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An increase in regulatory T cells in peripheral blood correlates with an adverse prognosis for malignant melanoma patients – A study of T cells and natural killer cells

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ABSTRACT

Malignant melanoma is a highly immunogenic tumour, and the immune profile significantly influences cancer development and response to immunotherapy. The peripheral immune profile may identify high risk patients. The current study showed reduced levels of $CD4^+$ T cells and increased levels of $CD8^+$ T cells in peripheral blood from malignant melanoma patients compared with controls. Percentages of peripheral $CD56^{dim}CD16^+$ NK cells were reduced and $CD56^{bright}CD16^-KIR3^+$ NK cells were increased in malignant melanoma patients. Late stage malignant melanoma was correlated with low levels of $CD4^+$ T cells and high levels of $CD56^{bright}CD16^-KIR3^+$ NK cells. Finally, high levels of Tregs in peripheral blood were correlated with poor overall survival and disease-free survival. The results indicate that changes in specific immune cell subsets in peripheral blood samples from patients at the time of diagnosis may be potential biomarkers for prognosis and survival. Further studies will enable clarification of independent roles in tumour pathogenesis.

1. Introduction

Malignant melanoma is one of the most common cancers worldwide with more than 100,000 new cases diagnosed and 16,500 deaths every year in Europe (https://ecis.jrc.ec.europa.eu, April 18th, 2023). Despite its aggressive nature, early stage malignant melanoma is efficiently treated with surgical removal of the tumour. However, early detection is crucial for the chances of recovery, as the 5-year survival rate drops to 9–28% if the patient is diagnosed with late stage malignant melanoma (Svedman et al., 2016). Malignant melanoma is considered a highly immunogenic tumour and the presence of lymphocytes is associated with prognosis (Neagu, 2012).

Natural Killer (NK) cells comprise 5–15% of human peripheral blood mononuclear cells (PBMCs) and are recognized for their potential to kill abnormal, cancerous or virus-infected cells in the absence of antigen specificity (Paul and Lal, 2017; Di Vito et al., 2019). The $CD56^{dim}CD16^+$ NK subtype constitutes at least 85–90% of peripheral NK cells and is classically associated with high cytotoxicity (Poli et al., 2009). The lack of or abnormal expression of HLA class I molecules on malignant cells induces NK cell activation and lysis through engagement with activating receptors (Di Vito et al., 2019). Common NK cell receptors include killer cell immunoglobulin-like receptors (KIRs) and killer cell lectin-like receptors (KLRs) such as the NKG2 receptor family comprising both inhibitory and activating receptors (Anfossi et al., 2006; Moretta et al., 1996). Furthermore, NK cells express the inhibitory receptor ILT2 through which they bind HLA-G and HLA-F (Liang et al., 2006). The $CD56^{bright}CD16^-$ NK subtype constitutes normally less than 10% of peripheral NK cells (Poli et al., 2009). The $CD56^{bright}CD16^-$ cells are less cytotoxic compared to $CD56^{dim}CD16^+$ cells and function mainly through secretion of cytokines and chemokines (Caligiuri, 2008; Melaiu et al., 2020). NK cell infiltration is generally associated with a longer patient survival in solid tumours (Nersesian et al., 2021). Furthermore,

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high cytotoxic activity of peripheral blood lymphocytes has been associated with reduced cancer risk (Imai et al., 2000). Peripheral $CD56^{bright}$ NK cells seem to inversely correlate with survival in malignant melanoma patients (de Jonge et al., 2019). Furthermore, an enrichment of cytotoxic $CD56^{dim}$ NK cells in tumour-free lymph nodes has been observed in malignant melanoma patients (Ali et al., 2014).

Regulatory T cells (Tregs) are an immunosuppressive subset of T cells involved in preservation of immune tolerance, which constitute 5–10% of the total $CD4^+$ T cell population in peripheral blood (Gershon et al., 1972; Sakaguchi et al., 1995; O'Shea and Paul, 2010). Tregs exert their function through cell-cell contact and by secretion of soluble factors (Jorgensen et al., 2019). Studies have showed an elevated presence of Tregs in peripheral blood of patients with metastatic melanoma compared with healthy controls (Jacobs et al., 2012). Although conflicting studies have been published, high infiltration of Tregs in the tumour is often associated with poor prognosis, whereas infiltration of cytotoxic T cells are associated with better survival rate in metastatic melanoma patients (Erdag et al., 2012; Brody et al., 2009; Knol et al., 2011; Ladanyi et al., 2010; Miracco et al., 2007; Jacquelot et al., 2016).

In the current study, we investigated the presence of specific T cell and NK cell phenotypes in peripheral blood of malignant melanoma patients at the time of diagnosis and surgical treatment compared to healthy controls, a possible correlation with tumour stage and thickness, and the association between specific immune cells and survival.

2. Materials and methods

2.1. Study cohort

Twenty-four patients diagnosed with malignant melanoma in 2014 and 2015 were recruited from Department of Plastic and Breast Surgery, Zealand University Hospital, Denmark. Four patients previously diagnosed with cancer were excluded. Peripheral blood samples were collected from the patients just before excision of the tumour ($n = 1$), at excision ($n = 2$), just after excision ($n = 4$), at a follow-up re-excision ($n = 2$), or just after re-excision ($n = 11$). The time of diagnosis was defined as the day of the initial excision. Six donors with benign skin lesions including seborrheic keratosis and compound melanocytic nevus were included as surgery controls. Peripheral blood samples were collected at the time of surgery in 2015. Twenty-nine anonymized healthy blood donors were included as healthy controls from Department of Clinical Immunology, The Blood Bank, Zealand University Hospital, Denmark. Peripheral blood samples were collected during a regular blood donation. Six samples were collected in 2014 and the rest were collected in 2020. A comparison of immune cell phenotypes from control samples collected in 2014 and 2020 indicated minor changes over time for $CD4^+$ T cells, $CD56^{dim}CD16^+ILT2^+$ and $CD56^{bright}CD16^-KIR3^+$ NK cells, however, only four controls from 2014 were included. The clinicopathological data including age, sex, histological tumour type, tumour thickness, detection of positive sentinel nodes, presence of ulceration, presentation of metastases and survival data was updated March 2023. All patients and donors provided informed consent and the study was approved by and carried out in compliance with the Danish Data Protection Agency, the Danish National Committee on Health Research Ethics (SJ-363), and ethical standards of the Helsinki declaration.

