Chapter 4

Enhanced expression of activation markers and PD-1 by CD4+ T cells of young but not old patients with metastatic melanoma

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Abstract

The biological behavior of melanoma is unfavorable in the elderly when compared to young subjects. Recent studies with checkpoint inhibitors have identified T cells as prominent players in the immune response against melanoma cells. We hypothesized that differences in T cell responses might underlie the distinct behavior of melanoma in young and old melanoma patients.

Therefore, we investigated the circulating T cell compartment of 34 patients with metastatic melanoma and 42 healthy controls, which were classified as either young (≤ 50 years) or old (≥ 65 years).

Absolute numbers of CD4+ T cells were decreased in young and old melanoma patients when compared to the age-matched control groups. Percentages of naive and memory CD4+ T cells were not different when comparing old melanoma patients to age-matched controls. Percentages of memory CD4+ T cells tended to be increased in young melanoma patients compared to young controls. Proportions of naive CD4+ T cells were lower in young patients than in age-matched controls, and actually comparable to those in old patients and controls. This was accompanied with increased percentages of memory CD4+ T cells expressing HLA-DR, Ki-67 and PD-1 in young melanoma patients in comparison to the age-matched controls, but not in old patients. The percentage of CTLA-4 expressing CD4+ T cells was similar in melanoma patients and controls. Proportions of FOXP3+Helios- regulatory T cells were increased in young and old melanoma patients when compared to their age-matched controls, whereas those of FOXP3+Helios+ regulatory T cells were similar. We observed no clear modulation of the circulating CD8+ T cell repertoire in melanoma patients.

In conclusion, we show that CD4+ T cells of young melanoma patients show strong signs of activation, whereas these signs are lacking in CD4+ T cells of old melanoma patients. These findings might explain the unfavorable behavior of melanoma in the elderly.

Introduction

Melanoma is an aggressive form of skin cancer with frequent metastases towards other organs. The incidence of melanoma in Europe is currently on the rise 1. Among adolescents and young adults, melanoma is the most prevalent type of cancer in women and ranks second in men 2. Nevertheless, melanoma is largely a disease of the elderly, as 43% of all newly-diagnosed patients are 65 years or older. In addition, the median age at diagnosis is 64 years for males and 57 for females 3. Importantly, the biological behavior of melanoma differs between young and old patients. Old patients more frequently present with unfavorable prognostic tumor factors as evidenced by a higher Breslow’s thickness, a higher occurrence of histological ulcerative tumors and a higher mitotic activity 4–6 and lastly a worse disease specific survival 6. Currently, it
is unclear why the biological behavior of melanoma differs in young and old patients.

Ample evidence indicates that the immune system plays a key role in the outcome of melanoma. Spontaneous regression occurs in 3.7–15% of primary melanomas. Even for metastatic melanoma, one in every 400 patients reaches a spontaneous complete remission. Immune checkpoint inhibitors like the anti-CTLA-4 antibody ipilimumab and the anti-PD-1 antibodies nivolumab and pembrolizumab have demonstrated remarkable efficacy in boosting T cell responses against metastatic melanoma. For the combination of ipilimumab and nivolumab a response rate as high as 61% was recently reported. Currently, no validated biomarkers are commonly used to select patients for treatment with checkpoint inhibitors.

Aging of the immune system might be a factor contributing to the unfavorable behavior of melanoma in the elderly. Both the innate and adaptive immune arms of the immune system are affected by aging. These changes have been linked to the increased susceptibility for infections and various types of cancer in the elderly. T cell responses might be compromised in the elderly due to various perturbations, such as reduced numbers and diversity of naïve T cells, skewing of the memory T cell receptor repertoire, poor cytokine secretion and functional exhaustion of the memory compartment. Moreover, numbers of regulatory T cells increase with ageing. Regulatory T cells inhibit immune responses and are essential for preventing autoimmunity. In the context of cancer, however, these cells may dampen anti-tumor responses. It might therefore be possible that aging of cellular immunity underlies the unfavorable behavior of melanoma in the elderly.

