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Blood Immune Cell Biomarkers in Patient With Lung Cancer Undergoing Treatment With Checkpoint Blockade

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Summary: Characterization of host immune cell parameters before and during immunotherapy is expected to identify predictive biomarkers for clinical outcome. We prospectively monitored blood immune cells from 35 patients with advanced non–small cell lung cancer undergoing checkpoint inhibitor monotherapy. The aim was to identify parameters correlating with better/worse outcome. Peripheral blood was serially collected before each infusion at the onset and at cycle 3 and 5 of immunotherapy. A complete leukocyte blood count, the lymphocytic subpopulations and the percentages of both HLA-DR^{low} monocytes and dendritic cells (DC) were monitored. Disease control was defined as partial/complete response and stable disease on computed tomography scan according to RECIST 1.1. The predictive value of the immune cell parameters investigated was evaluated by patients' survival analysis. Forty percent of patients showed a clinical response, and the global median overall survival was 7.0 months (95% confidence interval: 3.5–10.5). Patients with an initial neutrophil-to-lymphocyte ratio (NLR) ≥ 5.2, and/or an amount of HLA-DR^{low} monocytes $\geq 11\%$ and/or a total DC level $\leq 0.4\%$ of leukocytes did rarely respond to PD-1 inhibitor therapy. Otherwise, the immunotherapy-induced decrease of the neutrophil-to-lymphocyte ratio and/or HLA-DR^{low} monocytes and the increase of total DC frequencies were correlated with improved therapy response and prolonged overall survival. Blood values in the third cycle of immunotherapy did already reflect the effects observed. On the basis of the 3 immune cell parameters identified we created 3 different variants of scores that enable to stratify patients into groups of risk/therapy response. Our results warrant further investigation in larger prospective clinical trials for validation.

Key Words: biomarker, dendritic cells, flow cytometry, immune monitoring, lung cancer, HLA-DR^{low} monocytes, neutrophil-to-lymphocyte ratio, PD-1 inhibitor

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BACKGROUND

Despite the tremendous developments in early detection and novel treatment modalities, the overall survival (OS) of patients with lung cancer has not much improved during the past decades. However, current studies have shown benefits of immunotherapy in lung tumors,[1] in particular those targeting the immune-checkpoint proteins PD-1/PD-L1. By blocking the inhibitory signal between PD-1 on T cells and PD-L1 on tumor cells, T cells get back the capacity to attack cancer cells. The promising benefit was shown for selected patients with advanced non–small cell lung cancer (NSCLC) treated with the PD-1 inhibitors pembrolizumab or nivolumab in first-line or second-line settings (for review see Brahmer et al's study[2]). However, not all patients do respond to therapy, and some patients develop therapy-resistance at the beginning or in the course of treatment.

The identification of baseline characteristics of patients who will most benefit from treatment with immunotherapy remains an important challenge. Biomarker-driven selection of immunotherapy responders and nonresponders would minimize unnecessary exposure of patients to potentially permanent immune-related toxicities and reduce the financial burden for health systems because of these expensive treatments.[3] The optimal predictive biomarker should be easily applicable in clinical settings, cost–effective, and provide an accurate prediction of a patient's clinical response. Tissues that lack tumor-infiltrating lymphocytes (TILs) are unlikely to respond to immune-checkpoint inhibitors; therefore, the percentage of TIL has been shown to predict response to anti-PD-1 therapy in melanoma patients.[4] Furthermore, the response rate to checkpoint blockade tends to be proportional to the tumor mutational burden resulting in neoantigens recognized by T cells. Rizvi et al[5] showed that response to anti-PD-1 treatment correlated with high tumor mutational burden and neoantigen load in patients with NSCLC. However, cancers with similar mutational burden can have very different response rates to checkpoint blockade therapy indicating that additional mechanisms play an important role.[6] Factors that affect the choice of treatment in NSCLC lacking a driver mutation include the level of PD-L1 expression, the extent of disease, and histology; for example, for patients with PD-L1 expression $\geq 50\%$ of cancer cells, pembrolizumab monotherapy is a preferred treatment option (KEYNOTE-024 study[7]). As other predictive parameters for risk stratification and treatment strategies are urgently needed, several studies investigate the benefit of blood immune cells, such as monocytes,[8] neutrophils, or lymphocytes,[9–11] as biomarkers. Flow cytometry serves as a powerful analytical platform for the rapid characterization of individual cells within heterogenic cell populations. The aim of this study was to evaluate blood immune cells as potential predictive biomarkers for patients with lung cancer undergoing checkpoint blockade therapy. In addition to lymphocytic subpopulations, we focused especially on cells of the innate immune system,

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such as neutrophils, HLA-DR^{low} monocytes, representing a subtype of myeloid-derived suppressor cells (MDSC),[12] and dendritic cells (DC).

MATERIALS AND METHODS

Patient Cohort

This study was approved by the institutional review board of the Ärztekammer Sachsen-Anhalt (Germany). EDTA peripheral blood samples were obtained from 35 patients with advanced lung cancer treated within the Clinic of Internal Medicine, Hospital Martha-Maria Halle-Dölau, Halle, Germany. Patients prospectively enrolled met the following criteria: age >18 years, histologically confirmed the diagnosis of metastatic NSCLC, PD-L1 expression investigated by immunohistochemistry, adequate organ function, and capacity to make an informed decision. All patients were negative for epidermal growth factor receptor mutation or anaplastic lymphoma kinase translocation. Patients with a previous history of systemic immunosuppressive therapy or active autoimmune disease were excluded.

Enrolled patients received either pembrolizumab as monotherapy (Keytruda; MSD Merck Sharp & Dohme AG; 200 mg for chemotherapy-naïve patients, or 2 mg/kg for patients previously treated with chemotherapy) every 3 weeks, or nivolumab (Opdivo; Bristol-Myers Squibb SA; administered intravenously at a dose of 3 mg/kg) every 2 weeks. Agent choice was on the basis of the PD-L1 status and patients' previous treatment history (first- or second-line setting). Toxic effects were graded with the use of the National Cancer Institute Common Terminology Criteria for Adverse Events, version 4.0.

