained in RPMI 1640 (Life Technologies, California, USA) with 10% FBS (Gemini Bio-Products, California, USA). Recombinant IL-2 was obtained from NIH-BRB. Antibodies that were used for flow cytometry—IgG2, CD16, CD56, CD3, CD19, CD14, CD4, CD8 and MHC-class I—were purchased from BioLegend (San Diego, California, USA). Human NK cells and monocyte purification kits

were obtained from Stem Cell Technologies (Vancouver, BC, Canada).

Sample of patients with primary ovarian cancer

Effusion samples (ascites, pleural fluid) or solid tumour and peripheral blood were obtained from patients diagnosed with high-grade serous ovarian cancer after written informed consent was obtained. Associated clinical information was taken from medical charts. To confirm the presence of tumour cells in effusion samples, a review of clinical pathology reports from the day of the procedure was performed. If same-day pathology reports were not available, a report within 1 month of collection and from the same source was reviewed. Patients' characterisation is shown in online supplemental tables S1 and S2.

Determination of platinum sensitivity

Chemo-naive samples were collected from patients with a new diagnosis of high-grade serous ovarian cancer and who did not receive any chemotherapy treatments. To determine the platinum sensitivity of chemo-naive samples, disease recurrence was prospectively reviewed. Specimens were determined to be platinum-sensitive if the patient either did not experience disease relapse or if the disease relapsed ≥6 months after the final platinum-based chemotherapy infusion was administered. Specimens from patients who experienced disease relapse <6 months after the final platinum-based chemotherapy infusion were determined to be platinum-resistant.

Recurrent specimens were collected from patients who received primary treatment for high-grade serous ovarian cancer and subsequently experienced a relapse of the disease. To determine the platinum sensitivity of recurrent specimens, a retrospective review of treatment history was performed. Recurrent specimens were categorised as platinum-sensitive if at the time of collection disease relapse had occurred ≥6 months after the final platinum-based chemotherapy treatment. Specimens were determined to be platinum-resistant if at the time of collection the patient was observed to have disease progression while receiving platinum-based chemotherapy or if the patient had disease recurrence <6 months after their last platinum-based chemotherapy infusion.

Determination of treatment status

Chemo-naive samples are those collected from patients at the time of diagnosis and before the initiation of chemotherapy. Post-neoadjuvant samples are those collected from patients after 3–4 cycles of chemotherapy and at the time of interval cytoreductive surgery. Recurrent samples are those collected from patients who have experienced a relapse of disease.

Processing the samples of patients with primary ovarian cancer

Fresh tumour or effusion samples including ascites and pleural fluid were obtained and immediately processed at the lab. Cells were harvested by centrifugation, washed with dulbecco's modified eagle medium (DMEM), and treated with, red blood cell lysis buffer for samples containing blood. The harvested cells were either used fresh or aliquoted for cryopreservation in FBS/10% DMSO. Cryopreserved cells were kept in liquid nitrogen for storage and future use. Peripheral blood was collected in red-top and green-top vacutainer tubes.

Bacteria sonication

Gram-positive probiotic bacteria AJ2 (*Streptococcus thermophiles, Bifidobacterium longum, Bifidobacterium breve, Bifidobacterium infantis, Lactobacillus acidophilus, Lactobacillus plantarum, Lactobacillus casei* and *Lactobacillus bulgaricus*) and AJ4 (*S. thermophiles, L. acidophilus, L. plantarum* and *Lactobacillus paracasei*) were weighed and resuspended in RPMI 1640 containing 10% FBS at a concentration of 10 mg/1 mL. The bacteria were thoroughly vortexed, then sonicated on ice for 15 s, and set at a 60% amplitude. The sonicated samples were then incubated for 30 s on ice. After every five pulses, a sample was taken to observe under the microscope until at least 80% of cell walls were lysed. It was determined that approximately 20 rounds of sonication/incubation on ice were conducted to achieve complete sonication. Finally, the sonicated samples (sAJ4 and sAJ2) were aliquoted and stored in a −80°C freezer.

Purification of NK cells and monocytes from the peripheral blood

Peripheral blood was separated using Ficoll-Hypaque centrifugation, after which the white, cloudy layer, containing PBMCs was harvested. NK cells and monocytes were negatively selected from PBMCs using the EasySep Human NK cell enrichment and EasySep Human Monocytes enrichment kits, respectively. To assess the purity of NK cells and monocytes, we stained them with anti-CD45 and anti-CD3/CD16/CD56 antibodies for NK cells, and anti-CD45 and anti-CD14 antibodies for monocytes. We then performed flow cytometric analysis to quantify the percentage of positive cells in each population. Samples showing >95% purity were used in the study (online supplemental figure S1).