In the current study, we therefore investigated the circulating T cell compartments of young and old melanoma patients. For comparison, we recruited a cohort of aged-matched healthy controls. A comprehensive analysis of activation, proliferation and differentiation markers, checkpoint molecules and regulatory T cell transcription factors shows that CD4+ T cells of young melanoma patients show signs of an ongoing immune response, whereas these signs are lacking in CD4+ T cells of old melanoma patients.

Methods

Study subjects
Peripheral blood was obtained from 34 systemic treatment-naive, metastatic melanoma patients, who were either ≤ 50 years (n = 11) or ≥ 65 years (n = 18). For three patients, only lymphocyte true count could be performed due to logistic reasons. In addition, blood samples were obtained from 42 age-matched healthy controls that were young (n = 13) or old (n = 39). Health of the control subjects was assessed by health assessment question-
naries, physical examination and blood tests as previously described 11. Melanoma patients using immune-modulating drugs or having infections, other types of malignant disease or autoimmune disease were excluded from the study. Written informed consent was obtained from all study subjects and the study was approved by the medical ethical committee of the UMCG (identifier 2011.388 and 2012.375). The clinical trial registry identifier is NTR4539. The study was in line with the declaration of Helsinki.

Flow cytometry
Peripheral blood mononuclear cells (PBMC) were isolated by density centrifugation with Lymphoprep (Axis-Shield). PBMC or whole blood samples were stained with the following fluorochrome-conjugated monoclonal antibodies: CD3-eFluor605, CD4-eFluor450, CD27-APC-eFluor780, HLA-DR-eFluor780, Helios-eFluor450, FOXP3-PE (eBioscience), CD4-APC-H7, CD8-Perce, CD8-PE-Cy7, CD31-AF647, CD45RO-FITC, CD45RO-PE-Cy7, CCR7-PE-Cy7, Ki-67-Perce-Cy5.5, CTLA-4-BV421 (BD Biosciences), PD-1-PE, CD28-AF700 (Biolegend), CD161-PE (Miltenyi Biotec). Intracellular staining for FOXP3, Helios, Ki-67 and CTLA-4 was performed after cells were permeabilized with a FOXP3 staining buffer set according to instructions of the manufacturer (eBioscience). Whole blood samples were treated with BD lysis solution according to instructions of the manufacturer (BD Biosciences). Stained samples were analyzed on a LSR-II flow cytometer (BD Biosciences). Analysis was performed with Kaluza Flow Analysis Software (Beckman Coulter).

Statistics
Demographics and baseline characteristics of all patients were summarized using descriptive statistics. The Mann Whitney U Test was used to compare different groups. Analyses were performed with GraphPad Prism 5.0. Two-tailed $p$-values $< 0.05$ were considered significant.

Results

Subjects characteristics and lymphocyte numbers
Characteristics of the melanoma patients are shown in the supplemental Table. The time between development of metastases after discovery of the primary tumor was shorter in old compared to young patients, albeit not statistically significant. Markers of systemic inflammation – erythrocyte sedimentation rate and C-reactive protein – tended to be higher in young patients than in old patients. Absolute numbers of CD3+ T cells were lower in melanoma patients when compared to their aged-matched healthy controls (Table 1). This difference could be explained by a numerical decline of CD4+ T cells in melanoma patients, whereas numbers of circulating CD8+ T cells were similar in patients and controls. Absolute numbers of B cells were decreased in young and old melanoma patients compared to the aged-matched control. Numbers of NK cells were similar in patients and con-
Thus, absolute numbers of circulating CD4+ T cells and B cells are altered in patients with metastatic melanoma.