2.2. Preparation of samples and flow cytometry analyses

Peripheral blood mononuclear cells (PBMCs) were purified from the blood samples using Ficoll-Paque PLUS (VWR, Radnor, PA, USA, #17-1440-02) standard density gradient centrifugation. Aliquots of PBMCs were cryopreserved in FBS containing 10% dimethylsulfoxide (DMSO; Merck, #D2650) until flow cytometric analysis. Cryopreserved PBMCs were thawed in a water bath and transferred to 37°C pre-warmed RPMI 1640 medium (ThermoFisher, #72400054) supplemented with 20% fetal bovine serum (FBS; Merck, Darmstadt, Germany, #F9665) and centrifuged at 500 g for 10 min. Cells were resuspended to a concentration of 1×10^6 cells/mL in FACSFlow staining buffer (BD Biosciences, Franklin Lakes, NJ, USA, #342003) with 5% FBS. Live/Dead Fixable Violet Dead Cell Stain Kit (Life Technologies, Carlsbad, CA, USA, #L34955) was used according to manufacturer's protocol. Cells were washed once in FACSFlow (BD Biosciences, #342003), pelleted by centrifugation, and resuspended in staining buffer. Staining of Tregs and NK cells were performed for 15 min at room temperature with antibodies as listed in Table A1. Finally, samples were washed in 3 mL FACSFlow, pelleted by centrifugation, resuspended in 200 µL FACSFlow, and kept at 4°C until analyzed on a FACSCanto II flow cytometer (BD Biosciences). Analyses were performed using FACS Diva Software (BD Biosciences). The total T cell population was defined by CD3 expression. Single positive $CD4^-$ and CD8-expressing cells were selected, and HLA-G expression on each of these subsets was measured in relation to an isotype control allowing a threshold of 1% positive cells. The Treg population was defined as $CD3^+CD4^+CD25^+CD127^{lo}$. The total NK cell population was defined as $CD56^+CD3^-CD14^-CD20^-$ and NK subsets as $CD56^{dim}CD16^+$ and $CD56^{bright}CD16^-$. The percentage of NK subsets expressing the ILT2, KIR3 or NKG2C receptor was determined using isotype controls allowing a threshold of 1% positive cells.

2.3. Statistical analyses

The data were analyzed according to specific *a priori* hypotheses

Table 1
Clinicopathological characteristics of the malignant melanoma patients and the control group.

Variable		Controls* ($n = 35$)	Patients ($n = 20$)
Age** (years)	Median	49	63
	Range	26–82	26–84
	Unknown	6	
Sex			
	Male	15 (43%)	8 (40%)
	Female	15 (43%)	12 (60%)
	Unknown	5 (14%)	
Histology			
	SSMM		13 (65%)
	NMM		4 (20%)
	Desmoplastic		1 (5%)
	Unknown***		2 (10%)
Clinical stage			
	0-IA		4 (20%)
	IB		8 (40%)
	IIA-IIC		3 (15%)
	III-IV		5 (25%)
Clark level			
	I		2 (10%)
	II		0 (0%)
	III		6 (30%)
	IV		8 (40%)
	V		3 (15%)
	Unknown***		1 (5%)
Ulceration			
	Yes		3 (15%)
	No		17 (85%)
	Unknown		
Tumour thickness			
	0.01–1.50 mm		8 (40%)
	>1.50 mm		10 (50%)
	Unknown***		2 (10%)
Sentinel node			
	Negative		14 (70%)
	Positive		5 (25%)
	Unknown***		1 (5%)

*Comprise 29 healthy blood donors and 6 individuals who had undergone surgery for benign skin lesions; **Age at time of diagnosis; ***Could not be determined due to characteristics of the tumour such as anatomical locations. SSMM = superficial spreading malignant melanoma; NMM = nodular malignant melanoma.

based on theoretical aspects and previous observations and studies. Gaussian distribution of all variables was evaluated based on QQ-plots and Shapiro-Wilks tests. The blood donor control group included in 2014 and in 2020, and the blood donor control group and surgery control group were compared using unpaired *t*-test or Mann-Whitney test. Comparisons of the control group and the malignant melanoma patients with samples collected within one month from initial excision of the tumour were performed using unpaired *t*-test or Mann-Whitney test. One-way ANOVA or Kruskal-Wallis test was used to compare each of the parameters across multiple time points. Comparisons of immune cell phenotypes in patients with different tumour stages were performed using one-way ANOVA or Kruskal-Wallis test. Disease-free survival (DFS) and overall survival (OS) rates for clinical parameters (ulceration, sentinel node status, tumour thickness and age) and for low vs. high percentage of immune cell phenotypes divided based on the median expression value were estimated according to the Kaplan-Meier method. DFS was defined as the time from diagnosis until recurrence or death caused by malignant melanoma, and OS was defined as the time from diagnosis until death of any cause. Patients were censored at the date of their last contact with a medical physician. Unknowns were excluded from the survival analyses. *P*-values ≤ 0.05 were considered statistically significant. Statistical analyses were performed using GraphPad Prism® software (version 9.0.2) and IBM SPSS statistics (version 28).

