Ageing and immunity: unraveling the association between immunosenescence and frailty

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Blood-based immune biomarkers associated with Clinical Frailty Scale in older patients with melanoma receiving checkpoint inhibitor immunotherapy

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ABSTRACT

Introduction

Immunotherapy with checkpoint inhibition (ICI) is increasingly prescribed to older patients with cancer. High age, especially in combination with frailty, has been associated with immune senescence, which is the age-related decline in immune function, thereby possibly hindering ICI effectiveness. This cross-sectional study aimed to assess whether blood cell immune senescence markers are associated with age, frailty and response to anti-PD-1 treatment in older patients with metastatic melanoma.

Methods

In a prospective observational study, sixty patients with stage IIIC or IV melanoma undergoing anti-PD1 treatment were categorized into young (<65 years; n=22), old (>65 years) without frailty (n=19), and old with frailty (n=19). In-depth immune cell phenotyping was performed in baseline blood samples (prior to treatment) using multispectral flow cytometry and compared between groups and with immunotherapy treatment response. Antigen-presenting cell capacity was evaluated using mixed lymphocyte reaction and T cell proliferative potential was assessed using PHA proliferation assay.

Results

No significant differences in treatment response rates were observed across age groups. Older patients, irrespective of frailty, showed lower levels of naïve CD8+ T cells, with the old and frail group also exhibiting reduced tissue-resident effector memory CD8+ T cells and CD8+ mucosal-associated invariant T (MAIT) cells. These differences were not associated with treatment outcomes. T cell proliferation and antigen-presenting cell capacities did not differ across groups.

Conclusion

Several ageing and frailty-associated changes were detected among circulating immune cells in blood, but were not associated with response to immunotherapy in our study. While these findings suggest that the level of frailty and ageing may not necessarily preclude the efficacy of ICI therapy, further investigation is needed to fully understand the impact of frailty and ageing on immunotherapy.

INTRODUCTION

The incidence of melanoma has rapidly increased over the past decades. In 2022, there were 331 722 cases worldwide and over 8000 cases in the Netherlands (1, 2). Of the worldwide newly diagnosed melanoma patients, 66% is 60 and 29% is 75 years or older, respectively (2). In recent years, immunotherapy with immune checkpoint inhibition (ICI) has become a promising treatment for various cancers, including advanced melanoma. Immunotherapy has significantly extended patient survival, particularly with the use of ipilimumab and nivolumab combination (3). Recent studies demonstrated that high age is associated with deterioration of the immune system, called immune senescence (4-6). One of the contributors to immune senescence is the shrinkage of the thymus and bone marrow, as well as skewing of immune cells to the myeloid lineage, resulting in substantial changes in diverse immune compartments. This is reflected by a progressive decline in the frequency of naïve T cells along with the accumulation of terminally-differentiated memory T cells, which was shown to induce melanoma growth and metastasis. Moreover, T cells express an immunosenescent phenotype characterized by reduced expression of CD27 and CD28 and higher expression of CD57, a major marker of immunosenescence. This immunosenescent phenotype has been associated with resistance to immunotherapy treatment (7) (Figure S1). Additionally, there is a decline in the transition from stem cell to subsequent pro-B and pre-B cell stages, resulting in decreased numbers of peripheral B cells exported from the bone marrow, which may compromise the humoral immunity in older patients (8). Simultaneously, myeloid-derived suppressor cell (MDSCs) numbers increase with ageing (9, 10). These cells may suppress the priming and reactivation of antigen-specific immune cell responses.

Importantly, several studies suggest that immune senescence is linked with frailty, a clinical state characterized by a decline in functioning across multiple physiological systems, accompanied by increased vulnerability to stressors resulting in high risk of poor health outcomes, incident disability, hospitalization and mortality (11). Individuals can be categorized as robust, pre-frail, or frail, based on the extent of their physiological and functional impairments. Frailty can result from multiple factors, including socio-demographic aspects (such as poverty, living alone, and low educational levels), psychological issues (like depression), nutritional deficiencies (such as malnutrition), polypharmacy, and chronic diseases (including inflammatory conditions, cancer, endocrine disorders, and dementia), as well as low levels of physical activity. Frailty is recognized as a dynamic condition that fluctuates over time. As frailty exists on a continuum, minor differences in frailty scores can impact patient outcomes. Emerging research shows that an elevated lymphocyte count has

been associated with frailty and especially with low physical activity and grip strength (4). Additionally, it was shown that lower frequencies of naïve CD4+ T cells and higher proportion of central memory CD8+ T cells were predictive of higher scores of the frailty index (12). It is possible that the efficacy of ICI, which relies on the initiation and reactivation of tumor-specific immunity, may be hampered due to frailty (13). Only a few studies have investigated the relation between immunological ageing, frailty and immunotherapy efficacy (14-18). These studies suggest that the ageing of the immune system compromises the adaptive immune response, particularly affecting T cells, which may reduce the effectiveness of immunotherapy (19). While previous studies showed no significant differences in efficacy or side effects of ICIs between young and older patients, it is important to note that these studies did not take differences between patients in terms of frailty or immunological ageing into account (20). Balancing the potential benefits of treatment, such as an increase in survival or a reduction of symptoms, against potential harms, including adverse side effects and risk of complications for older patients, is crucial during the decision-making process. Thus, it is important to identify better biomarkers of response to immunotherapy in older adults, as frailty can increase the impact of potential adverse events and hamper the eligibility of patients for immunotherapy. The aim of the exploratory study is to identify markers of immune senescence in older patients with metastatic melanoma, their association with calendar age and frailty, and to determine whether these markers of immune senescence are associated with clinical response to anti-PD-1 treatment.

METHODS

Study population

The present cohort included a selection of patients from the prospective tumor-specific T-Cell IMMunity in patients with solid tumors study (TCIMM study). This prospective observational cohort study aimed to understand the immune factors related to the efficacy and side effects of immunotherapy in treated cancer patients by performing an in-depth analysis of systemic and intra-tumoral immune parameters using blood, tumor, intestinal and faecal samples.

The TCIMM study included patients aged 18 years or older, with a histological or cytological confirmed solid tumor, who received immunotherapy between 2015 and 2023 and had a WHO score of 0-2 at the time of study entry. Written informed consent was obtained from all participants. Patients presenting with severe anaemia (Hb < 6.0 mmol/L), human immunodeficiency virus (HIV) or chronic hepatitis B or C infection were excluded for safety reasons. Peripheral blood samples were drawn

at baseline (prior to treatment), during treatment and after treatment at 1 month, 3 months and 6 months. The study was approved by the Medical Ethics Committee of Leiden University Medical Center (Committee of Medical Ethics; NL59959.058.17). All patients signed informed consent.

For the current study, patients were eligible if they had irresectable stage IIIC or stage IV melanoma and started anti-PD1 treatment as first line monotherapy. In total, 60 patients were included. For the immunological analyses, patients were randomly selected, to avoid any potential sources of bias, and divided in 3 almost equally large groups of patients composed of young patients (<65 years) (n=22), old patients without frailty (n=19) and older patients with frailty (both >65 years) (n=19).

Frailty definition

The Clinical Frailty Scale (CFS) was used to define frailty in the cohort and was collected retrospectively. The clinical frailty scale is a 9-point scale that quantifies frailty based on function in individual patients. It is complemented by a visual chart to assist with the classification of frailty. Higher scores indicate increased frailty and associated risk (21). The validity of retrospective CFS assignment has been demonstrated in various studies (22-25). Patients with a CFS score of 4 or higher were classified as pre-frail or frail, thus categorized in the “older patients with frailty” group. Considering the relatively fit nature of the cohort with only few patients with a CFS of 5 or higher, a CFS cut-off of 4 instead of 5 was used to define frailty. However, previous studies have employed a CFS cut-off of 4 to define frailty, with the frail patients experiencing poorer outcomes, supporting the validity of our approach (26). Patients with a score of 0-3 were classified as non-frail.

Clinical data

All clinical data were registered from medical charts. This information about patient and tumor characteristics included comorbidity, defined by the Charlson Comorbidity Index (CCI) (27), tumor characteristics (superficially spreading, acro lentiginous, desmoplastic, nodular, lentigo maligna), stage of disease, and detailed information on ICI treatment as well as previous treatment (with a BRAF inhibitor, type of anti-PD-1 treatment) and outcome.

Response outcomes

Imaging assessment, including CT or PET CT or MRI (in case of cerebral metastases), was performed after 3 months and 6 months of treatment, or in some cases earlier if there were clinical signs of progression before these timepoints. Responses were evaluated according to the RECIST 1.1. For the current study, the radiological

response to treatment was categorized as follows: a partial or complete response observed at 3 months. Should the disease remain stable or present a mixed response at the 3-month time point, assessment was deferred to the 6-month mark. A partial or complete response at 6 months, or the persistence of stable disease at this time point, was classified as a response to treatment. In case progression occurred after either 3 or 6 months, the patient was classified as a non-responder to treatment.

Sample collection

The blood sampling of patients on anti-PD-1 immunotherapy was done at baseline as part of the study "Longitudinal analysis of tumor-specific T-cell immunity in patients with solid tumors" (NL59959.058.17). 100mL of blood from patients prior to immunotherapy was collected in sodium heparin tubes (BD Bioscience, Drachten, the Netherlands) and processed within 6 hours upon withdrawal. Peripheral blood mononuclear cells (PBMCs) were isolated and cryopreserved. Buffy coats from three healthy donors, after informed consent, was obtained from Sanquin (Leiden, the Netherlands) and the isolated PBMCs were used as third parties for the mixed lymphocyte reaction (MLR) assay (28).

Isolation of white blood cells

Viable PBMCs were purified by Ficoll (LUMC pharmacy, Leiden, the Netherlands) density gradient separation, washed with Phosphate buffered saline (PBS; Fresenius Kabi, Huis ter Heide, the Netherlands), cryopreserved in 80% fetal calf serum (FCS; Serana Europe, Pessin, Germany) and 20% dimethyl sulfoxide (DMSO; WAK-Chemie Medical, Steinbach, Germany) and stored in the vapor phase of liquid nitrogen until further use (28). The handling, immune assays and analysis of the PBMCs were done according to the standard operation procedures (SOPs) of the Leiden department of Medical Oncology by trained personnel (29).

Immune assays

The immune profiles between the 3 groups of patients in blood samples taken at baseline were compared, and it was investigated whether the obtained immune cell populations were associated with a response to the applied immunotherapy. We performed the following analyses.

Immunophenotyping of PBMCs

The cryopreserved PBMC samples were thawed in IMDM + 10% FCS, washed with Iscove's Modified Dulbecco's *Medium* (IMDM, Thermo Fisher Scientific, Eindhoven, the Netherlands) + 10% FCS and counted according to standard SOP, as published

previously (28). The samples of the 3 above-mentioned patient groups were divided equally between the 3 staining runs.

Immunophenotype of the PBMC was assessed by multispectral flow cytometry (AURORA, Cytek Biosciences, Amsterdam, the Netherlands) staining using our previously described 40-marker panel (28). The PBMCs were first stained with 1:2400 diluted LIVE-DEAD zombie UV fixable amine-reactive dye (Biolegend Europe, Amsterdam, the Netherlands) at room temperature (RT) for 20 minutes, after which the cells were washed with FACS buffer consisting of PBS+0.5% Bovine serum albumin (BSA, Sigma, St Louis, USA), and subsequently incubated with 50 µl PBS/0.5%BSA/5%Trustain FcX blocking solution (Biolegend) for 10 minutes on ice to block Fc receptors. Next, the cells were stained for 30 minutes at RT and in the dark with the cell surface antibodies in two consecutive rounds with three times washing with FACS buffer in between. Intracytoplasmic/nuclear staining was performed using the True-nuclear Transcription Factor Buffer set (Biolegend) according to manufacturers' instruction. Details on antibodies, titers and unmixing are listed in Table S1. After staining the cells were washed twice and stored in FACS buffer. Acquisition was done within 24 hours on a 5-laser Aurora CytekTM spectral analyzer (Cytek Biosciences). High-dimensional single cell data analysis was performed by opt-Distributed Stochastic Neighbor Embedding (optSNE) dimensionality reduction followed by FLOWSOM consensus metaclustering using the cloud-based OMIQ data analysis software (OMIQ, Boston, USA). OptSNE/FLOWSOM analysis were performed on the total CD45+ immune cell population, as well as on cellular subsets (T cells, natural killer (NK) cells, B cells and myeloid cells after gating on CD3+, CD3-CD56+, CD19+ and CD3-CD56-CD19-remaining cells, respectively). The different cell populations were visualized and quantified. Expression levels of each of the indicated markers were depicted for the individual cell populations and frequencies of CD3 T cells, NK cells, B cells and myeloid cells, and remaining cells were shown as percentage of total CD45.