<table>
<thead>
<tr>
<th></th>
<th>young HC (n = 13)</th>
<th>young melanoma patient (n = 13)</th>
<th>old HC (n = 28)</th>
<th>old melanoma patients (n = 18)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3+ counts · 10^9/L</td>
<td>1.12 (0.78–1.59)</td>
<td>0.89 (0.47–1.59)*</td>
<td>1.28 (0.55–2.34)</td>
<td>0.93 (0.50–2.28)*</td>
</tr>
<tr>
<td>CD4+ counts · 10^9/L</td>
<td>0.79 (0.18–0.99)</td>
<td>0.51 (0.24–0.95)</td>
<td>0.89 (0.33–1.43)</td>
<td>0.53 (0.31–1.21)**</td>
</tr>
<tr>
<td>CD8+ count · 10^9/L</td>
<td>0.32 (0.19–0.74)</td>
<td>0.30 (0.10–0.57)</td>
<td>0.34 (0.10–1.25)</td>
<td>0.24 (0.06–1.00)</td>
</tr>
<tr>
<td>B cell counts</td>
<td>0.19 (0.08–0.50)</td>
<td>0.12 (0.03–0.22)*</td>
<td>0.18 (0.06–0.50)</td>
<td>0.13 (0.04–4.19)b</td>
</tr>
<tr>
<td>NK cell counts</td>
<td>0.15 (0.06–0.44)</td>
<td>0.19 (0.06–0.37)</td>
<td>0.31 (0.07–0.65)</td>
<td>0.21 (0.03–0.51)</td>
</tr>
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**Table 1: True counts of peripheral lymphocyte subsets shown for young and old metastatic melanoma patients compared to age-matched healthy controls.**

Statistical significance is indicated as * p < 0.05 or ** p < 0.01. a p-value: 0.057. b p-value: 0.051. HC = healthy controls, NK = natural killer.

**T cell differentiation subsets**

We investigated if the lower number of CD4+ T cells in melanoma patients resulted from a decline of particular T cell differentiation subsets. Therefore, we further divided the CD4+ T cells compartment into CD45RO-CCR7+ naive (T_{Naive}), CD45RO+CCR7+ central memory (T_{CM}), CD45RO+CCR7- effector memory (T_{EM}) and CD45RO-CCR7- terminally differentiated (T_{TD}) cells (Figure 1A). Proportions of CD4+ T_{Naive} cells were decreased in young melanoma patients when compared to age-matched healthy controls (Figure 1B). Proportions of CD4+ T_{Naive} cells in young melanoma patients were actually similar to those in old patients and controls. We observed trends for increased proportions of CD4+ T_{CM} en T_{EM} cells in young melanoma patients versus age-matched controls (Figure 1C and D), whereas proportions of CD4 T_{TD} cells were similar in young patients and controls (Figure 1E). The percentages of all CD4+ T cell differentiation subsets were similar in old mela-
noma patients and age-matched controls. We obtained similar results when CD4+ T$_{\text{Naive}}$ and CD4 T$_{\text{TD}}$ cells were more stringently defined as CD45RO-CCR7-CD27+CD28+ and CD45RO-CCR7-CD27-CD28- cells, respectively (Supplemental Figure 1). Among CD8+ T cell differentiation subsets, we observed no differences between melanoma patients and healthy controls (data not shown).

As CD4+ T$_{\text{Naive}}$ cells were found reduced in young melanoma patients, we next determined if CD31+ thymic emigrant CD4+ T$_{\text{Naive}}$ cells or post thymically expanded CD31- central CD4+ T$_{\text{Naive}}$ cells were decreased in young melanoma patients (Figure 1F). Proportions of CD31+ thymic emigrant CD4+ T$_{\text{Naive}}$ cells were decreased in young patients when compared to age-matched controls (Figure 1G). Young melanoma patients were actually demonstrating similar low proportions of these cells, as old patients and controls. In contrast, proportions of post thymically expanded CD31- central CD4+ T$_{\text{Naive}}$ cells were comparable in young and old melanoma patients versus the age-matched controls (Figure 1H). Thus, the CD4+ T$_{\text{Naive}}$ cell compartment of young melanoma patients resembled those of old patients and controls, rather than that of young healthy controls.

