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ORIGINAL ARTICLE

Blood natural killer cells during treatment in recurrent ovarian cancer

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ABSTRACT

Objective: Recent research indicated favorable prognostic impact of intratumoral natural killer (NK) cells in ovarian carcinoma (OC). The role of NK cells during chemotherapy in OC is unknown. We investigated impact of NK cells in OC patients treated with palliative chemotherapy.

Methods: Participants receiving palliative chemotherapy for recurrent OC ($N = 72$) had prospectively blood samples at baseline and before cycle 2. NK cell counts were quantified by flow cytometry. NK cell activity was measured by the NK Vue® assay, estimating interferon-gamma production. Overall survival (OS) was the primary endpoint. Cutoffs were predefined, NK numbers ($\geq 184 \times 10^6$ cells/L vs. $< 184 \times 10^6$ cells/L) and NK activity (<200 pg/mL vs. ≥ 200 pg/mL).

Results: Median OS in patients with low vs. high NK cell count at baseline was 7.1 months vs. 15.6 months ($p = .028$), respectively, and before cycle 2 was 5.7 vs. 17.3 months, $p < .001$, respectively. The difference in restricted mean survival (ΔRMST) was 5.7 months (95% CI: 3.3–8.0) at cycle 2 vs. 2.5 months (95% CI: −0.6 to 5.6) at baseline, showing a significant difference with no overlap of confidence intervals. In multivariate analyses, low NK cell count remained significant with a hazard ratio (HR)=2.83, 95% CI: 1.53–5.22, $p = .001$ (baseline) and HR = 3.34, 95% CI: 1.67–6.71, $p = .001$ (before cycle 2). Patients with both low NK count and NK activity at baseline ($N = 20$) had median OS 6.5 months vs. 11.5 months in patients with either high activity, high count or both ($p = .007$). In parallel, patients with both low NK activity and count at cycle 2 ($N = 18$) had a median survival of 4.0 months vs. 15.4 months ($p < .001$).

Conclusions: A low blood NK cell count in recurrent metastatic ovarian cancer during chemotherapy is associated with unfavorable prognostic impact. Early increase in survival difference based on NK cell status suggests an association between NK cell count and treatment benefit.

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Introduction

Epithelial ovarian cancer (EOC) is a highly malignant disease with 152,000 deaths worldwide each year [1]. Current treatment strategy is surgery followed by adjuvant chemotherapy. Eventually, the majority of patients develop recurrence and the therapy becomes palliative with the aim of improving quality of life (QoL) and life prolongation. Chemotherapy options are numerous, but with the exception of second-line treatment of platinum sensitive disease, all treatment options have low response rates with low evidence of improved overall survival (OS) [2,3]. Commencement of chemotherapy is often based on the subjective evaluation by the physician in combination with patient preferences. Objective biomarkers supporting treatment decisions are needed. Recent years' research has established a clinical significance of the immune system in relation to cancer, making immune factors one of the hallmarks of cancer [4].

T-cells have been in focus in recent immune oncology research, but other immune cells may also play an important role in the eradication of tumors. In this context, natural killer (NK) cells have drawn much interest due to the high tumor killing potential seen in both *in vitro* and *in vivo* studies [5]. The NK cells were discovered in 1975 and defined as immune cells able to kill target cells without prior sensitization [6,7], but they also have important regulatory capacities [8]. Interferon-gamma (IFNG) is one of the key regulatory cytokines produced by the NK cells with important immune stimulating abilities and anti-tumor properties [9]. The clinical significance in human cancer, however, has been sparsely investigated [5,10].

A high level of intratumoral NK cells has been associated with improved prognosis in several cancers [11,12], and we have recently demonstrated a favorable prognostic role of intratumoral NK cells in a nationwide ovarian cancer cohort [13]. For solid tumors, on the other hand, the prognostic implications of NK cells in the peripheral blood are unclear

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[14–16]. A prognostic impact would support NK cells as an easily applicable biomarker in ovarian carcinoma (OC) guiding treatment decisions. Also, a clinical significance in OC might support research on the NK cell as a therapeutic target.

The aim of the present study was to investigate the prognostic impact of blood NK cell count and activity as well as their association with benefit of chemotherapy in recurrent ovarian cancer patients.

Material and methods

Patient cohort

The study cohort consisted of 72 patients with recurrent metastatic ovarian cancer undergoing palliative chemotherapy at the Department of Oncology, University Hospital of Southern Denmark, Vejle. The main inclusion criteria were histologically verified diagnosis, evaluable disease by the CA 125 Gynecological Cancer Intergroup (GCIG) criteria [17], age $\geq$ 18 years, adequate bone marrow and organ function, performance status (PS) $\leq$ 2, and life expectancy of at least three months. Patients gave signed, informed consent and were included in the study between December 2016 and October 2018. Palliative chemotherapy regimens were administered according to national guidelines. Treatment response was defined according to GCIG [17] by either CA 125 or the Response Evaluation Criteria in Solid Tumors (RECIST) 1.1. Imaging was conducted every 8–12 weeks depending on treatment regimen, and CA 125 blood samples were drawn at each treatment cycle. Blood was sampled prospectively at baseline 0–6 days prior to the first chemotherapy cycle and 0–3 days prior to treatment cycle 2.