Mixed Lymphocytes Reaction assay

The antigen-presenting capacity of PBMCs was determined in a Mixed Lymphocyte Reaction (MLR) assay as published previously (28, 30). The MLR assay is based on a third-party (allogeneic) T cell proliferation response. PBMCs from three healthy donors served as third parties for the MLR assay. The MLR assays were carried out in triplicate wells in round-bottomed 96-well plates to ensure efficient third-party PBMCs/patient APC (within PBMCs) contact. Irradiated PBMCs alone, as well as third-party PBMCs alone, were used as negative controls. Proliferation of cells was measured by the addition of ^{3}H-thymidine (50ul/ well, stock 10 µCi/mL, Perkin Elmer, Boston, USA) for 16-18 hours at 37°C, whereafter the cells were harvested on

MicroBeta glass fiber filter paper (Perkin Elmer). Incorporation of ^{3}H-thymidine was determined on a Wallac MicroBeta TRILUX 1450 LSC & Luminescence counter (Perkin Elmer). The proliferation of the third-party PBMCs is expressed as the stimulation index (SI) calculated as the ratio of the counts per minute of ^{3}H-thymidine in MLR co-culture to that in the third-party only (control) culture. A threshold of at least 3 is defined as a positive response. The number of positive responses out of the tested three third parties, as well as the strength of response (SI), were determined.

Phytohemagglutinin (PHA) proliferation assay

The proliferative potential of PBMCs was evaluated in a proliferation assay [26], using PHA stimulation and ^{3}H-thymidine incorporation (as described above). PBMCs from patients were cultured in quadruplicate wells in the presence or absence of PHA (1 µg/ml). The SI was calculated as the ratio of lymphocytes cultured with PHA over those of the unstimulated control cultured lymphocytes. To discard the outliers, we determined the mean (m) of the value of the replicate wells, then determined the most distant value (data point x). Next, we calculated the mean (m) and the standard deviation (s) of the replicate wells, excluding this data point x. We considered x an outlier when $x > m + 3s$ or $x < m - 3s$. Finally, the mean was recalculated excluding the outlier point x.

Statistical analysis

Statistical evaluation was performed using the statistical package SPSS version 25.0 and Graphpad version 9.3.1. First, the baseline clinical characteristics between the three groups of patients (young, old without frailty and old with frailty) were compared using chi-square tests. Second, the differences between the cell populations as identified by OMIQ analyses between the three groups were assessed. Cell population data were not normally distributed. All cell populations were presented as a percentage of the total CD45. For this, as well as for the PHA assay data, the Kruskal-Wallis tests were used. The MLR assay data were analyzed using Chi-square tests. Third, the association between the presence of frailty and response to treatment was assessed using the Chi-square for trend tests. Finally, the association between the cell populations and response to treatment was determined using Mann-Whitney tests. A p-value of <0.05 was defined as statistically significant.

Laboratory environment

Immunomonitoring of patients' PBMCs was performed in the laboratory of the department of Medical Oncology (LUMC) that operates under research conditions but uses standard operation procedures for all tests, with pre-established definitions of positive responses and trained personnel. This laboratory has been externally

and internally audited according to the reflection paper for laboratories that perform immunomonitoring and participated in all proficiency panels of the CIMT Immunoguiding Program (CIP; of which SHvdB and MJPW are

steering committee members; http://www.cimt.eu/workgroups/cip/) as well as many of the proficiency panels (including ICS gating and ELISPOT plate reading panels) of the USA-based Cancer Immunotherapy Consortium (CIC of the Cancer Research Institute) to validate its standard operating procedures (SOPs) (29).

RESULTS

Patient characteristics are summarized in Table 1. Sixty patients were enrolled in this study, including 22 patients < 65 years (young patients), 19 patients ≥ 65 years (old patients) without frailty and 19 patients ≥ 65 years and frail (old with frailty). The distribution of the CFS score in patients with frailty is displayed in Figure S2. The median age of young patients was 61 (interquartile range IQR 55-64), of old patients without frailty 73 (IQR 70-77) and of old patients with frailty 76 (IQR 72-81) years. Forty-three patients were male (71.7%). Fifty-nine patients had stage IV melanoma (98.3%) and one patient had an irresectable stage IIIC melanoma (1.7%). Sixteen patients had brain metastases (26.7%). Among the thirty-two patients with a BRAF mutation (53.3%), eight had previously received BRAF treatment. They were distributed equally across the three groups (n=2 in the young patient group, n=3 in the older patient group without frailty, and n=3 in the older patient group with frailty).

Twenty-four patients had a WHO score of 0 (40%), and twenty-nine had a WHO score of 1 (48.3%). Forty-five patients had a Charlson Comorbidity Index (CCI) of 0 (75%), thirteen patients had a score of 1 (21.7%), and three patients had a score of 3 (3.3%). Except for the WHO score and age, no major differences were observed between the three patient groups.

Table 1. Baseline patient characteristics

	All patients		<65 years		≥65 non-frail		≥65 (pre)-frail		p-value
Age, median (IQR)	70	(63-75.8)	61	(55-64)	73	(70-77)	76	(72-81)	
Sex, n (%)									
Male	43	(71.7)	15	(25)	15	(25)	13	(21.7)	0.696
Female	17	(28.3)	7	(11.7)	4	(6.7)	6	(10)	
WHO-performance status, n (%)									
0	24	(40)	13	(21.7)	9	(15)	2	(3.3)	0.008
1	29	(48.3)	8	(13.3)	6	(10)	15	(25)	
2	1	(1.7)	0	0	0	0	1	(1.7)	
Unknown	6	(10)	1	(1.7)	4	(6.7)	1	(1.7)	
Charlson score, n (%)									
0	45	(75)	19	(31.7)	12	(20)	14	(23.3)	0.491
1	13	(21.7)	3	(5)	6	(10)	4	(6.7)	
2	0	0	0	0	0	0		0	
3	2	(3.3)	0	0	1	(1.7)	1	(1.7)	
Stage, n (%)									
IIIC	1	(1.7)	0	0	0	0	1	(1.7)	0.334
IV	59	(98.3)	22	(36.7)	19	(31.7)	18	(30)	
BRAF mutation, n (%)									
No	26	(43.3)	7	(11.7)	11	(18.3)	8	(13.3)	0.072
Yes	32	(53.3)	15	(25)	6	(10)	11	(18.3)	
Unknown	2	(3.3)	0	0	2	(3.3)	0	0	
Brain metastases, n (%)									
No	44	(73.3)	17	(28.3)	14	(23.3)	13	(21.6)	0.815
Yes	16	(26.7)	5	(8.3)	5	(8.3)	6	(10)	

	All patients		<65 years		≥65 non-frail		≥65 (pre)-frail		p-value
Previous treatment BRAF inhibitor, n (%)									
No	52	(86.7)	20	(33.3)	16	(26.7)	16	(26.7)	0.763
Yes	8	(13.3)	2	(3.3)	3	(5)	3	(5)	
LDH (U/L), n (%)									
Normal (<250 U/L)	39	(65)	17	(28.3)	11	(18.3)	11	(18.3)	0.299
Elevated (>250 U/L)	18	(30)	4	(6.7)	7	(11.7)	7	(11.7)	
Missing	3	(5)	1	(1.7)	1	(1.7)	1	(1.7)	

Table 1. Patients baseline characteristics stratified into 3 groups: <65 years, young patient group; > 65 years non-frail, old patients without frailty group; > 65 years (pre)-frail, old patients with frailty group. Baseline characteristics differences between the three groups of patients were assessed using Chi-square tests.

Association between immune cell composition and calendar age and frailty

To study potential age- and/or frailty-related changes in the immune cell composition in metastatic melanoma patients, an in-depth immunophenotyping of pre-therapy isolated PBMCs was performed for 60 patients using a 40-marker spectral flow cytometry panel (Figure S3, Table S1). Simple optSNE dimensionality reduction analysis of the total CD45 population with the lineage markers CD3, CD19, CD56, and CD14 (Figure 1A-B) revealed the lowest frequencies of total B cells in old patients with frailty (p=0.0477) and a trend towards higher frequencies of total NK cells in old patients irrespective of frailty. No significant differences in the total frequency of CD3+ T cells and myeloid cells among the 3 patient groups were observed (Figure 1C).

To get insight into the phenotype and subsets of the circulating immune cell lineages and their association with age and frailty, pre-gated T cells, NK cells, B cells and myeloid cells were subjected to further detailed optSNE-FlowSOM cluster segmentation.

Analysis of the CD3+ T cells revealed 19 different subpopulations (Figure 2A), of which populations 10 and 17 were reduced in old patients with frailty (p=0.0234 and p=0.0008, respectively) and population 11 in old patients without and with frailty (p=0.0031 and p=0.0009, respectively) (Figure 2B). Population 11 comprises naïve CD8+CD45RA+CCR7+CD27+CD127+CD28+ T cells, and populations 10 and 17 comprise CD8+CD45RA-CCR7-CD45RO+CD103+ tissue resident-like effector memory T cells and CD8+CD45RO+KLRG-1+CD161++ mucosal-associated invariant T (MAIT) cells, respectively (Figure 2C).

