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Klinik für Innere Medizin II (Chefarzt: Prof. Dr. med. W. Schütte)

Untersuchungen zur Biomarkersuche bei Patienten mit Bronchialkarzinom unter Antikörper (Checkpointblockade)- Therapie

Habilitationsschrift

zur Erlangung des akademischen Grades
eines habilitierten Doktors der Medizin (Dr. med. habil.)
für das Fachgebiet Innere Medizin

kumulative Schrift vorgelegt
der Medizinischen Fakultät
der Martin-Luther-Universität Halle-Wittenberg

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Datum der Verteidigung: 21.01.2025

Referat

Die Immuncheckpointinhibitor-Therapie hat die Behandlung des Lungenkarzinoms in den vergangenen Jahren revolutioniert. Der einzige im klinischen Alltag etablierte prädiktive Biomarker ist bisher PD-L1. Dies führt dazu, dass nahezu alle Lungenkarzinompatienten eine Immuntherapie erhalten. Das Ziel dieser Arbeit war daher die Identifikation neuer Biomarker. Es erfolgte u.a. die Untersuchung der HLA-DR^{low} Monozyten, der Neutrophilen-Lymphozyten-Ratio (NLR) und der dendritischen Zellen (DC) im Blut. Die Untersuchungen erfolgten mittels Durchflusszytometrie vor Therapiebeginn und zur dritten Antikörpergabe. Es erfolgte die Korrelation der Werte mit dem progressionsfreien Überleben)(PFS) und dem Gesamtüberleben (OS) der Patienten. Mit einem niedrigen PFS waren eine hohe NLR und hohe HLA-DR^{low} Monozyten sowie niedrige DC assoziiert. Auf Grund der Ergebnisse können u.a. die NLR, die HLA-DR^{low} Monozyten und die DC als prädiktive Biomarker für eine primäre Therapieresistenz angesehen werden.

Schlüsselwörter: Lungenkarzinom – Immuncheckpointinhibitor-Therapie – PD-L1 – Biomarker-Durchflusszytometrie

Referat

Immune checkpoint inhibitor therapy has revolutionized the treatment of lung cancer in recent years. The only predictive biomarker that is established in clinical practice is PD-L1. As a result, almost all lung cancer patients receive immunotherapy. The aim of this work was therefore the identification of new biomarkers. Among other things, the HLA-DR^{low} monocytes, the neutrophil-lymphocyte ratio (NLR) and the dendritic cells (DC) in the blood were examined. The examinations were carried out using flow cytometry before the start of therapy and at the time of the third antibody administration. The values were correlated with the progression-free survival (PFS) and the overall survival (OS) of the patients. High NLR and high HLA-DR^{low} monocytes as well as low DC were associated with low PFS. Based on the present results, the NLR, the HLA-DR^{low} monocytes, the DC and the Slan+ monocytes, among others, can be regarded as predictive biomarkers for primary therapy resistance.

Key words: lung cancer – immune checkpoint inhibitor therapy – PD-L1 – biomarker – flow cytometry

Bibliografische Angaben

Möller, Miriam
Untersuchungen zur Biomarkersuche bei Patienten mit Bronchialkarzinom unter Antikörper (Checkpointblockade)-Therapie
Halle (Saale), Universität Halle-Wittenberg, Habilitation
104 Seiten, 2023

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Abkürzungsverzeichnis

AUC	Area under the ROC Curve
CR	komplette Remission
CT	Computertomografie
DC	Dendritische Zellen
G-CSF	Granulozyten-Kolonie-Stimulierender Faktor
HLA-DR	Human leukocyte antigen-DR isotype
IFN	Interferon
IL	Interleukin
LCT	lokale konsolidierende Therapie
mDC	myeloide dendritische Zellen
MDSC	myeloide Suppressorzellen
NK	natürliche Killerzellen
NLR	Neutrophilen-Lymphozyten-Ratio
NSCLC	Nicht-Kleinzelliges Lungenkarzinom
OS	Gesamtüberleben
PD-1	programmed cell death protein-1
pDC	plasmacytoide dendritische Zellen
PD-L1	programmed death-ligand 1
PFS	Progressionsfreies Überleben
PR	partielle Remission
SCLC	Kleinzelliges Lungenkarzinom
SD	stable disease
TMB	Tumor Mutational Burden

1. Einleitung

1.1. Epidemiologie

Lungenkarzinome gehören in Deutschland zu den häufigsten malignen Erkrankungen. Bei Männern sind sie die zweithäufigsten und bei Frauen die dritthäufigsten Krebserkrankungen. Weltweit und in Deutschland ist das Lungenkarzinom die häufigste krebsbedingte Todesursache. Die 5-Jahres-Überlebensrate in Deutschland beträgt bei Männern etwa 17% und bei Frauen 22%, somit zählt das Lungenkarzinom zu den prognostisch ungünstigsten Krebserkrankungen. Die Zahl der krebsbedingten Todesfälle ist bei Männern mit ca. 28.000/Jahr deutlich höher als bei Frauen (ca. 17.000/Jahr). Histopathologisch werden drei Haupttypen unterschieden. Das Adenokarzinom macht 43% aller Lungenkarzinome aus, das Plattenepithelkarzinom 22% und das Kleinzellige Lungenkarzinom 15% (Zentrum für Krebsregisterdaten 2019). Als Hauptrisikofaktor gilt das inhalative Rauchen. Ein Screening für Risikopatienten ist in Europa aktuell noch nicht etabliert. In der größten europäischen Studie, der NELSON Trial, wurden insgesamt etwa 15.000 Patienten randomisiert. Die Einschlusskriterien waren u. a. Alter zwischen 50 und 74 Jahren sowie eine positive Raucheranamnese (Ru Zhao et al. 2011). Bei den Patienten, bei denen ein Lungenkarzinom diagnostiziert wurde, lag der prozentuale Anteil der Patienten, die in kurativer Intention operiert werden konnten bei 67,7% in der Screeninggruppe und bei 24,5% im Kontrollarm. Die Überlebensdaten zeigen eine statistisch signifikante Reduktion der karzinomspezifischen Sterblichkeit von 2,6% vs. 3,3% zu Gunsten der Screeninggruppe (Hunger et al. 2021; Koning et al. 2020). Asymptomatische Risikopatienten profitieren von einem strukturierten Lungenkarzinom-Früherkennungsprogramm mittels jährlicher Low-Dose CT-Untersuchung. Die genauen Rahmenbedingungen sind noch durch das Bundesumweltministerium, den Gemeinsamen Bundesausschuss und evtl. weitere Fachkreise festzulegen.

1.2. Immuntherapie

1.2.1. Immuntherapie beim NSCLC

Noch vor einigen Jahren gab es bei der systemischen Tumortherapie des Lungenkarzinoms zwei große Therapiesäulen, zum einen die Chemotherapie, zum anderen die zielgerichtete Therapie. Dies wurde im Jahre 2015 um eine weitere Säule erweitert, die Immuntherapie. Die Immuntherapeutika werden auch als „Checkpointinhibitoren“ bezeichnet. Tumorzellen entziehen sich dem Immunsystem. Der PD-1 Rezeptor ist auf aktivierten T-Zellen zu finden. Bindet die Tumorzelle mit ihrem Liganden PD-L1 an den PD-1-Rezeptor der T-Zelle, so resultiert eine Inhibition der T-Zelle und die Tumorzelle entzieht sich so einer Elimination durch das Immunsystem.

Im Juli 2015 wurde das erste Immuntherapeutikum, Nivolumab, für das NSCLC (Plattenepithelkarzinom) nach Versagen einer Chemotherapie, unabhängig vom PD-L1-Status, zugelassen. Nivolumab nutzt als „Checkpoint“ den PD-1-Rezeptor. In der Studie zeigte sich eine signifikante Erhöhung des 1-Jahres-Überlebens von 24% auf 41% im Vergleich zur Chemotherapie mit Docetaxel. Im Vergleich der Nebenwirkungen war die Immuntherapie ebenfalls der Chemotherapie deutlich überlegen (Brahmer et al. 2015; Borghaei et al. 2015). Ende 2020 wurde dann Nivolumab in Kombination mit Ipilimumab plus zwei Zyklen platinbasierte Chemotherapie für das NSCLC unabhängig vom PD-L1 Status und der Histologie zugelassen. Besonders profitieren Patienten mit einem PD-L1 negativen Plattenepithelkarzinom von der doppelten Immuntherapie (Paz-Ares et al. 2021). Das zweite zugelassene Immuntherapeutikum war der PD-1-Inhibitor Pembrolizumab. Nach seiner Zulassung in der zweiten Therapielinie bei einer PD-L1-Expression von mindestens 1% der Tumorzellen (Herbst et al. 2016) kam auch zügig die Zulassung in der first-line Therapie bei Patienten mit einer hohen PD-L1-Expression von mindestens 50% auf Grund der Daten aus der Keynote-024 (Reck et al. 2016). Seit Herbst 2018 ist Pembrolizumab auch in Kombination mit einer platinhaltigen Chemotherapie und unabhängig vom PD-L1- Status in der Erstlinienbehandlung zugelassen (Gandhi et al. 2018; Paz-Ares et al. 2018). Weiterhin zugelassen ist auch der PD-L1- Inhibitor Atezolizumab in der First-line-Therapie in Kombination mit Paclitaxel, Carboplatin und Bevacizumab. Diese Kombination hat in der IMpower150 Studie besondere Wirkung gezeigt bei Patienten mit Lebermetastasen sowie bei Patienten mit EGFR- und ALK- Treiberalterationen nach Versagen der Thyrosinkinaseinhibitor Therapie (Socinski et al. 2018). Auch als Dreier-Kombination mit nab-Paclitaxel und Carboplatin ist Atezolizumab zugelassen (West et al. 2019) sowie als Monotherapie in der First-line bei hohem PD-L1 Status (TPS mind. 50% oder IC mind. 10%) (Herbst et al. 2020). Ab der Secondline-Therapie ist Atezolizumab unabhängig vom PD-L1-Status als Monotherapie zugelassen (Rittmeyer et al. 2017). Das vierte in Deutschland für das Lungenkarzinom zugelassene Immuntherapeutikum ist der PD-L1-Inhibitor Durvalumab. Die erste Zulassung erfolgte beim NSCLC für das Stadium III, als konsolidierende Therapie über 1 Jahr nach simultaner Radiochemotherapie (Antonia et al. 2017). Im metastasierten Stadium ist es seit 2023 als doppelte Immuntherapie in Kombination mit Tremelimumab plus Chemotherapie unabhängig von der Histologie und dem PD-L1 Status zugelassen. Eine besondere Wirksamkeit konnte diese Kombination bei Patienten mit KRAS und/oder STK11 Mutation zeigen (Johnson et al. 2023). Zuletzt wurde Cemiplimab als weiterer PD-1 Inhibitor beim NSCLC zugelassen. Die Gabe ist im Stadium III, bei Patienten, die eine Kontraindikation gegen eine simultan kombinierte Radiochemotherapie haben, sowie im Stadium IV bei hoher PD-L1 Expression (mind. 50%) als Monotherapie möglich (Sezer et al. 2021). Bei PD-L1 Expression von mindestens 1% kann die Gabe von Cemiplimab in Kombination mit einer platinbasierten Chemotherapie in den o.g. Stadien erfolgen (Makharadze et al. 2023).

Die aktuell beim NSCLC in Deutschland zugelassenen Immuntherapeutika sind in Tabelle 1 zusammengefasst.

Tabelle 1: Stadienabhängig zugelassene Immuntherapeutika beim NSCLC

Therapielinie	Nivolumab	Pembrolizumab	Atezolizumab	Durvalumab	Cemiplimab
Stadium III	Keine Zulassung	Keine Zulassung	Keine Zulassung	PD-L1 Status ≥ 1% nach Radiochemotherapie	PD-L1 Status ≥ 50% als Monotherapie oder PD-L1 Status ≥ 1% in Kombination mit Chemotherapie
First-line	In Kombination mit Ipilimumab plus Chemo-therapie	PD-L1 Status ≥ 50% als Monotherapie oder unabhängig vom PD-L1 Status in Kombination plus Chemotherapie	PD-L1 Status ≥ 50% als Monotherapie oder unabhängig vom PD-L1 Status in Kombination plus Chemotherapie	In Kombination mit Tremelimumab plus Chemotherapie	PD-L1 Status ≥ 50% als Monotherapie oder PD-L1 Status ≥ 1% in Kombination mit Chemotherapie
Second-line	Unabhängig vom PD-L1 Status zugelassen	Zugelassen bei PD-L1 Status ≥ 1%	Unabhängig vom PD-L1 Status zugelassen	Keine Zulassung	Keine Zulassung

1.2.2. Immuntherapie beim SCLC

Beim SCLC ist seit September 2019 die kombinierte Immun-Chemotherapie mit Carboplatin/Etoposid und Atezolizumab zugelassen (Horn et al. 2018). Die Zulassung einer weiteren kombinierten Immun-Chemotherapie folgte dann im Juli 2020 mit Durvalumab in Kombination mit Platin und Etoposid (Paz-Ares et al. 2019). Damit stehen aktuell für das SCLC zwei verschiedene Kombinationstherapien unabhängig vom PD-L1 Status zur Verfügung. Diese Zulassungen waren seit Jahrzehnten die ersten Fortschritte bei der Therapie des metastasierten SCLC, wobei nur etwa 10% der Patienten langfristig von der Kombination der Chemotherapie mit dem entsprechenden Immuntherapeutikum und der jeweiligen Erhaltungstherapie profitieren. Die aktuell in Deutschland zugelassenen Immuntherapeutika für das SCLC sind in Tabelle 2 dargestellt.

Tabelle 2: Zugelassene Immuntherapeutika beim SCLC

Therapielinie	Atezolizumab	Durvalumab
First-line Therapie	In Kombination mit Platin plus Etoposid zugelassen	In Kombination mit Platin und Etoposid zugelassen.

1.2.3. Nebenwirkungen der Immuntherapeutika

Mögliche Nebenwirkungen der Immuntherapeutika sind im Vergleich zu den Chemotherapeutika seltener und wenn, dann autoimmuner Natur und somit vollkommen verschieden gegenüber denen der letztgenannten. Immunbedingte Nebenwirkungen können grundsätzlich alle Organe oder Gewebe betreffen, am häufigsten die Haut, das Kolon, die Lunge, die Leber und endokrine Organe wie Hypophyse oder Schilddrüse. Andere Organe sind sehr selten betroffen, entsprechende Nebenwirkungen können aber sehr schwerwiegend oder sogar tödlich sein wie z.B. neurologische Störungen und Myokarditis. Die meisten dieser Nebenwirkungen sind jedoch leicht bis mäßig und reversibel, wenn sie frühzeitig erkannt und behandelt werden. Die Nebenwirkungen einer Therapie mit Immuncheckpointinhibitoren treten normalerweise innerhalb weniger Wochen oder Monate nach Behandlungsbeginn auf. Sie können aber auch jederzeit während der Behandlung auftreten, z.B. schon ein paar Tage nach der ersten Infusion oder manchmal erst 1 Jahr nach Ende der Therapie. Die häufigsten Nebenwirkungen sind Juckreiz, gastrointestinale Symptome (wie Diarrhö z.B. bei Kolitis), Pneumonitis sowie Funktionsstörungen der Schilddrüse. Die am häufigsten gemeldete unerwünschte Wirkung unter Anti-PD-1/PD-L1 Blockade ist Fatigue. Die Inzidenz dieser, deren Pathogenese in einzelnen Arzneimittelstudien kaum bekannt ist, beträgt 16-37% für Anti-PD-1 und 12-24% für Anti-PD-L1 (Naidoo et al. 2015). Nur bei einer Minderheit der Patienten kann die Fatigue auf eine Hypothyreose zurückgeführt werden. Für Nivolumab wurde bei 74-85% der Patienten eine behandlungsbedingte unerwünschte Wirkung dokumentiert, wobei 12-20% Grad 3 und 4 waren (Weber et al. 2017).