Generation of sNK cells

To generate OCs, monocytes were cultured in alpha-MEM media supplemented with M-CSF (25 ng/mL) for 21 days and RANKL (25 ng/mL) from day 6 to 21 days. The media were replenished every 3 days. For NK cell expansion, purified NK cells were activated with rh-IL-2 (5000 U/mL) and anti-CD16 mAb (3 µg/mL) for 18–20 hours before they were co-cultured with OCs and sAJ2 (OCs:NK:sAJ2; 1:2:4) in RPMI 1640 medium containing 10% human serum AB (Gemini Bio-Products, California, USA). The media were refreshed every 3 days with RPMI complete medium containing rh-IL-2 (5000 U/mL). The sNK cells were used on day 15 for cytotoxicity assay.

eSight

xCELLigence RTCA eSight (Agilent, USA) was purchased, and cell behaviour and cell function were studied using real-time biosensor impedance-based and image-based

measurements. The impedance-based xCELLigence technology uses proprietary microplates (E-Plates View 96) embedded with gold biosensors at the bottom of each well, which serve to non-invasively quantify cell behaviour. Over the course of an experiment, the biosensors monitor cell metrics such as proliferation, adhesion strength, changes in morphology, migration and differentiation. On day 1, 50 µL of the respective media was added to each well, and the machine was run once to measure the background. Subsequently, 5 * 10^3 (OVCAR8 and OVCAR4) and 1 * 10^4 (OSCSCs and OSCCs) target cells were seeded per well, and the machine was run overnight for adhesion. The impedance of each well was monitored every 15 min, and the images of the cells were acquired every hour. After incubating for 18–24 hours, different concentrations of effector cells were added to each well, with a twofold dilution for each target cell type. To perform the serial dilution, we used different E:T ratios for different cell types. For OVCAR8 and OVCAR4, we started with an E:T ratio of 5:1, using 2.5 * 10^4 effector cells, and then halved the ratio and the number of effector cells for each subsequent dilution, until we reached an E:T ratio of 0.625:1, using 3.125 * 10^3 effector cells. For OSCSCs and OSCCs, we followed the same procedure, but with an initial E:T ratio of 2.5:1, using 2.5 * 10^4 effector cells. Impedance readings were recorded at 15 min intervals, and images were captured at 60 min intervals for a period of 48–72 hours.

z-Movi

The workflow of z-Movi starts with the incubation of target cells in the z-Movi chip for 2 hours. Then, the effector cells can be flushed into the z-Movi chip and incubated for 5–10 min before starting the analysis. The intercellular binding activities can be monitored in real time on the application of an acoustic force ramp. Effector cells will detach from the monolayer in a force-dependent manner and accumulate at the acoustic node. Detaching of the fluorescently labelled effector cells can be followed from the top view of the flow channel.

Single-cell RNA sequencing

Single-cell RNA sequencing was performed using a 10X Chromium machine. Single-cell cDNA libraries were prepared using the 10X Chromium Single-Cell 3′ Reagent kit v3 and sequenced via Illumina Novaseq 6000 (Illumina) to a depth of around 30 000 reads per cell. Raw data from each sample were demultiplexed and aligned to a custom reference genome (GRCh38), and UMI counts were quantified using 10X Genomics CellRanger software (V.3.0.0) with default parameters. Single-cell clustering and cell cycle scoring were performed using the Seurat package (V.3.0). The RNAseq data presented in this study are deposited in the NCBI GEO repository, accession number GSE226160, https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE226160).[13]

Surface staining analysis

Staining was performed by labelling the cells with antibodies as described previously.[49–51] Flow cytometric analysis was performed using the Attune NxT flow cytometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA), and FlowJo V.10.4 (BD, Oregon, USA) was used for analysis. Beckman Coulter Epics XL cytometer (Brea, California, USA) was used, and results were analysed in the FlowJo vX software (Ashland, Oregon, USA).

ELISAs

Single ELISAs were performed as previously described.[51] To analyse and obtain the cytokine and chemokine concentration, a standard curve was generated by either two- or threefold dilutions of recombinant cytokines provided by the manufacturer.