The study was approved by the Regional Committee on Health Research Ethics for Southern Denmark (S-20160049) and the Danish Data Protection Agency (16/28860) and complies with the Helsinki II Declaration.

NK cell count

Venous blood was collected in BD vacutainer EDTA tubes (BD Biosciences, San Jose, CA, USA) and NK cell count was measured by flow cytometry. Surface staining of whole blood was performed in BD Trucount® Tubes using BD Multitest® CD3FITC/CD16 PE + CD56 PE/CD45 PerCP/CD19 APC (clones: SJ25C1, SK7, B73.1, NCAM16.2, 2D1) (BD Biosciences, San Jose, CA, USA). Erythrocytes were eliminated by treating the samples with BD FACS Lysing Solution (BD Biosciences, San Jose, CA, USA). All samples were analyzed within 2 h on a BD FACSCanto II equipped with a 488 nm- and a 633 nm laser (BD Biosciences, San Jose, CA, USA). Seven-color setup beads were used for daily quality control of the instrument and for compensation. FlowJo version X (Flowjo, Ashland, OR, USA) was used for data analyses. NK cells were defined as $CD45^{high}$Side $Scatter^{low}CD3^{-}CD56^{+}/CD16^{+}$ (Figure 1). A predefined cutoff level was based on the reference value from Bisset et al. [18] that used an identical definition of NK cells ($CD3^{-}CD56^{+}/CD16^{+}$) dividing patients in 'NK cell high ($\geq 184 \times 10^6$ cells/L)' and 'NK cell low ($< 184 \times 10^6$ cells/L)'; also median values were applied.

NK cell activity

NK Vue® (ATGen Co. Ltd, Seongnam, South Korea) is an assay using whole blood to measure NK cell activity [19]. One milliliter of venous blood was collected into NK Vue® tubes containing Na-heparin and PROMOCA® (ATGen Co. Ltd, Seongnam, South Korea). The samples were kept for 20–24 h at 37 °C during which time NK cells were activated by PROMOCA®, hence causing them to release IFNG [20]. Subsequently, the level of IFNG in the plasma was measured by the NK Vue® ELISA (ATGen). According to the manufacturer, a 200 pg/mL cutoff differentiates between abnormal (<200 pg/mL) and normal ($\geq$200 pg/mL) NK activity test results. The clinical relevance of this cutoff in metastatic cancer has been verified by Hansen et al. [21].

Statistical analysis

The primary end point of OS was calculated from the date of the baseline blood sample to the date of death. NK cell values obtained at treatment cycle 2 formed the basis of a secondary endpoint calculating survival from that time point. Kaplan–Meier's plots were applied to illustrate survival, and significance of differences was analyzed by the log rank test. Median survival was used for comparison of patients grouped by level of (1) NK cell count and (2) NK cell activity. To compare differences in survival time calculated from the two time points of baseline and before treatment cycle 2, the difference in restricted mean survival time (ΔRMST) was used as a quantitative measure of survival difference according to NK cell count and NK cell activity level. This was done solely to be able to test whether a survival difference at treatment cycle 2 was significantly higher than at baseline. To be able to directly compare survival differences at the two time points, ΔRMST was analyzed only for patients with values obtained at both time points ($N = 58$). Restricted mean survival time is mean survival time within a maximum time of observation. We selected 24 months as a suitable time frame for recurring ovarian cancer patients. Factors analyzed in univariate cox regression were: FIGO stage, primary surgical outcome, histology, platinum sensitivity, baseline PS and prior lines of chemotherapy. The independent prognostic implications of NK cell count and NK cell activity were investigated in multivariate Cox regression analysis including factors that had a significant ($p < .05$) prognostic impact in the univariate analyses. The proportional hazards assumption was tested on all Cox regression models.

Pearson's Chi-square test was used as a measure of association between NK cell variables and clinical factors. The potential association between NK cell count and NK cell activity was measured by Spearman's rank correlation. Association between treatment response and NK cell values was evaluated by logistic regression.