3. Results

3.1. Characteristics of the sample cohort and control groups

Characteristics of the patient and control groups are listed in Table 1. The mean age of the patient group at the time of diagnosis was 63 years and 49 years for the control group. Both groups comprised both females and males. Most patients was diagnosed with superficial spreading malignant melanoma, level III or IV tumours, tumour thickness above 1.5 mm and no signs of ulceration or detection of positive sentinel nodes. The number of samples in the analyses varies because of availability of sample material.

Differences in frequencies of T cell subsets were not related to undergoing minor surgery as no variables showed significant differences between the surgery control group and the blood donor control group. It was not possible to compare the NK subsets between the two groups because results from only two surgery controls were obtained due to low amounts of cells collected and stored for the remaining four surgery control patients. Based on these initial analyses, it was decided to merge the blood donor and surgery control groups as one control group for further analyses.

3.2. The percentage of $CD4^+$ T cells was reduced and the percentage of $CD8^+$ T cells was increased in malignant melanoma patients at the time of diagnosis compared with controls

The frequencies of immune cell phenotypes in peripheral blood of malignant melanoma patients within one month from excision of the tumour were compared to those of healthy controls. The percentage of $CD4^+$ T cells was significantly reduced in patients compared with controls, while the percentage of $CD8^+$ T cells was significantly increased ($P = 0.0086$ and $P = 0.0219$, respectively; Fig. 1a). There was a tendency towards higher percentage of $CD4^+CD25^+CD127^-$ Tregs in patients compared with controls (Fig. 1a). There was no significant difference between the percentage of HLA-G-expressing $CD4^+$ and $CD8^+$ cells between patients and controls (Fig. 1b).

3.3. The percentage of $CD56^{dim}CD16^+$ NK cells was reduced and the percentage of $CD56^{bright}CD16^-KIR3^+$ NK cells was increased in peripheral blood in malignant melanoma patients compared with healthy controls

A significant reduction in the percentage of $CD56^{dim}CD16^+$ cells was observed in patients compared with controls ($P = 0.0205$), while no difference was observed in the percentage of $CD56^{bright}CD16^-$ cells (Fig. 1c). The $CD56^{dim}CD16^+$ and $CD56^{bright}CD16^-$ cell subsets were investigated for expression of the KIR3, ILT2 and NKG2C receptors. There was a significant higher percentage of $CD56^{bright}CD16^-KIR3^+$ cells in patients compared with controls ($P = 0.0058$), but no significant differences between the expression of the other markers in both NK cell subsets (Fig. 1c).

For a subset of the patients, blood samples were collected at multiple time points (0–1 months, 1–6 months and 6–12 months) after excision of the tumour. No significant differences were observed in the frequencies of T cell and NK cell phenotypes between the different time points (data not shown).

3.4. Late stage malignant melanoma was correlated with low percentage of $CD4^+$ T cells and high percentage of $CD56^{bright}CD16^-KIR3^+$ NK cells

There was a significant association between low percentage of $CD4^+$ T cells and late stage ($P = 0.0175$) and between high percentage of $CD56^{bright}CD16^-KIR3^+$ NK cells and late stage ($P = 0.0231$; Fig. 2). Furthermore, the percentage of $CD4^+$ cells was negatively and the percentage of $CD8^+$ T cells was positively correlated with tumour thickness ($r_s = -0.5433$, $P = 0.0385$ and $r_s = 0.6345$, $P = 0.0129$, respectively; Fig. 3).

3.5. Ulceration, positive sentinel node and tumour thickness were correlated with poor disease-free survival

To investigate whether the immune cell phenotypes were associated with clinical parameters and survival, patients were divided into groups with low and high expression of the immune markers based on the median expression value. Expression of immune markers was not associated with known prognostic predictors such as sex, tumour thickness, ulceration or detection of positive sentinel lymph nodes (data not shown). Seven patients died within the follow-up period, four from disseminated malignant melanoma disease. One patient had experienced recurrence of the disease. Results confirmed an association between clinical outcome and the known prognostic predictors. Ulceration, positive sentinel nodes and tumour thickness were significantly associated with worse DFS ($P < 0.0001$, $P = 0.0083$ and $P = 0.0243$, respectively) and ulceration with OS ($P < 0.0001$; Fig. 4). There was a tendency towards association between worse OS and positive sentinel nodes, large tumour thickness and high age, and between DFS and high age (Fig. 4).

3.6. High levels of Tregs in peripheral blood were correlated with poor overall survival and disease-free survival

Of the investigated T cell and NK cell phenotypes only Tregs were significantly associated with DFS and OS ($P = 0.0304$, $P = 0.0180$; Fig. 5). There was a tendency towards an association between worse DFS and OS and high expression of NKG2C on $CD56^{bright}CD16^-$ cells (Fig. 5). No significant association was observed between survival and any of the other investigated immune phenotypes (Fig. A.1 and Fig. A.2). It was not possible to perform Cox proportional hazard analyses considering clinical parameters due to few events in the subgroups.

4. Discussion

The potential of immunotherapy-based treatments continues to gain importance. However, a favourable response of implemented therapies are limited to a subset of patients. Therefore, it is important to identify biomarkers that can be used for stratification of cancer patients and to improve individualized treatment strategies. As malignant melanoma is known as a highly immunogenic cancer, certain immune profiles of T cells and NK subsets and related markers were in the current study