Scheduled computed tomography or magnetic resonance imaging was performed every 9 weeks according to RECIST 1.1 criteria or with clinical worsening of the patient's condition. We defined a treatment benefit according to the following criteria: stable disease and partial/complete response. Treatment continued until confirmed disease progression, unacceptable toxicity, or withdrawal of consent. In most cases, patients who did not continue immunotherapy beyond the third cycle were patients, whose clinical conditions deteriorated.

Blood Samples, Flow Cytometry, and Antibody Staining

Peripheral blood samples (2.7 mL EDTA blood) were taken before each infusion: (i) at the day of treatment onset; (ii) at the third cycle; (iii) at the fifth cycle of immunotherapy. Blood was prepared within 4–6 hours to prevent the increase of the monocytic HLA-DR expression caused by phagocytosis. A complete leukocyte blood count was monitored. Flow cytometry samples were measured with a FACS CANTO II flow cytometer (BD Biosciences, Heidelberg, Germany). Data analyses were performed with BD FACS DIVA software. Cytometer Setup and Tracking (CST) Beads (BD Biosciences) were used daily to set standardized geometric mean fluorescence intensity (MFI) ranges in the fluorescence channels used. Absolute values of CD4$^+$ and CD8$^+$ T cells, B cells, and natural killer (NK) cells were determined using the BD Multitest IMK kit and BD Trucount tubes (BD Biosciences) with a no-wash procedure according to the manufacturer's instruction. Circulating DC populations were identified with the "Blood DC Enumeration Kit" (Miltenyi, Bergisch Gladbach, Germany) supplemented with the monoclonal antibody (mAb) CD16 for the detection of CD16$^+$ DC, and with an HLA-DR mAb for gating reasons. Briefly, aliquots of whole blood were labeled with a cocktail of mAb consisting of anti-CD14/CD19 PE-Cy5 plus anti-CD1c-PE as a marker for myeloid DC (mDC2), CD141/BDCA-3 APC (mDC1), and CD303/BDCA-2 FITC for plasmacytoid DC (pDC)[13] in addition to mAb CD16 PE-Cy7 (Biolegend, Fell, Germany) and HLA-DR V500 (BD Biosciences). After antibody incubation, red cell lysis, and 2 washing steps, the cells were fixed according to manufacturer's instructions. At least 1 million blood leukocytes were analyzed, and gating strategy is provided in Supplemental Figure 1 (Supplemental Digital Content 1, http://links.lww.com/JIT/A546).

Monocytic HLA-DR expression was quantified with mAb labeled on a protein/fluorophore ratio of 1/1 (QuantiBRITE reagents; BD Biosciences). The anti-HLA-DR 1/1 PE (clone L243)/anti-CD14 PerCP-Cy5.5 mAb was used according to the manufacturer's instruction. A standard curve for antigen quantification was established using multilevel calibrated QuantiBRITE beads. The measured geometric MFI of the gated population was converted into "antibody molecules bound per cell" (ABC) using Microsoft Excel spreadsheet. HLA-DR MFI values of ≤5000 ABC for the whole monocytes population have been designated as "immunoparalysis" in former studies, as the patients are at high risk of infectious diseases.[14] Taking an MFI of 5000 ABC as borderline value for a low HLA-DR intensity, the amount of HLA-DR^{low} monocytes was estimated as percentage of CD14$^+$ cells as recently described.[15]

Statistical Analyses

The statistical analysis was done using the commercial software SPSS 25.0 (SPSS Inc., Munich, Germany). Differences in the number of immune cells between patients with different responses to therapy were analyzed using ANOVA analysis. All *P*-values are exploratory. To evaluate correlations between neutrophil-to-lymphocyte ratio (NLR) or HLA-DR^{low} monocytes with other immune cell parameters, Spearman correlation coefficients were calculated. Survival was defined as the time from the first cycle of nivolumab/pembrolizumab to progression (according to RECIST) or death for progression-free survival (PFS), or death alone for OS. Survival analysis firstly comprised a descriptive presentation of the cumulative survival functions according to Kaplan-Meier. Differences among the curves were evaluated using the log-rank test. In addition, univariate Cox regression analysis was performed to examine the correlation of immune cell parameters with PFS and OS. Two-sided *P*-values of <0.05 were considered statistically significant. Predictor variables with a significant difference between the patients' groups with and without response to treatment were analyzed with receiver operating characteristic (ROC) curves to determine the overall strength of association [area under the ROC curve (AUC)], the optimal cutoff point for the prediction of therapy response (maximizing the sum of sensitivity and specificity), and the predictive values obtained with this cut point. In addition to a risk score indicating patients with a high probability of nonresponse (score variant A), 2 predictive scores were calculated (variants B and C), with higher score values indicating a higher probability of treatment response.

RESULTS

A total of 35 patients with NSCLC, who received at least 2 cycles of immunotherapy with an anti-PD-1 antibody, were enrolled in this study. Detailed characteristics of patients are provided in Table 1. The median age was 65 years (range, 24–85 y), 19 patients were male individuals. Most of the patients were current or former smokers. The majority of cancers were adenocarcinoma (66%). Information about tumor expression of PD-L1 was available for 34 patients, of which 23 had a PD-L1 expression $\geq 1\%$. Pembrolizumab was offered to 18/35 (51%) patients; the remaining 17 of 35 (49%) patients received nivolumab. The most frequently reported treatment-related adverse events were low in severity and included fatigue and hypothyroidism (in 5.7% of patients).