Figure 1. CD45 cell populations

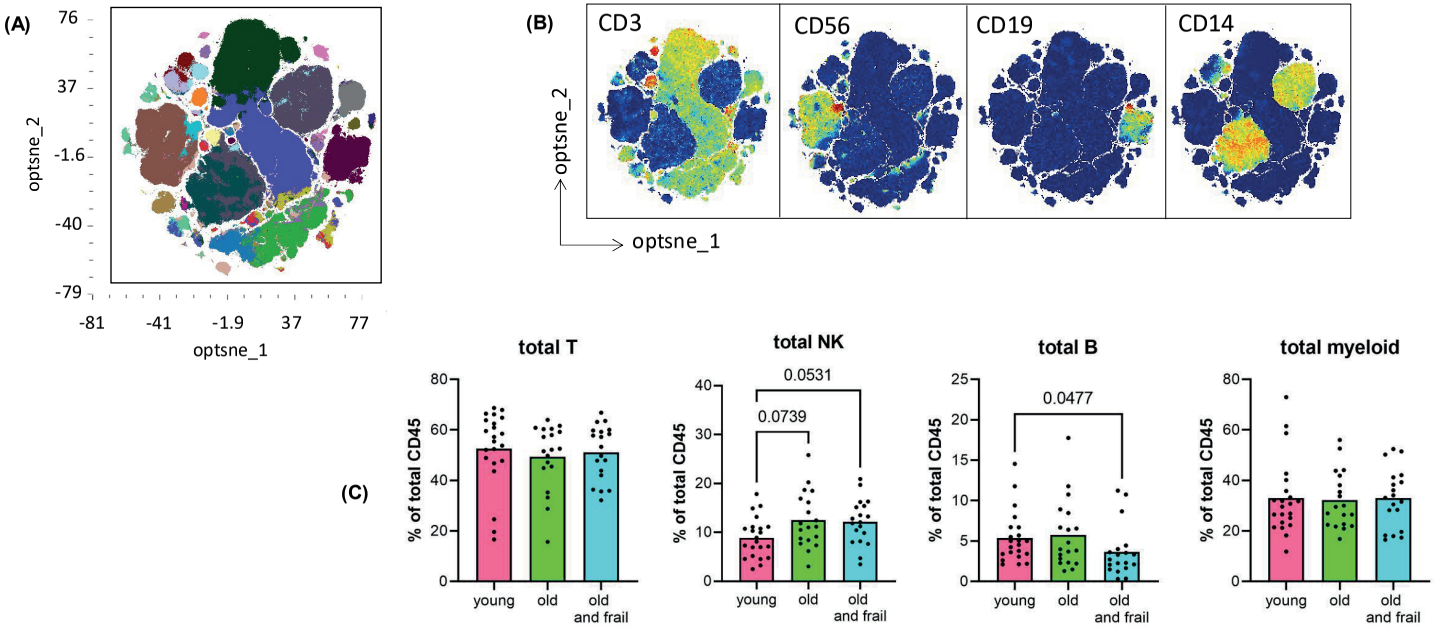
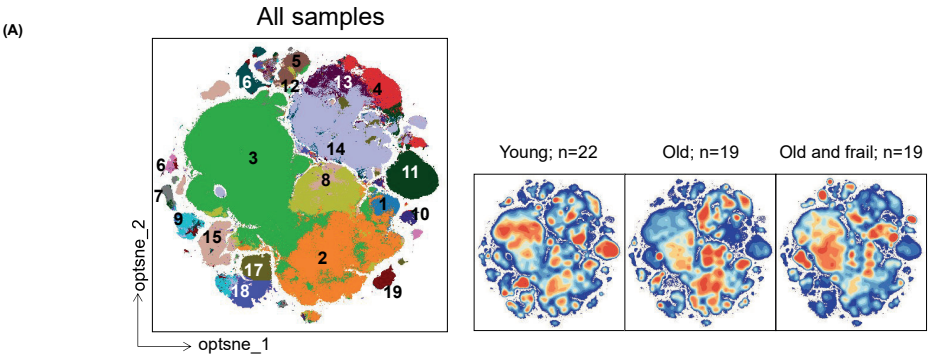
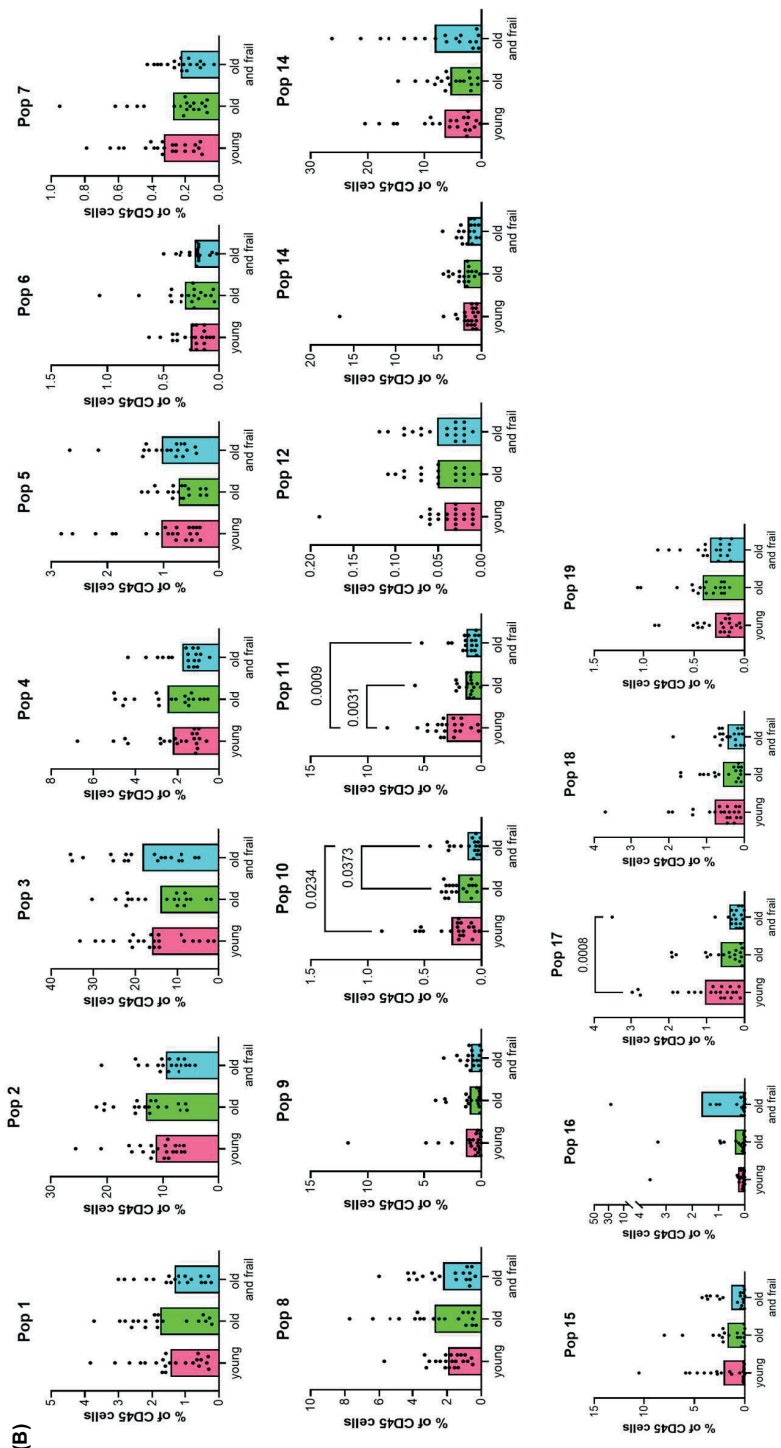


Figure 1. (A) Cluster partitions by FLOWSOM. (B) OptSNE plots visualizing contour plots. Contour plots show the staining intensity of individual cell markers used. (C) Frequencies of total T-cells, total NK cells, total B-cells, and total myeloid cells in young, old and old-frail patient groups. Cell populations are presented as a percentage of the total CD45 cells. Statistical differences were assessed with Kruskal-Wallis tests and $p<0.05$. Young, young patient group; old, old patient without frailty group; old and frail, old patients with frailty group.

Figure 2. CD3+ T cell populations





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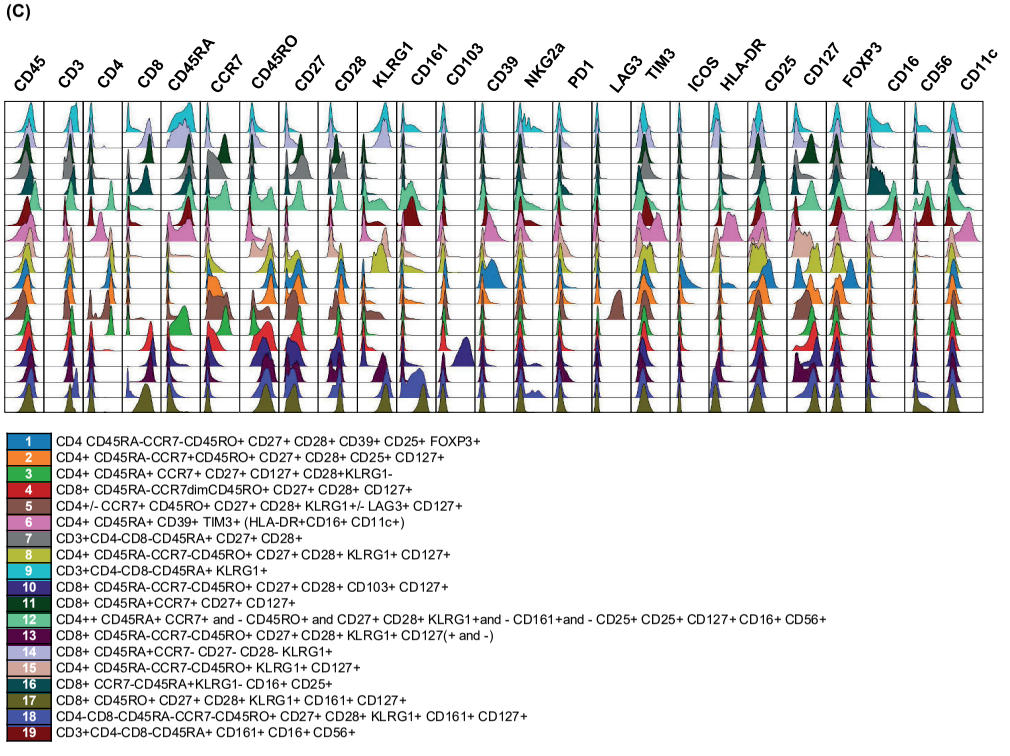


Figure 2. (A) Cluster partitions by FLOWSOM of PBMCs stained with antibodies for CD3+ T-cell markers. In total, 19 different clusters were defined (left). OptSNE plots visualizing contour plots of the 3 patient groups (right). (B) Frequencies of T cell populations in young, old and old-frail patient groups. Cell populations are presented as a percentage of the total CD45+ cells. Statistical differences were assessed with Kruskal-Wallis tests and $p<0.05$. (C) Expression levels of each of the indicated markers are depicted for the individual immune cell populations.

Sub-clustering of the NK cell, myeloid cell, and B cell populations revealed differences between the 3 patient groups only for the B cell subpopulations (Figures S4-S6). The CD19+ B cells comprised 8 different subpopulations (Figure S6A), of which populations 31 and 34 were reduced in both old and frail patients (Figure S6B). Population 31 (CD19+ HLADR+ CD27- CD86- CCR7+ CD45RA+ CD39dim) and population 34 (CD19+ HLADR+ CD27- CD86- CCR7+ CD45RA+ CD39dim CD1c+) both expressed CCR7, which in previous studies was shown to be expressed during B cell development [27,28], suggesting that these are naïve B cells.

No clear difference in antigen-presenting cells (APC) capacity upon ageing and frailty.

To evaluate the capacity of APCs to stimulate T cell responses upon ageing and frailty, a mixed lymphocyte reaction (MLR) assay was performed (Figure 3 and Table 2). In the young patient group, the APCs of 3 out of 22 patients (13.6%) were not able to induce T cell proliferation of any of the third-party donor PBMCs compared to 5 out of 19 (26.3%) in the old patient group without frailty and 6 out of 19 (31.6%) in the old patient group with frailty. Yet, these differences were not statistically significant (p=0.37). Also, not when the number of positive proliferation responses against the three different allogeneic PBMC donors was compared (p=0.73). In the young patient group, 15 (68.2%) patients had a proliferation response to ≥ 2 donors, compared to 11 (57.9%) patients in both old patient groups.

Figure 3. Antigen-presenting cell responses in young, old non-frail and old-frail patients

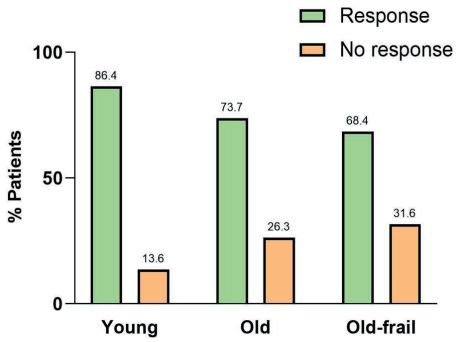


Figure 3. Mixed Lymphocytes Reaction assays were performed to evaluate the antigen-presenting cells (APCs) capacity based on a T-cell proliferative response of healthy donor PBMCs. The results for the MLR were expressed in terms of cell proliferation quantified by the stimulation index (SI). SI≥3 indicated a positive response and SI<3 indicated no response. Statistical differences were assessed using Chi-square tests and p<0.05.