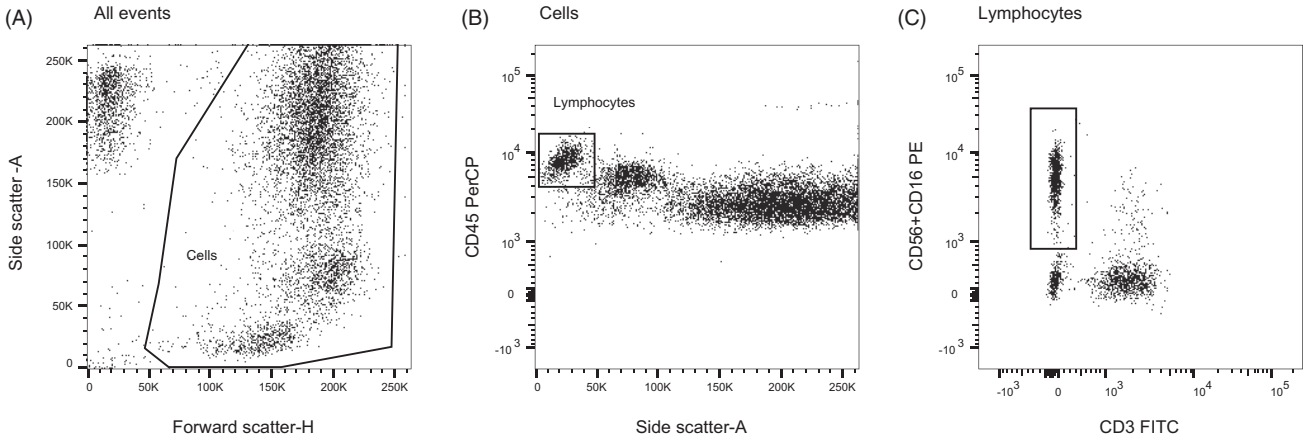

Figure 1. (A) Forward scatter and side scatter allow for the identification of the cell subset by size and granularity. (B) Lymphocytes are differentiated using side scatter and CD45 staining. (C) In the lymphocyte subset, the NK cells are distinguished by the CD3–CD56+/CD16+ phenotype.

Descriptive, correlational, survival and regression analyses were performed using STATA version 15® (StataCorp, College Station, TX, USA).

Results

Patients

The majority of patients ($N = 61$, 86%) had high grade serous carcinoma (HGSC). One to five treatment lines had been administered prior to inclusion; 30 patients (43%) had received only one line of therapy. Two-thirds of the patients were platinum resistant. Baseline patient characteristics according to NK cell count results appear from Table 1.

We analyzed the prognostic value of NK cell count ($N = 69$, $N = 61$) and NK cell activity ($N = 66$, $N = 62$) at baseline and treatment cycle 2, respectively. Missing blood values were caused by missed sampling, technical error in the sample preparation, early end of treatment, or death. The median OS was 8.9 months (95% CI: 7.4–15.6 months). For the 21 patients still alive at time of analysis, the median follow-up time was 19.3 months (range 11.7–33.3 months). Twenty-one patients (29%) achieved a treatment response according to GCIG criteria [17] six of which only according to CA-125 and seven only according to RECIST 1.1. The remaining eight had response both according to CA-125 and RECIST 1.1.

NK cell count and survival

Median blood NK cell count at baseline was 173×10^6 cells/L (range 36–608×10^6 cells/L). Median blood NK cell count at cycle 2 was 171×10^6 cells/L (range 25–530×10^6 cells/L). NK cell count at baseline was associated with OS. Patients with a low (predefined cutoff: $<184 \times 10^6$ cells/L) ($N = 36$) and a high baseline NK cell count ($\geq 184 \times 10^6$ cells/L) ($N = 33$) had a median OS of 7.1 months and 15.6 months ($p = .028$), respectively (Figure 2); using median as cutoff provided similar results 7.3 months vs. 13.0 months ($p = .047$), respectively. Comparing OS based on cycle 2, NK cell level (cut off 184×10^6 cells/L) showed an even more pronounced difference in OS (median 5.7 vs. 17.3 months, $p < .001$) (Figure 2); using median as cutoff provided similar results 5.4 months vs. 17.3 months ($p < .001$), respectively. NK cell counts in individual patients and change from baseline to cycle 2 are depicted in spaghetti and spider plots in Figure 3. ΔRMST values were calculated for the group of patients ($N = 58$) that have samples at both baseline and at the 2nd cycle. The ΔRMST was 5.7 months (95% CI: 3.3–8.0) at cycle 2 vs. 2.5 months (95% CI: −0.6 to 5.6) at baseline, showing a significant difference with no overlap of confidence intervals.

Patient survival was also analyzed based on shifts between categories from baseline to cycle 2. In terms of survival, the categories 'low to high' ($N = 7$) and 'high to high' ($N = 20$) performed equally well (median survival: 17.5 and 18.0 months, respectively), just as the categories 'high to low' ($N = 8$) and 'low to low' ($N = 23$) had similar poor survival (median survival: 6.8 months and 7.4 months, respectively).