### Expression of activation and proliferation markers by circulating CD4+ T cells

We studied the activation status of CD4+ T cells in the young and old melanoma patients by determining the percentage of HLA-DR expressing cells (Figure 2A). Percentages of HLA-DR expressing cells were increased among CD4+ T cells of young melanoma patients when compared to those in young controls (Figure 2A). Proportions of HLA-DR expressing CD4+ T cells in young melanoma patients resembled those in old patients and controls.

In addition, we determined the percentage of proliferating CD4+ T cells by analyzing these cells for expression of Ki-67 (Figure 2B). The percentage of Ki-67 expressing cells was higher in young melanoma patients than in age-matched healthy controls. In contrast, no modulation of Ki-67 was observed in old melanoma patients when compared to their age-matched controls.

We also assessed CD4+ T cells for expression of CD161, a killer cell lectin-like receptor that identifies a population of highly pro-inflammatory cells (Figure 2C). Young melanoma patients showed an increase of CD161 expressing CD4+ T cells compared to young controls (Figure 2C). In contrast, the percentage of CD161 expressing cells was similar in old melanoma patients and age-matched controls. Thus, circulating CD4+ T cells of young melanoma patients show clear signs of an ongoing immune response, whereas these signs are lacking in CD4+ T cells of old melanoma patients.

### PD-1 and CTLA-4 expression by CD4+ T cells

We determined if CD4+ T cells of young and old melanoma patients show increased expression of the checkpoint molecules PD-1 and CTLA-4. Percentages of PD-1 expressing cells were increased in young melanoma patients when compared to age-matched controls (Figure 3A). In contrast, the percentage of PD-1 expressing CD4+ T cells was not modulated in old melanoma patients. The percentage of CTLA-4 expressing cells CD4+ T cells was similar in melanoma patients and controls, both in young subjects and old subjects.
Lymphocyte profile in melanoma patients

(Figure 3B). Thus, the CD4+ T cell compartment of young melanoma patients, but not old melanoma patient, shows increased expression of the checkpoint inhibitor PD-1 but not CTLA-4.