In der Keynote-024-Studie wurde Pembrolizumab, welches alle 3 Wochen in einer fixen Dosis von 200 mg verabreicht wurde, mit einer Chemotherapie auf Cisplatin-Basis als Erstlinientherapie bei metastasierten NSCLC-Patienten verglichen (Tumor-PD-L1-Expression ≥ 50%). Eine behandlungsbedingte Toxizität wurde bei 73,4% (jede Nebenwirkung) festgestellt und 26,6% der Patienten hatten eine Toxizität 3. Grades oder höher (Reck et al. 2016).

Bei Hypothyreose wird ab einem TSH-Wert über 10 µU/ml die Einleitung einer entsprechenden Substitution empfohlen. Für alle anderen Nebenwirkungen gilt, leichte unerwünschte Wirkungen (Grad 1-2) werden in der Regel symptomatisch behandelt, eine Therapieunterbrechung ist nicht notwendig. Bei persistierenden Beschwerden entsprechend einem Grad 2 kann eine Unterbrechung im Verlauf notwendig sein. Bei schweren oder persistierenden Nebenwirkungen muss eine orale oder intravenöse Applikation von Kortikosteroiden erfolgen. Die genauen Dosierungen, die dazu notwendig sind, können nicht angegeben werden, da entsprechende Studien fehlen. Zum Teil sind auch weitere Immunsuppressiva notwendig (z.B. TNF-Alpha-Antagonisten), deren Einsatz ist jedoch anhand von Studien noch nicht ausreichend untersucht. Bei schweren Nebenwirkungen muss die

Immuntherapie dauerhaft beendet werden. Zur Behandlung von immuntherapievermittelten Nebenwirkungen existieren spezielle Therapiebücher und Leitfäden, an denen man sich orientieren kann, wie z.B. die ESMO Guidelines (Haanen et al. 2017).
Unerwünschte Wirkungen sind möglicherweise sogar ein Prädiktor für ein längeres Gesamtüberleben (OS). Dies konnte zumindest unter Therapie beim malignen Melanom gezeigt werden. Patienten mit immunvermittelter Kolitis und Diarrhö, egal welchen Grades, zeigte ein verlängertes OS (HR 0,53; $p<0,01$) (Abu-Sbeih et al. 2019).
Eine weitere Besonderheit der Immuntherapie stellt die so genannte „Pseudoprogression“ dar. Dies ist keine Nebenwirkung im eigentlichen Sinne. So kann man zum Teil zunächst ein Wachstum des Tumors in den ersten radiologischen Kontrolluntersuchungen unter der Therapie feststellen. Man könnte also radiologisch einen Progress vermuten. Dieser lässt sich im Falle eines Pseudoprogresses jedoch klinisch ganz und gar nicht feststellen. Patienten mit einem Pseudoprogress fühlen sich meist klinisch besser, was im Gegensatz zum radiologischen Befund steht. Das scheinbare Tumorwachstum ist jedoch auf eine Einwanderung von Immunzellen ins Tumorgewebe zurückzuführen, was wiederrum zu einer Zunahme des Volumens ohne Proliferation der Tumorzellen führt. Es handelt sich um eine Art Entzündungsreaktion. Eine Unterscheidung zwischen Tumor- und Immunzellen ist radiologisch jedoch nicht möglich. In diesem Fall muss man sich also zwingend an der Klinik des Patienten orientieren. Geht es ihm besser, nehmen seine Schmerzen ab, sein Appetit zu oder ähnliches, so sollte die Immuntherapie bis zum nächsten Staging oder bis zur Verschlechterung der Klinik zunächst fortgeführt werden. Handelt es sich um einen Pseudoprogress, so kann man beim nächsten Staging meist eine Remission feststellen.
Im Gegensatz dazu steht die so genannte „Hyperprogression“. Dies stellt ein übermäßiges Wachstum des Tumors unter Checkpointblockade dar. Der Unterschied im Vergleich zur Pseudoprogression ist, dass es sich um einen echten Progress handelt, der scheinbar noch ausgeprägter ist, als wenn man den Patienten gar nicht spezifisch behandelt hätte, also über einen normalen Progress hinausgeht. Die Klinik des Patienten zeigt in der Regel eine Symptom- und Allgemeinzustandsverschlechterung. Radiologisch zeigt sich eine Volumenzunahme des Tumors und es können auch neue Läsionen hinzukommen. Dies ist eine eindeutige Abgrenzung zum Pseudoprogress. Immunzellen können nur dort einwandern, wo schon Tumorzellen vorhanden sind, daher zeigt sich die Entstehung neuer Läsionen nie bei einem Pseudoprogress. Die Häufigkeit einer Hyperprogression variiert in der Literatur, häufig wird sie jedoch bei bis etwa 10% der Behandelten angegeben. Bei einer Hyperprogression sollte die Immuntherapie unverzüglich abgebrochen werden und auf eine Chemotherapie umgestellt werden, sofern das beim Patienten möglich ist.

1.2.4. Prädiktive Marker

Obwohl die Immuntherapeutika die Behandlung des NSCLC revolutioniert haben, gibt es einige Patienten, die nicht auf eine Immuntherapie ansprechen und auch solche, die mit einer Hyperprogression auf die Therapie reagieren. In der Erstlinie sprechen durchschnittlich etwa 50-75% der Patienten an, in der Zweitlinie nur noch rund 20-40% (ohne Immuntherapie in der Erstlinie).
Als aktuell nahezu einziger etablierter Marker gilt PD-L1. Dieser hilft uns allerdings im klinischen Alltag nur unzureichend weiter. Schließt man alle Patienten mit behandelbarer Treiberalteration aus, bekommen so gut wie alle Patienten in der ersten Therapielinie eine Immuntherapie, sei es als Monotherapie oder in Kombination mit einer Chemotherapie. Doch wie können wir Patienten im Vorfeld besser selektionieren? Als ein weiterer potenzieller Marker ist „tumor mutational burden“, kurz TMB, seit einigen Jahren im Gespräch. Bei TMB handelt es sich um die Mutationslast des Tumors, im Speziellen um die Anzahl der nicht synonymen, tumor-spezifischen Mutationen im Exom einer Tumorprobe. Die Varianz, die bereits in der Keimbahn DNA nachweisbar ist, bleibt somit unberücksichtigt (Lawrence et al. 2013; Schumacher und Schreiber 2015). Tumore mit hoher TMB können eine hohe Neoantigenlast aufweisen, welche zu einer hohen Immunogenität des Tumors und einer erhöhten T-Zellreaktivität sowie Antitumorantwort führen kann (Kim und Chen 2016; Liontos et al. 2016; Sharma und Allison 2015; Giannakis et al. 2016). Hier zeigte sich in Studien ein besonders gutes Ansprechen auf eine kombinierte Immuntherapie mit Nivolumab und Ipilimumab (Hellmann et al. 2018). TMB als Marker hat sich bisher auch noch nicht in der klinischen Praxis etabliert, da Studien darauf hindeuten, dass er eher prognostischer als prädiktiver Natur ist.
Beim SCLC existieren aktuell keinerlei relevante prädiktive Marker, auch eine PD-L1 Testung ist hier nicht sinnvoll. Umso wichtiger wäre es in Zukunft relevante Biomarker mit prädiktivem Wert zu identifizieren, um Patienten mit SCLC im Vorfeld der Therapie besser selektionieren zu können, da nur wenige Patienten wirklich von der zusätzlich zur Chemotherapie gegebenen Checkpointblockade profitieren.

1.2.5. Dauer der Immuntherapie

Eine weitere Frage beschäftigt sich mit der Dauer der Immuntherapie. Wie lange diese bei fehlendem Progress und guter Verträglichkeit gegeben werden sollte, ist bisher nicht geklärt. In den Studien wurden die Immuntherapien nach zwei Jahren beendet, die Zulassung sieht jedoch die Fortsetzung bis zum Progress vor.
In einer explorativen Analyse der Checkmate-153 zeigte sich ein signifikanter Vorteil für Patienten, die dauerhaft bis zum Progress oder Auftreten einer nicht akzeptablen Toxizität mit Nivolumab

behandelt wurden, gegenüber Patienten, bei denen die Therapie nach einem Jahr beendet wurde. Eingeschlossen wurden Patienten mit vorbehandeltem metastasierten NSCLC. Es wurden insgesamt 1428 Patienten eingeschlossen, von diesen hatten 252 Patienten nach einem Jahr noch keinen Progress unter Nivolumabtherapie und wurden somit in zwei Gruppen randomisiert. Die eine Gruppe erhielt Nivolumab weiter, bei der anderen wurde die Therapie zunächst beendet. Das PFS in der Gruppe mit fortgesetzter Immuntherapie betrug 24,7 Monate versus 9,4 Monate bei Beendigung der Therapie nach einem Jahr (HR 0,56). Das mediane OS ist bei fortgesetzter Therapie noch nicht erreicht, gegenüber 32,5 Monate bei beendeter Nivolumabgabe (HR 0,61) (Waterhouse et al. 2020). Dies ist die erste randomisierte Studie mit dem Ziel, die Dauer einer Immuntherapie zu untersuchen. Sie zeigt eindeutig, dass eine fortgesetzte Gabe signifikant besser ist, als die Therapie nach einem Jahr zu beenden. Wie die Situation nach zwei Jahren oder länger aussieht, ist allerdings unklar (Waterhouse et al. 2020). Es herrscht zumindest Einigkeit, dass eine Immuntherapie ohne Progress und relevante Nebenwirkungen für mindestens zwei Jahre gegeben werden sollte, entsprechend der Dauer in den Zulassungsstudien. Danach kann diese auch in Abhängigkeit vom Patientenwillen z.B. bei geringeren Nebenwirkungen (die den Patienten aber stärker belasten) oder ähnlichem großzügiger beendet werden. Auch eine Wiederaufnahme bei Progress ist dann möglich.

1.3. Stadienabhängige Therapie

1.3.1. Stadium I

Im Stadium I steht, wenn funktionell und technisch operabel, die alleinige operative Therapie an erster Stelle. Damit kann ein 5-jahres Überleben von etwa 68-92% erreicht werden (Goldstraw et al. 2016). Im Stadium IB sollte eine EGFR-Testung (Exon 19 und 21) erfolgen. Bei positivem Befund wird eine adjuvante Therapie mit Osimertinib über 3 Jahre empfohlen (Wu et al. 2020).
Sollte der Patient inoperabel sein, kann eine stereotaktische Strahlentherapie die Operation, ohne schlechteres Outcome ersetzen (MacMahon et al. 2017). Sollte ein malignitätsverdächtiger pulmonaler Rundherd > 8 mm eine Größenprogredienz im zeitlichen Verlauf aufweisen sowie eine pathologische Glukosestoffwechselsteigerung in der FDG PET-CT Untersuchung, so ist eine stereotaktische ablative Strahlentherapie ohne histologische Sicherung bei Inoperabilität des Patienten möglich (MacMahon et al. 2017).

1.3.2. Stadium II

Im Stadium II steht ebenfalls, wenn funktionell und technisch operabel, die operative Therapie an erster Stelle. Damit kann ein 5-jahres Überleben von etwa 53-60% erreicht werden (Goldstraw et al. 2016). Es sollte sich bei fehlenden Kontraindikationen eine adjuvante Systemtherapie anschließen.

Durch eine adjuvante Chemotherapie erhöht sich die 5-Jahres Überlebensrate um etwa 4% (Burdett et al. 2015). Bei Patienten mit gutem ECOG (0/1) soll die Gabe von 4 Zyklen cisplatinhaltiger Kombinationschemotherapie erfolgen (Burdett et al. 2015). Es sollte eine EGFR-Testung erfolgen (Exon 19 und 21). Bei positivem Befund wird eine adjuvante Therapie mit Osimertinib über 3 Jahre empfohlen (Wu et al. 2020). Es bleibt dem Behandler überlassen ob im Vorfeld eine adjuvante Chemotherapie durchgeführt wird oder nicht. Neuste Daten deuten jedoch darauf hin, dass eine adjuvante Chemotherapie keine Vorteile im Gesamtüberleben bringt. Es sollte ebenfalls eine Testung auf PD-L1 erfolgen. Bei einer PD-L1 Expression ≥ 50 % muss noch die Testung bezüglich einer ALK-Translokation durchgeführt werden. Patienten mit einer PD-L1 Expression ≥ 50 % (ohne EGFR- oder ALK-Alteration) sollte nach R0 Resektion und durchgeführter adjuvanter Chemotherapie noch eine adjuvante Immuntherapie mit Atezolizumab über 1 Jahr angeboten werden (Felip et al. 2021).
Eine weitere Möglichkeit ist die neoadjuvante Immunchemotherapie. Bei Patienten mit PD-L1 Status ≥ 1% kann eine neoadjuvante Therapie mit Nivolumab plus platinbasierte Chemotherapie über 3 Zyklen erfolgen. Im Anschluss sollte innerhalb von 6 Wochen die Operation erfolgen. Eine adjuvante Systemtherapie ist in diesem Fall nicht notwendig (Forde et al. 2022).

1.3.3. Stadium III

Das Stadium IIIA ist von den Therapieoptionen das wohl heterogenste Stadium des NSCLC. Prinzipiell sollte immer eine Operabilität geprüft werden. Es sollte sich bei fehlenden Kontraindikationen eine adjuvante Systemtherapie analog dem Stadium II anschließen. Ebenfalls möglich ist analog dem Stadium II eine neoadjuvante Immunchemotherapie (siehe Kapitel 1.3.2.).
Sollte keine Operabilität gegeben sein, ist eine Radiochemotherapie die Behandlung der Wahl. Diese sollte bei gutem ECOG möglichst simultan durchgeführt werden (bessere 5-Jahre Überlebensrate bei tendenziell stärkeren Nebenwirkungen). Für Patienten mit einem schlechteren ECOG kommt alternativ die sequenzielle Therapie in Betracht. Sollte auf den Tumorzellen immunhistochemisch eine PD-L1 Expression nachgewiesen werden (PD-L1 mind. 1%), dann sollte sich eine konsolidierende Immuntherapie mit Durvalumab für ein Jahr anschließen. Hier konnte in der PACIFIC Studie eine signifikante Verlängerung des progressionsfreien Überlebens (HR 0,52; Median 11,2 Monate) und des Gesamtüberlebens gezeigt werden (Antonia et al. 2017).
Die Stadien IIIB und IIIC gelten üblicherweise als inoperabel. In einzelnen Fällen kommt trotzdem eine operative Therapie in Betracht, in den meisten Fällen ist aber analog des inoperablen Stadiums IIIA eine Radiochemotherapie mit anschließender konsolidierender Immuntherapie bei PD-L1 Positivität der Goldstandard.

1.3.4. Stadium IV

Im Stadium IV unterscheidet man das Stadium IVA, welches eine Oligometastasierung, eine intrapulmonale Metastasierung und eine Pleurakarzinose sowie einen malignen Pleuraerguss oder Perikarderguss beinhaltet, von einer multipel metastasierten (Stadium IVB) Erkrankung. In einem oligometastasierten Stadium (z. B. singuläre Nebennierenmetastase) sollte immer ein atypisch kuratives Konzept geprüft werden, z. B. mit Radiochemotherapie des Primärtumors und der LK-Metastasen sowie OP oder Bestrahlung der singulären Metastase. Gomez und Mitarbeiter haben in einer kontrollierten, multizentrischen Phase II Studie Patienten im Stadium IV mit maximal 3 Metastasen untersucht. Die Patienten hatten einen ECOG-Perfomance-Status von maximal 2 und wurden nach abgeschlossener systemischer Therapie in zwei Gruppen randomisiert, vorausgesetzt, dass sie keinen Progress innerhalb der ersten 3 Monate nach Beginn der systemischen Therapie hatten. Die erste Gruppe erhielt im Anschluss an die Chemotherapie entweder eine Erhaltungstherapie oder eine Verlaufskontrolle, während die zweite Gruppe eine lokale konsolidierende Therapie aller noch messbaren Tumorläsionen inklusive aller Metastasen erhielt. Die Erstlinientherapie in beiden Gruppen bestand aus mindestens vier Zyklen einer Platindoublette oder einer entsprechenden zielgerichteten Therapie bei EGFR mutierten oder ALK-translozierten Patienten über mindestens 3 Monate. Bei den Patienten aus der Gruppe mit lokaler konsolidierender Therapie (LCT) wurde entweder eine Operation oder eine Radiatio oder eine Kombination aus beiden Verfahren durchgeführt. Die Entscheidung hierfür wurde durch ein Multidisziplinäres Team gefällt und es wurden alle tumorbefallenen Stellen, inklusive Primärtumor, Lymphknoten und Metastastasen berücksichtigt. Im Anschluss daran erhielten die Patienten entweder eine systemische Erhaltungstherapie oder nur eine Verlaufskontrolle (Gomez et al. 2016). Im Jahr 2019 wurden die Langzeitdaten des Gesamtüberlebens präsentiert. Die Studie wurde nach Randomisierung von insgesamt 49 Patienten vorzeitig beendet, auf Grund eines signifikant besseren progressionsfreien Überlebens (PFS) und Gesamtüberlebens in der LCT-Gruppe. Das PFS in der LCT-Gruppe betrug 14,2 Monate vs. 4,4 Monate in der Kontrollgruppe. Das Gesamtüberleben (OS) betrug in der LCT-Gruppe im Median 41,2 Monate vs. 17 Monate in der Kontrollgruppe. Auch das Überleben nach Tumorprogress war deutlich länger bei den Patienten, die lokal konsolidierend behandelt wurden (LCT-Gruppe 37,6 Monate vs. Kontrollgruppe 9,4 Monate) (Gomez et al. 2019).