NK cell activity and survival

The median blood NK activity at baseline was 131 pg/mL (range 0–7300). At cycle 2, the median activity was 189 pg/mL (range 0–4153). Based on the predefined cutoff, 38 (58%) patients had an abnormally low NK cell activity (<200 pg/mL) at baseline and 32 (51%) had an abnormally low activity at cycle 2. No statistical significant association between NK activity and OS was found (Figure 4). The ΔRMST was not statistically significant higher at cycle 2 compared to baseline, as the 95% CI overlapped.

There was no correlation between NK cell count and NK cell activity, neither at baseline (Spearman's rho = 0.1310) nor before treatment cycle 2 (Spearman's rho = 0.1296).

Patients with both low activity and count at baseline ($N = 20$) had a median survival of 6.5 months vs. 11.5 months in patients with either high activity, high count or both ($p = .007$). In parallel, patients with both low activity and count at cycle 2 ($N = 18$) had a median survival of 4.0 months vs. 15.4 months ($p < .001$).

Table 1. Baseline patient demographics and disease characteristics.

	All patients $n = 72$	NK cell count low[a] Baseline $n = 36/69$	NK cell count low Cycle 2 $n = 33/61$	NK activity low[b] Baseline $n = 38/66$	NK activity low Cycle 2 $n = 32/62$
Median age (range)	69 (47–92)	67 (47–84)	71 (56–84)	69 (47–92)	69 (54–92)
p Value[c]		.521	.414	.900	.271
Median CA-125 kU/L (range)	322 (6–30,072)	257 (6–30,072)	425 (13–4797)	546 (6–10,325)	488 (6–4403)
p Value[c]		.443	.404	.442	.476
Histology					
High grade serous carcinoma	61 (86%)	29 (81%)	25 (89%)	32 (85%)	25 (78%)
Low grade serous carcinoma	4 (5%)	1 (3%)	2 (7%)	2 (5%)	2 (6%)
Endometrioid	4 (5%)	3 (8%)	0 (0%)	2 (5%)	2 (6%)
Mucinous	3 (4%)	3 (8%)	1 (4%)	2 (5%)	3 (9%)
p Value[c]		.096	.230	.645	.341
Previous lines of chemotherapy					
1	32 (45%)	15 (42%)	16 (57%)	15 (39%)	13 (40%)
2–3	31 (43%)	17 (47%)	11 (39%)	20 (53%)	14 (44%)
4–5	9 (12%)	4 (11%)	1 (4%)	3 (8%)	5 (16%)
p Value[c]		.228	.080	.232	.441
Platinum sensitive					
Yes	24 (33%)	14 (39%)	10 (36%)	13 (34%)	9 (29%)
No	48 (67%)	22 (61%)	18 (64%)	25 (66%)	23 (71%)
p Value[c]		.307	.654	.860	.131
Performance status					
0–1	46 (64%)	22 (61%)	20 (71%)	24 (63%)	24 (71%)
2	26 (36%)	14 (39%)	8 (29%)	14 (37%)	8 (29%)
p Value[c]		.632	.658	.840	.659
Treatment regimen					
Carboplatin	10 (14%)	5 (14%)	4 (13%)	6 (16%)	1 (3%)
Carboplatin + Liposomal dox.	13 (18%)	8 (22%)	5 (15%)	5 (13%)	7 (22%)
Carboplatin + Paclitaxel	1 (1%)	0 (0%)	0 (0%)	0 (0%)	1 (3%)
Liposomal doxorubicin	14 (20%)	8 (22%)	5 (13%)	8 (21%)	7 (22%)
Topotecan	16 (22%)	9 (25%)	6 (19%)	9 (23%)	7 (22%)
Treosulfan	12 (17%)	3 (8%)	10 (31%)	8 (21%)	8 (25%)
Paclitaxel	2 (3%)	1 (3%)	0 (0%)	1 (3%)	1 (3%)
Gemcitabine	2 (3%)	0 (0%)	2 (6%)	0 (0%)	0 (0%)
Vinorelbine	1 (1%)	1 (3%)	0 (0%)	0 (0%)	0 (0%)
Bevacizumab	1 (1%)	1 (3%)	1 (3%)	1 (3%)	0 (0%)
p Value[c]		.454	.103	.453	.066

[a]NK cell count $< 184 \times 10^6$ cells/L.
[b]NK activity < 200 pg/mL.
[c]Pearson Chi square test.

Treatment response and NK cells

No association between baseline NK cell count and treatment response was found by logistic regression ($p = .447$). However, at cycle 2, the odds ratio for achieving a response was 3.38 (95% CI: 1.06–10.75, $p = .040$) in favor of high NK cell count. Treatment response was not associated with NK cell activity ($p = .413$ and $p = .471$).