Regulatory T cells
Finally, we questioned if numbers of regulatory T cells are modulated in melanoma patients. Therefore, we assessed the proportions of FOXP3+Helios+ and FOXP3+Helios- regulatory T cells in the peripheral CD4+ T cell compartment of patients and controls (Figure 4A). The proportions of FOXP3+Helios+ regulatory T cells were, irrespective of age, comparable in melanoma patients and healthy controls (Figure 4B). In contrast, we observed a clear increase of FOXP3+Helios- regulatory T cells in young and old melanoma patients when compared to their age-matched controls (Figure 4C). Thus, we observed preferential expansion of Helios-negative regulatory T cells in patients with metastatic melanoma.
Figure 1: CD4+ T cell differentiation subsets in melanoma patients and controls.  
(A) Representative flow cytometric staining of CD45RO and CCR7 in CD4+ T cells in melanoma patients and  
age-matched controls. (B) Percentages of CD45RO-CCR7+ CD4+ T<sub>Naive</sub> cells, (C) CD45RO+CCR7+ CD4+ T<sub>CM</sub>  
cells, (D) CD45RO+CCR7+ CD4+ T<sub>EM</sub> and (E) CD45RO-CCR7+ CD4+ T<sub>TD</sub> cells in young controls (n = 13), young  
patients (n = 11), old controls (n = 39) and old patients (n = 15). (F) Representative flow cytometric staining for  
CD31 in CD4+ T cells in melanoma patients and healthy controls. (G) Percentages of CD31+ thymic emigrant  
CD4+ T<sub>Naive</sub> cells and (H) CD31- central CD4+ T<sub>Naive</sub> cells in the same patients and controls. Statistical signifi-  
cance is indicated as * p < 0.05, ** p < 0.01 and *** p < 0.001.
Figure 2: Activation and proliferation of CD4+ T cells in melanoma patients and controls. (A) Left panel: representative staining of HLA-DR on CD4+ T cells of patients and controls. Right panel: percentages of HLA-DR4+ CD4+ T cells in young controls (n = 12), young patients (n = 10), old controls (n = 34), old patients (n = 15). (B) Left panel: representative staining of intracellular Ki-67 in CD4+ T cells of patients and controls. Right panel: percentages of Ki-67+ CD4+ T cells in young controls (n = 10), young patients (n = 10), old controls (n = 10), old patients (n = 10). (C) Left panel: representative staining of CD161 on CD4+ T cells of patients and controls. Right panel: percentages of CD161+ CD4+ T cells in young controls (n = 13), young patients (n = 11), old controls (n = 39) and old patients (n = 15). Statistical significance is indicated as * p < 0.05, ** p < 0.01 and *** p < 0.001.
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Figure 3: Expression of checkpoint molecules by CD4+ T cells of melanoma patients and controls.
(A) Left panel: representative staining for PD-1 on CD4+ T cells of patients and controls. Right panel: percentages of PD-1+ CD4+ T cells in young controls (n = 10), young patients (n = 10), old controls (n = 10), old patients (n = 10). (B) Left panel: representative staining of intracellular CTLA-4 in CD4+ T cells of patients and controls. Right panel: percentages of CTLA-4+ CD4+ T cells in the same donors as mentioned at (A). Statistical significance is indicated as ** p < 0.01.

Figure 4: Regulatory T cell frequencies in melanoma patients and controls.
(A) Representative staining for intracellular FOXP3 and Helios in CD4+ T cells of patients and controls. (B) Percentages of FOXP3+Helios+ regulatory T cells and (C) FOXP3+Helios- regulatory T cells in young controls (n = 10), young patients (n = 10), old controls (n = 10), old patients (n = 10). Statistical significance is indicated as * p < 0.05 and *** p < 0.001.
Discussion

We here provide evidence for a poor CD4+ T cell response in the peripheral blood of old melanoma patients, whereas CD4+ T cells of young melanoma patients showed prominent signs of activation, proliferation and differentiation. The notion of an ongoing immune response in young melanoma patients is further substantiated by the decrease of thymic emigrant CD4+ TNaive cells and the concomitant expansion of TCM and inflammatory TEM when compared to age-matched controls. Interestingly, proportions of CD4+ TNaive cells in young melanoma patients were comparable to those in the old patients and controls, suggesting a melanoma-induced immune response. Thus, our findings suggest poor activation of peripheral CD4+ T cells in old melanoma patients, whereas the CD4+ TNaive cell pool shows signs of premature contraction in young melanoma patients. The latter finding may be due to chronic stimulation with melanoma antigens.

The reduced activation status of circulating CD4+ T cells in old melanoma patients might contribute to the worse biological behavior and survival of melanoma in the elderly. CD4+ T cells play a central role in anti-tumor responses and empower tumor-specific CD8+ T cells to gain their full cytotoxic phenotype. It remains to be elucidated why CD4+ T cells respond poorly to melanoma in the elderly. Both CD4+ T cell inherent changes and functional impairment of antigen presenting cells are likely relevant. Remarkably, therapeutic melanoma trials with checkpoint inhibitors directed to CTLA-4 or PD-1 that prospectively stratify patients for age to assess for differences in outcome report that the response is independent of age. One explanation for the latter finding might be the substantial selection bias in these therapeutic studies towards fit elderly with a more indolent disease course.