Im Stadium IVB steht die palliative systemische Therapie im Vordergrund. Strahlentherapie und Operation stehen hier als Instrumente z. B. zur Schmerztherapie oder lokalen Kontrolle einzelner Beschwerden verursachender Metastasen zur Verfügung. Die systemische Therapie richtet sich im Wesentlichen nach der Histopathologie und Molekularpathologie. Die mediane Überlebenszeit in

diesem Stadium beträgt 8 – 18 Monate. Durch die neuen Immuntherapien ist im weiteren Verlauf mit einer Verlängerung dieser Zeitspanne zu rechnen, jedoch gibt es aktuell schon einen deutlichen Unterschied zu Patienten mit einer vorliegenden behandelbaren Treiberalteration. Erkenntnisse aus drei unabhängigen retrospektiven Analysen zeigen ein Langzeitüberleben mit medianem Gesamtüberleben > 4 Jahre durch den sequenziellen Einsatz von 1st/2nd Generation EGFR-Tyrosinkinaseinhibitoren gefolgt von T790M-spezifischen Inhibitoren (Shimamura et al. 2022; Okamoto et al. 2018; Lin et al. 2016). Aktuell wird bei allen Patienten mit NSCLC die Detektion von behandelbaren Treiberalterationen empfohlen. Zusätzlich sollte bei allen Patienten der PD-L1-Status geprüft werden. Falls eine therapierelevante Treiberalteration gefunden wird, sollte diese mit einer gezielten Therapie behandelt werden. Falls nicht, sollte eine kombinierte Chemo-/ Immuntherapie in der First-line-Therapie gegeben werden. In der Regel beinhaltet diese die 4-malige Gabe einer Chemo- und Immuntherapie (bei Kombination von Nivolumab + Ipilimumab zwei Zyklen Chemotherapie) mit einer anschließenden Immun-Erhaltungstherapie bis zum Eintreten einer Krankheitsprogression. Eine Ausnahme stellen die Tumore mit einer hohen PD-L1-Expression dar (≥ 50%), hier ist auch die alleinige Gabe einer Immuntherapie möglich. Ob diese Patienten mit einer Kombinationstherapie oder einer Immunmonotherapie behandelt werden sollten, ist nicht sicher geklärt und bleibt dem Behandler überlassen. Es wird jedoch von einigen Experten favorisiert, bei jüngeren Patienten mit einem gutem ECOG und/oder hohem Therapiedruck (z. B. durch einen schnell wachsenden Tumor in Gefäßnähe o. ä.) eher eine Kombinationstherapie zu wählen.

In der Second-line-Situation erleben aktuell die Therapien aus einer Kombination von Docetaxel und einem Angiogenesehemmer ihre Renaissance. Als Kombinationspartner stehen entweder Ramucirumab intravenös, unabhängig von der Histologie des Tumors oder Nintedanib oral für Patienten mit Adenokarzinom, zur Verfügung. Nach 4 Zyklen Kombinationstherapie ist dann wieder eine Erhaltungstherapie mit dem jeweiligen Angiogenesehemmer möglich.

Bei Progress unter der Second-line-Therapie gibt es keine klaren Empfehlungen mehr. Hier wird von vielen Experten nochmals eine Immuntherapie eingesetzt, aber auch die orale Gabe von Erlotinib beim Adenokarzinom und Afatinib beim Plattenepithelkarzinom ist möglich. In weiteren Therapielinien sollten dann Substanzen zum Einsatz kommen, welche im Vorfeld noch nicht verwendet wurden, im Zweifel können jedoch auch Substanzen eingesetzt werden, welche bereits in einer früheren Therapielinie zum Einsatz kamen, jedoch schon länger nicht mehr gegeben wurden. Da keine klaren Richtlinien diesbezüglich existieren, bleibt dies jedem Behandler selbst überlassen. Im Falle eines Tumorprogresses sollte jedoch auch immer eine Rebiopsie diskutiert werden, da es häufiger als gedacht zu Transformationen des Tumors zu einem anderen Subtyp kommen kann. Als

Beispiel sei hier die Transformation eines Adenokarzinoms zu einem Kleinzelligen Lungenkarzinom genannt. Da sich die Therapien der verschiedenen histologischen Subtypen deutlich voneinander unterscheiden, sollte im Zweifel bei einem Tumorprogress immer eine Rebiopsie erfolgen. Zusammenfassend kann man sagen, dass wir durch die fortlaufende Entwicklung in der Therapie des Lungenkarzinoms mit Zulassung und Testung immer weiterer Substanzen und Substanzklassen, immer näher zu einer individualisierten Therapie jedes einzelnen Patienten kommen. Grundsätzlich gilt, dass möglichst jeder Lungenkrebspatient in einem Lungenkrebszentrum mit Therapieentscheidung in einem interdisziplinären Tumorboard behandelt werden sollte, um jedem Patienten die Chance auf eine bestmögliche Lungenkrebstherapie zu bieten.

1.4. Zielstellung

Nicht alle Patienten profitieren von einer Therapie mit Immuncheckpointinhibitoren. Bisher ist der einzige etablierte prädiktive Biomarker PD-L1. Es wäre wünschenswert, dass weitere Biomarker zur Verfügung stehen, um im Vorfeld der Therapie eine bessere Patientenselektion zu erreichen, da aktuell nahezu jeder Patient ohne therapierbare Treiberalteration und ohne Kontraindikation im Stadium IV eine Immuntherapie erhält. Eine bessere Patientenselektion hätte den Vorteil den Patienten, die nicht von einer Immuntherapie profitieren, mögliche Nebenwirkungen der Checkpointblockadetherapie zu ersparen. Ebenfalls sehr wertvoll wäre eine Ressourcenschonung und wirtschaftliche Entlastung bei aktuell sehr hohen Therapiekosten für die Checkpointinhibitoren. Folgende Zielstellungen wurden im Rahmen dieser Habilitation untersucht:

a) Identifikation von Biomarkern zur Detektion des Ansprechens von Patienten mit NSCLC unter alleiniger Immuntherapie mit Pembrolizumab als First- oder Second-line Therapie oder mit Nivolumab als Second-line Therapie.
b) Identifikation von Biomarkern zur Detektion des Ansprechens von Patienten mit NSCLC unter First-line Therapie mit einer kombinierten Immunchemotherapie.
c) Identifikation von Biomarkern zur Detektion des Ansprechens von Patienten mit SCLC unter First-line Therapie mit einer kombinierten Immunchemotherapie.
d) Immunzellmonitoring bei Patienten mit NSCLC und Langzeitansprechen unter Therapie mit Immuncheckpointinhibitoren.

2. Ergebnisse/Originalarbeiten

Den hier dargestellten Ergebnissen liegen folgende eigene Originalarbeiten zu Grunde, welche sich im Anhang befinden und die ausgewiesenen Abbildungen beinhalten.

2.1. Blutimmunzellbiomarker bei Patienten mit NSCLC unter alleiniger Checkpointinhibitortherapie

Miriam Möller, Steffi Turzer, Wolfgang Schütte, Barbara Seliger, Dagmar Riemann
Blood Immune Cell Biomarkers in Patient With Lung Cancer Undergoing Treatment With Checkpoint Blockade; J Immunother. 2020 Feb-Mar; 43(2): 57–66. Published online 2019 Oct 4. doi: 10.1097/CJI.0000000000000297; **IF = 4.456**

Dagmar Riemann, Wolfgang Schütte, Steffi Turzer, Barbara Seliger, **Miriam Möller**
High PD-L1/CD274 Expression of Monocytes and Blood Dendritic Cells Is a Risk Factor in Lung Cancer Patients Undergoing Treatment with PD1 Inhibitor Therapy; Cancers 2020, 12(10), 2966; https://doi.org/10.3390/cancers12102966 (registering DOI); https://www.mdpi.com/2072-6694/12/10/2966; **IF = 6.639**

2.2. Blutimmunzellbiomarker bei Patienten mit NSCLC unter einer kombinierten Immunchemotherapie

Miriam Möller, Steffi Turzer, Georgi Ganchev, Andreas Wienke, Wolfgang Schütte, Barbara Seliger, Dagmar Riemann; Blood Immune Cell Biomarkers in Lung Cancer Patients Undergoing Treatment with a Combination of Chemotherapy and Immune Checkpoint Blockade; Cancers 2022, 14(15), 3690; doi.org/10.3390/cancers14153690; **IF = 6.639**

2.3. Blutimmunzellbiomarker bei Patienten mit SCLC unter einer kombinierten Immunchemotherapie

Dagmar Riemann, Steffi Turzer, Georgi Ganchev, Wolfgang Schütte, Barbara Seliger, **Miriam Möller**; Monitoring Blood Immune Cells in Patients with Advanced Small Cell Lung Cancer Undergoing a Combined Immune Checkpoint Inhibitor/Chemotherapy; Biomolecules 2023, 13(2), 190; https://doi.org/10.3390/biom13020190 (registering DOI); Received: 6 December 2022 / Revised: 11 January 2023 / Accepted: 13 January 2023 / Published: 17 January 2023; **IF = 4,569**

2.4. Monitoring von Blutimmunzellmarkern bei Patienten mit NSCLC und Langzeitüberleben unter Therapie mit Immuncheckpointinhibitoren

Miriam Möller, Wolfgang Schütte, Steffi Turzer, Barbara Seliger, Dagmar Riemann; Blood immune cells as biomarkers in long-term surviving patients with advanced non-small cell lung cancer undergoing a combined immunotherapy;

2.1. Blutimmunzellbiomarker bei Patienten mit NSCLC unter alleiniger Checkpointinhibitortherapie

Die Charakterisierung von Immunzellparametern im Blut soll dazu dienen mögliche prädiktive Biomarker für das Ansprechen auf eine Immuntherapie zu identifizieren. Diese Parameter wurden zunächst vor Therapiestart und unter laufender alleiniger Immuntherapie mit Nivolumab (Second-line) oder Pembrolizumab (First-line oder Second-line) bei Patienten mit NSCLC im fortgeschrittenen oder metastasierten Stadium bestimmt. Es erfolgte die Abnahme eines kleinen EDTA-Blutröhrchens mit Bestimmung des kompletten Leukozytenblutbildes, den Lymphozytensubpopulationen sowie der prozentualen Bestimmung der HLA-DR^{low} Monozyten und der Dendritischen Zellen (DC). Die erste Bestimmung erfolgte vor Beginn der Therapie, die zweite zum 3. Zyklus und die dritte zum 5. Zyklus der Immuntherapie. Das klinische Ansprechen auf die Therapie wurde definiert als komplette (CR) oder partielle Remission (PR) sowie als stabile Befundsituation im CT (stable disease - SD) entsprechend RECIST 1.1. Zudem wurden das PFS und das OS der Patienten evaluiert und mit den im Blut gemessenen Parametern korreliert. Insgesamt 35 Patienten mit NSCLC, denen mindestens 2 Zyklen Immuntherapie appliziert wurden, konnten in diese Studie eingeschlossen werden. Die Patientencharakteristika sind in Tabelle 1 zusammengefasst. Etwa 40% der Patienten zeigten ein klinisches Ansprechen und das globale mediane OS betrug 7,0 Monate (95% CI 3,5- 10,5). Patienten mit einer initialen Neutrophilen-Lymphozyten-Ratio (NLR) ≥ 5,2 und/oder einer Rate von HLA-DR^{low} Monozyten ≥ 11% und/oder einem Level von DC ≤ 0,4% zeigten ein signifikant schlechteres Ansprechen auf die Immuntherapie (Tabelle 2, Abbildung 1). Auf der anderen Seite zeigten Patienten mit einem immuntherapieinduzierten Abfall der NLR und/oder der HLA-DR^{low} Monozyten und/oder dem Anstieg der DC unter Immuntherapie ein signifikant besseres Ansprechen auf die Gabe der Immuncheckpointinhibitoren, welches auch verbunden war mit einem verlängerten PFS und OS (Tabelle 2, 3, Abbildung 1,2). Vergleichbar mit anderen Studien hatten Patienten die nie geraucht haben eine schlechtere Prognose (El-Osta und Jafri 2019). Insgesamt 5 von 35 Patienten waren Nie Raucher, lediglich ein Patient zeigte ein klinisches Ansprechen auf die Checkpointinhibition (stable disease). Auf Grund der niedrigen Patientenzahl wurde diese Gruppe nicht separat ausgewertet.

Auf der Basis der drei identifizierten Immunzellparameter wurden drei Scores entwickelt, um die Patienten in Risikogruppen bezüglich des Therapieansprechens einzuteilen. Beim Risiko-Score A wurde 1 Punkt gegeben, wenn der Patient Nie Raucher war. Ein Punkt erhielt der Patient jeweils für das Vorhandensein einer NLR ≥ 5,2, HLA-DR^{low} Monozyten ≥ 11% und DC ≤ 0,4% der Leukozyten. Mit mindestens einem Risikofaktor (Score 1 Punkt) wiesen 89% der Patienten kein Ansprechen auf eine Immuntherapie auf. Dies zeigt eine hohe Sensitivität dieses Scores. Die Hinzunahme des Risikofaktors Nie Raucher erfolgte auf Grund der Tatsache, dass Nie Raucher generell eine niedrigere Rate an HLA-DR^{low} Monozyten aufweisen, jedoch meistens trotzdem ein schlechteres Ansprechen auf die Immuntherapie zeigen (Riemann et al. 2019).

Als zweites wurde ein prätherapeutischer Score entwickelt (Risiko- Score B). Der Patient erhielt 1 Punkt für eine positive Raucheranamnese sowie jeweils einen Punkt für das Vorhandensein einer NLR < 5,2, HLA-DR^{low} Monozyten < 11% und DC > 0,4% der Leukozyten. Zusätzlich wurden eine Thrombozytose (Pedersen und Milman 1996) und ein hohes Alter (Tas et al. 2013) als bekannte Risikofaktoren berücksichtigt. Der Patient erhielt 1 Punkt bei Thrombozyten < 400.000/µl und 1 Punkt für ein Alter < 75 (Score maximal 6). Insgesamt 95% der Patienten mit einem Score von 6 zeigten ein Therapieansprechen unter Checkpointinhibition (Tabelle 4).

Der dritte Score zeigte ebenfalls eine starke Assoziation bezüglich eines Therapieansprechens und wurde zum Zeitpunkt der 3. Antikörpergabe bestimmt (Risiko-Score C). Die prätherapeutischen Werte der drei identifizierten Biomarker wurden gleich 100% gesetzt. Der Patient erhielt jeweils 1 Punkt für eine Stabilität der 3 Immunparameter im Blut (< 10% Abweichung vom Ausgangswert). Jeweils 2 Punkte wurden gegeben für eine Verbesserung der Parameter von ≥ 10% zum Ausgangsbefund (Abnahme von NLR und HLA-DR^{low} Monozyten sowie Zunahme der DC) (Score maximal 6). Zum Zeitpunkt der dritten Antikörpergabe wiesen 91% der Patienten mit mindestens 4 Punkten ein Ansprechen auf die Therapie auf. Es konnten jeweils signifikante Unterschiede sowohl im PFS als auch im OS zwischen den verschiedenen Risikogruppen der 3 Scores nachgewiesen werden (Abbildung 3).