Table 2. Antigen-presenting cells responses in young, old non-frail and old-frail patients

	All patients	Young	Old	Old-frail	p-value
Age (median, IQR)	70 (63-75.8)	61 (55-64)	73 (70-77)	76 (72-81)	
Stimulation Index, n (%)					
Yes	46 (76.7)	19 (86.4)	14 (73.7)	13 (68.4)	0.37
No	14 (23.3)	3 (13.6)	5 (26.3)	6 (31.6)	
Number of responses, n (%)					
≥2	37 (61.7)	15 (68.2)	11 (57.9)	11 (57.9)	0.73
<2	23 (38.3)	7 (31.8)	8 (42.1)	8 (42.1)	

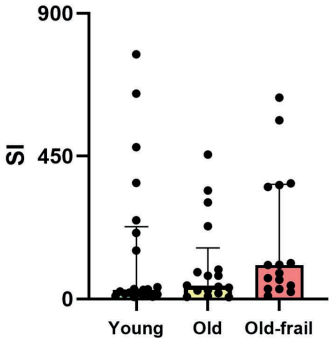
Abbreviations: SI, stimulation index; n, number; IQR: interquartile range

Table 2. Mixed Lymphocytes Reaction assays were performed to evaluate the antigen-presenting cells (APCs) capacity based on a T-cell proliferative response of healthy donor PBMCs. The results for the MLR were expressed in terms of cell proliferation quantified by the stimulation index (SI). SI≥3 indicated a positive response and SI<3 indicated no response. Statistical differences were assessed using Chi-square tests and p<0.05.

No clear difference in PBMCs proliferative capacity upon ageing and frailty.

To evaluate the proliferative capacity of T cells, PBMCs were stimulated with PHA (Figure 4). No significant differences were observed between the three patient groups, although a trend was observed of a stronger proliferation from young to old and frail old patients. The median (IQR) SI in the young patient group was 29 (95% C.I. 12-228) compared to 42 (95% C.I. 22-161) in the old patient group without frailty and 107 (95% C.I. 37-361) in the old patient group with frailty (p=0.09).

Figure 4. Antigen-presenting cells responses in young, old non-frail and old frail patients



SI PHA	Young	Old	Old-frail	p-value
Median (IQR)	28.51 (12.23-227.93)	42.47 (22.44-160.89)	107.09 (37.18-361.37)	
Mean (SD)	28.51 (228.13)	109.5 (136.43)	260.04 (370.09)	0.09

Abbreviations: SI, stimulation index; IQR, interquartile range; SD, standard deviation

Figure 4. PHA proliferation assays were performed to evaluate the patients' lymphocytes' proliferative potential. Statistical differences were assessed using Kruskal-Wallis tests and p<0.05.

Immune correlates to clinical response

Since there was no difference in the clinical response of young and old (frail or not) patients, we divided the patients into two groups based only on their clinical outcome in order to determine whether there is a difference in the immune profile between clinical responders and non-responders. While the different T, B, and NK cell populations did not vary substantially between the responders and non-responders, we did observe a correlation between clinical response and the myeloid subpopulations (Figures S7-S10).

In the T cell subpopulations, population 6 was significantly associated with treatment response (p=0.0368) (Figure S7). This cell population comprises both T cell and myeloid markers and was found to be CD4+CD45RA+ CD39+ CD68+ TIM3+ (HLA-DR+CD16+CD11c+). Emerging studies have demonstrated the expression of CD4 in myeloid progenitor cells, and therefore, this cell population is defined as a CD4-expressing myeloid cell subset [29].

Analysis of the frequencies of myeloid cell populations in relation to treatment responses revealed significant differences in cell population 40, with lower frequencies in patients with a clinical response (p=0.0491). Both cell populations 46 (p=0.0461) and 47 (p=0.017) were higher in these responding patients. Population 40 expressed HLA-DR, but could not be further defined. Population 46 comprised HLA-DR+ CD14dim/+CD11c+ CD16++ CD86+ TIM3+ dendritic cells. The frequency of population 47 was lower in the old patient group without frailty compared to the young patient group (p= 0.04), but only expressed CD123+, and thus could not be further defined (Figure S5).

Frailty may not impact the clinical response to checkpoint therapy in older patients

Figure 5 shows the clinical response to treatment between the young, old patients without frailty and the old patients with frailty. The clinical response was a bit higher in the young patient group (13 out of 22 patients, 59.1%) when compared to the old patient group with frailty (9 out of 19 patients, 47.4%), but there were no statistically significant differences between the three groups of patients. In addition, no

association with treatment response and ageing or frailty was observed (p=0.45). This demonstrates that frailty in older patients does not preclude them from a successful immunotherapeutic treatment.

Figure 5. Response treatment between young, old, and old-frail groups

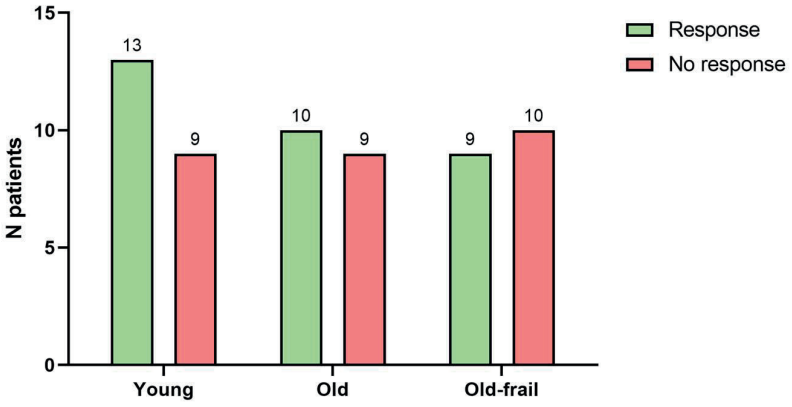


Figure 5. Comparison of response to treatment between young, old, and old-frail patient groups. No statistical differences were observed using the Chi-square for trend tests.

DISCUSSION

In this study, we observed lower frequencies of CD8+ naïve T cells in older compared to young patients, irrespective of the presence of frailty, confirming the loss of naïve T cells with age as observed in other groups focusing on immune senescence in older patients (4, 5). In addition, we observed a frailty-associated loss of effector CD8+ tissue resident-like memory (CD8+ TRM) T cells, CD8+ MAIT cell populations and of B cells, the latter of which is in line with earlier observations on declining B cells during ageing, but not with that of increasing MDSCs with ageing (8). Moreover, we detected higher frequencies of NK cells in older patients, irrespective of clinical frailty. However, none of these age and frailty-related immune cell differences were associated with differences in the clinical response to immunotherapy.

In contrast to previous studies in old non-cancer patients that showed a correlation between frailty and decreased levels of total T cells (5, 31), our study in melanoma patients did not reveal significant differences in total T cell frequencies between the young and older patients, regardless of frailty status. Age-dependent changes in the naïve and memory T cell pools have been widely reported in frail patients without cancer (32). Consistent with previous research, we observed lower numbers

of CD8+ naïve T cells in older melanoma patients compared to young patients, irrespective of the presence of frailty. Moreover, prior studies have shown lower frequencies of circulating naïve CD4+ T cells in old patients with frailty from nursing homes and in the general practice (4, 5, 12), results that we did not observe in our study. Our results revealed a significant decrease in CD8+ MAIT cells with age independent of frailty, which is in line with a previous study that demonstrated a gradual decline in percentage and number of CD8+ MAIT cells from young to older individuals (33). In our study, we have identified a subpopulation of senescent CD8+ T cells, characterized by the phenotype CD8+CD45RA+CCR7-CD27-CD28-KLRG-1+ (cell population 14); however, no significant differences were observed between the patient groups, diverging from previous findings that have shown a correlation with frailty and loss of CD28 and CD27 markers in older patients (34-36). The failure to detect senescence-linked changes in immune cell populations in older patients can potentially be attributed to the relatively small age difference between young and old patients. Plus, the sample size of our cohort was relatively small, which may have resulted in underpowering of the study. Another explanation may be that patient inclusion in this observational cohort led to a relatively healthy cohort, as the frailest patients may be underrepresented in the study, which might be the reason for the uniform distribution of the senescence-associated cell frequencies across these age groups. Additionally, no significant differences were observed between population 14 and the response rates. Hui et al. demonstrated that CD28 was the primary target for PD-1-mediated inhibition, suggesting that the abundance of T cells lacking CD28 expression may correlate with the absence of efficacy of anti-PD-1/PD-L1 therapy (37). The absence of observed differences in response rates among our study groups may be attributed to the uniform distribution of the senescent cell frequencies across the groups, which may have resulted in only minor differences in immunological profiles between patients, thereby hampering the associations with response to treatment. The lack of correlation between immune senescence markers and response may also suggest that immune senescence per se does not interact with the effectiveness of immune checkpoint inhibitors (38).

Our results did not reveal statistically significant differences in response rates among the study participants. However, there appears to be a trend of declining responses with advancing age and increasing frailty. Although this trend could hold clinical significance, the small participant numbers might not allow for statistical confirmation. The observed lack of correlation may result from the study's insufficient power or the cross-sectional design at baseline, which did not capture the dynamic changes in frailty over time. Although categorizing patients into three groups (young, old fit, and old frail) allowed clear comparison across distinct age and fitness profiles, longitudinal assessments and treating the Clinical Frailty Scale as a continuous

variable could have provided more nuanced insights into the relationship between frailty and treatment outcomes.

We did not observe age- or frailty-dependent differences in myeloid-derived suppressor cells (MDSCs). MDSCs potently suppress T cell activity, leading to the immune escape of malignant tumors, thus promoting resistance to checkpoint inhibitor treatments (39, 40). Verschoor et al. reported a significant increase in number of circulating myeloid cells, especially in MDSCs, in older and frail patients compared to young patients (41). Our results of the MLR did not show significant differences in relation to ageing and frailty, suggesting no problems with APC function, as would have been the case when higher frequencies of MDSC would be present (42). This supports our observation that they were not prominently present among our patients. However, a trend of impaired function of APCs in the old patients without and with frailty in comparison to the young patients can be observed, implying that immune cells, in the older and frail individuals, may be less efficient at capturing and presenting antigens.

The main strengths of this paper include the use of validated methods according to standard operating procedures (SOPs) and of a large panel of markers for flow cytometry. Moreover, analyses included frailty, rather than only using calendar age.

However, this study also has its limitations. First, the lack of specific antibodies for subpopulations of myeloid cells, as well as for NK cells and B cells, did not allow us to identify all the cell subsets. Therefore, it is important to remain cautious regarding the naming of cell populations. Marker expression can overlap and vary within different degrees, which cannot be nuanced when naming those subpopulations. Secondly, the sample size was low, decreasing statistical power. For this reason, we chose not to correct for multiple testing. Additionally, we included patients pre-treated with BRAF inhibitors; however, their distribution was nearly uniform across the three groups, and they were not outliers with respect to the identified immune cell populations, ensuring they did not skew the study's outcomes. Finally, there was a relatively small age difference between young and old patients, potentially leading to a lack of power in our study. Moreover, a CFS score of 4 or higher was used to include both pre-frail and frail patients. This decision was made in light of the cohort's relatively fit nature, with only few patients with a CFS of 5 or higher, to ensure adequate sample sizes and comparability across groups. Although a CFS score of 5 or higher is typically used to define frailty, our approach is supported by precedents in the literature that have utilized similar cut-offs in comparable populations (25). We acknowledge that this may impact the study's power and the robustness of its conclusions. Additionally, incorporating measures of plasma cytokines could have potentially strengthened the classification of frail and non-frail older adults; however, the resources did not

allow for further experimentation. Considering the variation and importance of the tumor microenvironment, investigating immune senescence markers in relation to immunotherapy responses should be further studied at the tissue level.

Several ageing- and frailty-associated changes were detected among circulating immune cells in blood, but were not associated with response to immunotherapy in our study. While these findings suggest that the level of frailty and ageing may not necessarily preclude the efficacy of ICI therapy, further investigation is needed to fully understand the impact of frailty and ageing on immunotherapy.