Independent prognostic relevance of NK cell count

NK cell count remained an independent prognostic marker with OS as the endpoint in the Cox multivariate analysis. Patients with a low number of NK cells at baseline had significantly poorer OS compared to those with a high number of NK cells, hazard ratio (HR)=2.83, 95% CI: 1.53–5.22, $p = .001$ (Table 2). The same applied to low NK cell count compared to high NK cell count before treatment cycle 2, HR = 3.34, 95% CI: 1.67–6.71, $p = .001$ (Table 2).

NK cell count was also tested with the medians as cutoff in the multivariate analysis with similar results, baseline: HR = 2.66, 95% CI: 1.45–5.00, $p = .002$; before second treatment: HR = 3.40, 95% CI: 1.68–6.85, $p = .001$.

Discussion

Many factors are potential contributors to the poor treatment outcome in ovarian cancer. Cancer cell drug-resistance mechanisms have traditionally been considered fundamental [22], and recent years cancer research has demonstrated the importance of reduced immune surveillance in cancer [23]. In the current study, we investigated the significance of an essential player in immune surveillance; the NK cell. Only few studies have evaluated the significance of blood NK cells during chemotherapy [12,14,21,24–26]. Nevertheless, a favorable prognostic role has been found in renal cell carcinoma [12], breast cancer [26] and lymphoma [25]. In ovarian cancer, results are both limited and conflicting, as one report has shown a high density of intratumoral NK cells to be an unfavorable prognostic marker [27] while a more recent and larger study performed by our research group has pointed to a positive prognostic impact [13], although based on tissue and not as a circulating marker.

The quantification of NK cells was performed using flow cytometry, which is widely accessible and therefore makes the potential of measuring the number of NK cells an appealing approach for clinicians. The data described were arrived at using an antibody multi test approved for clinical use allowing the separation of NK cells with a $CD45^{high}$Side