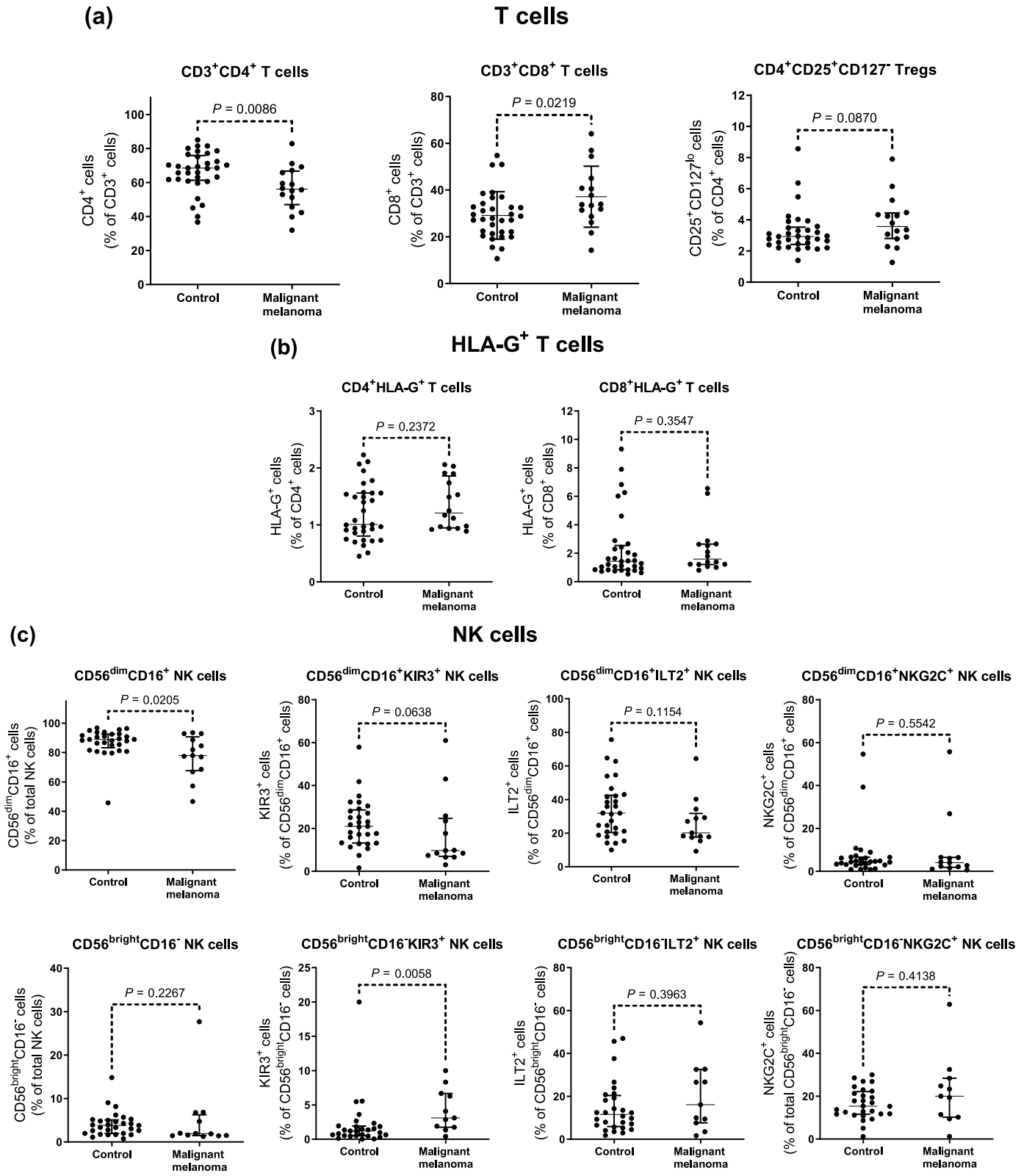

Fig. 1. T cell and NK subsets in peripheral blood of healthy controls and malignant melanoma patients. Percentage distribution of immune cell phenotypes in peripheral blood samples collected within one month from excision of the tumour from malignant melanoma patients ($n = 16$ for T cells; $n = 11$–13 for NK cells) and from healthy controls ($n = 33$ for T cells; $n = 28$–29 for NK cells). (a) $CD3^+CD4^+$ T cells, $CD3^+CD8^+$ T cells, and Tregs defined as $CD4^+CD25^+CD127^-$ cells. (b) $CD4^+HLA\text{-}G^+$ T cells and $CD8^+HLA\text{-}G^+$ T cells. (c) $CD56^{dim}CD16^+$, $CD56^{dim}CD16^+KIR3^+$, $CD56^{dim}CD16^+ILT2^+$, $CD56^{dim}CD16^+NKG2C^+$, $CD56^{bright}CD16^-$, $CD56^{bright}CD16^-KIR3^+$, $CD56^{bright}CD16^-ILT2^+$, and $CD56^{bright}CD16^-NKG2C^+$ NK cells. Based on flow cytometric analyses. Groups were compared using unpaired *t*-test with Welch's correction when appropriate, where error bars show the mean ± SD ($CD3^+CD8^+$ and $CD56^{bright}CD16^-NKG2C^+$ cells) or Mann-Whitney test, where error bars show the median with interquartile range (all others). Each dot represents one individual.