^{51}Cr release cytotoxicity assay

The ^{51}Cr release cytotoxicity assay was performed as previously described.[52] Briefly, different ratios of PBMCs or NK cells and ^{51}Cr–labelled OSCSCs or primary patient high-grade serous ovarian cancers were incubated for 4 hours. Then, the supernatants were harvested from each sample, and the released radioactivity was counted using the gamma counter. The percentage-specific cytotoxicity was calculated as follows:

$$\%\text{cytotoxicity} = \frac{\text{Experimental cpm} - \text{spontaneous cpm}}{\text{Total cpm} - \text{spontaneous cpm}}$$

LU $30/10^6$ is calculated by using the inverse of the number of PBMCs or NK cells needed to lyse 30% of tumours×100.

Gene set enrichment plot

Paired-sample DESeq2 analysis[53] was performed on 18 samples of patients with paired ovarian tumour [32] to identify the genes with differential expression between chemo-naive samples and samples of patients with recurrent ovarian tumour. The list of these differentially expressed genes was evaluated for the enrichment of a representative MSigDB MHC-class I antigen processing gene set, within either chemo-naive or recurrent samples. Enrichment visualisation consisted of a waterfall plot denoting the gene rank when sorted by signed log p-value (positive (negative) for up- (down-) regulation), a mountain plot indicating enrichment score and a ladder plot.

GSEA-squared

GSEA[54 55] was performed on the ranked list of differentially expressed genes between chemo-naive and recurrent ovarian tumor samples using the MSigDB C2CP, C5GO and Hallmark gene sets.[56 57] GSEA-squared enrichment analysis was performed as previously described in ref.[58] table 1 using the following categories and associated keywords:

Gene set names containing the term ‘MHC’ and excluding the term ‘CLASS_II’ are denoted as MHC-class

Table 1 [58]

Category	Keywords
Cell cycle	CELL_CYCLE, MITOTIC, DNA_REPLICATION, CHROMOSOME_SEGREGATION, SPINDLE, CELL_DIVISION, REPLICATION, E2F, STRAND, G2
Differentiation	DIFFERENTIATION
Immune	INFLAM, IMMUN, INTERLEUKIN, LEUKOCYTE, TNF, MHC, CYTOKINE, CHEMOKINE, ANTIGEN, LYMPH

I-related and are highlighted in the plot with bright green triangles.

Statistical analyses

An unpaired or paired, two-tailed Student's t-test was performed for experiments with two groups. One-way analysis of variance with a Bonferroni post-test was used to compare different groups for experiments with more than two groups. Duplicate or triplicate samples were used for assessment. For gene sets, statistical analysis was performed in the R statistical environment (V.4.2.1) using the fgsea (V.1.25.1) and stats (V.4.2.1) packages. Visualisations were generated in the R statistical environment (V.4.2.1) using the ggplot2 (V.3.4.2) and fgsea (V.1.25.1) packages. The following symbols represent the levels of statistical significance within each analysis: ****p<0.0001, ***p<0.001, **p=0.001–0.01 and *p=0.01–0.05.

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Contributors SH- Y, P- CC, KK, YJ, TS, GAD, NAM and AM performed the experiments and data collection. FE and YJL performed analysis. KK, TS, and FE prepared figures. KK performed statistical analysis and prepared manuscript. SH-Y, TS, FE, GAD, AO, SMK, TGG, SM and AJ reviewed and edited the manuscript. SMK, TGG, SM and AJ supervised the project. SMK, TS, GAD, TGG, SM and AJ received funding for project. Prism GraphPad was used to generate graphs and statistical analysis.

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Patient and public involvement Patients and/or the public were not involved in the design, or conduct, or reporting, or dissemination plans of this research.

Patient consent for publication Not applicable.

Ethics approval Protocols allowing the collection and use of human specimens for this study were approved by the UCLA Office of the Human Research Protection Program (IRB# 10-0727 and IRB# 20-1659). Written informed consents, approved by the UCLA Institutional Review Board (IRB#11-000781 and #10-0727), were obtained from healthy individuals and patients with ovarian cancer, and all procedures were approved by the UCLA-IRB.

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Data availability statement All data relevant to the study are included in the article or uploaded as supplementary information.