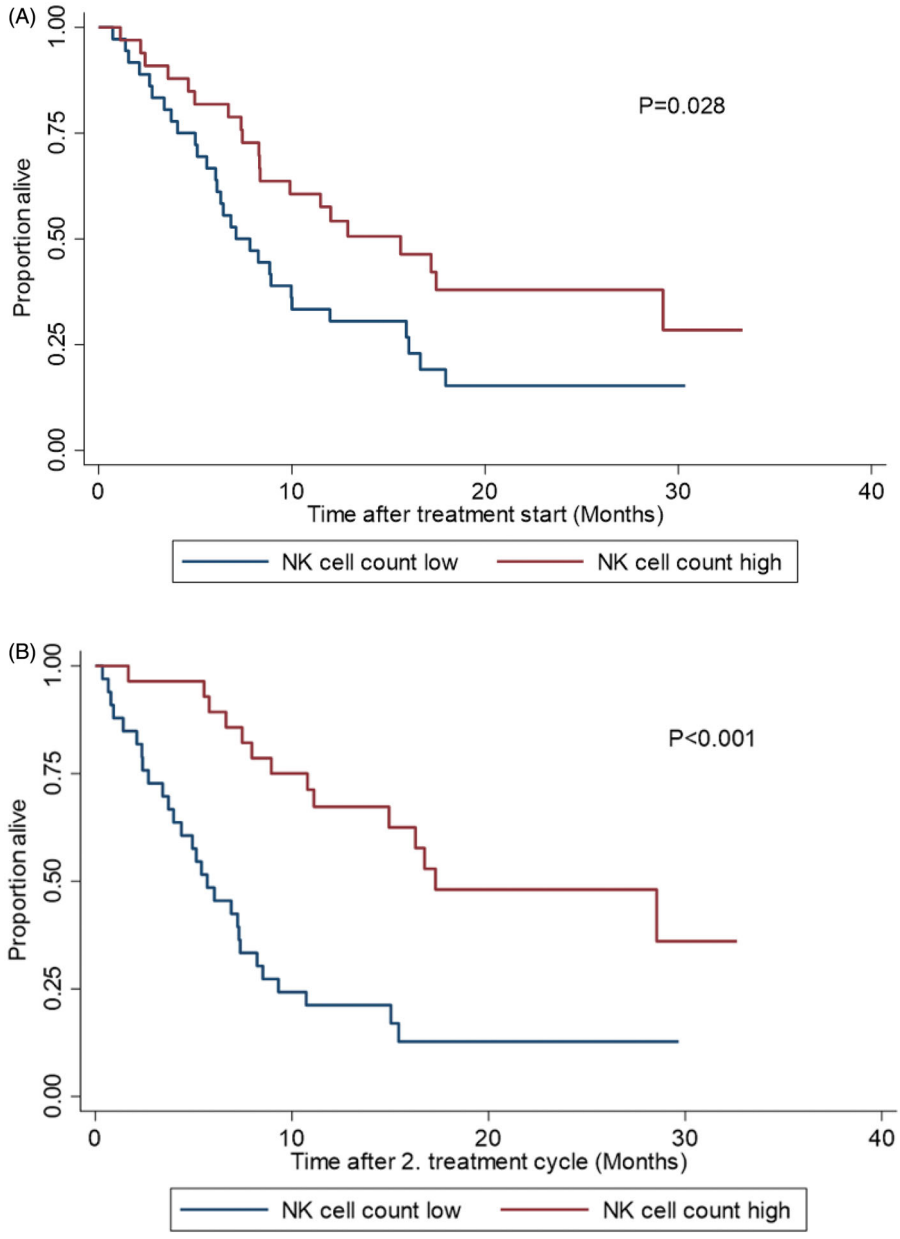

Figure 2. Kaplan–Meier's curves with OS as endpoint. (A) Baseline NK cell count. (B) Cycle 2 NK cell count. NK cell count high vs. low was defined by the cut-off of 184×10^6 cells/L.

Scatter^{low}CD3$^-$CD56$^+$/CD16$^+$ phenotype. Unfortunately, this standardized setup omits the possibility of distinguishing between the most regulatory CD56bright NK cell subset producing vast numbers of cytokines, including IFNG, and the more cytotoxic CD56dim NK cells subset, which is more involved in the direct killing of the target cells [28,29]. It cannot be ruled out that more powerful results could be obtained by dividing NK cells into distinct subsets, nor can it be ruled out that the quantity of cells in subsets may correlate with the NK cell activity measured in this study.

Traditionally, measurement of NK cell activity has been performed by either ^{51}Cr-release assay or CD107a expression, which are reliable yet time consuming methodologies. The cumbersome nature of these techniques may partly explain the sparse clinical research in the field. So far, NK cell activity has mainly been associated with the incidence of cancer [19,30–33] while prognostic implications have only been investigated in few studies [24,34]. The present investigation is the first to measure NK cell activity by a recently developed, simple blood test using IFNG as read out and correlate it to the clinical outcome in ovarian cancer patients treated with chemotherapy. Confirming the previous studies in cancer, we found the median level of IFNG to be below the 200 pg/mL threshold defining an abnormally low NK cell activity in both baseline samples and prior to 2nd treatment cycle. When analyzing these groups in further detail we did see a tendency toward a better OS in patients with IFNG above the same threshold at both time points; however, this was not statistical significant. This result was somewhat unexpected given that (1) NK cell count in the same samples was found to be of prognostic value in terms of OS and (2) compared to cell count high activity was expected to be of greater value as both anergic and exhausted NK cells may constitute a substantial proportion of the total. However,