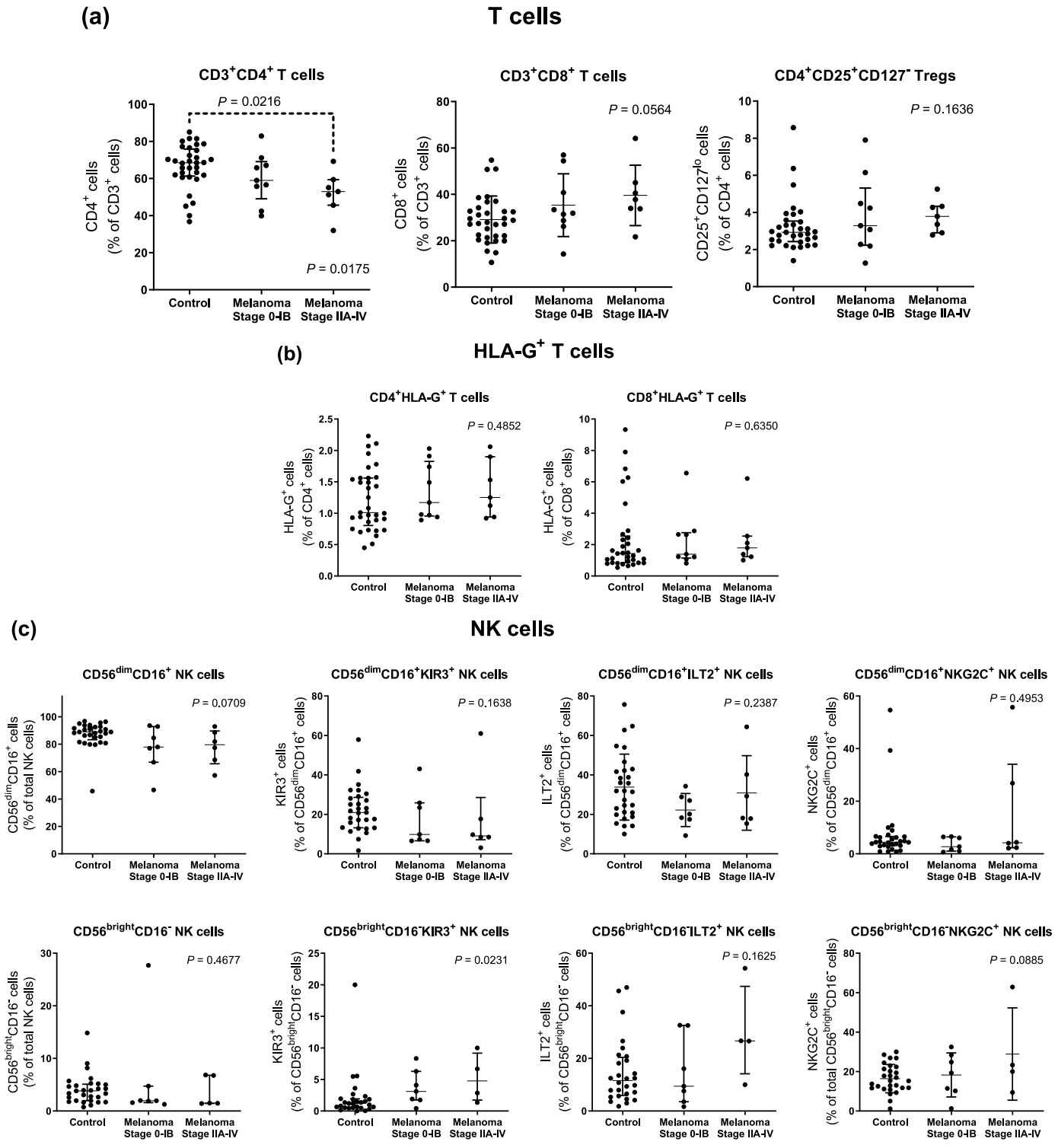

Fig. 2. T cell and NK subsets in peripheral blood of malignant melanoma patients with different clinical stages. Percentage distribution of immune cell phenotypes in peripheral blood samples from healthy controls ($n = 33$ for T cells; $n = 28$–29 for NK cells) and malignant melanoma patients divided into groups of stage 0-IB ($n = 9$ for T cells; $n = 7$ for NK cells) and stage IIA-IV tumours ($n = 7$ for T cells; $n = 4$–6 for NK cells). (a) $CD3^+CD4^+$ T cells, $CD3^+CD8^+$ T cells, and Tregs defined as $CD4^+CD25^+CD127^-$ cells. (b) $CD4^+HLA\text{-}G^+$ T cells and $CD8^+HLA\text{-}G^+$ T cells. (c) $CD56^{dim}CD16^+$, $CD56^{dim}CD16^+KIR3^+$, $CD56^{dim}CD16^+ILT2^+$, $CD56^{dim}CD16^+NKG2C^+$, $CD56^{bright}CD16^-$, $CD56^{bright}CD16^-KIR3^+$, $CD56^{bright}CD16^-ILT2^+$, and $CD56^{bright}CD16^-NKG2C^+$ NK cells. Based on flow cytometric analyses. The groups were compared using an ANOVA analysis, where error bars show the mean ± SD ($CD3^+CD8^+$, $CD4^+HLA\text{-}G^+$, $CD56^{dim}CD16^+ILT2^+$, and $CD56^{bright}CD16^-NKG2C^+$ cells) or a Kruskal-Wallis test, where error bars show the median with interquartile range (all others). Each dot represents one individual. Post-hoc analyses of immune cell phenotypes with a significant association between the groups were made, and the significant pairwise comparisons are represented on the graph with a dotted line.

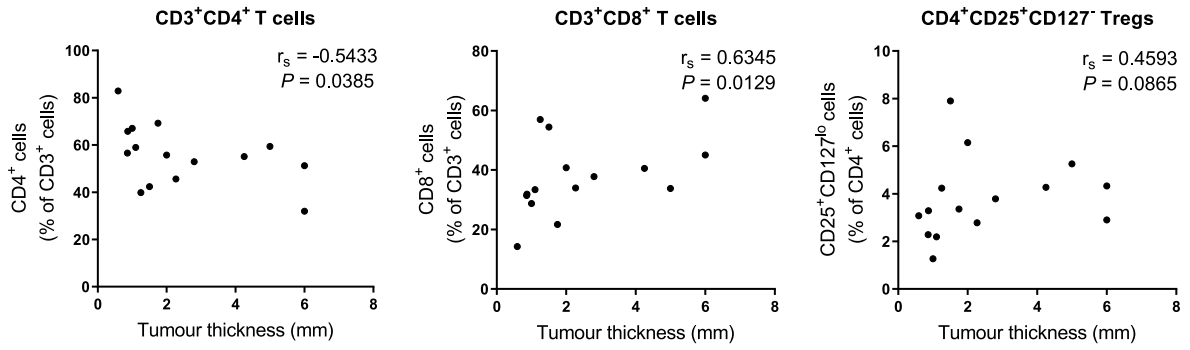

Fig. 3. Correlation between tumour thickness and immune cell phenotypes. Correlation between tumour thickness and $CD3^+CD4^+$ T cells, $CD3^+CD8^+$ T cells, and Tregs defined as $CD4^+CD25^+CD127^-$ cells, in peripheral blood samples collected within one month from excision of the tumour from malignant melanoma patients. Based on flow cytometric analyses. Correlation analyses were performed by Spearman's analysis ($n = 15$ for T cells; $n = 10$–12 for NK cells). Each dot represents one individual. The coefficient r_s and *P*-values are indicated on the graph for each immune cell phenotype.