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REFERENCES

1 Siegel RL, Kratzer TB, Giaquinto AN, *et al*. Cancer statistics, 2025. *CA Cancer J Clin* 2025;75:10–45.
2 Kurnit KC, Fleming GF, Lengyel E. Updates and New Options in Advanced Epithelial Ovarian Cancer Treatment. *Obstet Gynecol* 2021;137:108–21.
3 Roett MA, Evans P. Ovarian cancer: an overview. *Am Fam Physician* 2009;80:609–16.
4 Torre LA, Trabert B, DeSantis CE, *et al*. Ovarian cancer statistics, 2018. *CA Cancer J Clin* 2018;68:284–96.
5 Latifi A, Abubaker K, Castrechini N, *et al*. Cisplatin treatment of primary and metastatic epithelial ovarian carcinomas generates residual cells with mesenchymal stem cell-like profile. *J Cell Biochem* 2011;112:2850–64.
6 Thakur B, Ray P. Cisplatin triggers cancer stem cell enrichment in platinum-resistant cells through NF-κB-TNFα-PIK3CA loop. *J Exp Clin Cancer Res* 2017;36:164.
7 Steg AD, Bevis KS, Katre AA, *et al*. Stem cell pathways contribute to clinical chemoresistance in ovarian cancer. *Clin Cancer Res* 2012;18:869–81.
8 Zhang S, Balch C, Chan MW, *et al*. Identification and characterization of ovarian cancer-initiating cells from primary human tumors. *Cancer Res* 2008;68:4311–20.