At the time of data cut off, the mean follow-up time was 9.7 months (range, 1–26 mo), and 7 patients continued to receive anti-PD-1 inhibitors. Nine patients stopped treatment before the third cycle in most cases because of clinical worsening. The rate of confirmed objective response was 40% for all patients, and most patients without a disease control died within 4–5 months. The global median OS was 7.0 months [95% confidence interval (CI), 3.5–10.5]. The 6 patients with an age more than 75 years had a tendency to poorer survival (5.2 ± 0.8 compared with 12.9 ± 2.0 mo; $P = 0.078$). Comparable with other studies,[16] never smokers had low responsiveness to the immunotherapy, with only 1 clinical response observed (stable disease). Because of the low number of 5 patients, this group was not evaluated separately. Comparing survival data of patients in first-line with those of second-line monotherapy setting, no significant difference could be observed for OS and PFS in Kaplan-Meier curves, though a tendency to better survival of patients in first-line setting was observed after 10 months (data not shown).

TABLE 1. Patient Characteristics

Age at start of immunotherapy (y), n	
Median	65
Range	24-85
> 75 y	6 (17)
Sex, n (%)	
Male	19 (54)
Female	16 (46)
Histology, n (%)	
Adenocarcinoma	23 (66)
Squamous cell carcinoma	7 (20)
Adenosquamous	5 (14)
Smoking status	
Current or former smokers	30 (86)
Never smokers	5 (14)
PD-L1 expression, n (%)	
< 1%	11 (31)
1%-49%	9 (26)
≥ 50	14 (40)
Missing	1
Blood neutrophils	
≥ 10,000/μL	5 (14)
Blood thrombocytes	
> 400,000/μL	5 (14)
Liver metastasis	
n	3
Therapy setting, n (%)	
First-line monotherapy	14 (40)
Second-line monotherapy	21 (60)
Clinical response, n (%)	
Progression (P)	21 (60)
Disease stabilization (S)	7 (20.0)
Partial/complete response (R)	7 (20.0)

PD-L1 indicates programmed death-ligand 1.

Table 2 summarizes the initial immune cell parameters of patients (i) with a PFS ≤ 1 month, (ii) which were progressors with a PFS > 1 month, and (iii) which showed a clinical response (stable disease or partial/complete response). Data are mainly expressed as cells/μL blood, which allows a better comparison of values with known reference ranges. For the initial values, a high number of neutrophils ($> 10{,}000$ cells/μL) was associated with a very low PFS (≤ 1 mo). Furthermore, patients with a high percentage of HLA-DR^{low} monocytes ($> 9\%$ of monocytes) and low percentages of pDC, CD1c$^+$ mDC, and CD141$^+$ mDC (with total DC $\leq 0.4\%$ of leukocytes) showed the lowest PFS. Also for the absolute counts of pDC and mDC (cells/μL) significant differences were observed between the 3 groups (data not shown). CD141$^+$ mDC were rarely detected in patients with lung cancer, but progressors with a low PFS also showed the lowest initial percentages. There was no clear difference for the lymphocyte counts and for lymphocytic subpopulations between the 3 patients' groups (Table 2). Kaplan-Meier curves for the OS of patients with > 400 compared with ≤ 400 CD4$^+$ T cells showed a tendency to better survival for patients with higher amount of helper T cells, but only after 11 months ($P = 0.307$, data not shown).

Furthermore, the initial values of NLR, HLA-DR^{low} monocytes, and total DC did not differ between the groups "clinical response" and "progression with a PFS > 1 month." However, Kaplan-Meier curves of Figure 1 illustrate that patients with an NLR at therapy onset ≥ 5.2 ($P = 0.003$), a percentage of HLA-DR^{low} monocytes ≥ 11 ($P = 0.004$), and a total DC frequency $\leq 0.4\%$ of leukocytes ($P = 0.001$) had a significantly lower PFS. Furthermore, data of univariate prognostic factor analysis (Kaplan-Meier and Cox regression) showed significant differences in the OS of patients as provided in Table 3.

Never smokers had lower amounts of HLA-DR^{low} monocytes (2.9 ± 1.9 vs. $7.2 \pm 5.5\%$ of monocytes in never smokers vs. ever-smokers), as already described.[15] Despite a tendency to higher neutrophil counts in ever smokers, the NLR was not different between never smokers and ever smokers. Supplemental Table 1 (Supplemental Digital Content 1, http://links.lww.com/JIT/A546) compares data of NLR, HLA-DR^{low} MDSC, and total DC frequencies for patients receiving first-line versus second-line monotherapy of checkpoint blockade. In both therapy settings, patients with a PFS ≤ 1 month had the highest NLR, the highest percentages of HLA-DR^{low} monocytes and the lowest frequencies of total DC. Furthermore, in both settings, a clinical response was associated with an increase of DC levels, and stable or decreasing values of NLR and HLA-DR^{low} MDSC at the time point of third cycle. Therefore, data of both patient's groups were pooled in further analyses. As we did not investigate patients after the fifth cycle of immunotherapy, we cannot exclude a difference in the blood parameters between first-line and second-line settings beyond cycle 5.

At the time point of cycle 3, clear differences were observed between responders and nonresponders (Table 2), for example, patients with a clinical response had significantly lower neutrophil counts (resulting in a lower NLR) and lower HLA-DR^{low} monocytes. Otherwise, therapy responders were