hypothesized to be prominent in malignant melanoma patients compared with healthy controls. Furthermore, it was hypothesized that the markers could be related to DFS and OS for the malignant melanoma patient cohort. The current study comprises a unique experimental design for a prospective clinical study with relatively long follow up exploiting easily accessible sample material. To our knowledge, no previous studies have focused on both T cell and NK cell phenotypes in peripheral blood from malignant melanoma patients.

A previous study showed unaffected lymphocyte counts following minor to moderate surgery confirming that changes observed in the current study was not due to the surgical procedure itself (Salo, 1992). The current study showed a significant association between DFS and the following known prognostic markers: ulceration, detection of positive sentinel nodes and tumour thickness, and also between OS and ulceration as previously described (De Vries et al., 2011; Svedman et al., 2016). Although the association between age and survival outcome was not significant, there was a strong tendency towards high age being associated with poor survival. Differences in division of tumour thickness into multiple strata might explain why tumour thickness was not associated with OS (Melsted et al., 2017).

We identified a significant decrease in $CD4^+$ T cells and increase in $CD8^+$ T cells in malignant melanoma patients compared with healthy controls indicating a shift in the balance of helper T cells and cytotoxic T cells in peripheral blood. The percentages of $CD4^+$ and $CD8^+$ T cells were both associated with tumour thickness and the decrease in $CD4^+$ T cells with advance stage malignant melanoma. Previous studies have demonstrated that a high frequency of tumour-infiltration $CD4^+$ T cells was associated with short progression-free survival and that high infiltration of $CD8^+$ T cells as well as low infiltration of Tregs were associated with prolonged OS in metastatic malignant melanoma (Erdag et al., 2012; Jacquelot et al., 2016). Results in the current study could indicate a tendency towards low percentage of $CD4^+$ T cells and high percentages of $CD8^+$ T cells being associated with shorter DFS and OS, however, it was not significant. A heterogeneous immune profile specific for either the local tumour microenvironment, peripheral blood or adjacent lymph nodes might account for this controversy. The metastatic capacity is also most likely to accompany a unique immune profile.

Previous studies showed an elevated level of peripheral Tregs in malignant melanoma patients compared with healthy controls and in late stage melanoma compared with early stage (Jandus et al., 2008; McCarter et al., 2007; Cesana et al., 2006; Correll et al., 2010). Our results indicate a tendency towards higher percentage of $CD4^+CD25^+CD127^-$ Tregs in malignant melanoma patients compared with healthy controls and in stage IIA-IV malignant melanoma compared with stage 0-Ib malignant melanoma. Differences in the definition of Tregs by CD25, CD127 and FOXP3 expression patterns might account for the lack of significance in the current study. The frequency of Tregs in cancer in general is a controversial prognostic parameter. We have previously found that the level of tumour-infiltrating Tregs correlates with tumour thickness (Melsted et al., 2017). This could not be confirmed in the current study. Most interestingly, we observe a significant association between high percentage of peripheral Tregs and worse DFS and OS, which has also been shown in previous studies on malignant melanoma and other solid cancers (Gerber et al., 2014; Shang et al., 2015).

Much attention has been drawn towards the immunosuppressive HLA-G protein and its potential as an immune checkpoint. Clinical phase I trials with an HLA-G inhibitor used for treatment of patients with solid tumours have been initiated (CancerDiscovery, 2020). Similar to previously reported results, HLA-G-expressing T cells account for 1.6% of total $CD4^+$ T cells and 3.3% of total $CD8^+$ T cells in peripheral blood of healthy individuals in our study (Carosella et al., 2015). However, there was no difference in HLA-G expression on peripheral T cells from malignant melanoma patients compared with controls and no associations with survival outcomes in the current study. Differences in HLA-G expression might be more prominent locally in the tumour.

Our results showed a reduced level of $CD56^{dim}CD16^+$ NK cells in patients compared with controls in accordance with a published study (Ali et al., 2014). However, no significant difference was observed for $CD56^{bright}CD16^-$ NK cells. In previous studies, frequencies of circulating and tumour-infiltrating $CD56^{dim}$ and $CD56^{bright}$ NK cells have been found to be unaltered between healthy control and patients independent of disease stage and are suggested to have limited cytotoxic potential (Mirjacic Martinovic et al., 2014; Fregni et al., 2013). One study has shown a negative correlation between patient survival and circulating $CD56^{bright}CD16^-$ NK cells in malignant melanoma patients (de Jonge et al., 2019). No association between NK cell subtypes and survival was observed in the current study. NKG2C expression may characterize a distinct subtype of NK memory cells, and NK memory cells may be important in cancer development (Keppel et al., 2013; Pal et al., 2017; Sabry et al., 2018). In the current study, we observe a significant higher level of $CD56^{bright}CD16^-KIR3^+$ cells in patients compared with controls and a tendency towards fewer $CD56^{dim}CD16^+$ $KIR3^+$ cells. Furthermore, the KIR3 expression level on $CD56^{bright}CD16^-$ cells was associated with stage but not with survival. Previous studies have reported increased levels of KIR3DL in the total NK cell population in peripheral blood of pancreatic, gastric, colon, and kidney cancer patients (Peng et al., 2013; Al Omar et al., 2010). These studies did not investigate whether expression differed between $CD56^{dim}$ and $CD56^{bright}$ NK cell subtypes. The antibody used in the current study are specific for both inhibitory receptor KIR3DL1 and activating receptor KIR3DS1 (Shimasaki et al., 2020). The presented results show no significant differences in expression of ILT2 or NKG2C receptors on $CD56^{bright}CD16^-$ and $CD56^{dim}CD16^+$ NK cells, and the expression was not associated with disease stage or with survival. Messaoudene et al. also showed comparable levels of NKG2C expression on NK cell subtypes in metastatic melanoma