9 Ryschich E, Nötzel T, Hinz U, *et al*. Control of T-cell-mediated immune response by HLA class I in human pancreatic carcinoma. *Clin Cancer Res* 2005;11:498–504.
10 Pandha H, Rigg A, John J, *et al*. Loss of expression of antigen-presenting molecules in human pancreatic cancer and pancreatic cancer cell lines. *Clin Exp Immunol* 2007;148:127–35.
11 Chovatiya N, Kaur K, Huerta-Yepez S, *et al*. Inability of ovarian cancers to upregulate their MHC-class I surface expression marks their aggressiveness and increased susceptibility to NK cell-mediated cytotoxicity. *Cancer Immunol Immunother* 2022;71:2929–41.
12 Kaur K, Kozlowska AK, Topchyan P, *et al*. Probiotic-Treated Super-Charged NK Cells Efficiently Clear Poorly Differentiated Pancreatic Tumors in Hu-BLT Mice. *Cancers (Basel)* 2019;12:63.
13 Kaur K, Chen PC, Ko MW, *et al*. Sequential therapy with supercharged NK cells with either chemotherapy drug cisplatin or anti-PD-1 antibody decreases the tumor size and significantly enhances the NK function in Hu-BLT mice. *Front Immunol* 2023;14:1132807.
14 Tseng H-C, Arasteh A, Paranjpe A, *et al*. Increased lysis of stem cells but not their differentiated cells by natural killer cells; de-differentiation or reprogramming activates NK cells. *PLoS One* 2010;5:e11590.
15 Perera Molligoda Arachchige AS: Human NK cells: From development to effector functions. *Innate Immun* 2021;27:212–29.
16 Poli A, Michel T, Thérésine M, *et al*. CD56bright natural killer (NK) cells: an important NK cell subset. *Immunology* 2009;126:458–65.
17 Bui VT, Tseng HC, Kozlowska A, *et al*. Augmented IFN-γ and TNF-α Induced by Probiotic Bacteria in NK Cells Mediate Differentiation of Stem-Like Tumors Leading to Inhibition of Tumor Growth and Reduction in Inflammatory Cytokine Release; Regulation by IL-10. *Front Immunol* 2015;6:576.
18 Tseng H-C, Bui V, Man Y-G, *et al*. Induction of Split Anergy Conditions Natural Killer Cells to Promote Differentiation of Stem Cells through Cell-Cell Contact and Secreted Factors. *Front Immunol* 2014;5:269.
19 Bern MD, Parikh BA, Yang L, *et al*. Inducible down-regulation of MHC class I results in natural killer cell tolerance. *J Exp Med* 2019;216:99–116.
20 Jewett A, Kos J, Kaur K, *et al*. Natural Killer Cells: Diverse Functions in Tumor Immunity and Defects in Pre-neoplastic and Neoplastic Stages of Tumorigenesis. *Mol Ther Oncolytics* 2020;16:41–52.
21 Tseng HC, Cacalano N, Jewett A. Split anergized Natural Killer cells halt inflammation by inducing stem cell differentiation, resistance to NK cell cytotoxicity and prevention of cytokine and chemokine secretion. *Oncotarget* 2015;6:8947–59.
22 Aparicio-Pages MN, Verspaget HW, Pena AS. Lamers CB: Natural killer cell activity in patients with adenocarcinoma in the upper gastrointestinal tract. *J Clin Lab Immunol* 1991;35:27–32.
23 Duan X, Deng L, Chen X, *et al*. Clinical significance of the immunostimulatory MHC class I chain-related molecule A and NKG2D receptor on NK cells in pancreatic cancer. *Med Oncol* 2011;28:466–74.
24 Peng Y-P, Zhu Y, Zhang J-J, *et al*. Comprehensive analysis of the percentage of surface receptors and cytotoxic granules positive natural killer cells in patients with pancreatic cancer, gastric cancer, and colorectal cancer. *J Transl Med* 2013;11:262.
25 Jewett A, Man Y-G, Tseng H-C. Dual functions of natural killer cells in selection and differentiation of stem cells; role in regulation of inflammation and regeneration of tissues. *J Cancer* 2013;4:12–24.
26 Kaur K, Cook J, Park SH, *et al*. Novel Strategy to Expand Super-Charged NK Cells with Significant Potential to Lyse and Differentiate Cancer Stem Cells: Differences in NK Expansion and Function between Healthy and Cancer Patients. *Front Immunol* 2017;8:297.
27 Tseng H-C, Kanayama K, Kaur K, *et al*. Bisphosphonate-induced differential modulation of immune cell function in gingiva and bone marrow in vivo: role in osteoclast-mediated NK cell activation. *Oncotarget* 2015;6:20002–25.
28 Breznik B, Ko M-W, Tse C, *et al*. Infiltrating natural killer cells bind, lyse and increase chemotherapy efficacy in glioblastoma stem-like tumorospheres. *Commun Biol* 2022;5:436.
29 Kaur K, Topchyan P, Kozlowska AK, *et al*. Super-charged NK cells inhibit growth and progression of stem-like/poorly differentiated oral tumors *in vivo* in humanized BLT mice; effect on tumor differentiation and response to chemotherapeutic drugs. *Oncoimmunology* 2018;7:e1426518.
30 Chiang J, Chen P-C, Pham J, *et al*. Characterizing hepatocellular carcinoma stem markers and their corresponding susceptibility to NK-cell based immunotherapy. *Front Immunol* 2023;14:1284669.
31 Kaur K, Safaie T, Ko M-W, *et al*. ADCC against MICA/B Is Mediated against Differentiated Oral and Pancreatic and Not Stem-Like/Poorly Differentiated Tumors by the NK Cells; Loss in Cancer Patients due to Down-Modulation of CD16 Receptor. *Cancers (Basel)* 2021;13:239.