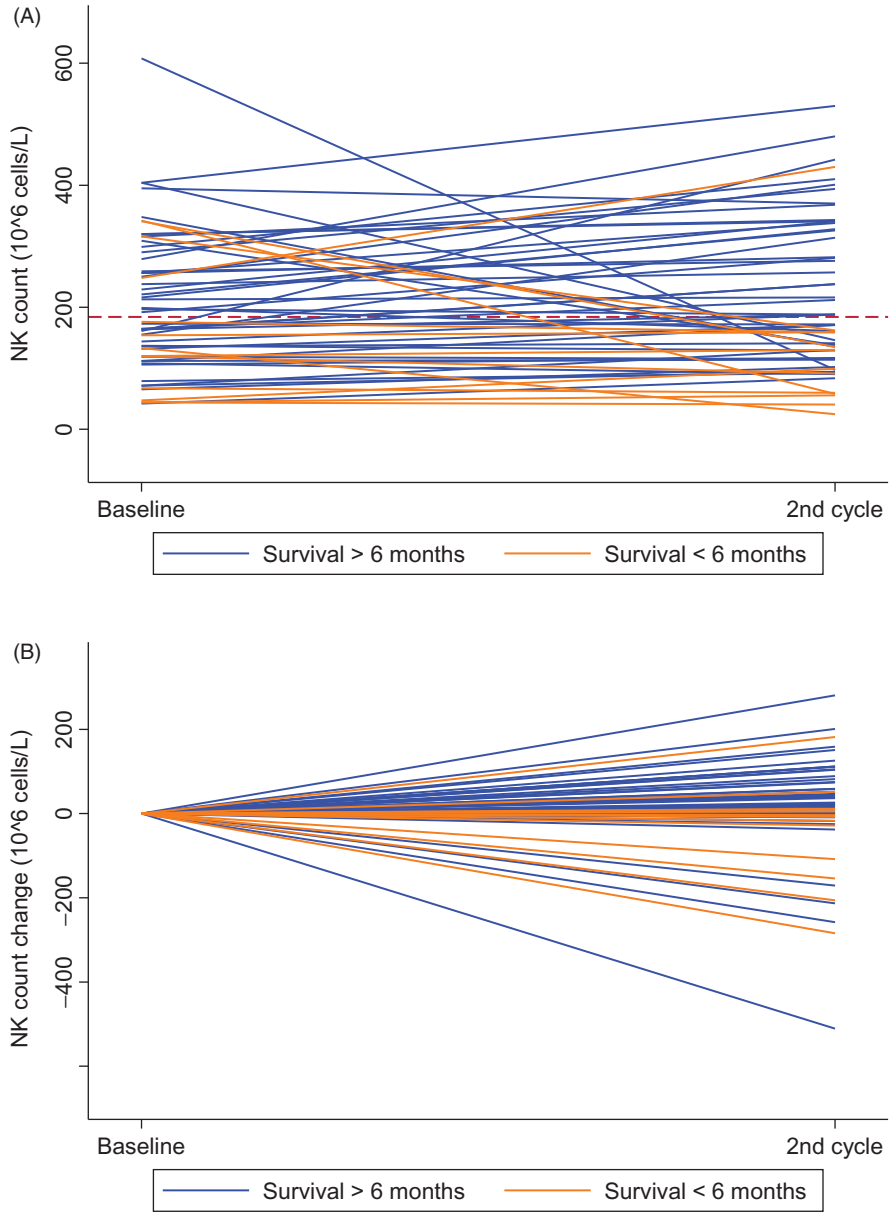

Figure 3. (A) Spaghetti's plot showing individual patient NK cell counts at baseline and at cycle 2. Patients with short survival <6 months marked with yellow color; patients with survival >6 months marked with blue color. The red reference line marks the cut off value of 184×10^6 cells/L applied in the study. (B) Spider plot showing change in individual patient NK cell counts from baseline to cycle 2. Patients with short survival <6 months marked with yellow color; patients with survival >6 months marked with blue color.

there are possible explanations for this outcome. First, we have studied the activity in a highly malignant type of cancer in which most therapies have only limited effect if any all. In turn, the lack of therapy response may cause a continuation of immune inhibition by the tumor, e.g., through release of tumor growth factor-β known to abrogate the effectiveness of NK cells. This may hamper the use of immune activity as a prognostic marker in this particular entity. Moreover, in this study, we limited the NK cell activity to be expressed by the ability to produce IFNG although this cell type emits a number of chemokines and cytokines important for establishing an immune response against cancer. An analysis of any of these may have prompted a different outcome. Furthermore, we did not measure the elemental cytotoxic effect of the NK cells which may be decisive in this context. Conceivably, instead of the regulatory effect actually measured, the cytotoxic potential may be the NK cell activity of prognostic value. For these reasons, caution is needed in interpreting these results and further comprehensive studies are needed to assess the potential of NK cell activity as a prognostic or predictive marker and importantly also as an evaluation tool in future immunotherapy targeting related functions.

Treatment of recurrent ovarian cancer remains a therapeutic challenge with an overall low response rate and decreasing QoL. So far, treatment decisions have mainly been based on clinical evaluation, and better objective methods of patient selection are needed. The data presented here indicate that the level of NK cells holds potential in this respect. A low level seems to imply a poor prognosis with limited survival time. This should be considered in the discussion on treatment with the patient.