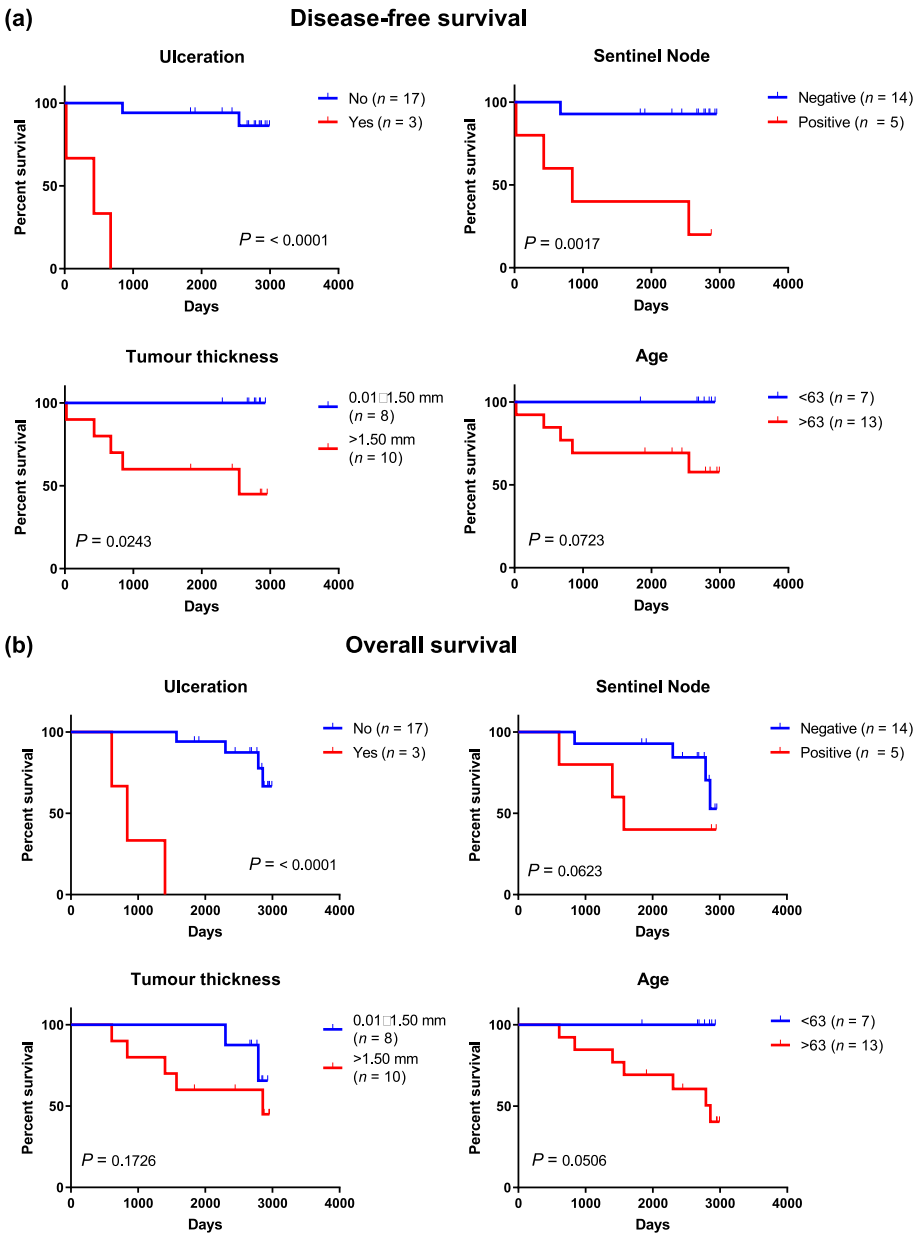

Fig. 4. Disease-free survival and overall survival analysis of known clinical prognostic predictors. (a) Disease-free survival or (b) overall survival analysis of clinical parameters for malignant melanoma patients including ulceration (no, yes), sentinel node status (negative, positive), tumour thickness (below or above 1.50 mm), and age (divided based on the median age at diagnosis). Survival rates were estimated according to the Kaplan-Meier method. *P*-values are indicated on the graph for each analysis.

(Messaoudene et al., 2014).

Strengths of the present study are simultaneous investigations of T and NK cells with an extensive characterization of different phenotypes and comparisons with a healthy control group. Furthermore, the follow-up time for survival analyses was long. A larger cohort of malignant melanoma patients would have provided the opportunity to investigate the presented immune cell phenotype more extensively in relation to clinical parameters.

In addition to the well-described approaches and use of immune checkpoint inhibitors against CTLA-4 and PD-1, therapies targeting inhibitory receptors NKG2A, ILT2 and KIRs have also been suggested (Khan et al., 2020). Blocking of NKG2A inhibition of T cells and NK cells may hold great prospects in multiple hematological malignancies and are currently under clinical trial in advanced gynaecological cancers showing great potential (Khan et al., 2020; Tinker et al., 2019). Whether success of therapy response in cancers can be predicted using peripheral immune profiles will be interesting to elucidate further.