Although the CD4+ T cells of young melanoma patients showed clear signs of activation and proliferation, these subjects all had metastatic disease. This means that their immune system has still failed to prevent disease progression and the degree of activation is therefore proven to be insufficient. Remarkably, we observed low proportions of CD4+ TNaive cells in young melanoma patients in comparison to age-matched controls. The proportions of these cells were actually comparable to those in old patients and controls. Interestingly, this premature contraction of the CD4+ TNaive cell pool in young melanoma patients could be entirely attributed to a decrease of CD31+ thymic emigrant CD4+ TNaive cells. Although it is unclear if this premature contraction has developed due to or prior to disease, it likely compromises CD4+ T cell immunity against the full spectrum of melanoma antigens.

We observed increased expression of PD-1 on circulating CD4+ T cells in young melanoma patients. This is an interesting finding, as PD-1 blocking therapy have proven successful in melanoma patients. PD-1 is an inhibitory receptor expressed by memory T cells and an early marker of exhausted T cells. The increased expression of PD-1 on CD4+ T cells in young patients likely mirrors the activation of these cells. In contrast, we observed no clear modulation of CTLA-4 in CD4+ T cells of melanoma patients and healthy controls. Baseline signatures of peripheral blood biomarkers are studied to
predict response to immune checkpoint inhibitors. For example, decreasing levels of CD4+CD25+FOXP3+ regulatory T cells during ipilimumab therapy are associated with a favorable response. Whether peripheral baseline PD-1 or CTLA-4 expression levels are useful to incorporate in a predictive biomarker signature is currently unclear.

FOXP3 is the hallmark transcription factor of regulatory T cells. In addition, expression of Helios appears to boost the regulatory functions of these suppressive cells. We found percentages of FOXP3+Helios- regulatory T cells to be increased in melanoma patients, irrespective of their age. We observed no increase of FOXP3+Helios+ regulatory T cells. The increase of FOXP3+Helios- regulatory T cells in melanoma patient might indicate that these cells develop in the presence of inflammatory cytokines. The interpretation of this finding is not complete clear: less regulatory function by Helios negativity in the context of inflammation might support an ongoing anti-tumor response. However, the precise function of FOXP3+Helios- regulatory T cells needs additional research in the context of inflammation and cancer.

We are aware that our findings do not necessarily reflect immune responses at the tumor site in melanoma patients. Brisk tumor-infiltrating lymphocytes and high lymphocyte tumor distribution and density in melanoma are associated with improved disease-specific survival. A study with tumor tissue samples from 147 metastatic melanoma patients showed an independent positive association between overall survival and higher counts of CD8+ T cells and PD-1 expressing cells. CD4+ T cell and regulatory T cell counts were not predictive of survival. However, these cells may primarily fulfill their functions outside the tumor site, for instance in surrounding secondary or tertiary lymphoid structures. It would therefore be interesting to study CD4+ T cells in lymphoid tissues of melanoma patients.

In conclusion, we provide evidence that circulating CD4+ T cells in young patients with metastatic melanoma are strongly activated, whereas CD4+ T cells of old melanoma patients seem relatively dormant. This difference might contribute to unfavorable behavior of melanoma in the elderly. In addition, our findings suggest premature contraction of the CD4+ T_Naive cell compartment in young patients with metastatic melanoma.
References


Supplemental Figure 1:
(A) Percentages of CD27+CD28+CD45RO-CCR7+ CD4+ T_{Naive} cells and (B) CD27-CD28-CD45RO-CCR7- CD4+ T_{TD} cells in young controls (n = 13), young patients (n = 11), old controls (n = 39) and old patients (n = 15).
Statistical significance is indicated as * p < 0.05 and *** p < 0.001.

Supplemental Table: Baseline characteristics of young and old metastatic melanoma patients prior to systemic treatment.
The values shown represent a median unless otherwise indicated. LDH = lactate dehydrogenase, S-100B = S100 calcium binding protein B, ESR = erythrocyte sedimentation rate, CRP = C-reactive protein, OS = overall survival.