DECLARATIONS

Clinical trial number

Not applicable.

Ethics approval and consent to participate

The study conducted in accordance to the Declaration of Helsinki and was approved by the Medical Ethics Committee of Leiden University Medical Center (Committee of Medical Ethics; NL59959.058.17). All patients signed informed consent.

Consent for publication

Not applicable.

Availability of data and material

Not applicable.

Competing interests

No conflicts of interest to disclose.

Authors contributions

Estelle Tran Van Hoi carried out the research and analysis under the supervision of Johanneke E.A. Portielje. Simon P. Mooijaart, Diana Van Heemst and Nienke A. de Glas. Saskia J. Santegoets performed analysis and contributed to interpretation of the results. Asli Özkan contributed to the patients follow-up. Marije Slingerland, Elizabeth M.E. Verdegaal, Ellen Kapiteijn, Marij J.P. Welters and Sjoerd H. van der Burg contributed in the design and implementation of the TCIMM study. Marije J.P. Welters, and Nienke A. de Glas conducted the present research.

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Single stain	Laser	Detector	Fluor	Antigen	Characteristics of markers in immunosenescence field [1-3]		Clone	Lot 1	Unmixing
s1	UltraViolet laser	UV2	BUV395	CD45RA	Naïve and terminally differentiated effector T cells marker	Lost as T cells become central or effector memory cells; ratio of naïve to these memory T cells is a hallmark of immunosenescence	HI100	2077243	with beads
s2		UV4	Zombie UV	L/D	Distinguishes live from dead cells	(to be able to only analyse viable cells as dead cells can non-specifically bind antibodies)	NA		with cells
s3		UV7	BUV496	CD16	NK cells marker and is expressed on monocytes and some macrophages	Changes in expression levels can indicate altered immune functions in ageing	3G8	1099494	with cells
s4		UV9	BUV563	CD39	Exhausted T cells marker, also expressed in activated antigen-specific CD4 T cells in the tumour [4]	Can lead to increased immunosuppression with age	TU66	2069477	with beads
s5		UV10	BUV615	ICOS	A costimulatory molecule for T cell activation and function	May be involved in the functional decline and activation of T cells in ageing	DX29	2077268	with beads
s6		UV11	BUV661	CD1c	Marker of dendritic cells	Involved in antigen presentation; initiation of immune responses can decline with age	F10/21A3	2189988	with cells
s7		UV14	BUV737	CD86	Costimulatory molecule on antigen-presenting cells	Essential for effective T cell activation, which can decline with age	2331 (FUN-1)	1228590	with beads
s8		UV16	BUV805	CD8	Cytotoxic T cells marker	Accumulation of senescent CD8+ T cells is a hallmark of immunosenescence	SK1/HIT8a	1200765	with cells
s9	violet laser	V1	BV421	CD161	Expressed on a subset of T cells and NK cells	CD161 is involved in regulating immune responses[5], potentially implicated in age-related immune alterations	HP-3G10	B334269	with beads
s10		V2	SB436	CD123	Plasmacytoid dendritic cells marker	Numerical and functional decline in pDC is associated with ageing	6H6	2305276	with beads
s11		V3	PacBlue	CD15	Neutrophils marker	Neutrophil functions may decline with ageing, although the neutrophils are hardly present after gradient isolation of blood sample	W6D3	B273508	with beads
s12		V4	BV480	CD33	Early myeloid cells marker	Increased proportions are associated with ageing	P67.6	276608	with beads
s13		V6	BV510	CD11c	Dendritic cells marker	Involved in antigen presentation, initiation of immune responses can decline with age	B-Ly6	1344885	with cells
s14		V7	PacOrange	CD3	T cells marker	Ratio of different T cell subsets changes with age, can indicate immunosenescence	UCHT1	539078	with cells
s15		V8	BV570	CD45RO	Memory T cells marker	Increased proportions suggest an aged immune repertoire	UCHL1	B354623	with beads
s16		V10	BV605	CD163	M2-like macrophages marker	M2 macrophages may increase with age, can promote tumour [6, 7]	GHI/61	B339795	with beads
s17		V11	BV650	PD1	Immune checkpoint inhibitor marker	Increases on T cells as they become activated and exhausted, a hallmark of immunosenescence	EH12.2H7	B341605	with beads
s18		V13	BV711	CD103	Integrin expressed on tissue resident memory T cells	Tissue resident marker, can be upregulated upon activation[8]	Ber-ACT8	B328658	with beads
s19		V14	BV750	CD56	NK cells marker (can also be expressed in T cells)	Changes in NK cell numbers and function are associated with ageing	5.1H11	B348832	with beads
s20		V15	BV785	CD28	Costimulatory molecule for T cells activation	Loss of CD28 in T cells is a hallmark of immunosenescence	CD28.2	B344136	with cells
s21	blue laser	B1	BB515	CD141	Expressed in subset of dendritic cells	CD141+ DC may decrease in number with age	1A4	1166028	with beads
s22		B2	AF488	Foxp3	Transcription factor for Treg cells	Treg Foxp3+ cells may increase in number with age	259D	B315166	with beads
s23		B3	Spark Blue 550	CD14	Monocytes/macrophages marker	Phenotype and function of monocytes and macrophages change with age	63D6	B321401	with cells
s24		B8	PerCP	CD45	Leukocytes marker	Decreased proportions with age	Hi30	B331249	with beads
s25		B9	PerCP/Cy5.5	CD11b	Myeloid cells marker	Changes in expression might reflect shifts in innate immunity with age	ICRF44	B328101	with cells
s26		B10	PerCP/eF710	CD274/PD-L1	PD-1 Ligand marker	Its expression on tumor cells and tumor associated macrophages can contribute to the immunosuppressive environment seen in ageing	MIH5	2246625	with beads

Table S1. Panel markers for flow cytometry

For each antibody, the conjugated fluorochrome, clone name and supplier are given.

s27	yellow green laser	YG1	PE	CLec9a	Expressed in subset of dendritic cells	CLEC9A+ DC (cDC1) may decrease in number with age [6]	8F9	B309940	with cells
s28		YG2	CF568	CD4	Helper T cells marker	Changes in the CD4+ T cell compartment are associated with immunosenescence	C4-206	21C0304	with cells
s29		YG3	PE/Dazzle 594	CD206	Activated macrophages marker	Activated macrophages increase with age	15-Feb	B329923	with beads
s30		YG4	PE/Fire640	CD25	Expressed on activated T cells and Treg cells	Involved in cell proliferation and regulatory functions that may change with age	M-A251	B332511	with cells
s31		YG5	PE/Cy5	Tim3	Immune checkpoint molecule	Increased expression is associated with T cell exhaustion in ageing	F38-2E2	B353724	with beads
s32		YG6	PE/Fire700	CD127	Memory T cells and effector T cells marker, also expressed in a subset of Treg cells	Memory and effector T cells increase in number with age	AO19D5	B348589	with beads
s33		YG9	PE/Cy7	KLRG1	Expressed on senescent T cells	Marker of terminal differentiation and senescence in T cells	SA231A2	B330891	with cells
s34		YG10	PE/Fire810	HLA-DR	MHC class II molecule marker	Its expression on T cells can mean activated T cells. Its increased expression can be associated with inflammation and ageing	L243	B341939	with cells
s35	red laser	R1	APC	NKG2a	NK cells marker and immune checkpoint molecule on T cells	Increased proportions with age	Z199	200056	with beads
s36		R2	Alexa647	CD68	Macrophages marker	Decreased proportions with age	Y1/82A	B311503	with beads
s37		R3	Spark NIR 685	CD19	B cells marker	Changes in B cell subsets are hallmarks of immunosenescence	HIB19	B324543	with beads
s38		R4	APC/R700	Lag3	Immune checkpoint molecule	Contributes to immune exhaustion and increases with age	T47-530	1114707	with cells
s39		R7	APC/Fire750	CCR7	Involved in cell migration to lymph nodes, expressed in various cell types	Loss of CCR7 expression on T cells is associated with immunosenescence	G043H7	B338294	with cells
s40		R8	APC/Fire810	CD27	Costimulatory molecule	Loss of CD27 expression on T and B cells is a hallmark of immunosenescence	QA17A18	B332527	with beads

Continued Table S1. Panel markers for flow cytometry

For each antibody, the conjugated fluorochrome, clone name and supplier are given.

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Figure S1. Conceptual overview of the interplay between immune cell response and melanoma growth with immunosenescence

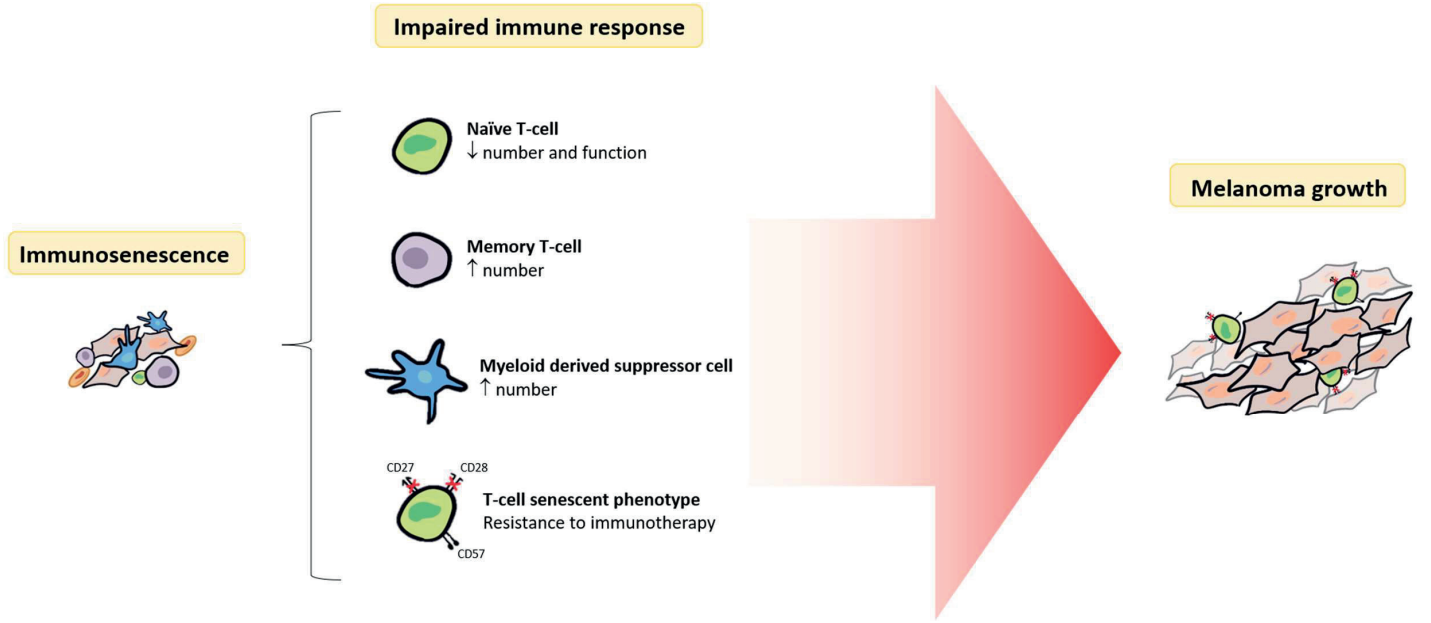


Figure S1. In melanoma, immunosenescence leads to reduced cell number and function of naïve T cells and memory T cells, inducing melanoma growth and metastasizing process. Moreover, T cells express an immunosenescent phenotype characterized by reduced expression of CD27 and CD28 and higher expression of CD57, a major marker of immunosenescence. This immunosenescent phenotype has been associated with resistance to immunotherapy treatment.