TABLE 2. Blood Immune Cells Before and During Anti-PD-1 Antibody Monotherapy

	Onset of Treatment				Time Point of Cycle 3		
	PFS ≤1 mo	**Progressive Disease with PFS >1 mo**	**Clinical Response**	***P***	**Progressive Disease**	**Clinical Response**	***P***
N	9	12	14		14	14	
Leukocyte counts (cells/μL)	**11586 ± 3186**	8495 ± 2603	8597 ± 2262	0.009	10276 ± 3934	**7544 ± 1851**	0.027
Neutrophil counts (cells/μL)	**9442 ± 3110**	5739 ± 2117	6214 ± 1948	0.002	8165 ± 3680	**5068 ± 1662**	0.008
Lymphocyte counts (cells/μL)	1351 ± 625	1460 ± 653	1459 ± 519		1218 ± 623	1634 ± 725	
NLR	7.1 ± 3.3	4.8 ± 3.1	5.0 ± 2.9		9.18 ± 6.96	**4.08 ± 3.13**	0.019
$CD3^+$ T cells (cells/μL)	923 ± 442	998 ± 518	989 ± 401		829 ± 574	1072 ± 487	
$CD4^+$ T cells (cells/μL)	514 ± 215	518 ± 265	577 ± 252		390 ± 226	**607 ± 281**	0.033
$CD8^+$ T cells (cells/μL)	365 ± 242	395 ± 263	355 ± 222		345 ± 290	401 ± 262	
NK cells (cells/μL)	136 ± 65	173 ± 113	227 ± 143		168 ± 132	280 ± 164	
HLA-DR^{low} MDSC (% of monocytes)	9.6 ± 8.3	5.4 ± 5.0	5.8 ± 2.5		11.3 ± 11.5	**3.9 ± 2.6**	0.028
Total DC (% of leukocytes)	**0.42 ± 0.34**	0.83 ± 0.26	0.87 ± 0.35	0.009	0.53 ± 0.45	**1.29 ± 0.63**	0.001
$CD16^+$ DC (% of leukocytes)	0.34 ± 0.34	0.59 ± 0.22	0.60 ± 0.315		0.42 ± 0.33	**1.01 ± 0.61**	0.005
pDC (% of leukocytes)	**0.033 ± 0.02**	0.098 ± 0.051	0.119 ± 0.054	0.001	0.09 ± 0.08	0.105 ± 0.05	
$CD1c^+$ mDC (% of leukocytes)	**0.049 ± 0.028**	0.125 ± 0.068	0.146 ± 0.068	0.002	0.09 ± 0.11	**0.168 ± 0.066**	0.029
$CD141^+$ mDC (% of leukocytes)	**0.003 ± 0.002**	0.008 ± 0.007	0.012 ± 0.009	0.015	0.006 ± 0.007	0.009 ± 0.006	

For the onset of treatment, parameters are shown in the 3 patient groups "PFS <1 month," "progressive disease with PFS >1 month," and "clinical response."

At the time point of cycle 3, values are given for the groups "progressive disease" and "clinical response."

Bold values highlight significant differences in 1-way ANOVA.

ANOVA indicates analysis of variance; DC, dendritic cell; mDC, myeloid dendritic cell; MDSC, myeloid-derived suppressor cell; NK, natural killer; NLR, neutrophil-to-lymphocyte ratio; pDC, plasmacytoid dendritic cell; PFS, progression-free survival.

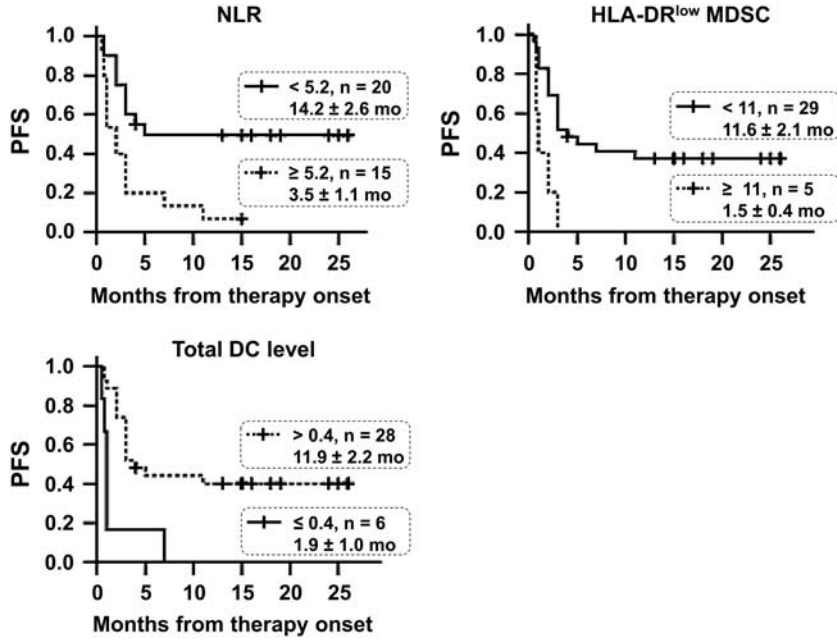

FIGURE 1. PFS for patients grouped below and above cutpoint for the parameters NLR (HR, 3.0; $P = 0.009$), HLA-DR^{low} monocytes (HR, 3.85; $P = 0.011$) and total DC levels (HR, 4.35; $P = 0.003$), estimated at the onset of checkpoint therapy. In Kaplan-Meier plots, patients with censored values are denoted by tick marks. Patient number (n) is given for each group and the mean ± standard error of the estimated PFS. DC indicates dendritic cell; HR, hazard ratio; NLR, neutrophil-lymphocyte ratio; MDSC, myeloid-derived suppressor cell; PFS, progression-free survival.