Als ein weiterer prädiktiver Biomarker für das Ansprechen auf eine Immuntherapie konnte die PD-L1/CD274 Expression auf Monozyten und Dendritischen Zellen gefunden werden. Patienten mit einer hohen PD-L1/CD274 Expression auf Monozyten und DC-Subpopulationen, gemessen im peripheren Blut, zeigten ein deutlich schlechteres Ansprechen auf eine Immuntherapie. Eine niedrige PD-L1/CD274 Expression auf Monozyten und DC korrelierte hingegen mit einem verlängerten PFS und OS.

2.2. Blutimmunzellbiomarker bei Patienten mit NSCLC unter einer kombinierten Immunchemotherapie

Die im Kapitel 2.1. identifizierten prädiktiven Biomarker wurden bisher nur bei Gabe einer Monoimmuntherapie für das NSCLC untersucht. Da die meisten Patienten auf Grund aktueller Studiendaten eine kombinierte Immunchemotherapie in der First-line Therapie bekommen, wurde die Untersuchung der entsprechenden Biomarker in der nächsten Studie ausgeweitet. Insgesamt 90 Patienten mit fortgeschrittenem oder metastasierten NSCLC konnten in diese Studie eingeschlossen werden. Die Patientencharakteristika sind in Tabelle 1 zusammengefasst. Die Patienten erhielten eine kombinierte Immunchemotherapie, entweder mit Pembrolizumab oder Atezolizumab. Im Vergleich zur alleinigen Immuntherapie zeigte sich in Kombination mit einer Chemotherapie ein Therapieansprechen von knapp 75%, oft jedoch nur für wenige Monate.
Es erfolgte die Abnahme jeweils eines EDTA-Röhrchens vor Therapieeinleitung sowie zum Zeitpunkt der 3. Antikörpergabe. Patienten mit einer NLR ≥ 6,1, HLA-DR^{low} Monozyten ≥ 22%, einer Häufigkeit von Slan + nicht-klassischen Monozyten < 0,25% und/oder DC ≤ 0,14% der Leukozyten korrelierten mit einer schlechteren Prognose. Die Hazard Ratio bezüglich des PFS betrug 2,097 (1,208 -3,640) für die NLR, 1,964 (1,046 -3,688) für die HLA-DR^{low} Monozyten, 3,202 (1,712 – 5,99) für die Slan + nicht-klassischen Monozyten und 2,596 (1,478-4,56) für die DC. Patienten ohne einen dieser Risikofaktoren wiesen das beste PFS auf. Alle Patienten mit Langzeitüberleben unter der Checkpointinhibitortherapie hatten keine entsprechenden Risikofaktoren.
Des Weiteren korrelierten niedrige Zellzahlen an natürlichen Killerzellen (NK) mit einem kürzeren PFS (Cutoff 200 Zellen/µl). Frauen wiesen generell eine niedrigere Rate an NK-Zellen auf und zeigten entsprechend ein schlechteres PFS im Vergleich zu Männern.
Zum Zeitpunkt der zweiten Blutentnahme zeigten Patienten mit fehlendem Therapieansprechen eine Erhöhung der Neutrophilen Granulozyten, wohingegen Patienten mit einem klinischen Ansprechen eine Erniedrigung der Neutrophilen aufwiesen (Abbildung 1). Zusätzlich gab es bei Patienten mit Tumorprogress einen Anstieg der HLA-DR^{low} Monozyten, wobei Patienten mit einem PFS ≥ 12 Monate einen Abfall aufwiesen. Bezüglich der die Slan + nicht-klassischen Monozyten und der DC konnten keine signifikanten Unterschiede für Patienten mit Progress oder nur kurzzeitigem Therapieansprechen nachgewiesen werden. Lediglich Patienten, die ein PFS von mindestens 12 Monaten aufwiesen, zeigten einen Anstieg der entsprechenden Parameter, obwohl sie in der Regel bereits mit höheren Ausgangswerten gestartet sind (Abbildung 1).

Keine Unterschiede im PFS oder OS fanden sich im Vergleich der verschiedenen histologischen Typen des NSCLC, ebenfalls gab es keine Unterschiede im Vergleich des Raucherstatus, des Alters (< 75 und ≥ 75) und der Anzahl der Metastasen (≥ 3 Metastasen und < 3 Metastasen).
Hingegen wiesen Patienten mit einer hohen PD-L1 Expression auf den Tumorzellen ein signifikant besseres PFS auf (PD-L1 ≥ 50% und < 50%). Ebenfalls zeigten Männer, wie bereits erwähnt, ein signifikant besseres PFS im Vergleich zu Frauen.
Um die geschlechtsspezifischen Unterschiede genauer zu evaluieren wurden klinische Parameter sowie die Ergebnisse des Immunmonitorings von Männern und Frauen miteinander verglichen. Frauen zeigten deutlich häufiger einen Nichtraucher Status (23,1% vs. 9,4%). Histopathologisch wiesen Frauen seltener eine plattenepitheliale Histologie auf (7,7% vs. 39,1%). Interessanterweise hatten 65,4% der Frauen mindestens 3 Metastasen gegenüber nur 45,3% der Männer. Im Vergleich der PD-L1 Expression wiesen nur 12,5% der Frauen eine hohe Expression ≥ 50% auf gegenüber 25% der Männer. Keine geschlechtsspezifischen Unterschiede konnten bezüglich des Alters gefunden werden, genau wie bezüglich der meisten evaluierten Blutparameter.

2.3. Blutimmunzellbiomarker bei Patienten mit SCLC unter einer kombinierten Immunchemotherapie

In dieser Studie wurden nun ausschließlich Patienten mit fortgeschrittenem oder metastasierten kleinzelligen Lungenkarzinom unter kombinierter Immunchemotherapie eingeschlossen. Patienten mit SCLC sind ein besonderes Patientengut. Über Jahrzehnte gab es keine relevanten Neuerungen in der Therapie des SCLC. Viele Studien zur Evaluation einer Immuntherapie bei dieser Krebsart scheiterten, bis zur Zulassung der Immunchemotherapie mit Atezolizumab oder Durvalumab in der ersten Therapielinie. Daher war es besonders spannend zu evaluieren, ob sich die in den vorangegangenen Studien identifizierten Biomarker ebenfalls auf die Therapie des SCLC übertragen lassen. Insgesamt 40 Patienten mit fortgeschrittenem oder metastasierten SCLC konnten eingeschlossen werden. Die Patientencharakteristika sind in Tabelle 1 zusammengefasst. Es wurde die Anzahl an T-, B- und NK-Zellen sowie die HLA-DR^{low} Monozyten, die Slan+ nicht klassischen Monozyten und die DC bestimmt. Die Abnahme eines kleinen EDTA-Blutröhrchens erfolgte analog der anderen Studien vor Therapiebeginn und zur 3. Antikörpergabe. Das globale mediane OS betrug 10,4 +/- 1,1 Monate. Insgesamt 10 Patienten (25%) wiesen ein OS ≥ 12 Monate auf. Insgesamt 15% der Patienten zeigten initial einen Tumorprogress, was mit höheren Ausgangswerten für NLR und HLA-DR^{low} Monozyten korrelierte sowie niedrigeren Werten für NK-Zellen und DC. Es scheint zunächst eine hohe Ansprechrate von 85% zu geben, häufig ist das Therapieansprechen jedoch nur

von kurzer Dauer. Patienten ohne Therapieansprechen hatten ein deutlich verkürztes OS von 3,2 +/- 2,0 Monaten. Risikofaktoren für ein verkürztes OS waren das Vorhandensein von Leber-/Hirnmetastasen, eine zu Beginn erhöhte NLR ≥ 6,1, HLA-DR^{low} Monozyten ≥ 21%, Slan + nicht klassische Monozyten < 0,12% und/oder CD1c+ myeloide DC < 0,05% der Leukozyten. Lymphozyten Subpopulationen korrelierten nicht mit dem OS. Patienten mit 0-2 Risikofaktoren hatten ein signifikant besseres OS im Vergleich zu Patienten mit 3-5 Risikofaktoren (Abbildung 1).

Um besser zu verstehen, ob die basalen Blutimmunzellparameter mit dem Überleben korrelieren, wurden die Überlebenszeitanalysen nur für die 34 Patienten mit initialem Therapieansprechen wiederholt. Die 6 Patienten mit initialer Therapieresistenz wurden ausgeschlossen. Erneut zeigten Patienten mit Leber-/Hirnmetastasen, einer NLR ≥ 6,1 und Slan+ nicht klassische Monozyten < 0,12% ein schlechteres OS. Es zeigten sich keine signifikanten Unterschiede für HLA-DR^{low} Monozyten und die verschiedenen Subgruppen der DC. Daher legen unsere Ergebnisse nahe, dass die initiale Erhöhung der HLA-DR^{low} Monozyten möglicherweise auf eine primäre Therapieresistenz bezüglich der Immunchemotherapie der SCLC-Patienten hinweist. Im Gegensatz dazu könnten Slan+ nicht klassische Monozyten einen Faktor, der besonders wichtig ist für ein langanhaltendes Therapieansprechen und Überleben repräsentieren. Weitere Faktoren wie das Vorhandensein von Leber-/Hirnmetastasen spielen ebenfalls eine wichtige Rolle. Die 11 SCLC-Patienten ohne Leber-/Hirnmetastasen mit einer NLR < 6,1 wiesen ein medianes OS von 16,9 Monaten (13,4 -20,3) auf. Mit mindestens einem von beiden Risikofaktoren war das OS mit 8,2 Monaten deutlich kürzer (p < 0,001).

Alle Patientenmerkmale und die Höhe der Immunzellparameter im Blut wurden zwischen den Patienten mit SCLC dieser Studie und den Patienten mit NSCLC (siehe Abschnitt 2.2.) verglichen. Patienten mit SCLC wiesen häufiger Leber-/Hirnmetastasen auf (55% vs. 26%). Des Weiteren zeigten sie höhere Werte für NLR, die niedrigste Anzahl an DC und geringere NK-Zellen. Somit hatten deutlich mehr SCLC-Patienten mehr initiale Risikofaktoren (Leber-/Hirnmetastasen, NLR ≥ 6,1, HLA-DR^{low} Monozyten ≥ 21%, Slan + nicht klassische Monozyten < 0,12%, CD1c+ myeloide DC < 0,05% der Leukozyten) im Vergleich mit den NSCLC-Patienten. Insgesamt 45% der Patienten mit SCLC hatten 3-5 Risikofaktoren, gegenüber 24% der Patienten mit NSCLC (Abbildung 3).

2.4. Monitoring von Blutimmunzellmarkern bei Patienten mit NSCLC und Langzeitüberleben unter Therapie mit Immuncheckpointinhibitoren

In den vorangegangenen Studien wurden initiale Blutproben und Blutproben zum Zeitpunkt der 3. Antikörpergabe entnommen. Es konnten dabei die in den oberen Abschnitten genannten Feststellungen getroffen werden. Interessant scheint ob ein Blutimmunzellmonitoring im Verlauf auch sinnvoll ist um einen möglichen Progress schon frühzeitig, vor der radiologischen Progression, zu diagnostizieren. Insgesamt 12 der 90 Patienten mit NSCLC unter First-line Immunchemotherapie wiesen ein Langzeitüberleben unter Immuntherapie-Erhaltung auf, welches definiert wurde als OS ≥ 12 Monaten (Patienten aus Studie Abschnitt 2.2.). Bei diesen 12 Patienten erfolgte im Verlauf eine 3. Blutentnahme. In den meisten der Patienten konnte weiterhin eine niedrige NLR und HLA-DR^{low} Monozyten in Kombination mit stabil hohen Werten für Slan+ nicht klassische Monozyten und DC nachgewiesen werden. Zwei der Patienten zeigten jedoch einen Anstieg der immunsuppressiven Marker (NLR und HLA-DR^{low} Monozyten) kombiniert mit einem Abfall der Slan+ nicht klassichen Monozyten und DC. Bei einem der beiden Patienten korrelierte dies mit einer klinischen Tumorprogression, was zeigt, dass ein regelmäßiges Immunmonitoring unter laufender Therapie mit Immuncheckpointinhibitoren durchaus sinnvoll sein könnte.

3. Diskussion

In den letzten Jahren hat sich das Verständnis für Mechanismen und Signalwege, die unser Immunsystem regulieren deutlich verbessert. Dies hat den Grundstein für neue Therapieoptionen in der Krebsbehandlung gelegt. Dies bringt gleichzeitig die Herausforderung mit sich, mögliche prädiktive Biomarker zu identifizieren, um das Therapieansprechen für die verschiedenen Patienten vorhersagen zu können. Dies vermeidet zum einen unnötige Therapien für Patienten und somit auch eine unnötige Toxizität die diese Therapien mit sich bringen könnten, zum anderen ist dies ein Mittel um die hohen Therapiekosten zu senken und die Wirtschaftlichkeit zu verbessern (Ventola 2017).

Beim Lungenkarzinom, sowohl NSCLC als auch SCLC, hat in den letzten Jahren die Immuntherapie immer mehr Einzug gehalten. Doch nicht jeder Patient profitiert von einer Therapie mit Immuncheckpointinhibitoren. Beim NSCLC ist aktuell der einzig klinisch etablierte prädiktive Biomarker PD-L1, beim SCLC existieren bisher gar keine entsprechenden Marker. Für eine Patientenselektion ist jedoch das Vorhandensein von prädiktiven Markern von entscheidender Bedeutung. Diese sollten wenn möglich hoch spezifisch und sensitiv sein, möglichst leicht zu bestimmen sein und die Bestimmung sollte möglichst wenig kostenintensiv sein. Auf dieser Grundlage haben wir mehrere Immunzellparameter im peripheren Blut bestimmt und mit dem

Therapieansprechen auf eine entsprechende Immuntherapie korreliert. Die Bestimmung im peripheren Blut in einer kleinen EDTA-Monovette ist im klinischen Alltag besonders leicht handelbar und bietet keinerlei zusätzliche Risiken für den Patienten, bei dem eine Blutentnahme sowieso vor jeder Therapie erfolgt. Im Falle von Markern, die auf Gewebeproben bestimmt werden, besteht immer die Gefahr, dass die Tumorprobe für eine entsprechende Analyse nicht mehr ausreicht und ggf. eine erneute Gewebeentnahme notwendig wäre. Dies ist daher eindeutig ein Vorteil des Blutimmunzellmonitorings. Als Nachteil sei erwähnt, dass eine Blutprobe nie das Tumormikroenvironment in Gänze widerspiegeln kann.

In unserer ersten Studie (vergleiche Abschnitt 2.1.) die zu Beginn der Einführung der Immuntherapie durchgeführt wurde, wurden hauptsächlich Patienten untersucht die eine Second-line Immuntherapie ohne zusätzliche Chemotherapie erhielten. Dies begründet auch das eher geringe Therapieansprechen von insgesamt 40% der Patienten. Es konnten drei Marker identifiziert werden, die je nach Höhe, mit einem besonders niedrigen oder hohen PFS/OS korrelieren. Mit Hilfe dieser drei Marker und einem weiteren Risikofaktor, dem Nie Rauchen, konnten 3 Scores entwickelt werden mit deren Hilfe eine Vorhersage des Therapieansprechens relativ genau möglich war. In Zukunft wäre es sinnvoll unsere Scores mit anderen Scores zu vergleichen die ebenfalls für Patienten unter Immuntherapie entwickelt wurden, wie beispielsweise dem Gustave Roussy Immunscore (beinhaltet NLR, Laktatdehydrogenase und Serum-Albumin Konzentration) oder mit dem Royal Marsden Hospital prognostic score (beinhaltet Laktatdehydrogenase, Albumin und Anzahl der Metastasen) (Minami et al. 2019).

Eine höhere prätherapeutische NLR korrelierte mit einer schlechteren Prognose, dies konnte bereits bei Patienten mit verschiedenen soliden Tumoren unter Immuncheckpointtherapie gezeigt werden (Sacdalan et al. 2018). In unserer Analyse war ein Cutoff von 5,2 optimal, um die Patienten in verschiedene Risikogruppe einzuteilen. In verschiedenen anderen Studien konnten als Cutoff-Punkte 5,0 (Bagley et al. 2017) und 5,9 (Soyano et al. 2018) gefunden werden.