Figure S2. Distribution of Clinical Frailty Scale score in patients with frailty

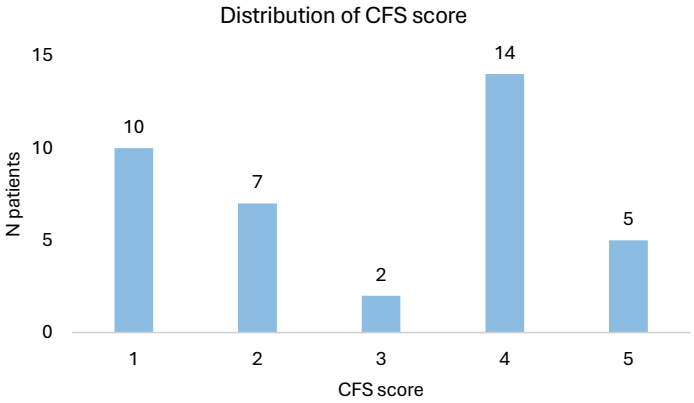


Figure S3. Frequencies of CD3+ T cell populations between patients having response/no response to treatment

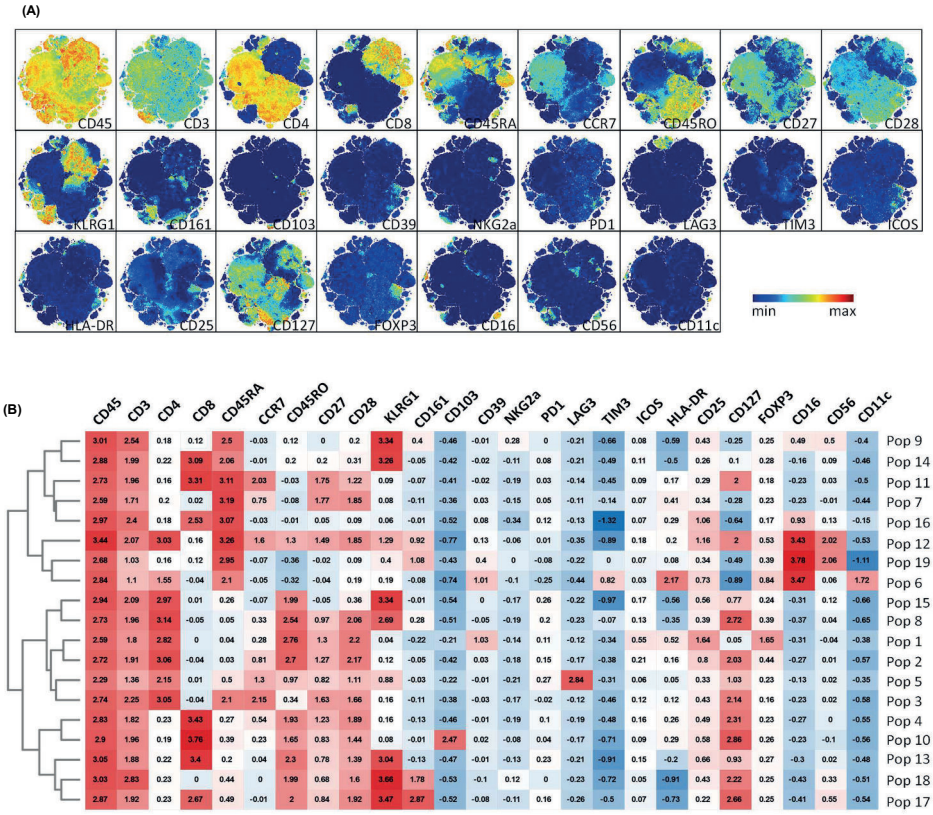


Figure S3. (A) OptSNE plots visualizing contour plots with the staining intensity of the individual markers used. (B) Heat map (FlowSOM plugin output) of the relative fluorescent intensity for the markers associated with the identified T cell subpopulations.

Figure S4. NK cell populations

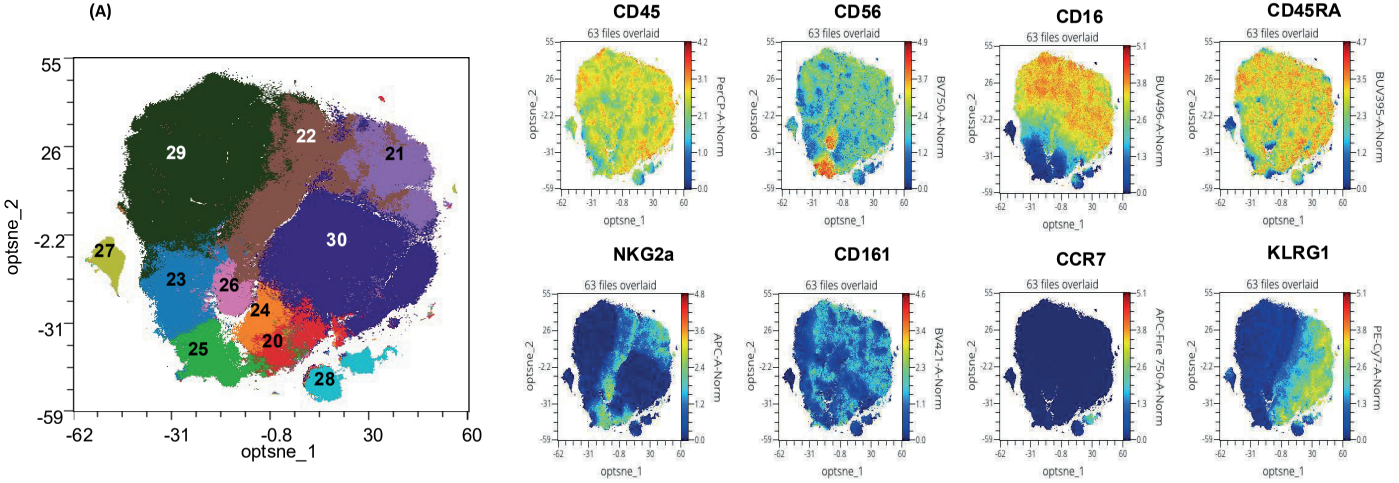
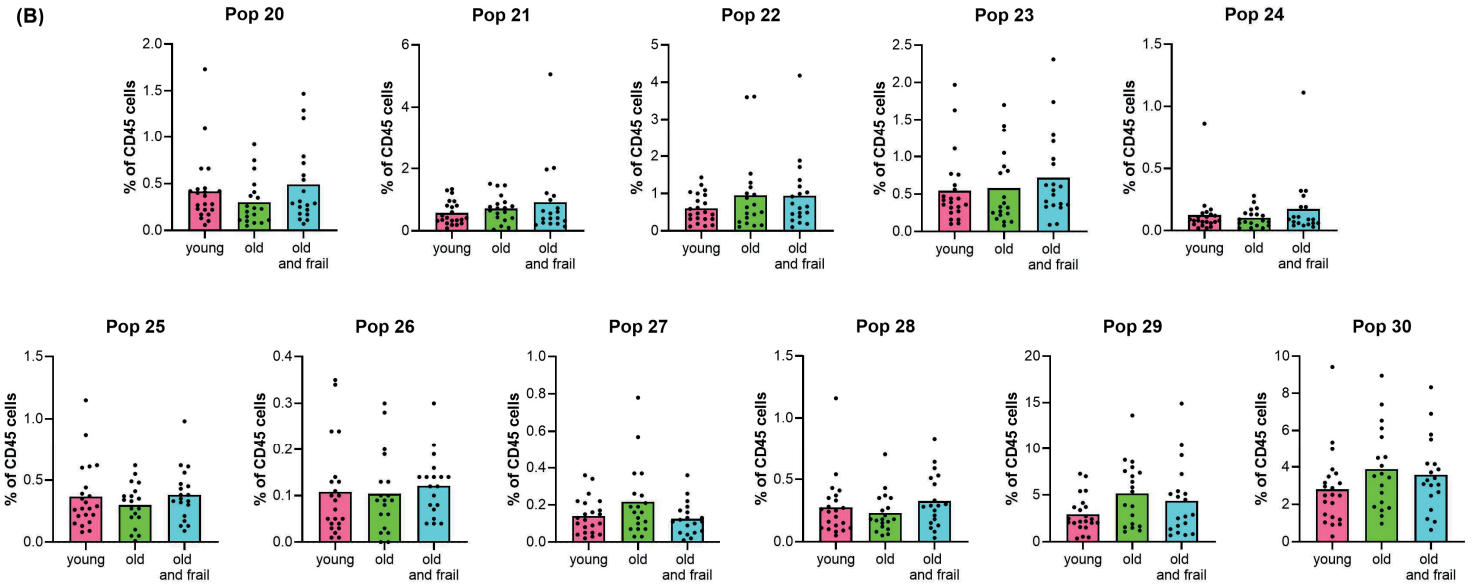


Figure S4. (A) Cluster partitions by FLOWSOM of PBMCs stained with antibodies for CD56+ NK cell markers. In total, 11 different clusters were defined (left). OptSNE plots visualizing contour plots of the 3 patient groups (right).

Figure S4. Continued



(B) Frequencies of NK cell populations in young, old and old-frail patient groups. Cell populations are presented as a percentage of the total CD45+ cells. Statistical differences were assessed with Kruskal-Wallis tests and $p<0.05$.

Figure S4. Continued

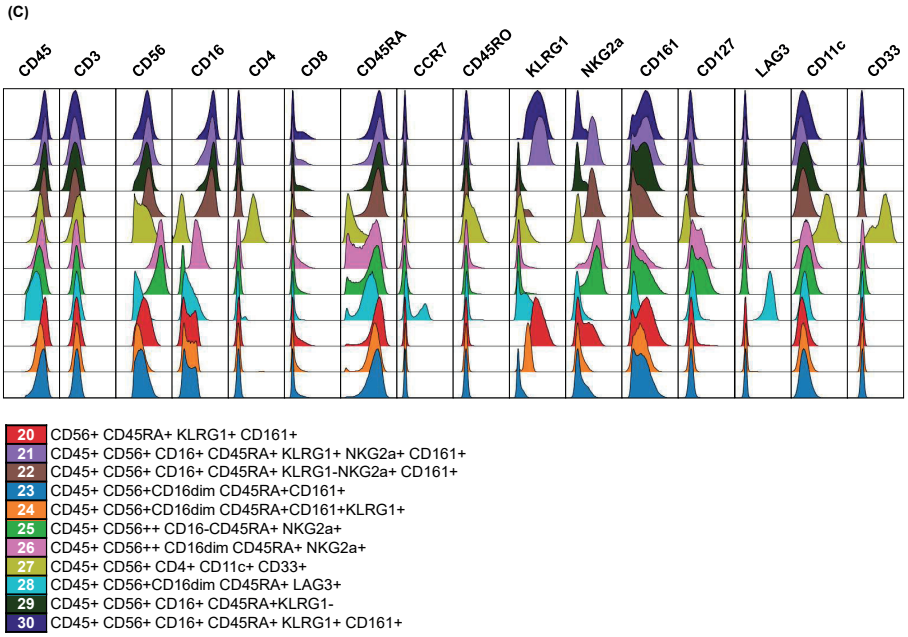


Figure S4. (C) Expression levels of each of the indicated markers are depicted for the individual cell populations.