TABLE 3. Relationship Between Blood Immune Cell Parameters With Patient's OS

			Kaplan-Meier			Cox Regression		
Variable	**Cutoff Point**	**N**	**% Censored**	**OS Time (mo)**	**Log-rank Test**	**HR**	**95% CI**	***P***
NLR	< 5.2	20	50.0	13.16	0.017			0.030
	≥ 5.2	15	13.3	6.49		2.504	1.090-5.753	
HLA-DR^{low} MDSC (%)	< 11	29	41.4	11.78	0.020			0.039
	≥ 11	5	0	4.2		2.944	1.055-8.215	
Total DC (%)	≤ 0.4	6	16.7	2.83	0.005	3.726	1.291-10.75	0.015
	> 0.4	27	40.7	12.03				
Score variant A	< 1	12	75.0	21.25	< 0.001			
	≥ 1	20	10.0	6.77		7.291	2.087-25.47	0.002
Score variant B	< 5.5	23	13	6.96	< 0.001			
	> 5.5	10	80	22.6		9.516	2.157-41.99	0.003
Score variant C	< 3.5	15	20	10.0	0.004			
	> 3.5	10	80	22.2		6.577	1.453-29.78	0.015

Data of univariate prognostic factor analysis (Kaplan-Meier and Cox regression) are shown.
HR with 95% CI and *P*-values are provided.
CI indicates confidence interval; DC, dendritic cell; HR, hazard ratio; MDSC, myeloid-derived suppressor cell; NLR, neutrophil-to-lymphocyte ratio; OS, overall survival.

often patients with higher percentages of mDC and with a higher number of CD4$^+$ T cells (Table 2). With the values at the onset of therapy set to 100%, patients with a partial/complete response showed a decrease of NLR (to 57 ± 25% of onset values), a decrease of HLA-DR^{low} MDSC (to 60 ± 30% of onset values), and an increase of total DC (to 192 ± 124% of onset values) (Supplemental Table 2, Supplemental Digital Content 1, http://links.lww.com/JIT/A546). Patients with partial/complete response had also the highest increase in lymphocyte counts, especially in NK cells and CD4$^+$ T cells. In contrast, in patients with tumor progression, an increase of both the NLR (to 200 ± 154% of onset values) and HLA-DR^{low} MDSC (to 267 ± 238 of onset values) and a decrease of total DC amounts (to 62 ± 40% of onset values) were observed. Patients with stable disease had values between the 2 options.

Figure 2 illustrates the time course of selected blood immune cell markers in the 3 patient groups (progression,

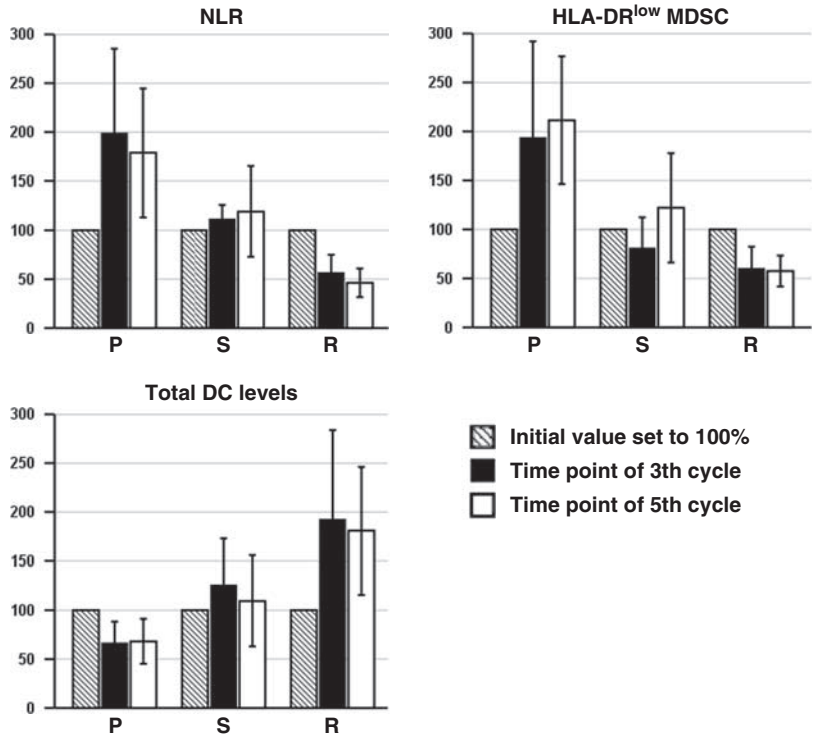

FIGURE 2. Time course of blood immune cell markers in the patients' groups progression (P), stable disease (S) and partial/complete response (R) with values at the onset of checkpoint therapy set to 100%. Mean values and error bars (95%) are displayed. DC indicates dendritic cell; NLR, neutrophil-lymphocyte ratio; MDSC, myeloid-derived suppressor cell.

stable disease, and partial/complete response) with initial values set to 100%. In most cases, a clinical response was associated with stable or decreasing values of NLR and HLA-DR^{low} monocytes, respectively, whereas the percentages of total DC increased. The effect was more pronounced in the group "partial/complete response" compared with "stable disease." Therapy response–associated changes of immune cells could already be observed at cycle 3, often with no clear further improvement at the time point of fifth cycle (Supplemental Table 2, Supplemental Digital Content 1, http://links.lww.com/JIT/A546). B-cell counts were an exception, showing a significant increase only after the fifth treatment cycle. These data suggest that checkpoint therapy-induced changes in immune cells, at least of the innate immune system, can be already monitored at the time point of cycle 3 of immunotherapy.

A high NLR significantly correlated with low percentages of total DC (Supplemental Table 3, Supplemental Digital Content 1, http://links.lww.com/JIT/A546). Within the NLR value, neutrophil counts had a strong effect on DC levels. In most cases, the correlation became more obvious in cycle 3 compared with values at the onset of checkpoint blockade therapy. As an exception, initial pDC amounts inversely correlated with initial NLR values (-0.582, $P < 0.001$), but this correlation was lost during checkpoint blockade therapy. In addition, lymphocytes, especially T cells, were positively correlated with the amounts of total DC (Supplemental Table 3, Supplemental Digital Content 1, http://links.lww.com/JIT/A546), with comparable correlations for both CD4$^+$ and CD8$^+$ T cells (data not shown). No significant correlation was found between the amount of HLA-DR^{low} MDSC and the percentages of total DC (data not shown).