Neutrophile Granulozyten sind dafür bekannt, die Tumorgenese sowie das Tumorwachstum und die Metastasierung zu fördern. Weiterhin sollen sie die Angiogenese stimulieren und die Immunsuppression fördern (Moses und Brandau 2016). In verschiedenen Tumoren ist eine hohe Anzahl der Neutrophilen Granulozyten im Blut und in Tumorgewebeproben assoziiert mit einer Progression der malignen Erkrankung und einem schlechten Outcome (Kasuga et al. 2001). In unseren Analysen zeigte sich eine negative Korrelation zwischen der Anzahl an Neutrophilen Granulozyten und den DC. Dies könnte daran liegen, dass die DC eine entscheidende Rolle in der Kontrolle der Homöstase der Neutrophilen im peripheren Blut haben. Die DC beeinflussen die

Mobilisation der Neutrophilen aus dem Knochenmark und sind zuständig für die Rekrutierung und Apoptose dieser Zellreihe (Jiao et al. 2014).

Bei Patienten mit Lungenkarzinom in einem frühen Tumorstadium die eine primär operative Therapie bekommen zeigte sich eine Korrelation zwischen Neutrophilen und dem Prozentsatz an HLA-DR^{low} Monozyten als eine wichtige Subpopulation der myeloiden Supressorzellen (MDSC) (Riemann et al. 2019). Diese Beobachtung konnte in den späten Tumorstadien im Rahmen unserer aktuellen Studien nicht gemacht werden. Eine Erhöhung der HLA-DR^{low} Monozyten konnte in verschiedenen Tumortypen gezeigt werden (Greten et al. 2011). Zusätzlich konnten eine Erhöhung von löslichen inflammatorischen Faktoren sowie tumorassoziierten extrazellulären Vesikeln die Bildung von MDSC bedingen (Filipazzi et al. 2012). Diese monozytischen Zellen können die T-Zell-Funktion bei Tumorpatienten unterdrücken, wie es bereits bei Patienten mit Sepsis für die HLA-DR^{low} Monozyten beschrieben wurde (Döcke et al. 1997). In einer anderen Studie konnte gezeigt werden, dass erhöhte Prozentsätze von monozytischen MDSC mit einer schlechteren Prognose bei chemotherapienaiven NSCLC-Patienten assoziiert sind (Vetsika et al. 2014). Daten von Patienten mit malignem Melanom konnten einen Zusammenhang zwischen der Resistenz auf Immuncheckpointinhibitoren und MDSC belegen (Weber et al. 2018).

Platinhaltige Chemotherapeutika, das Rückgrat aller Chemotherapien beim metastasierten NSCLC, können die Erhöhung der Antigenpräsentation der Tumorzellen und die Förderung der Migration der T-Zellen in den Bereich des Tumormikroenvironmentes sowie die Verringerung der HLA-DR^{low} MDSC bewirken (Biasi et al. 2014; Galluzzi et al. 2015). Es konnte bereits in der Vergangenheit gezeigt werden, dass eine Chemotherapie einen immunogenen Zelltod induzieren kann, die Tumor Antigenität verbessern kann, immunsuppressive Signalwege unterbrechen kann und die T-Zellantwort verbessern kann (Yan et al. 2018). Interessanterweise ist die Expression der Immuncheckpointmoleküle wie PD-1 und PD-L1 in den Tumorläsionen verknüpft mit dem Ansprechen des Patienten auf eine Chemotherapie beim NSCLC (Ye et al. 2021).

Daher war es besonders spannend in der zweiten Studie (Vergleiche 2.2) Patienten unter Immunchemotherapie mit Hilfe von den o.g. Blutimmunzellparametern zu monitoren. Ergänzt haben wir diese durch die Slan+ CD16+ nicht-klassischen Monozyten, welche ein vergleichbares Verhalten in Bezug auf PFS und OS wie die DC zeigten. Die Gabe einer kombinierten Immunchemotherapie in der First-line Therapie erhöhte das Therapieansprechen unserer Patienten auf knapp 75% im Vergleich zu 40% der Patienten aus unserer ersten Studie. Aktuell ist dies die Standardtherapie beim metastasierten NSCLC und scheint vor allem den frühen Progress unter alleiniger Immuntherapie in gewissem Maße zu verringern.

Patienten mit einer NLR ≥ 6,1 hatten eine schlechtere Prognose. Die NLR, die Neutrophile und Lymphozyten kombiniert, ist ein anerkannter, aber bisher nicht in der klinischen Praxis etablierter Marker, der die Prognose von Lungenkarzinompatienten unter Therapie widerspiegeln kann (Ushio et al. 2022; Sacdalan et al. 2018; Gu et al. 2015). Bezüglich dieser Ratio scheinen allerdings die Neutrophilen eine entscheidendere Rolle zu spielen als die Lymphozyten. Betrachtet man ausschließlich die Anzahl der Lymphozyten, so zeigen sich keinerlei signifikante Unterschiede zwischen den Patienten die auf die Therapie ansprechen und denen die nicht ansprechen. Schaut man sich die Lymphozyten Subpopulationen an, so korreliert nur die Anzahl der NK-Zellen mit der Prognose der Patienten. Eine geringere Anzahl an NK-Zellen (Cutoff 200 Zellen/µl) korrelierte in unserer Studie mit einem schlechteren PFS, dies konnte bereits in einigen anderen Studien gezeigt werden (Mazzaschi et al. 2020; Youn et al. 2020). In unserer Studie korrelierte eine hohe Anzahl an Neutrophilen mit der Anzahl an Monozyten sowie mit dem Prozentsatz von HLA-DR^{low} Monozyten MDSC, dies konnte in der Vergangenheit ebenfalls schon beschrieben werden für verschiedene Tumorstadien (Riemann et al. 2019). Es ist bekannt das HLA-DR^{low} Monozyten die Lymphozytenfunktion in Tumorpatienten supprimieren (Greten et al. 2011; Vetsika et al. 2014), vergleichbar mit der Situation in einer Sepsis (Döcke et al. 1997) oder einem Polytrauma (Hershman et al. 1990).

Wir untersuchten die HLA-DR^{low} MDSC und und die Slan+ nicht klassischen Monozyten als zwei Subpopulationen der Monozyten mit verschiedenen Eigenschaften. Beide Typen von Monozyten zeigten eine inverse Korrelation in unserer Studie. Monozyten im Blut können in drei verschiedene Subpopulationen eingeteilt werden. Zum einen in die klassischen (CD14hoch CD16-), zum anderen in die intermediären (CD14hoch CD16+) und zuletzt in die nicht-klassischen (CD14niedrig/negativ CD16+) Monozyten. Diese verschiedenen Subpopulationen zeigen transkriptomische Unterschiede welche zu verschiedenen Spezialisierungen und Funktionen der jeweiligen Zellen führt (Passlick et al. 1989; Gren et al. 2015). CD16+ Monozyten können wiederrum eingeteilt werden in Slan- und Slan+ Subpopulationen, wobei die letzteren als nicht klassische Monozyten bezeichnet werden (Hofer et al. 2015; Hofer et al. 2019). Obwohl die Zellen monozytären Ursprungs sind, können Slan+ nicht klassische Monozyten sehr schnell die Funktionen von DC übernehmen und sich zu Makrophagen differenzieren (Ahmad et al. 2019). Nicht klassische Monozyten werden als proinflammatorische Population mit antitumorösen Eigenschaften betrachtet (Hanna et al. 2015). Slan+ Monozyten können NK-Zellen via IL-12 aktivieren und die Interaktion zwischen Slan+ Zellen und NK-Zellen fördert die Differenzierung von naiven T-Lymphozyten in Interferon (IFN)-gamma-produzierende Th1 Zellen (Gerosa et al. 2002).

In unserer Studie konnten keine signifikanten Unterschiede zwischen Patienten mit kurzzeitigem Therapieansprechen und initialem Tumorprogress bezüglich der initialen Werte für Slan+ Zellen und DC gefunden werden. Jedoch zeigte sich eine deutliche Korrelation der beiden Werte mit einem Langzeitansprechen (PFS ≥ 12 Monate). Diesbezüglich konnte sogar eine deutlichere Korrelation im Vergleich zur NLR nachgewiesen werden. Eine inverse Korrelation bestand zwischen Neutrophilen und Slan+ sowie Neutrophilen und DC. Einige Patienten hatten sehr niedrige Raten an DC im Blut, was zu einer schlechteren Funktion ihres Immunsystems und der damit verbundenen schlechteren Prognose beitragen könnte. Im Vergleich zu gesunden Menschen weisen NSCLC-Patienten signifikant weniger DC im Blut auf (Tabarkiewicz et al. 2008; Riemann et al. 2019). Ein Mangel an aktivierten CD103+ DC in Gewebeproben von Melanompatienten wurde in Zusammenhang mit einem schlechteren Ansprechen auf eine Immuntherapie diskutiert (Salmon et al. 2016). Auf der anderen Seite waren DC Gensignaturen deutlich mit einem Therapieansprechen auf eine Therapie mit Atezolizumab beim NSCLC assoziiert (Mayoux et al. 2020). Die Anzahl an DC und deren Expression auf coinhibitorischen Molekülen wie PD-L1 kann das Therapieansprechen und dadurch bedingt das Überleben des Patienten beeinflussen. Patienten unter alleiniger Immuntherapie mit einer höheren PD-L1/CD274 Expression auf Subtypten der DC und Monozyten zeigten ein signifikant schlechteres Überleben (Vergleiche 2.1.). Das Verstehen und Modulieren der DC Anzahl sowie das Verändern der funktionellen Aktivität könnte entscheidend dazu beitragen die Effektivität der Immuntherapien zu erhöhen (Murgaski et al. 2019).

Beim metastasierten NSCLC zeigt sich ein Vorteil im Ansprechen auf eine Immuntherapie in Abhängigkeit des PD-L1 Status (Brahmer et al. 2017). Dies konnte in unserer Studie ebenfalls bestätigt werden für Patienten mit einer PD-L1 Expression ≥ 50%. Des Weiteren fanden wir weibliches Geschlecht als unabhängigen Risikofaktor für ein schlechteres Ansprechen auf eine Immuntherapie, was bereits in anderen Studien für verschiedene histologische Tumortypen beschrieben war (Conforti et al. 2018). Es wird diskutiert, dass Männer und Frauen grundsätzlich auf verschiedene Art und Weisen auf Immuntherapien ansprechen, unabhängig von Histologie, Art und Setting der Therapie (Conforti et al. 2018). In Tierversuchen konnte bereits gezeigt werden, dass die PD-1/PD-L1 Expression u.a. von Sexualhormonen moduliert werden könnte (Polanczyk et al. 2007). In einer Studie konnte gezeigt werden, dass es bei Frauen zu einer höheren Rate an Hyperprogressionen kommt (Kanjanapan et al. 2019). Eine andere Arbeitsgruppe beschrieb eine niedrigere Anzahl an CD33hoch Monozyten im Blut von Frauen. Auf Grund dieser Tatsache diskutierten sie das schlechtere Ansprechen auf eine Immuntherapie (Olingy et al. 2022). Vergleicht man Männer und Frauen in unserer Studie, so zeigt sich bei Frauen eine im Durchschnitt niedrigere PD-L1 Expression auf den Tumorzellen, Frauen waren häufiger als Männer Nie Raucher und Frauen hatten vor Therapiebeginn häufiger schon mehr als 3 Metastasen im Vergleich zu Männern. Im

Vergleich der Blutimmunzellparameter wiesen Frauen zu Beginn eine niedrigere Anzahl an NK-Zellen auf, wobei eine niedrige Anzahl an NK-Zellen in dieser Studie auch mit einem schlechteren PFS korrelierte.

Um zu schauen ob unsere bisherigen Ergebnisse sich ebenfalls auf Patienten mit SCLC unter Immunchemotherapie übertragen lassen, schlossen wir bei unserer dritten Studie (vergleiche 2.3.) 40 Patienten mit fortgeschrittenem und metastasierten SCLC ein. Das SCLC unterscheidet sich deutlich vom NSCLC, da es sich um einen hochaggressiven neuroendokrinen Tumor handelt. Dieser zeichnet sich durch sein schnelles Wachstum und seine frühzeitige Metastasierung aus. Initiale Studien zur Etablierung einer Immuntherapie in der Zweitlinie, ähnlich wie beim NSCLC, scheiterten und führten in Deutschland nicht zu einer Zulassung der entsprechenden Substanzen. Im Verlauf gelang endlich die Etablierung einer Immuntherapie in der Erstlinie in Kombination mit Platin und Etoposid. Jedoch profitieren nicht viele der Patienten dauerhaft von einer Immuntherapie (Hamilton und Rath 2019), obwohl man meinen könnte das dies so sein müsste, da das SCLC eine der höchsten TMB Raten aufweist und dies bisher in Verbindung mit einem verbesserten Ansprechen auf eine Immuntherapie gebracht wurde (Chalmers et al. 2017). Es gibt zwar initial eine hohe Rate an Patienten mit partieller Tumorremission unter der systemischen Therapie, jedoch kommt es im Verlauf sehr schnell zur Resistenzentwicklung mit Progression des Tumors. Die Mechanismen der Resistenzentwicklung beim SCLC sind bisher noch zu wenig verstanden. Es scheinen sowohl intrinsische Faktoren auf Seite des Tumors (u.a. zu wenig Tumorantigene, gestörte Antigenpräsentation), als auch extrinsische Faktoren (Fehlen von T-Zellen, inhibitorische Immuncheckpoints, immunsuppressive Zellen) bei der Resistenzentwicklung beteiligt zu sein (Sharma et al. 2017). Daher wären beim SCLC prädiktive Biomarker besonders bedeutend, um eine entsprechende Patientenselektion zu erreichen, zumal auch PD-L1 in diesen Tumoren keinen prädiktiven Wert besitzt.

In dieser Studie wurden ähnliche Ergebnisse im Vergleich zu den beiden NSCLC Studien dokumentiert, es gab jedoch einige Besonderheiten. Eine hohe NLR vor Beginn der Therapie war sehr stark assoziiert mit einer primären Therapieresistenz sowie einem schlechteren OS. Das Vorhandensein von Leber-/Hirnmetastasen korrelierte bei SCLC-Patienten sehr stark mit einem schlechten OS, dies war jedoch bei NSCLC-Patienten kaum der Fall. Alle SCLC-Patienten mit fehlendem Therapieansprechen hatten zu Beginn der Therapie Leber-/Hirnmetastasen sowie eine hohe Rate an HLA-DR^{low} MDSC. Die Rate an HLA-DR^{low} MDSC scheint in erster Linie assoziiert zu sein mit einer primären Therapieresistenz als mit einer längerfristigen Resistenzentwicklung. Eine niedrigere NLR war verbunden mit höheren Raten an Slan+ nicht klassischen Monozyten und korrelierte mit einem verbesserten OS. Bei Patienten mit einem klinischen Therapieansprechen bewirkte die Immunchemotherapie einen Anstieg der CD3+ T-Zellen zum Zeitpunkt der 2.

Blutentnahme (zum 3. Therapiezyklus). Trotzdem konnte kein Zusammenhang zwischen der Anzahl der Lymphozyten Subpopulationen und dem Überleben gefunden werden.

Vergleicht man SCLC- und NSCLC-Patienten hatten SCLC-Patienten zu Beginn der Therapie niedrigere Werte von DC und mehr Risikofaktoren (Vorhandensein von Leber-/Hirnmetastasen, hohe NLR, hohe Anzahl an HLA-DR^{low} MDSC, niedrige Werte für Slan+ nicht klassische Monozyten und niedrige Werte für DC (CD1c+ MDC).