32 Patch A-M, Christie EL, Etemadmoghadam D, *et al*. Whole-genome characterization of chemoresistant ovarian cancer. *Nature New Biol* 2015;521:489–94.
33 Kaur K, Ko M-W, Ohanian N, *et al*. Osteoclast-expanded super-charged NK-cells preferentially select and expand CD8+ T cells. *Sci Rep* 2020;10:20363.
34 Sadeghi S, Chen P-C, Jewett A, *et al*. Chapter 14 - combination of nk cell immunotherapy with chemotherapy and radiation enhances nk cell therapy and provides improved prognosis in cancer patients and in humanized blt mouse model system. In: Jewett A, Fong Y, eds. *NK cells in cancer immunotherapy: successes and challenges*. Academic Press, 2023: 301–20.
35 Kaur K, Sanghu J, Memarzadeh S, *et al*. Exploring the Potential of Natural Killer Cell-Based Immunotherapy in Targeting High-Grade Serous Ovarian Carcinomas. *Vaccines (Basel)* 2024;12:677.
36 Kaur K, Jewett A. Supercharged NK Cell-Based Immuotherapy in Humanized Bone Marrow Liver and Thymus (Hu-BLT) Mice Model of Oral, Pancreatic, Glioblastoma, Hepatic, Melanoma and Ovarian Cancers. *Crit Rev Immunol* 2023;43:13–25.
37 Kozlowska AK, Topchyan P, Kaur K, *et al*. Differentiation by NK cells is a prerequisite for effective targeting of cancer stem cells/poorly differentiated tumors by chemopreventive and chemotherapeutic drugs. *J Cancer* 2017;8:537–54.
38 Jewett A, Bonavida B. Target-induced inactivation and cell death by apoptosis in a subset of human NK cells. J Immun 1996;156:907–15.
39 Kaur K, Ko M-W, Chen F, *et al*. Defective NK cell expansion, cytotoxicity, and lack of ability to differentiate tumors from a pancreatic cancer patient in a long term follow-up: implication in the progression of cancer. *Cancer Immunol Immunother* 2022;71:1033–47.
40 Ko M-W, Kaur K, Safaei T, *et al*. Defective Patient NK Function Is Reversed by AJ2 Probiotic Bacteria or Addition of Allogeneic Healthy Monocytes. *Cells* 2022;11:697.
41 Kaur K, Nanut MP, Ko M-W, *et al*. Natural killer cells target and differentiate cancer stem-like cells/undifferentiated tumors: strategies to optimize their growth and expansion for effective cancer immunotherapy. *Curr Opin Immunol* 2018;51:170–80.
42 Romee R, Rosario M, Berrien-Elliott MM, *et al*. Cytokine-induced memory-like natural killer cells exhibit enhanced responses against myeloid leukemia. *Sci Transl Med* 2016;8:357ra123.
43 Terrén I, Orrantia A, Astarloa-Pando G, *et al*. Cytokine-Induced Memory-Like NK Cells: From the Basics to Clinical Applications. *Front Immunol* 2022;13:884648.
44 Simonetta F, Alvarez M, Negrin RS. Natural Killer Cells in Graft-versus-Host-Disease after Allogeneic Hematopoietic Cell Transplantation. *Front Immunol* 2017;8:465.
45 Zhang P, Yang S, Zou Y, *et al*. NK cell predicts the severity of acute graft-versus-host disease in patients after allogeneic stem cell transplantation using antithymocyte globulin (ATG) in pretreatment scheme. *BMC Immunol* 2019;20:46.
46 Ojo EO, Sharma AA, Liu R, *et al*. Membrane bound IL-21 based NK cell feeder cells drive robust expansion and metabolic activation of NK cells. *Sci Rep* 2019;9:14916.
47 Larson RC, Kann MC, Bailey SR, *et al*. CAR T cell killing requires the IFNγR pathway in solid but not liquid tumours. *Nature New Biol* 2022;604:563–70.
48 Tseng HC, Inagaki A, Bui VT, *et al*. Differential Targeting of Stem Cells and Differentiated Glioblastomas by NK Cells. *J Cancer* 2015;6:866–76.
49 Jewett A, Cavalcanti M, Bonavida B. Pivotal role of endogenous TNF-alpha in the induction of functional inactivation and apoptosis in NK cells. *J Immunol* 1997;159:4815–22.
50 Jewett A, Bonavida B. Interferon-alpha activates cytotoxic function but inhibits interleukin-2-mediated proliferation and tumor necrosis factor-alpha secretion by immature human natural killer cells. *J Clin Immunol* 1995;15:35–44.
51 Jewett A, Bonavida B. Target-induced inactivation and cell death by apoptosis in a subset of human NK cells. *J Immunol* 1996;156:907–15.
52 Jewett A, Wang MY, Teruel A, *et al*. Cytokine dependent inverse regulation of CD54 (ICAM1) and major histocompatibility complex class I antigens by nuclear factor kappaB in HEp2 tumor cell line: effect on the function of natural killer cells. *Hum Immunol* 2003;64:505–20.
53 Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol* 2014;15.

54 Korotkevich G, Sukhov V, Budin N, *et al*. Sergushichev A: Fast gene set enrichment analysis. *bioRxiv* 2021;2021:060012.
55 Subramanian A, Tamayo P, Mootha VK, *et al*. Gene set enrichment analysis: A knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci USA* 2005;102:15545–50.
56 Liberzon A, Birger C, Thorvaldsdóttir H, *et al*. The Molecular Signatures Database (MSigDB) hallmark gene set collection. *Cell Syst* 2015;1:417–25.
57 Liberzon A, Subramanian A, Pinchback R, *et al*. Molecular signatures database (MSigDB) 3.0. *Bioinformatics* 2011;27:1739–40.
58 Balanis NG, Sheu KM, Esedebe FN, *et al*. Pan-cancer Convergence to a Small-Cell Neuroendocrine Phenotype that Shares Susceptibilities with Hematological Malignancies. *Cancer Cell* 2019;36:17–34.