In summary, initial values and therapy-induced changes in the NLR, the percentage of HLA-DR^{low} monocytes, and the frequency of total DC might be predictive biomarkers for a clinical response to checkpoint blockade therapy. Predictor variables with a significant difference between the patients' groups with and without response to treatment were analyzed with ROC curves to determine the overall strength of association (AUC), the optimal cutoff point for the prediction of therapy response (maximizing the sum of sensitivity and specificity), and the predictive values obtained with this cut point. ROC curve statistics for the prediction of therapy response by immune cell parameters are given in Table 4. The consideration of single parameters evaluated at onset of therapy, such as an NLR ≥ 5.2, HLA-DR^{low} monocytes $\geq 11\%$ of monocytes, or total DC $\leq 0.4\%$ of leukocytes, resulted in unsatisfactory AUC values <0.7. Therefore, score variants were created that might enable to stratify patients into different groups of clinical response before/during antibody treatment (Supplemental Table 4, Supplemental Digital Content 1, http://links.lww.com/JIT/A546). As a risk score (variant A), with 1 point given for being never smoker, having a NLR ≥ 5.2, a percentage of HLA-DR^{low} MDSC $\geq 11\%$, and total DC level $\leq 0.4\%$ of leukocytes, each (maximum 4 points), the AUC in predicting the progress of tumor disease was 0.762. Already with 1 adverse factor (this means a score of 1 point), 89% of patients were nonresponders to therapy (score with high sensitivity). We included never-smoker status as a risk factor to make up for the fact that HLA-DR^{low} monocytes were always lower in never smokers, as already described.[15] As a pretherapeutic score for clinical response (variant B), 1 point was given for smoking history, for having an NLR <5.2, HLA-DR^{low} MDSC <11%, and total DC levels >0.4% each. In addition, we excluded thrombocytosis[17] and old age[18] as 2 further known risk factors for patients with lung cancer in this score: 1 point was given for platelets <400,000/μL blood, and 1 point for an age less than 75 years (score with a maximum of 6 points, Supplemental Table 4, Supplemental Digital Content 1, http://links.lww.com/JIT/A546). With an AUC in predicting therapy response being 0.821, this score (cut point 5.5) had a high specificity: 95% of patients with cancer with a pretherapeutic score of 6 points did respond to therapy (Table 4). Another strong association was found for a therapy-response score monitored at the time point of cycle 3 (variant C), with pretherapeutic values set to 100%. The AUC in predicting therapy response was 0.857 (Table 4). In this score, 1 point was given for a constant value (ie, <10% change in comparison with the initial value) with respect to the 3 main parameters (NLR, HLA-DR^{low} MDSC, and total DC frequency) each; 2 points were given for an "improvement" $\geq 10\%$ of the initial value (this means: a decrease in case of both NLR and HLA-DR^{low} MDSC and an increase with respect to total DC amounts). The maximum value of this score variant was also 6 points, and the cutoff point was >3.5. At the time point of the third cycle, 91% of patients with 4 points (this means either with stable values of all 3 parameters and at least an "improvement" in 1 marker, or with an "improvement" in 2 of the 3 parameters) did respond to therapy. Out of the 10 patients with ≥ 4 points in score C, only 1 patient showed a PFS of <5 months. Both PFS and OS were significantly different for patients grouped according to these scores. Kaplan-Meier curves illustrating PFS and OS for the 3 score variants are shown in Figure 3, and univariate

TABLE 4. Receiver Operating Characteristic Curve Analysis for the Prediction of Therapy Response by Several Single Immune Cell Parameters and 3 Different Score Variants

Prediction Method	N	Cutoff Point	AUC	95% CI	*P*	Sensitivity	Specificity	PPV	NPV
Initial NLR	35	≥ 5.2	0.679	0.497-0.860	0.077				
Initial % of HLA-DR^{low} MDSC	34	$\geq 11\%$	0.625	0.438-0.812	0.221				
Initial % of total DC	34	$\leq 0.4\%$	0.630	0.438-0.823	0.209				
Initial % of pDC	34	≤ 0.06	0.689	0.511-0.868	0.064				
Risk score variant A	33	> 0.5	0.762	0.592-0.932	0.012	88.9%	57.1%	81.0%	83.0%
Response score variant B	33	> 5.5	0.821	0.666-0.977	< 0.002	64.3%	94.7%	90.0%	78.0%
Response score variant C	25	> 3.5	0.857	0.710-1.000	0.003	64.3%	90.9%	77.0%	70.0%

The prediction performance for scores is provided.

AUC indicates area under the ROC curve; CI, confidence interval; DC, dendritic cell; MDSC, myeloid-derived suppressor cell; NPV, negative predictive value; PPV, positive predictive value; ROC, receiver operating characteristic.