Es konnte bereits gezeigt werden, dass das Gesamtüberleben für Patienten mit SCLC und Neutrophilie schlecht ist (Liu et al. 2020; Deng et al. 2017). Während chronisch inflammatorischer Prozesse, wie beim Vorhandensein einer Krebserkrankung, gibt es ein permanentes Signal zur Rekrutierung von Neutrophilen und Monozyten aus dem Knochenmark. Tumorzellen produzieren Granulozyten-Kolonie-stimulierenden Faktor (G-CSF) welcher die Balance von Retention und Freisetzung der Neutrophilen im Knochenmark verschiebt, was wiederrum zu einer Erhöhung der Neutrophilen im Blut führt (Jablonska et al. 2017).

Unser Ergebnis, dass eine hohe NLR mit einer schlechteren Prognose bei SCLC-Patienten assoziiert ist, deckt sich mit den Ergebnissen anderer Arbeitsgruppen (Winther-Larsen et al. 2021). Im Vergleich zu NSCLC-Patienten wiesen Patienten mit SCLC zu Beginn schon höhere NLR-Raten auf. In dieser Studie zeigte sich eine positive Korrelation zwischen Anzahl der Monozyten und Prozentsatz der HLA-DR^{low} MDSC, wie bereits in verschiedenen Tumorstadien des NSCLC beschrieben (Riemann et al. 2019). Ebenfalls zeigte sich eine starke Korrelation von HLA-DR^{low} MDSC und einer Therapieresistenz. Patienten mit primärer Therapieresistenz wiesen die höchsten Raten an HLA-DR^{low} MDSC auf. In Patienten mit primären Leber-/Hirnmetastasen konnten beispielsweise hohe Mengen an HLA-DR^{low} MDSC gefunden werden. Während die Rate in gesunden Patienten bei etwa 2,6 +/- 2,5% liegt (Riemann et al. 2019), konnten in Patienten mit progressivem SCLC 10-fach höhere Werte ermittelt werden. Dies könnte bedeuten, dass solche Patienten bereits vor Einleitung einer Therapie ein so paralytisches Immunsystem aufweisen, dass ein Ansprechen auf Immuntherapeutika eigentlich nicht mehr möglich ist.

Vergleicht man weiterhin die DC so zeigt sich ein Anstieg aller DC-Subpopulationen in Patienten mit einem klinischen Tumoransprechen zum dritten Therapiezyklus. Die einzige Ausnahme stellen die Slan+ nicht klassischen Monozyten dar, welche über die Zeit stabil bleiben. Vergleicht man SCLC- und NSCLC-Patienten so weisen beide Gruppen gleiche Werte an Slan+ nicht klassischen Monozyten auf, jedoch SCLC-Patienten deutlich niedrigere Werte an DC. Der Median beim SCLC betrug 0,10% der Leukozyten gegenüber dem NSCLC mit 0,20%. Diese Ergebnisse der deutlich geringeren DC stimmen mit denen einer anderen Arbeitsgruppe überein (Afifi und Helal 2009).

Unsere bisherigen Erkenntnisse könnten dazu beitragen vor Einleitung einer Therapie oder nach Applikation von zwei Therapiezyklen ein mögliches Ansprechen auf die Therapie vorherzusagen. Interessant war es auch im Verlauf die entsprechend identifizierten Parameter zu monitoren, um einen Progress im Langzeitverlauf früher vorhersagen zu können. Es scheint sich zwar unter der Immuntherapie im Verlauf ein Plateau zu entwickeln, jedoch gibt es trotzdem Patienten, die erst nach bereits längerer Gabe der Immuntherapie progredient werden. In unserer zweiten Studie wiesen 12 von insgesamt 90 Patienten ein Langzeitansprechen auf (PFS ≥ 12 Monate). Von diesen Patienten wurde im Verlauf eine 3. Blutprobe entnommen. Bei insgesamt zwei Patienten konnte eine Verschlechterung der beschriebenen Immunzellparametern nachgewiesen werden. Einer dieser beiden Patienten wies kurz nach Abnahme dieser Blutprobe tatsächlich einen Progress auf, der andere hatte keine Verschlechterung seiner Krebserkrankung litt jedoch an einer progredienten Herzinsuffizienz, an der er ein Jahr nach der 3. Blutentnahme verstarb. Dies könnte zum einen daraufhin deuten, dass ein Blutimmunzellmonitoring durchaus sinnvoll ist, um frühzeitig einen Progress unter laufender Therapie und bei initialem Ansprechen zu erkennen, zum anderen, dass eine Verschlechterung der Werte auch andere Ursachen wie Komorbiditäten haben kann. Es wäre sicherlich sinnvoll in weiteren Studien ein entsprechendes Monitoring im Langzeitverlauf zu testen und den optimalen Abstand zwischen den Blutanalysen zu ermitteln.

OPEN

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) $\geq$ 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

(*J Immunother* 2019;00:000–000)

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,

Received for publication June 18, 2019; accepted August 26, 2019.
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Supplemental Digital Content is available for this article. Direct URL citations appear in the printed text and are provided in the HTML and PDF versions of this article on the journal's website, www.immunotherapy-journal.com.

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.

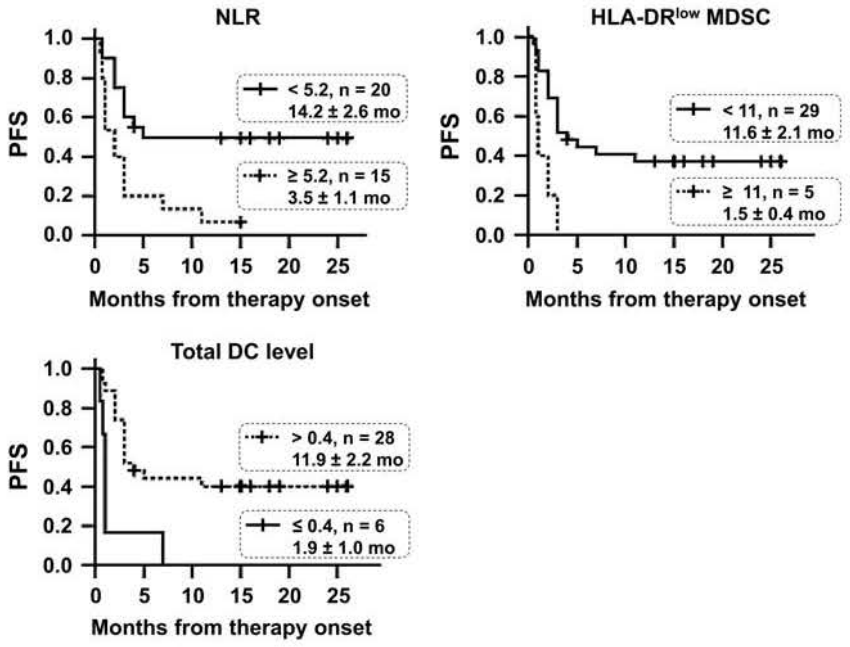

FIGURE 1. PFS for patients grouped below and above cutpoint for the parameters NLR (HR, 3.0; $P=0.009$), HLA-DR^{low} monocytes (HR, 3.85; $P=0.011$) and total DC levels (HR, 4.35; $P=0.003$), estimated at the onset of checkpoint therapy. In Kaplan-Meier plots, patients with censored values are denoted 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,

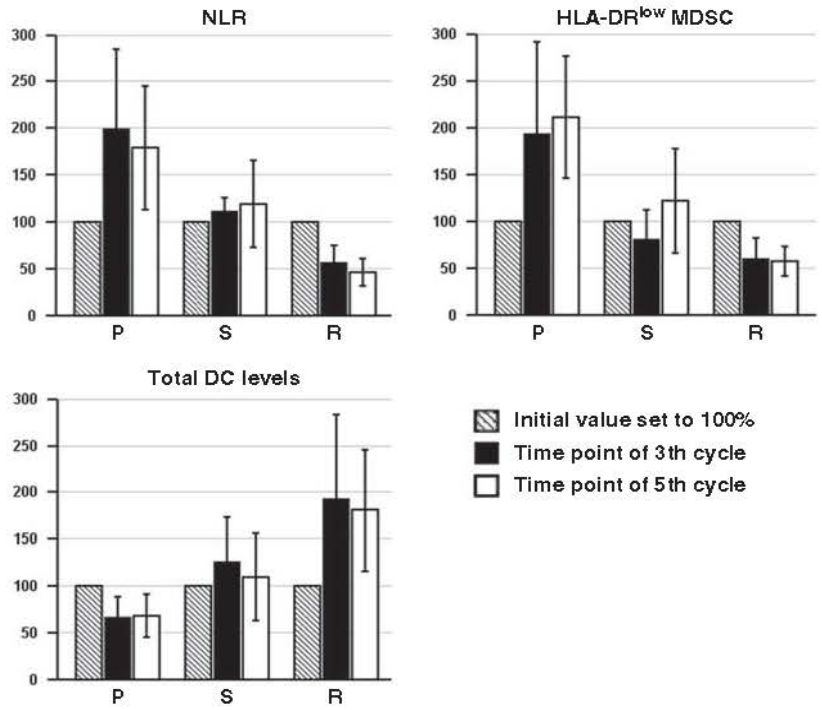

FIGURE 2. Time course of blood immune cell markers in the patients' groups progression (P), stable disease (S) and partial/complete response (R) with values at the onset of checkpoint therapy set to 100%. Mean values and error bars (95%) are displayed. DC indicates dendritic cell; NLR, neutrophil-lymphocyte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

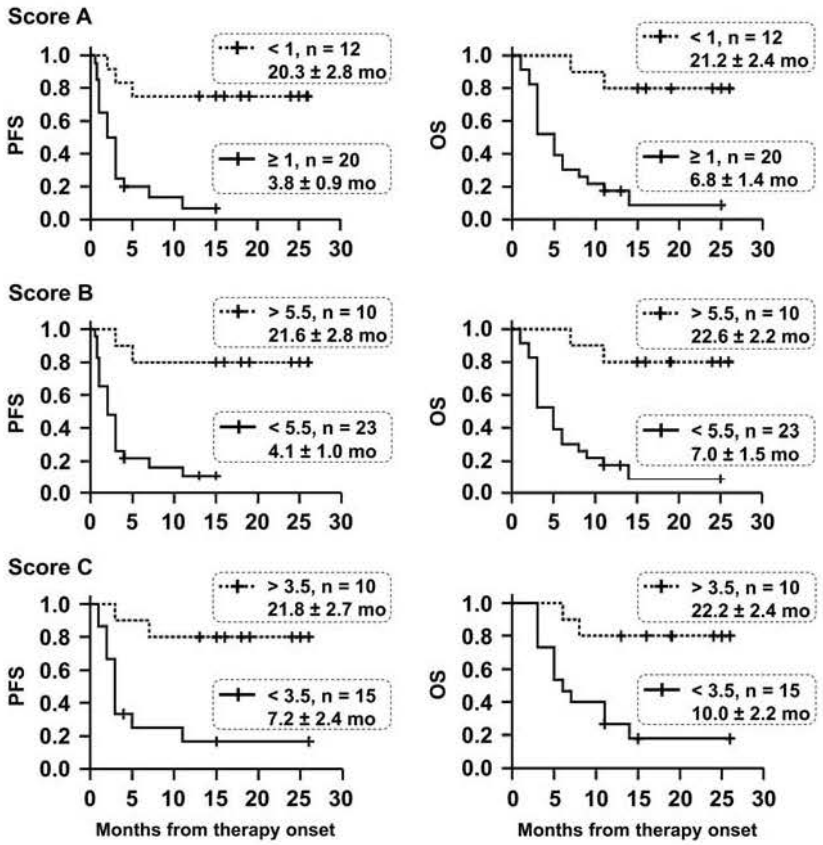

FIGURE 3. PFS and OS for patients grouped below and above cutpoint of 3 score variants (A, B, C), with the patient number and mean survival time ± standard error. Tick marks indicate censored observations. OS indicates overall survival; PFS, progression-free survival.

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 $\leq$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 $\geq$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 $\geq$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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High PD-L1/CD274 Expression of Monocytes and Blood Dendritic Cells Is a Risk Factor in Lung Cancer Patients Undergoing Treatment with PD1 Inhibitor Therapy

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Received: 25 August 2020; Accepted: 9 October 2020; Published: 13 October 2020

Simple Summary: Tumor cells can evade destruction via immune cells by expressing coinhibitory membrane molecules, which suppress antitumoral immune responses. Immune checkpoint inhibitor therapy acts by blocking these inhibitory pathways. Although this type of immunotherapy has shown promising results for selected cancer patients during recent years, an important challenge remains to identify baseline characteristics of patients who will mostly benefit from such therapy. The aim of our study was to assess the expression of the coinhibitory molecule PD-L1/CD274 on different antigen-presenting cells (monocytes and dendritic cell subsets) in the peripheral blood of 35 patients with nonsmall cell lung cancer undergoing checkpoint inhibitor therapy. CD274 expression correlated with therapy response and the survival of patients. Tumor patients with high CD274 expression levels of antigen-presenting cells in blood rarely responded to checkpoint inhibitor therapy. Our results implicate that a high CD274 expression in monocytes and dendritic cell subsets is a risk factor for therapy response.

Abstract: The aim of this study was to investigate the expression of the coinhibitory molecule PD-L1/CD274 in monocytes and dendritic cells (DC) in the blood of lung cancer patients undergoing PD1 inhibitor therapy and to correlate data with patient's outcome. PD-L1/CD274 expression of monocytes, $CD1c^+$ myeloid DC (mDC) and $CD303^+$ plasmacytoid DC (pDC) was determined by flow cytometry in peripheral blood at immunotherapy onset. The predictive value of the PD-L1/CD274-expression data was determined by patients' survival analysis. Patients with a high PD-L1/CD274 expression of monocytes and blood DC subpopulations rarely responded to PD1 inhibitor therapy. Low PD-L1/CD274 expression of monocytes and DC correlated with prolonged progression-free survival (PFS) as well as overall survival (OS). The highest PD-L1/CD274 expression was found in $CD14^+HLA\text{-}DR^{++}CD16^+$ intermediate monocytes. Whereas the PD-L1/CD274 expression of monocytes and DC showed a strong positive correlation, only the PD-L1/CD274 expression of DC inversely correlated with DC amounts and lymphocyte counts in peripheral blood. Our results implicate that a high PD-L1/CD274 expression of blood monocytes and DC subtypes is a risk factor for therapy response and for the survival of lung cancer patients undergoing PD1 inhibitor therapy.

Keywords: PD-L1/CD274; PD1 inhibitor therapy; lung cancer; flow cytometry; immune monitoring; dendritic cells; blood monocytes; $CD16^+$ monocytes; survival analysis

1. Introduction

Promising benefits of immunotherapy, in particular those targeting the immune checkpoint proteins PD1 and PD-L1, have been shown in lung cancer patients in recent studies. Immune checkpoints are proteins that restrict physiologic immune cell responses in order to maintain immune homeostasis and protect host tissues from unnecessary damage due to excessive inflammation. Programmed cell death 1 ligand 1 (PD-L1), also known as B7-H1 and CD274, is a transmembrane protein expressed on the surface of antigen-presenting cells [1]. After binding to its cognate receptor PD1/CD279 on T cells, PD-L1/CD274 exerts regulatory actions via a negative costimulatory effect on T cell functions to inhibit cytokine secretion, facilitate apoptosis of activated T cells and induce T-cell anergy [1]. Since many tumors can express PD-L1/CD274, the rationale of the PD-L1 pathway blockade is to inhibit the immunosuppressive PD-L1/PD1 interaction between tumor cells and T cells that hampers the activity of $CD4^+$ and $CD8^+$ T cells thereby enhancing T cell-mediated antitumor activities [2,3]. Selected patients with advanced non-small cell lung cancer (NSCLC) profit from the treatment with the PD1 inhibitors pembrolizumab or nivolumab in first-or second-line settings. However, treatment with immune checkpoint inhibitors is associated with a unique pattern of immune-related adverse effects [4]. Furthermore, durable responses are only observed in a minority of patients and primary, adaptive and acquired therapy resistances are common [4–6].

An important challenge remains to identify the baseline characteristics of patients who will mostly benefit from immunotherapy treatment. Multicolor flow cytometry represents a powerful tool to characterize individual cells within heterogeneous cell populations. Our recent results of the characterization of blood immune cells in lung cancer patients undergoing checkpoint blockade therapy showed a poor survival for patients with a high neutrophil-to-lymphocyte-ratio (NLR), a high amount of HLA-DR^{low} monocytes and a low frequency of dendritic cells (DC) [7]. Since the PD-L1/CD274 expression of antigen-presenting cells might lead to an inhibition of antitumor responses following the presentation of tumor antigens to T cells, the aim of this study was to evaluate PD-L1/CD274 expression of blood monocytes and DC subpopulations in lung cancer patients undergoing PD1 inhibitor therapy with respect to their effect on therapy response and prognosis.