Figure S5. Myeloid cell populations

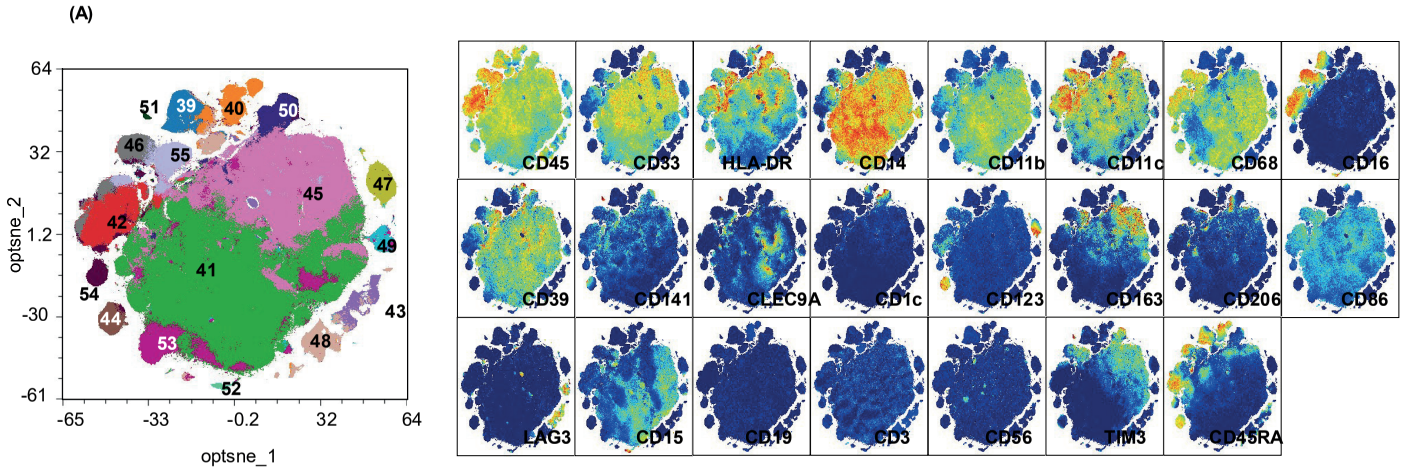


Figure S5. Continued

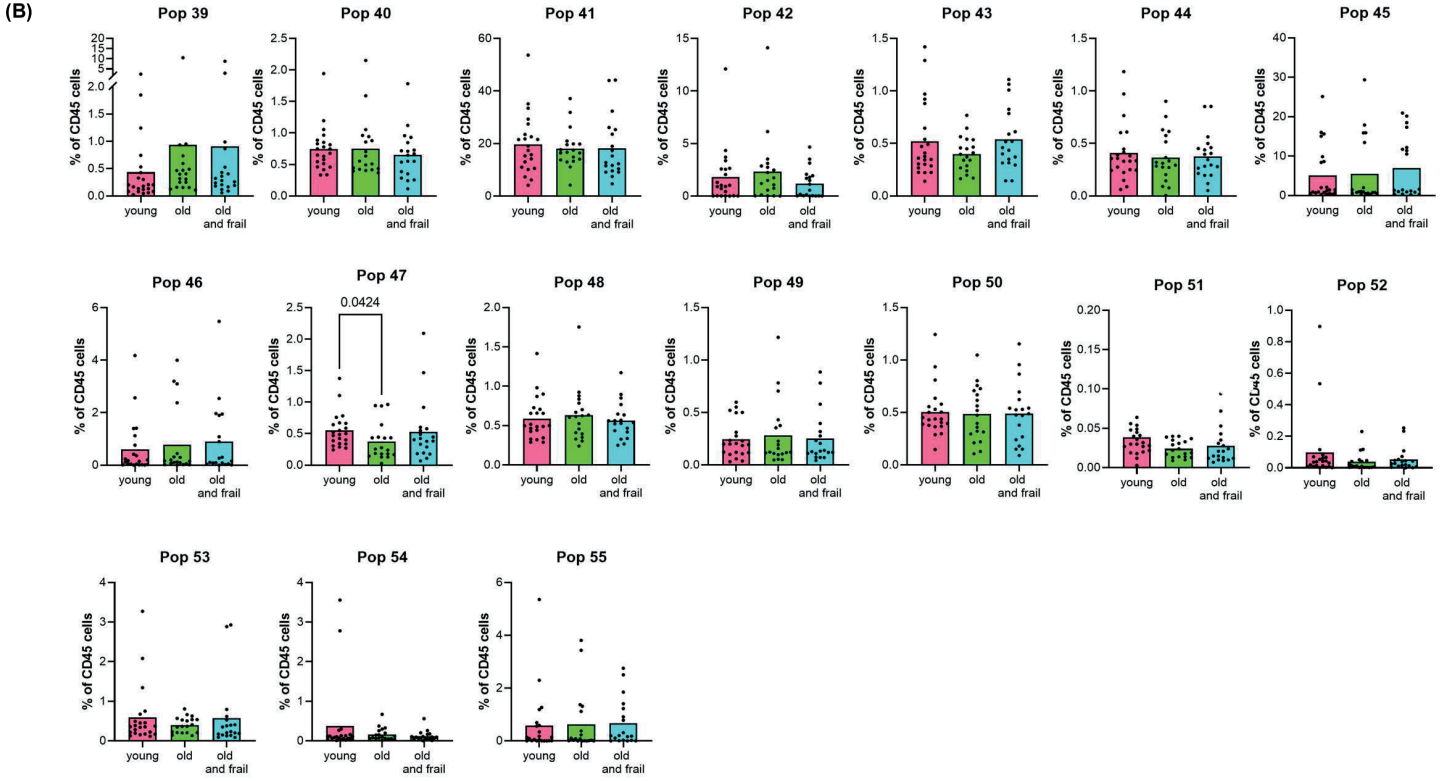


Figure S5. (A) Cluster partitions by FLOWSOM of PBMCs stained with antibodies for myeloid cell markers. In total, 17 different clusters were defined (left). OptSNE plots visualizing contour plots of the 3 patient groups (right). (B) Frequencies of myeloid cell populations in young, old and old-frail patient groups. Cell populations are presented as a percentage of the total CD45+ cells. Statistical differences were assessed with Kruskal-Wallis tests and $p<0.05$.

Figure S5. Continued

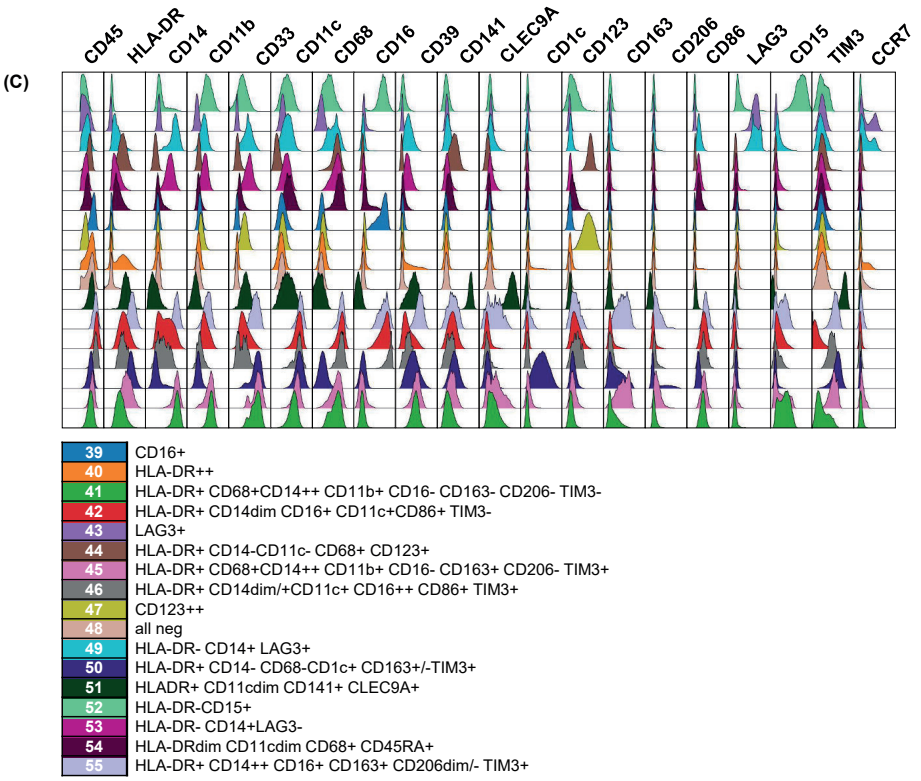


Figure S5. (C) Expression levels of each of the indicated markers are depicted for the individual cell populations.

Figure S6. B cell populations

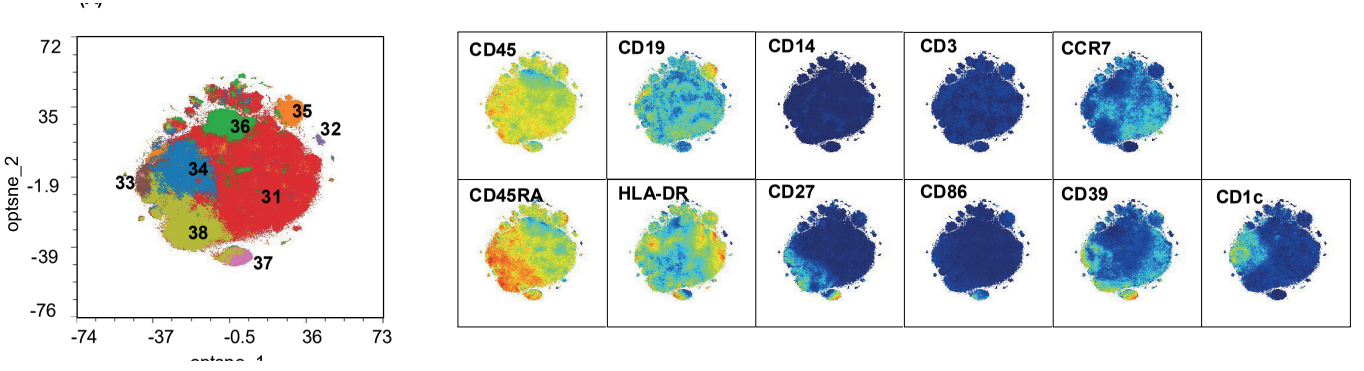
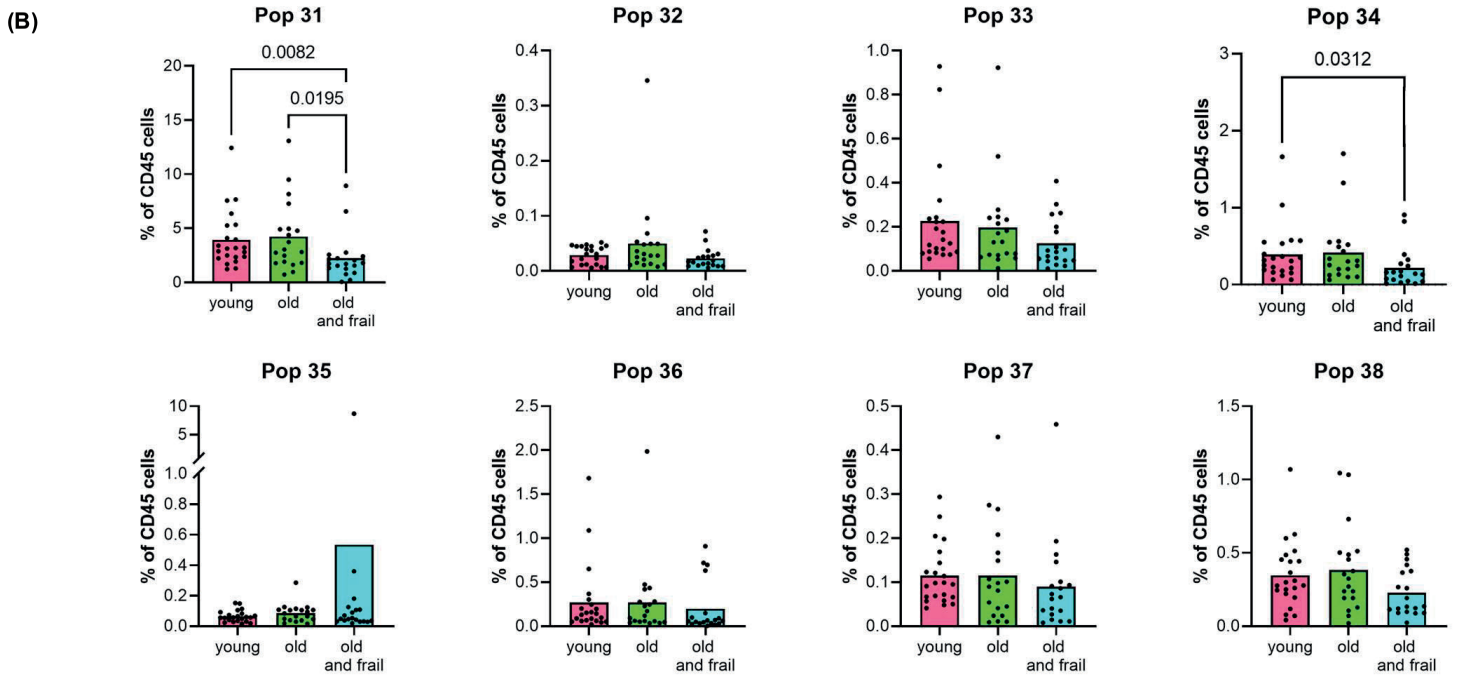