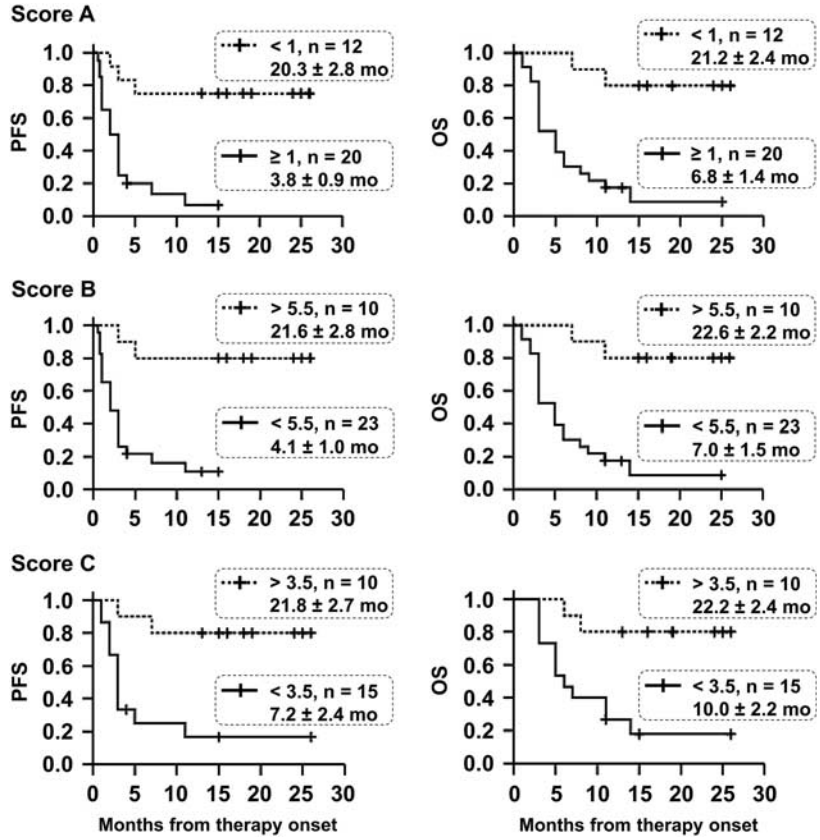

FIGURE 3. PFS and OS for patients grouped below and above cutpoint of 3 score variants (A, B, C), with the patient number and mean survival time ± standard error. Tick marks indicate censored observations. OS indicates overall survival; PFS, progression-free survival.

prognostic factor analysis for OS (Kaplan-Meier and Cox regression) is provided in Table 3.

DISCUSSION

During the past few decades, our understanding of the mechanisms and pathways regulating the immune system's response to cancer has been increased considerably and is the basis for new therapeutic options. However, challenges existing in the field of cancer immunotherapy include the inability to predict treatment efficacy and patients' clinical response, the urgent need for additional biomarkers, the development of resistance to cancer immunotherapies, and high treatment costs.[19] Future advances in cancer immunotherapy are expected to overcome and resolve many of these obstacles. Recently, immune-checkpoint inhibition has changed the therapeutic approach for patients with lung cancer, although not all of the patients with metastatic disease benefit from these immunotherapies. Unfortunately, reliable biomarkers to predict treatment benefit are scarce.

In this study, a high NLR and/or high levels of HLA-DR^{low} MDSC and/or a low frequency of total DC were identified as adverse factors for clinical response and survival in patients with NSCLC undergoing checkpoint inhibitor monotherapy. At the onset of immunotherapy, a high amount of neutrophils, and a corresponding high NLR were associated with a very low PFS (often ≤ 1 mo), this means, these patients were resembling never responders with primary resistance. Otherwise, initial neutrophil counts did not differ between patients with clinical response compared with patients with tumor progression after continuing immunotherapy for >1 month. A similar picture was observed for HLA-DR^{low} monocytes (initial high percentages associated with a very short PFS) and the total DC levels (initial low DC frequencies associated with short PFS). However, already at the time point of cycle 3 of immunotherapy, clear differences were observed between therapy responders and nonresponders, for example, patients with a partial/complete response showed a decrease of either the NLR and/or HLA-DR^{low} MDSC, and an increase of total DC. All the 3 immune cell parameters correlated both with patient's PFS and with OS in univariate prognostic factor analysis.

NLR, HLA-DR^{low} monocytes, and total DC frequency were used to establish score variants, on the one hand for patients starting therapy, and on the other hand, for patients at the time point of cycle 3. Already with 1 point, this means that out of 4 adverse factors (being never-smoker, NLR ≥ 5.2, HLA-DR^{low} monocytes $\geq 11\%$, and total DC $\leq 0.4\%$ of leukocytes), patients rarely responded to immunotherapy and had a poor OS (median, 5 mo, hazard ratio, 7.29). This risk prediction score is usable in routine clinical practice at therapy onset. Using the changes of the 3 main parameters (NLR, HLA-DR^{low} MDSC, and total DC amounts) at the time point of the third cycle in comparison with values at therapy onset, a clinical response score was proposed. Patients with a score of ≥ 4 points (eg, with an "improvement" $\geq 10\%$ of the initial value in 2 out of the 3 parameters tested) showed often a

survival time of > 20 months (hazard ratio, 6.6). However, our findings are hypotheses generating and have to be confirmed in prospective studies with larger patient cohorts. Furthermore, our scores should be compared with other scores developed for patients undergoing checkpoint inhibitor therapy, such as the Gustave Roussy immune score (with NLR, lactate dehydrogenase and serum albumin concentration), or the Royal Marsden Hospital prognostic score (including lactate dehydrogenase, albumin, and number of metastases).[20]

A higher pretreatment NLR has been shown to correlate with poor outcome in patients with different solid cancers receiving checkpoint inhibitor therapy (for review see[10]). In our analysis, a cutoff point of 5.2 was optimal for the separation of prognosis groups, a value similar to the cutoff point of 5.0 used by Bagley et al,[11] or 5.9 used by Soyano et al.[9] Neutrophils are known to facilitate tumorigenesis, promote tumor growth and metastasis, stimulate tumor angiogenesis, and mediate immunosuppression.[21] In several tumor types, the number of neutrophils in blood and tumor tissues is associated with disease progression and poor patients' outcome, for example, Kasuga et al[22] described leukocytosis being linked to poor prognosis in NSCLC. In an earlier study, a positive correlation between NLR and the percentage of regulatory T cells in lung cancer undergoing surgery of the primary tumor was described by our group.[15] In the current analyses, neutrophil counts negatively correlated with total DC frequencies. Despite the obvious view that neutrophils can negatively affect DC concentration, one might also speculate that a decrease of DC levels results in an increase in neutrophil counts, as in mice, conventional DCs play an important role in controlling peripheral neutrophil homeostasis by affecting bone marrow mobilization, or recruitment and apoptosis of neutrophils.[23]