2. Results

Table 1 shows the detailed characteristics of the 35 NSCLC patients of this study who received at least two cycles of PD1 inhibitor therapy. Pembrolizumab was offered to 18 of the 35 patients (51%), in seven cases (39%) as first-line- and in 11 (61%) as a second-line treatment. The remaining 17/35 (49%) patients received nivolumab. The mean follow-up time was 9.7 months (1–26 months) at the time of the data cut-off. The nine patients who stopped immunotherapy before the third cycle experienced a clinical worsening in most cases. Seven patients continued immune checkpoint inhibitor therapy. The tumor objective response rate was 40% for all patients with a median overall survival (OS) of 6.0 months and a 95% confidence interval (CI) of 3.2–8.8 months.

Table 2 summarizes the initial counts of monocytes, lymphocytes and blood DC subtypes as well as the CD274 expression of monocytes and DC subtypes in patients experiencing a clinical response (stable disease or remission) or a tumor progression. Between therapy responders and nonresponders, we did not observe differences between the pretherapeutic counts of monocytes and lymphocytes, respectively. Additionally, the amount of $CD14^+CD16^+$ monocytes and $CD14^+$HLA-$DR^{++}CD16^+$ intermediate monocytes did not reveal significant differences, though with a high standard deviation. However, the higher the number of blood DC, the better the patient responded to therapy.

Table 1. Patient characteristics.

Parameters	Characteristics	*N* (%)
Age at start of immunotherapy, years *n* (%)	Median Range >75 years	65 24–85 6 (17)
Sex, *n* (%)	Male Female	19 (54) 16 (46)
Histology, *n* (%)	Adenocarcinoma Squamous cell carcinoma mixed	23 (66) 7 (20) 5 (14)
Smoking status	Current or former smokers Never smokers	30 (86) 5 (14)
PD-L1/CD274 tumor expression, *n* (%)	<1% ≥1–49% >49 Missing	11 (31) 9 (26) 14 (40) 1
Response, *n* (%)	Stop after 2 treatment cycles Progressive disease after ≥3 cycles disease stabilization Partial remission	9 (25.7) 12 (34.3) 7 (20.0) 7 (20.0)

Table 2. Pretherapeutic counts of monocytes, lymphocytes and blood dendritic cells (DC) subtypes as well as programmed cell death 1 ligand 1 (PD-L1)/CD274 expression of monocytes and DC subtypes in the patients' groups "clinical response" (n = 14) and "progression" (n = 20). The p-value of the Student's t-test, the area under the ROC curve (AUC) showing the discrimination capability of the marker with respect to progression-free survival (PFS), as well as the cut-point value (Youden index method), are shown (MFI, mean fluorescence intensity).

Immune Cell Subtypes	Clinical Response	Progression	*p*-Value	AUC	Cut-off Value
Leukocytes (cells/µL)	8597 ± 2262	9600 ± 3175			
Neutrophils (cells/µL)	6214 ± 1948	7326 ± 3140			
Monocyte counts (cells/µL)	626 ± 160	672 ± 261			
$CD14^+CD16^+$ monocytes (% of monocytes)	23.6 ± 19.3	16.4 ± 11.6			
$CD14^+$HLA-$DR^{++}CD16^+$ monocytes (% of monocytes)	8.3 ± 3.8	7.7 ± 4.3			
Lymphocytes (cells/µL)	1459 ± 520	1413 ± 628			
$CD303^+$ pDC counts (cells/µL)	10.6 ± 6.2	5.9 ± 3.9	0.009	0.745	7.01
$CD303^+$ pDC (% of leukocytes)	0.119 ± 0.054	0.070 ± 0.050	0.011	0.769	0.061
$CD1c^+$ mDC (cells/µL)	13.2 ± 8.5	9.3 ± 8.6			
$CD1c^+$ mDC (% of leukocytes)	0.146 ± 0.068	0.089 ± 0.064	0.018	0.755	0.104
$CD141^+$ mDC (% of leukocytes)	0.0122 ± 0.009	0.006 ± 0.001	0.019		
Monocytic CD274 intensity (MFI)	450 ± 180	757 ± 468	0.027	0.750	480
CD274 intensity of pDC (MFI)	398 ± 194	609 ± 331	0.042	0.722	440
$CD274^+$ pDC (% of pDC)	12.5 ± 11.0	24.5 ± 15.4	0.022	0.730	16.0
CD274 intensity of $CD1c^+$ mDC (MFI)	433 ± 214	633 ± 322	0.041	0.705	450
$CD274^+$ mDC (% of $CD1c^+$ mDC)	15.09 ± 13.77	26.0 ± 18.11	0.062		

Human blood DC are a rare heterogeneous cell population that comprise approximately 1% of peripheral blood mononuclear cells. DC are broadly defined as antigen-presenting cells with a high expression of MHC class II molecules that lack other leukocyte lineage markers (CD3, CD14, CD19

and CD56) [8]. With respect to their lineage origin, they can be classified into two major subsets: plasmacytoid DC (pDC) as the major producers of type-I interferon (IFN), and myeloid lineage DC (mDC). Based on their expression of CD1c and CD141, two further mDC subsets have been described [9] and were investigated in this study. Most patients with advanced lung cancer had very low amounts of blood DC, with the lowest values observed for $CD141^+$ mDC. Despite measuring >10 E6 leukocytes by flow cytometry, often only <100 events of $CD141^+$ mDC could be detected. Due to the poor statistics, we focused on pDC and $CD1c^+$ mDC in our further investigations. pDC counts were especially low in nonresponders, with 2.6 ± 1.2 cells/µL in progressors with progression-free survival (PFS) ≤1 month, 6.9 ± 3.8 cells/µL in progressors with PFS >1 month, 9.0 ± 5.3 cells/µL in stable disease and 12.1 ± 7.0 cells/µL in remission. In addition, the percentage of $CD1c^+$ mDC showed significantly higher values in therapy responders (Table 2).

With respect to PD-L1/CD274 expression, monocytes had slightly higher intensities than DC subtypes (Table 2). Within the monocytic population, the proportion of $CD14^+HLA\text{-}DR^{++}CD16^+$ intermediate monocytes (8.0 ± 4.1% of monocytes) had the highest PD-L1/CD274 expression (mean fluorescence intensity (MFI) of 1179 ± 660). Otherwise, no difference could be observed in the PD-L1/CD274 expression between $CD1c^+$ mDC and pDC (Table 2, Figure 1).

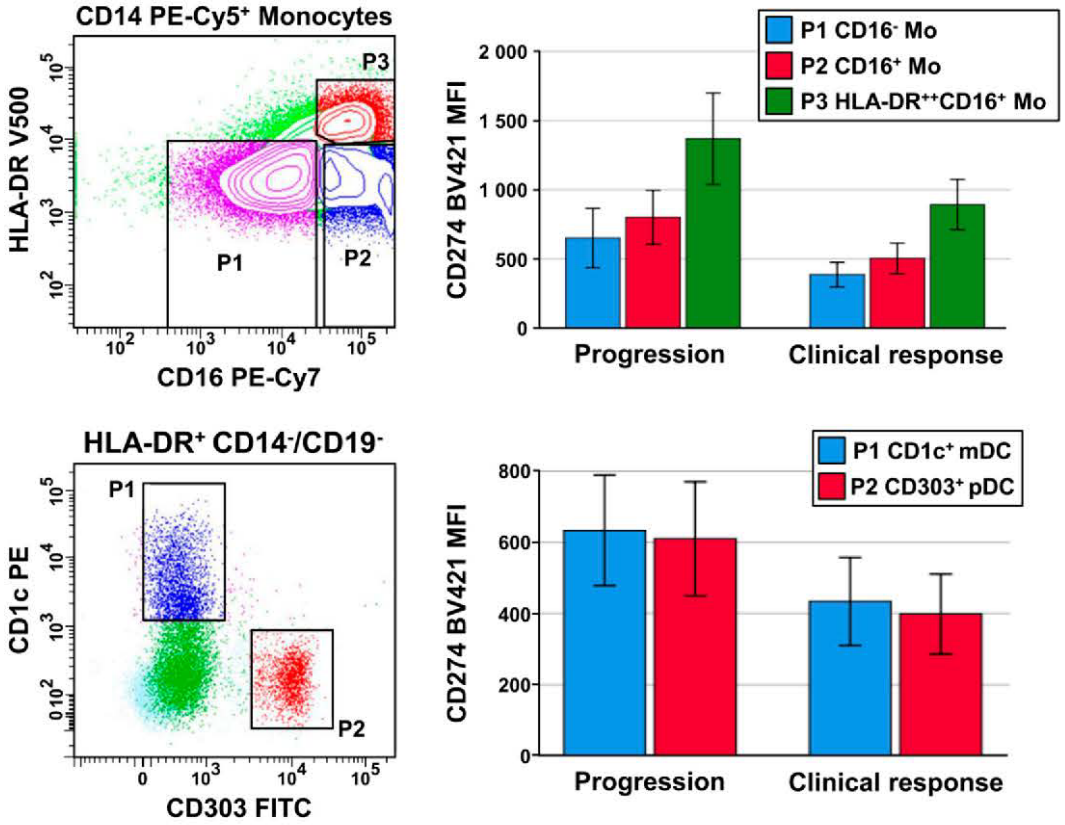

Figure 1. Gating strategy and PD-L1/CD274 mean fluorescence intensity (MFI) for $CD14^+$CD16-negative classical monocytes, $CD14^+CD16^+$ monocytes and $CD14^+HLA\text{-}DR^{++}CD16^+$ intermediate monocytes (upper part), and for $CD1c^+$ myeloid DC (mDC) and $CD303^+$ plasmacytoid DC (pDC) (lower part of the picture). Bars illustrate mean value and standard error with a significant difference between the outcome "progression" and "clinical response" with respect to the PD-L1/CD274 intensity of $CD14^+$CD16-negative monocytes ($p = 0.029$), $CD14^+CD16^+$ monocytes ($p = 0.034$), $CD14^+HLA\text{-}DR^{++}CD16^+$ monocytes ($p = 0.027$), $CD1c^+$ mDC ($p = 0.041$) and pDC ($p = 0.042$).

A high PD-L1/CD274 expression of monocytes and of DC subtypes was associated with a poor response to therapy. In patients responding to therapy compared to patients with progression, all the monocytic subgroups had a significantly lower PD-L1/CD274 expression (Figure 1). With respect to

pDC, the percentage of PD-L1/CD274$^+$ pDC was 31.9 ± 20.4 in patients with PFS ≤1 month, 21.1 ± 12.2 in progressors with a PFS >1 month and 12.5 ± 11.0 for patients with a clinical response. ROC analysis resulted in AUC values >0.700 (Table 2). With the cut-off points estimated by the Youden index method, univariate Kaplan–Meier and Cox regression analyses were performed for both PFS and OS, as given in Table 3.

Table 3. Relationship between initial pDC counts and PD-L1/CD274 expression of monocytes and DC subtypes, respectively, with patient's progression-free survival (PFS) (**A**) and overall survival (OS) (**B**). Data of univariate prognostic factor analysis (Kaplan–Meier and Cox regression) are shown (HR, hazard ratio; CI, confidence interval; MFI, mean fluorescence intensity).

A	Cut-Off Value	*n*	Kaplan–Meier			Cox Regression		
			% Censored	PFS Time (Months)	Log-Rank Test	HR	95% CI	*p*-Value
Blood pDC counts (cells/µL)	≤7.0	18	11.1	3.65 ± 1.236	0.002	3.455	1.427–8.365	0.006
	>7.0	17	52.9	15.46 ± 2.76				
Monocytic CD274 expression (MFI)	<480	16	56.3	16.00 ± 2.87	0.007	3.116	1.242–7.814	0.015
	≥480	18	11.1	4.62 ± 1.64				
CD274 MFI of pDC	≤440	19	47.4	13.95 ± 2.65	0.026	2.414	1.029–5.660	0.043
	>440	14	14.3	5.23 ± 2.08				
CD274$^+$ pDC (% of pDC)	≤16	17	58.8	16.66 ± 2.73	0.001	4.14	1589–10,784	0.004
	>16	14	7.1	3.32 ± 0.96				
CD274 MFI of CD1c$^+$ mDC	<450	15	53.3	15.01 ± 3.04	0.031	2.464	0.997–6.086	0.051
	≥450	18	16.7	6.57 ± 2.12				
B	**Cut-Point**	***n***	**Kaplan–Meier**			**Cox Regression**		
			% Censored	OS Time (Months)	Log-RankTesHR		95% CI	*p*-Value
Blood pDC counts (cells/µL)	≤7.0	18	11.1	5.94 ± 1.27	0.002	3.548	1.477–8.523	0.005
	>7.0	17	52.9	16.8 ± 2.44				
Monocytic CD274 expression (MFI)	<480	16	56.3	17.06 ± 2.61	0.004	3.343	1.334–8.376	0.010
	≥480	18	11.1	6.83 ± 1.63				
CD274 MFI of pDC	≤440	19	47.4	15.21 ± 2.41	0.028	2.397	1.024–5.607	0.044
	>440	14	14.3	7.57 ± 2.04				
CD274$^+$ pDC (% of pDC)	≤16	17	58.8	17.78 ± 2.44	0.001	4.011	1.532–10.501	0.005
	>16	14	7.1	6.5 ± 1.54				
CD274 MFI of CD1c$^+$ mDC	<450	15	53.3	16.33 ± 2.72	0.035	2.441	0.989–6.023	0.053
	≥448	18	16.7	8.61 ± 1.99				

Patients with a higher PD-L1/CD274 expression of monocytes and DC subtypes, respectively, showed a significantly poorer survival. Figure 2 illustrates that patients with an initial value of >7.01 pDC/µL blood, ≤16% CD274$^+$ pDC, a monocytic CD274 intensity of <480 and a CD274 intensity of CD1c$^+$ mDC ≤450 had a significantly longer PFS.

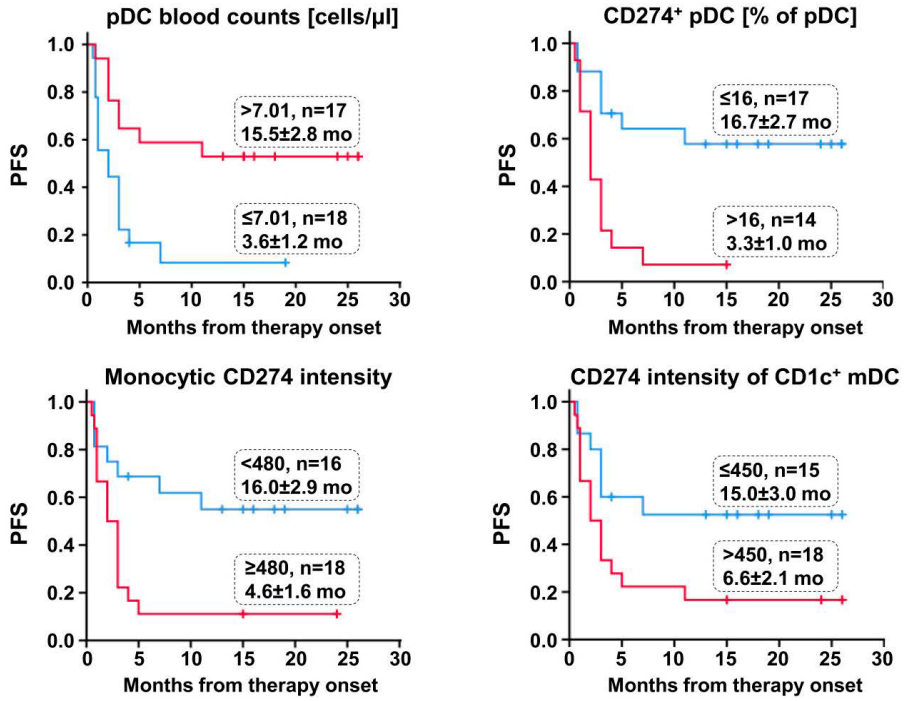

Figure 2. Kaplan–Meier curves showing progression-free survival (PFS) for patients undergoing PD1 inhibitor therapy and categorized with pDC blood counts, the percentage of CD274$^+$ pDC, monocytic CD274 expression (mean fluorescence intensity (MFI)) and CD274 expression of CD1c$^+$ mDC (MFI). Tick marks indicate censored observations. Cut-off point, patient number (*n*) and the mean ± standard error of the estimated PFS are given for each group. Survival statistics are shown in Table 3.