5. Conclusion

In summary, the study confirms that a high percentage of Tregs is correlated with poor OS and DFS. The percentage of peripheral $CD56^{bright}CD16^{-}$ NK cells is increased in malignant melanoma patients and correlated with late stage malignancy. The peripheral cytotoxic $CD56^{dim}CD16^{+}$ NK cell subsets was reduced in malignant melanoma patients compared with healthy controls. Changes in the peripheral immune cell profile of malignant melanoma patients at the time of diagnosis may be potential biomarkers for prognosis and survival. The mechanisms of immune regulation promoted by this immune profile should be elucidated in future studies.

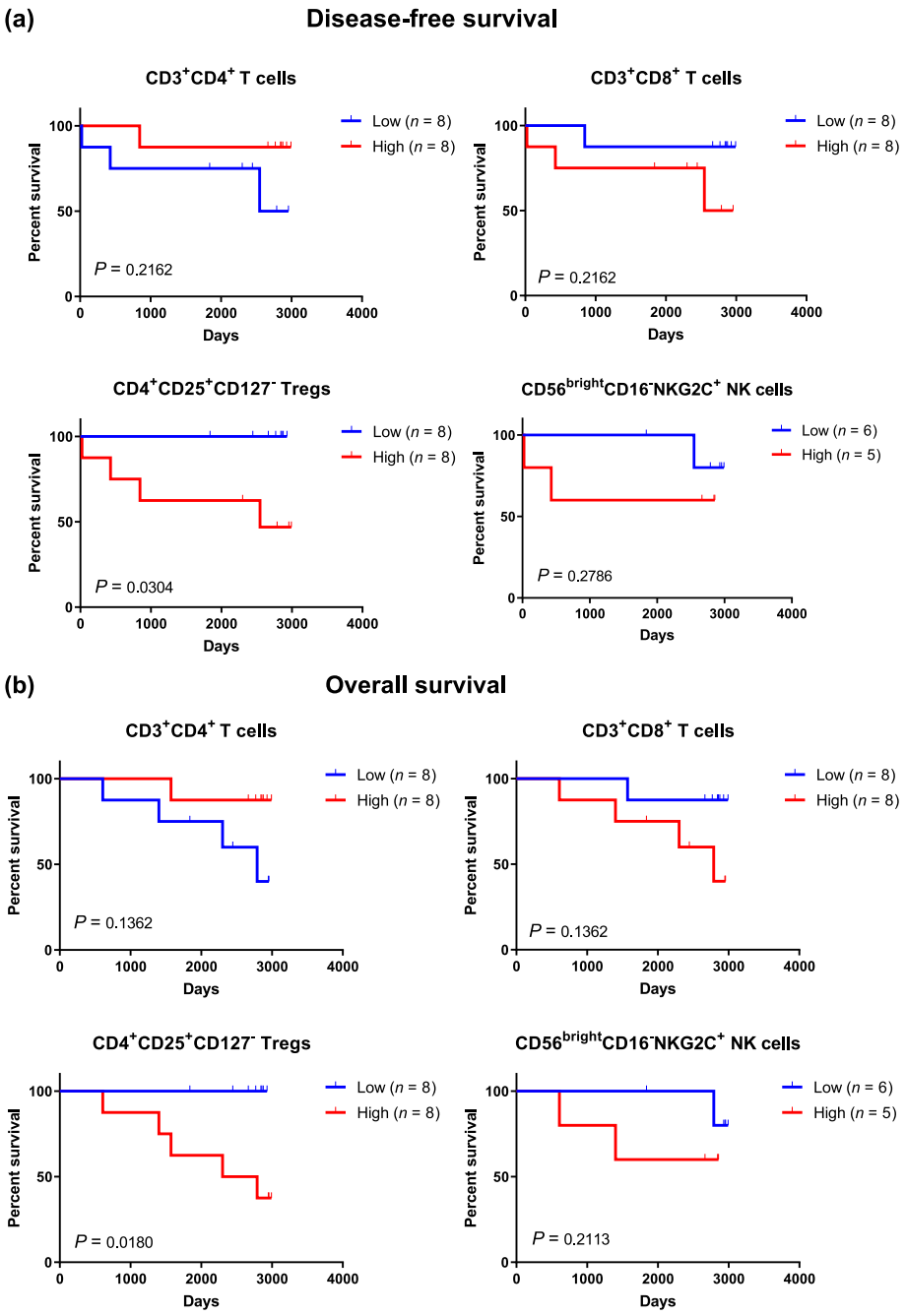

Fig. 5. Disease-free survival and overall survival analysis of T cell and NK subsets in peripheral blood of malignant melanoma patients. (a) Disease-free survival or (b) overall survival analyses comparing low vs. high percentage of $CD3^{+}CD4^{+}$ T cells, $CD3^{+}CD8^{+}$ T cells, Tregs defined as $CD4^{+}CD25^{+}CD127^{-}$ cells, and $CD56^{bright}CD16^{-}NKG2C^{+}$ NK cells in peripheral blood samples collected within one month from excision of the tumour from malignant melanoma patients. Based on flow cytometric analyses. Groups of low and high percentage were divided based on the median expression values for each of the immune cell phenotypes. Survival rates were estimated according to the Kaplan-Meier method. *P*-values are indicated on the graph for each analysis.

CRediT authorship contribution statement

Nanna Heldager Pedersen: Data curation, Formal analysis, Visualization, Writing – Original Draft, Writing – Review & Editing. **Helene Bjerregaard Jeppesen**: Investigation, Data curation, Formal analysis, Writing – Original Draft. **Gry Persson**: Methodology, Supervision. **Sophie Bojesen**: Investigation, Data curation. **Thomas Vauvert F. Hviid**: Conceptualization, Methodology, Supervision, Project administration, Funding acquisition, Formal analysis, Writing – Original Draft, Writing – Review & Editing.

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Availability of data and materials

The data and material used and analyzed in the study are available from the corresponding authors upon reasonable request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The authors do not have permission to share data.

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Appendix A. Supplementary data

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