In patients with lung cancer undergoing surgery of the primary tumor, neutrophil counts correlated with the percentage of HLA-DR^{low} monocytes, as an important subpopulation of MDSC.[15] However, this observation could not be confirmed in late tumor stages in this study. An increase of HLA-DR^{low} monocytes has been described in several tumor types (for review see[24]). In addition to soluble inflammatory factors, tumor-derived extracellular vesicles could contribute to the generation of MDSC.[25] These monocytic cells might suppress T-cell function in patients with cancer, as already described for HLA-DR^{low} monocytes in sepsis.[26] Furthermore, HLA-DR^{low} MDSC suppresses NK cell functions in patients with hepatocellular carcinoma, inhibiting autologous NK cell cytotoxicity and cytokine secretion in coculture.[27] In the literature, monocytic HLA-DR expression is rarely quantitatively determined, which hampers the comparability of data. In our investigation, the QuantiBRITE system with multilevel calibration beads and an HLA-DR-specific antibody with a 1/1 fluorochrome-to-protein ratio was used, an approach to reduce variability, leading to highly reproducible results across cytometers and institutions.[14] Using the geometric mean representing 5000 ABC as borderline value for "low" monocytic HLA-DR intensity, 2.3% HLA-DR^{low} monocytes can be found in an age-matched control group,[15] and 6.6% (range, 0.8%–26.1%) in patients with metastatic NSCLC in this study, with lower values in never smokers. This value is similar to the 9.4% HLA-DR^{low} monocytes reported by Huang et al[28] in the blood of patients with metastatic NSCLC, and to the 7.7% HLA-DR^{low} monocytes estimated by Chen et al[29] in patients with squamous cell carcinoma. Increased percentages of monocytic MDSC have been associated with worse response to treatment in patients with inoperable chemotherapy-naïve NSCLC confirming their value as biomarker.[30] Data on melanoma patients revealed that MDSC can contribute to patient resistance to immune-checkpoint inhibition (for review see[31]). Early phase clinical trials are running to date to improve outcome in patients with cancer undergoing checkpoint blockade therapy by reducing MDSC-mediated immunosuppression.[31] It is interesting to note that platinum agents, the backbone of chemotherapy for metastatic NSCLC, can not only increase antigen presentation by cancer cells and promote T-cell trafficking into the tumor microenvironment, but can also diminish HLA-DR^{low} MDSC.[32,33] Meanwhile, checkpoint blockade therapy has been combined with chemotherapy in patients with lung cancer (KEYNOTE-021[34]), and future studies might show possible effects of this therapy combination on the proportion of HLA-DR^{low} monocytes in treated patients.

Patients with NSCLC with low initial values of blood DC (both pDC and mDC) had a low PFS in this study, illustrating the value of blood DC as a putative biomarker. Furthermore, patients with partial/complete clinical response showed the highest immunotherapy-associated increase of mDC frequencies. In our investigations, DC amounts were positively correlated with the number of both CD4$^+$ and CD8$^+$ T cells. Human blood DC comprise ~1% of circulating mononuclear cells and have been classically defined as antigen-presenting leukocytes with a high expression of MHC class II (HLA-DR) molecules that lack other leukocyte lineage markers (such as CD3, CD14, CD19, and CD56). On the basis of their lineage origin, they can be divided into 2 major subsets, pDC as the major producers of type 1 interferon and mDC. Defined by the expression of CD16, CD1c/BDCA-1, and CD141/BDCA-3, 3 phenotypically distinct subsets of mDC have been described[35] and were analyzed in this study. Therapy response was especially associated with the increase of CD16$^+$ DC, whereas CD141$^+$ mDC could rarely be detected in patients with NSCLC in this study. Several DC dysfunctions have been described in cancers,[36] and the paucity of activated CD103$^+$ DC in melanoma lesions has been discussed to limit checkpoint blockade efficacy.[37] Otherwise, intratumoral CD141/BDCA-3$^+$ mDC correlates with intratumoral NK cell numbers and both innate cell types correlate with responsiveness to anti-PD-1 immunotherapy in melanoma patients.[38] These observations emphasize that understanding and modulating DC metabolism and activity might help to improve the efficacy of T-cell–centric immunotherapies in patients with tumor.[39]

The therapeutic activity of immune-checkpoint inhibitors is the result of a complex interplay between multiple factors in the tumor microenvironment and the immune system. Different mechanisms of immune suppression are known to prevent effective antitumor immunity, including increased secretion of immunosuppressive cytokines, enhanced differentiation of immune effector cells to a regulatory phenotype, and an influx of MDSC.[40] Considerable efforts are being devoted to elucidate the mechanisms controlling the development of primary and acquired resistance to checkpoint inhibitor therapy.[6] By understanding the resistance mechanisms involved, strategies can be developed to overcome resistance and treatment failure. The establishment of a standardized strategy to evaluate immune-related responses in patients receiving immune-checkpoint inhibitors will be extremely important in the future.

Biomarkers from blood sample collection are easier to handle than tumor tissues or TILs, and accumulating evidence demonstrates the potential predictive value of an increased NLR.[9–11] In this study, we confirm that NLR and the frequency of HLA-DR^{low} MDSC can predict PFS and OS in patients undergoing checkpoint inhibitor therapy, and we identified the amount of total DC as an additional predictive surrogate marker for therapy response in patients with lung cancer.

CONCLUSIONS

In conclusion, adverse factors which highlight patients with primary resistance to checkpoint blockade monotherapy are: (i) a high NLR value, (ii) high percentages of HLA-DR^{low} MDSC, and (iii) low DC frequencies at the onset of therapy. Otherwise, patients with partial/complete clinical response are characterized by the reduction of neutrophils and an increase of lymphocytes, resulting in a declining NLR. Furthermore, partial/complete clinical response is accompanied by a decrease of HLA-DR^{low} monocytes and an increase of total DC amounts. On the basis of these results, we propose score variants that categorize patients into different groups of risk or clinical response. Prospective evaluation and external validation of these scores are warranted and might help to aid patient selection in future immunotherapy trials.

CONFLICTS OF INTEREST/FINANCIAL DISCLOSURES

All authors have declared that there are no financial conflicts of interest with regard to this work.

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