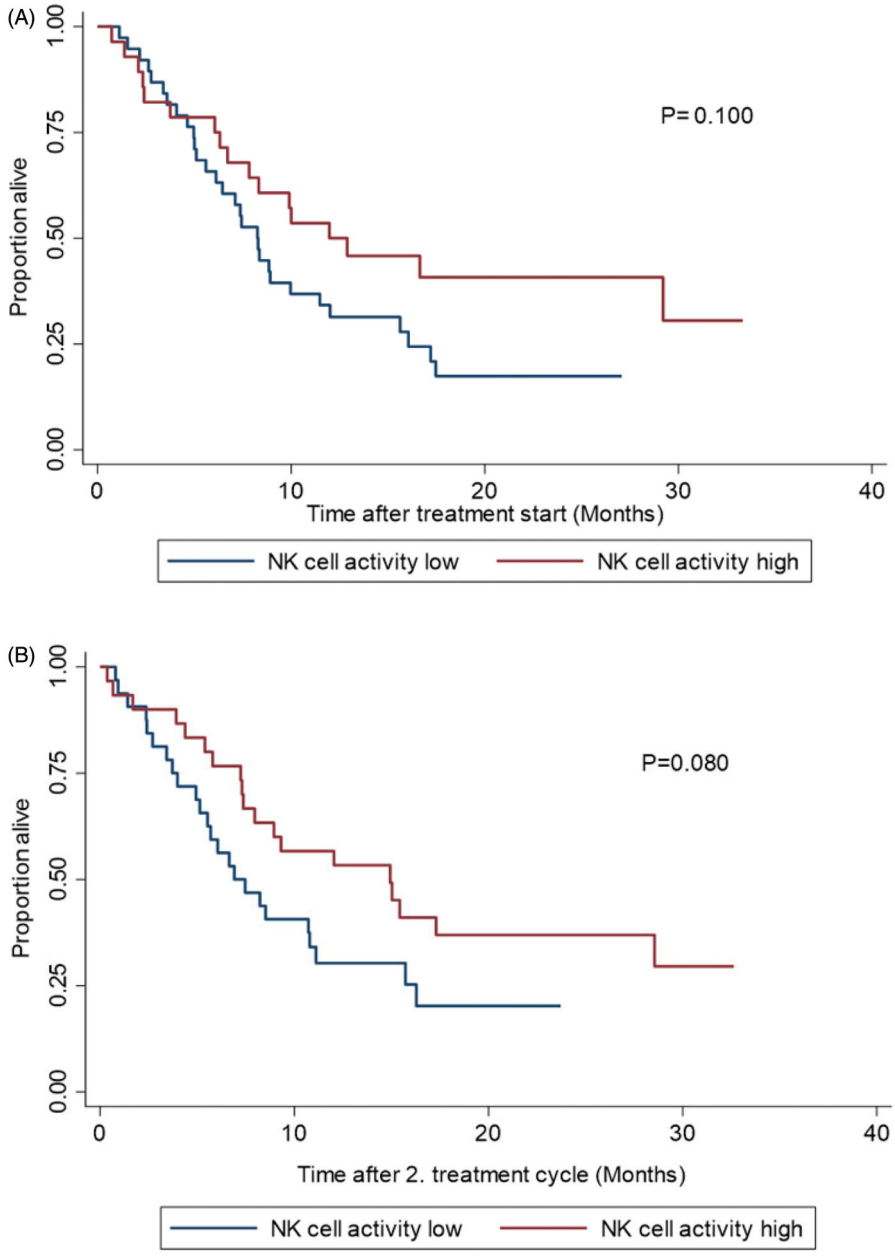

Figure 4. Kaplan–Meier's curves OS. (A) Baseline NK cell activity. (B) Cycle 2 NK cell activity. Abnormally low NK cell activity was defined as <200 pg/mL IFNG measured by the NK Vue® assay.

Table 2. Multivariate Cox regression results.

	OS Baseline		OS Cycle 2	
	HR (95% CI)	*p* Value	HR (95% CI)	*p* Value
Platinum sensitive				
No	Ref.		Ref	
Yes	0.52 (0.27–0.99)	.045	0.55 (0.27–1.10)	.092
Prior lines of chemotherapy				
1–3	Ref.		Ref.	
4–5	4.70 (2.03–10.90)	<.001	2.33 (0.96–5.67)	.062
Performance status				
0–1	Ref.		Ref.	
2	3.16 (1.71–5.86)	<.001	2.58 (1.34–4.97)	.005
NK cell count				
$\geq 184 \times 10^6$ cells/L	Ref.		Ref.	
$<184 \times 10^6$ cells/L	2.83 (1.53–5.22)	.001	3.34 (1.67–6.71)	.001

Variables not statistically significant in the univariate analysis were not included in the multivariate analysis.

Ineffective treatment should be terminated as early as possible, ideally after the first treatment cycle. At present no markers are available for that purpose. Our data show that the difference in RMST according to NK cell count increases significantly after the first cycle, which indicates a relation to treatment and may serve to identify a group of patients with a very poor prognosis after the first cycle. Furthermore, a significant association between treatment response and NK cell count at the second cycle was found. This study was not designed to find predictive markers, but the results point toward NK cells as potential predictors of treatment outcome. This could allow for early termination of ineffective treatment. Future studies are needed though, to investigate NK cells as a predictive marker.

A prognostic impact of blood NK cell count as well as intratumoral NK cell density in ovarian cancer puts NK cell

based treatment in perspective, especially with the recent disappointing results of T cell based PD-L1 checkpoint inhibition [35]. Although studies have been conducted on NK cell based treatment in cancer [36,37], they are few compared to the number of studies based on either modulation of T cell activity or cellular transfer therapy with T cells. Despite a great potential, no breakthrough results have yet appeared in the line of NK cell based therapy [36,37], but the prognostic impact in metastatic ovarian cancer presented here supports further research in NK cell based cancer treatment.

Conclusions

The present study is the first to show an unfavorable prognostic impact of low blood NK cell count in recurrent metastatic ovarian cancer. Identifying patients with a poor prognosis could support decisions on initiation of chemotherapy. In addition, an early increase in restricted mean survival suggests an association between NK cell count and treatment benefit. This could influence future treatment strategies in terms of ending or changing inefficient chemotherapy at an early stage.

Acknowledgments

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Disclosure statement

ATGen Co. Ltd. did not interfere with the study design, analyses, data interpretation or writing of the manuscript completion.

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Data availability statement

Individual participant data are not allowed to be shared according to the Danish data protection law, since a rule of total anonymization never could be upheld, due to the nature of the information in the dataset.

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