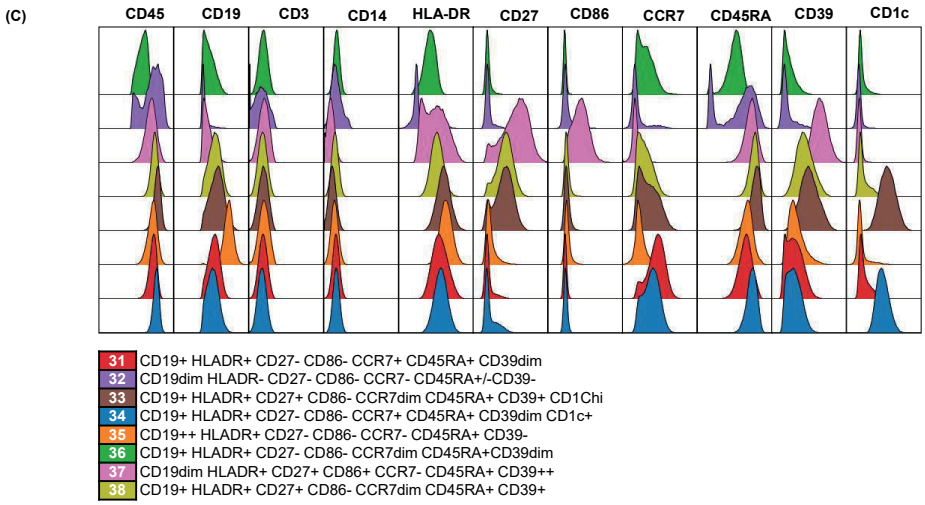
Figure S6. (A) Cluster partitions by FLOWSOM of PBMCs stained with antibodies for CD19+ B cell markers. In total, 8 different clusters were defined (left). OptSNE plots visualizing contour plots of the 3 patient groups (right).

Figure S6. Continued



(B) Frequencies of B cell populations in young, old and old-frail patient groups. Cell populations are presented as a percentage of the total CD45+ cells. Statistical differences were assessed with Kruskal-Wallis tests and p<0.05.

Figure S6. Continued



(C) Expression levels of each of the indicated markers are depicted for the individual cell populations.

Figure S7. Frequencies of CD3+ T cell populations between patients having a response/no response to treatment

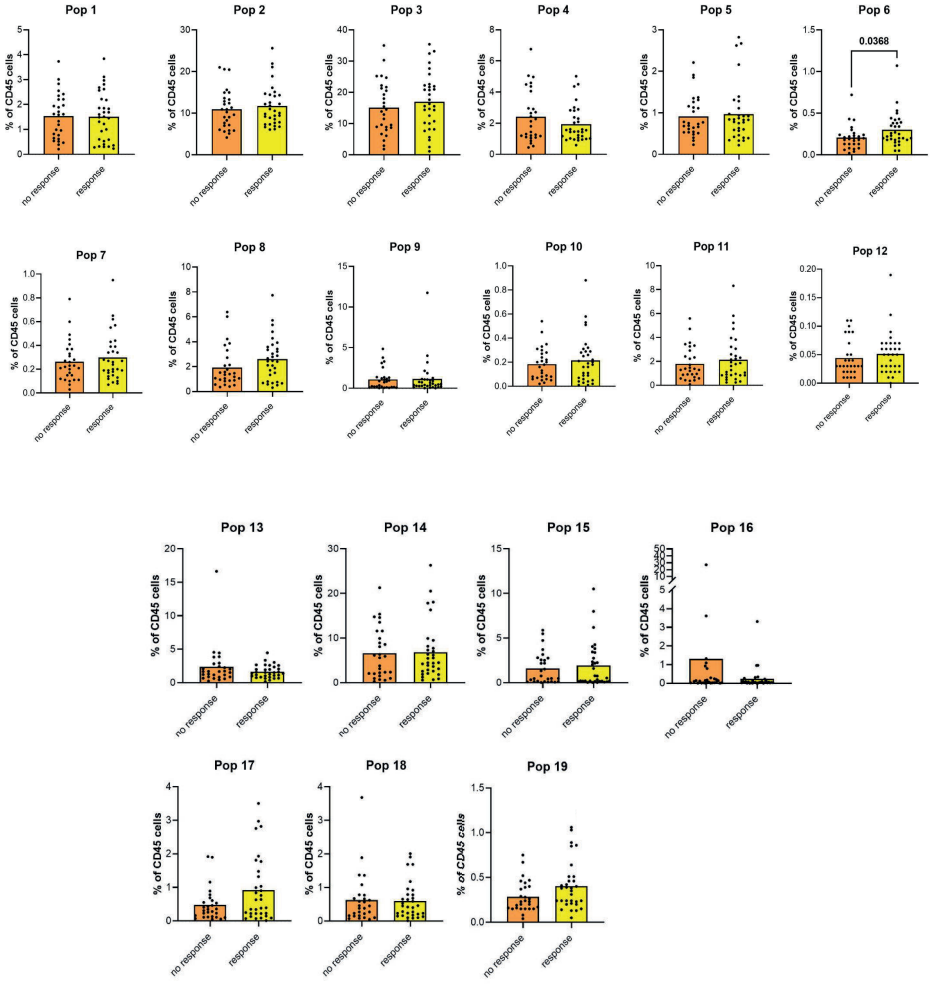


Figure S7. Frequencies of CD3+ T cell populations between the different responses to treatment. Cell populations are presented as a percentage of the total CD45+ cells. Statistical differences were assessed with Mann-Whitney tests and p<0.05.

Figure S8. Frequencies of NK cell populations between patients having a response/no response to treatment

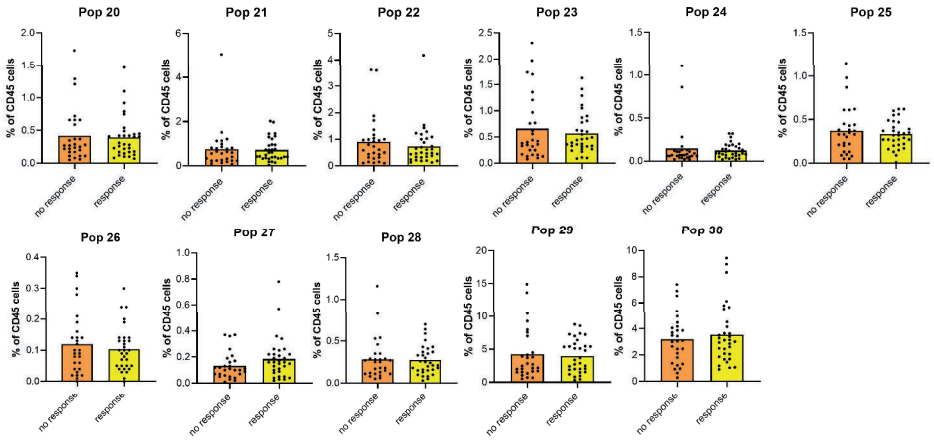


Figure S8. Frequencies of NK cell populations between the different responses to treatment. Cell populations are presented as a percentage of the total CD45+ cells. Statistical differences were assessed with Mann-Whitney tests and p<0.05.

Figure S9. Frequencies of myeloid cell populations between patients having a response/no response to treatment

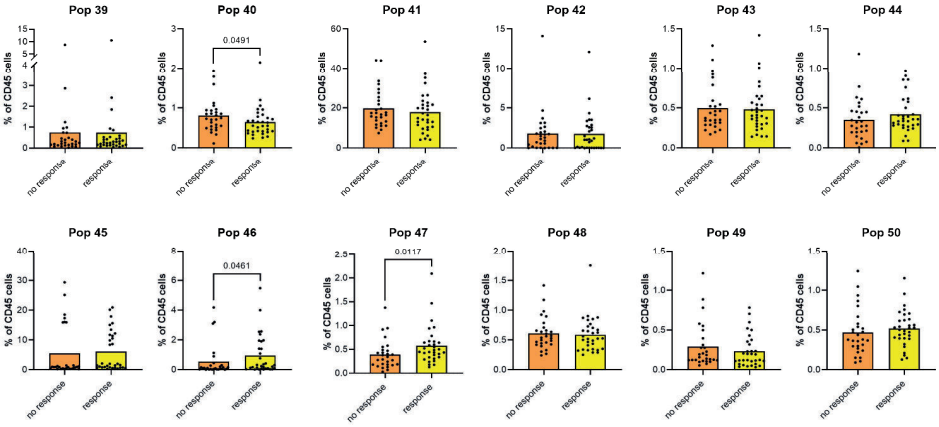


Figure S9. Frequencies of myeloid cell populations between the different responses to treatment. Cell populations are presented as a percentage of the total CD45+ cells. Statistical differences were assessed with Mann-Whitney tests and p<0.05.

Figure S10. Frequencies of B cell populations between patients having a response/no response to treatment

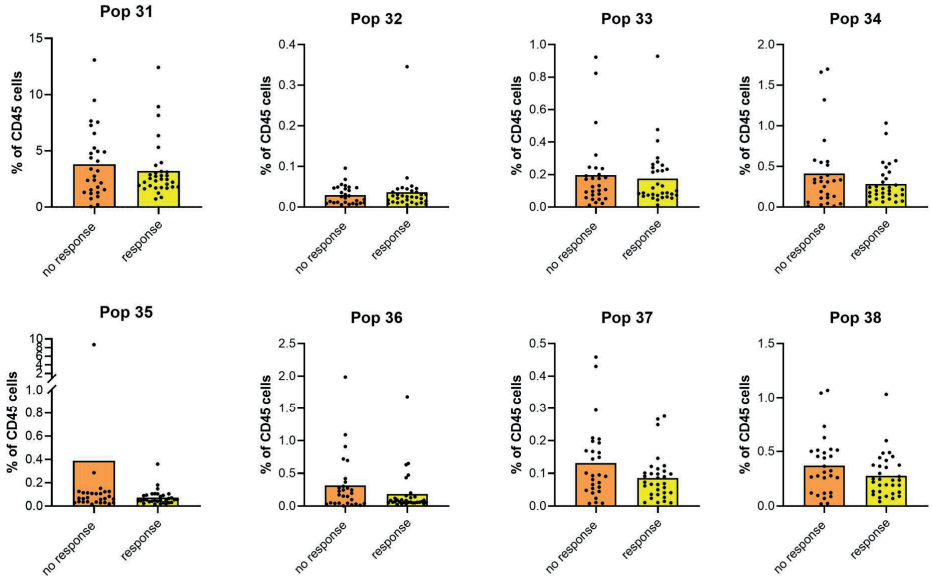


Figure S10. Frequencies of B cell populations between the different responses to treatment. Cell populations are presented as a percentage of the total CD45+ cells. Statistical differences were assessed with Mann-Whitney tests and p<0.05.