A positive correlation between the PD-L1/CD274 intensities of monocytes and DC subtypes was observed (Table 4). In contrast, no correlation of the PD-L1/CD274 intensities of monocytes and DC, respectively, with the PD-L1/CD274 tumor status (provided by the Department of Pathology) was observed, though this value was already evaluated at the time point of histotype assignment. Furthermore, a high PD-L1/CD274 expression of pDC significantly correlated with low amounts of both pDC and CD1$^+$ mDC (Table 4). Furthermore, the percentage of PD-L1/CD274$^+$ pDC inversely correlated with the number of blood lymphocytes, with similar results for T cells, B cells and NK cells. In contrast, monocytic PD-L1/CD274 intensity did neither correlate with the amount of pDC or CD1c$^+$ mDC, nor with lymphocyte counts.

Table 4. Association of PD-L1/CD274 expression of monocytes and DC subtypes with other immune cell markers, analyzed by Spearman's rank correlation. Correlation coefficient (CC) and *p*-values are shown.

Correlation of	CC	*p*-Value
Monocytic CD274 Expression with		
CD274 expression of pDC	0.954	<0.001
CD274 expression of CD1c$^+$ mDC	0.861	<0.001
CD274$^+$ pDC (% of pDC) with		
Proportion of pDC (% of leukocytes)	–0.523	0.003
Proportion of CD1c$^+$ mDC (% of leukocytes)	–0.416	0.022
Lymphocytes (cells/µL)	–0.632	<0.001

3. Discussion

Checkpoint inhibition has complemented the therapeutic approach for patients with advanced lung cancer, although not all the patients benefited from it [4]. Understanding the reasons for patients'

variability in response to therapy and developing reliable biomarkers to predict patients, who are likely to respond to therapy, remains a challenge. PD-L1/CD274 expression in tumor tissues has emerged as one such candidate biomarker of therapy response, since patients with PD-L1/CD274-expressing advanced tumors have a higher objective response rate and improved PFS and OS as compared to the negative subgroups [10]. In NSCLC, the positive prognostic value of PD-L1/CD274 expression was independent of age, stage and histotype [11].

The primary rationale of checkpoint blockade therapy was to inhibit the immunosuppressive PD-L1/PD1 interaction between tumor cells and T cells that hampers the activity of $CD4^+$ and $CD8^+$ T cells [1]. However, in recent studies PD-L1/CD274 expression by tumor tissues was associated with the presence of tumor-infiltrating lymphocytes, which could be involved in better immunotherapy-triggered prognosis [12,13]. PD-L1/CD274 is expressed at low levels on a wide range of cells and its expression can be upregulated in response to various stimuli (review in [14]). In the context of tumor microenvironments, cells including macrophages, DC, myeloid-derived suppressor cells, regulatory T cells and endothelial cells can upregulate PD-L1/CD274 due to inflammation responses. Since the primary function of coinhibitory receptor/ligand pairs is to attenuate the magnitude and duration of immune responses in order to minimize collateral tissue damage during a host immune response, PD-L1/CD274 expression of antigen-presenting cells might contribute to tumor escape.

In this study, PD-L1/CD274 expression of monocytes and blood DC subtypes in NSCLC patients undergoing PD1 inhibitor therapy was investigated. A high expression of this molecule was found to be a poor prognostic factor. Our results are in contrast to data from murine tumor models, where PD-L1/CD274-expressing antigen-presenting cells, rather than tumor cells, demonstrated essential antitumor effects of anti-PD-L1 monotherapy. A positive response to checkpoint inhibitor therapy has been associated with a high expression of PD-L1/CD274 on tumor-infiltrating immune cells indicating a role for PD-L1/CD274-expressing immune cells in suppressing antitumor responses, which are reinvigorated on checkpoint blockade therapy [15]. However, PD-L1/CD274-expressing monocytes and blood DC kept a significant negative impact on prognosis in this study. One could postulate that the onset of checkpoint inhibitor therapy was too late to reverse the pronounced immune suppression demonstrable in some of the NSCLC patients with advanced tumor stages. Adenocarcinoma was the most common tumor type in this study and an aggressive and early progressing nonsquamous NSCLC has been described, which might even represent a distinct disease entity [16]. Otherwise, the functional consequences of a PD-L1/CD274 expression could be affected by binding partners or molecules coexpressed with this molecule on antigen-presenting cells. Furthermore, besides PD1, PD-L1/CD274 can also bind to CD80 on activated T cells, thereby delivering another inhibitory signal [17,18], which is not inhibited by anti-PD1 antibody therapy. CD80 has been shown to interact with PD-L1/CD274 in cis on antigen-presenting cells to disrupt PD-L1/PD1 binding [19], and CD80 expression might differ between antigen-presenting cells in blood and tumor tissue. Furthermore, factors mediating PD-L1/CD274 expression of blood immune cells might exert pleiotropic immunosuppressive functions. These include for example immunosuppressive cytokines, such as IL-10 and IL-27, as well as the activation of different (oncogenic) signal transduction pathways, such as myc and phosphatidylinositol 3-kinase/Akt [20,21].

We observed that the percentage of PD-L1/$CD274^+$ pDC inversely correlated with lymphocyte counts and pDC numbers. Very low amounts of DC were found in some of the patients with advanced lung cancer, which might contribute to the disturbed immune functions and poor prognosis. Several tumor-derived factors could be responsible for the decline of DC, e.g., increased serum levels of IL-10 correlate with profound numerical deficiency and immature phenotype of circulating DC subsets in patients with hepatocellular carcinoma [22]. NSCLC patients with low pretherapeutic values of blood pDC had a poor therapy response. In settings of cancer, pDC-derived type-I IFNs can promote antitumoral immunity through their direct activity on both tumor and immune cells [23]. pDC secrete a multitude of other inflammatory cytokines and chemokines and can act as antigen-presenting cells, although with lower efficacy than conventional DC [24]. Our earlier results in lung cancer patients

showed that blood DC numbers decrease with age and tumor stage [25]. In addition, an increase of blood DC levels could be found in such patients, which did respond to checkpoint inhibitor therapy [7].

Tumors develop multiple strategies that lead to immune suppression thereby preventing effective antitumor immunity, such as the increased secretion of immunosuppressive metabolites and cytokines, e.g., IL-10 and TGF-β, enhanced differentiation of immune effector cells to a regulatory phenotype, as well as an accumulation of immunosuppressive cells, such as myeloid-derived suppressor cells [3]. Depending on the signals received from the microenvironment, DC can either activate adaptive immune responses or mediate immune tolerance. Immunogenic DC are characterized by a high expression of costimulatory molecules and the production of proinflammatory cytokines, whereas tolerogenic DC express low levels of costimulatory molecules and produce immunomodulatory cytokines. DC treated with lung cancer cell culture supernatants significantly downregulated the expression of MHC class II molecules and of the costimulatory molecules CD40 and CD80, but upregulated the inhibitory molecule PD-L1/CD274 [26]. Signals generated from inhibitory checkpoint molecules might contribute to the inhibitory properties of DC in cancer patients. Furthermore, PD-L1/CD274 silencing on DC could enhance T-cell responses leading to tumor clearance [2], which is in accordance with several studies demonstrating the advantages of knocking down PD-L1/CD274 regarding the efficacy of DC vaccine therapy [27,28]. Whereas a negligible PD-L1/CD274 expression of blood pDC and mDC of healthy donors has been described [29], blood DC of lung cancer patients show a clear PD-L1/CD274 expression in this study, thereby confirming the data of blood DC in patients with ovarian cancer [2] and melanoma [30]. Similarly, monocytes in healthy controls express only a small amount of PD-L1/CD274, whereas monocytes in cervical cancer patients show an increased expression [31]. Our results show that PD-L1/CD274 expression of monocytes and DC was positively correlated suggesting common ways of regulation in the different cell types. PD-L1/CD274 expression can be upregulated by a substantial number of mediators (for a review see [14,20,32]). As an example, PD-L1/CD274 expression of monocytes and DC has been found upregulated in response to the presence of T cells producing immune-stimulating cytokines, such as IFNs [33,34]. Other known inductors on monocytes and/or DC are IL-17 [35], TNF-α [36], IL-10 [20] and TGF-β [37]. Human blood contains several forms of soluble or extracellular PD-L1, included, e.g., in exosomes and microvesicles [38], which could be involved in the induction of PD-L1/CD274 expression on antigen-presenting cells. In advanced NSCLC, high levels of soluble PD-L1/CD274 correlated with nivolumab treatment failure [39], and serum with a high proportion of PD-L1/CD274$^+$ exosomes, have been shown to inhibit in vitro IL-2 and IFN-γ production by CD8$^+$ T cells [40].

Monocytic PD-L1/CD274 expression, which was also a poor risk factor in our study, did neither correlate with lymphocytic nor with blood DC counts. Our results show that CD14$^+$CD16$^+$ intermediate monocytes with a high HLA-DR intensity expressed the highest PD-L1/CD274 levels. Monocytes egress from the bone marrow as a uniform population of CD14$^+$CD16-negative cells, a proportion of which subsequently differentiates to become intermediate (CD14$^+$CD16$^+$ and high amounts of HLA-DR) and "non-classical monocytes" monocytes (CD14dimCD16$^+$) [41]. Intermediate monocytes show a high phagocytic activity and produce IL-10 [42]. Since IL-10 is known to inhibit HLA-DR expression [43] and the intermediate monocytes express a rather high HLA-DR intensity, IL-10 might not be the responsible inductor of monocytic PD-L1/CD274 expression. Future investigation will show whether IL-10 is involved in the high PD-L1/CD274 expression of monocytes and blood DC subtypes observed in lung cancer patients with a poor therapy response to anti-PD1 therapies.

Although the efficacy of immune checkpoint inhibitors is well-established in oncology, there is increasing evidence that their use may also be effective in several noncancer acute and chronic inflammatory conditions, including sepsis, burns and chronic infections [44]. An increased frequency of PD-L1/CD274-expressing monocytes is an independent risk factor for infectious complications in acute pancreatitis [45]. In sepsis, high monocytic PD-L1/CD274 expression has been correlated with increased T-cell apoptosis, lymphopenia, and T-cell dysfunction [46]. Whereas PD1 expression on T cells was not a reliable "danger signal" for immune suppression in septic patients, monocytic

PD-L1/CD274 intensity was an independent predictor of 28-day mortality in septic shock patients [47]. PD-L1/CD274 expression of $CD14^+CD16^+$ intermediate monocytes has been described upon hepatitis C virus (HCV) infection. The upregulation of monocytic PD-L1/CD274 intensity was associated with defective HCV-specific T-cell responses, while the inhibition of monocyte-associated PD-L1/CD274 expression enhanced the frequency of IFN-γ-producing HCV-specific T cells and the production of Th1 cytokines [48]. PD-L1/CD274 expression of DC was also increased in HCV-infected patients and this increase was associated with an impaired allostimulatory capacity of DC [49].

Despite notable and durable clinical responses, basic and clinical studies are still required to determine the exact mechanism of checkpoint inhibitor immunotherapy, and the appropriate selection of patients. Currently, major efforts are being made to elucidate the mechanisms involved in the development of primary and acquired resistance to checkpoint inhibitor therapy [50]. By understanding the resistance mechanisms involved, strategies can be designed to overcome resistance and treatment failure. PD-L1/CD274 expression of monocytes and blood DC could be involved in cancer-induced immune suppression and can be used as a blood biomarker for poor response to PD1 inhibitor therapy. The factors responsible for PD-L1/CD274 expression of blood immune cells, as well as the role of PD-L1/CD274-expressing $CD14^+$HLA-$DR^{++}CD16^+$ intermediate monocytes, needs to be clarified in future investigations. Considering that cancer immunotherapy is the most actively evolving therapy for lung cancer, we believe that this study has some important findings, which should be further pursued by confirmatory and extended studies.

4. Materials and Methods

4.1. Patient Cohort

The institutional review board of the Ärztekammer Sachsen-Anhalt approved this study (No. 96018). A 2.7 mL volume peripheral blood was collected from the 35 prospectively enrolled patients with the criteria: histologically confirmed diagnosis of metastatic NSCLC, age >18 years, adequate organ functions, medical decision-making capacity, available PD-L1/CD274 status determined by immunohistochemical analysis, epidermal growth factor receptor (EGFR) wild-type, negative for anaplastic lymphoma kinase (ALK) translocation, no previous history of systemic immunosuppressive therapy and no active autoimmune disease. Patients enrolled received either pembrolizumab as monotherapy (200 mg in chemotherapy-naïve patients, 2 mg/kg for patients previously treated with chemotherapy, every 3 weeks) or nivolumab intravenously administered (3 mg/kg every 2 weeks). PD-L1 tumor status and patients' treatment history determined the choice of agent (first-line or second-line setting). Every 9 weeks or with clinical worsening of the patient's condition, scheduled computed tomography (CT) or magnetic resonance imaging was performed. A treatment benefit was defined as complete/partial remission, and stable disease on CT scan according to RECIST 1.1. Patients with progressive disease at the first CT scan were categorized as no disease control. Treatment continued until confirmed disease progression, unacceptable toxicity, or withdrawal of consent.

4.2. Antibody Staining and Flow Cytometry

Peripheral blood from NSCLC patients was taken on the day of immunotherapy start. At first, the complete leukocyte blood count was monitored, then antibody staining of whole blood was performed. The "Blood DC Enumeration Kit" of company Miltenyi Biotec (Bergisch Gladbach, Germany) was supplemented with the monoclonal antibody (mAb) CD16 for the detection of $CD16^+$ monocytes, and with an HLA-DR mAb for better gating possibilities. In brief, whole blood samples were labeled with the mAbs CD303 FITC as a pDC marker [51], CD1c phycoerythrin (PE) for mDC (conventional DC2), CD14/CD19 PE-Cy5, CD141 allophycocyanin (APC) for mDC (cDC1), CD16 PE-Cy-7 (BioLegend, Koblenz, Germany), HLA-DR V500 and CD274 BV421 (BD Biosciences, Heidelberg, Germany). According to the manufacturer's instruction, mAb incubation, red cell lysis, two washing steps and cell fixation were performed. Samples were measured with a FACS CANTO II flow cytometer

(BD Biosciences) with FACS DIVA™ software. To set standardized geometric MFI ranges in the fluorescence channels used, Cytometer Setup and Tracking Beads (BD Biosciences) were used daily. At least 1×10^6 blood leukocytes were analyzed. The gating strategy for DC subpopulations was described earlier [7]. Monocytes were gated in a CD14/SSC plot, and a CD16/HLA-DR plot was used to identify $CD14^+CD16^+$ and $CD14^+HLA\text{-}DR^{++}CD16^+$ monocytes (Figure 1). BV421 histograms were used to estimate the CD274 MFI and the percentage of CD274-positive DC, with CD274 staining of B cells serving as a control

4.3. Statistical Analysis

The commercial software SPSS 25.0 (SPSS Inc., Munich, Germany) was used for all statistical analyses. ANOVA analysis and a Student's *t*-test were used to investigate the differences in immune cell numbers between responders and nonresponders to therapy. All *p*-values are exploratory. Spearman correlation coefficients (CC) were calculated to investigate correlations between PD-L1/CD274 expression of monocytes and DC, respectively, with immune cell parameters. For survival analysis, PFS was defined as the time from the first PD1 inhibitor treatment to tumor progression or death, OS was the duration of survival after starting immunotherapy. Survival analysis firstly comprised a descriptive representation of the cumulative survival functions according to Kaplan-Meier. The log-rank test was used to identify differences among the survival curves. To examine the correlation of immune cell values with PFS and OS, Cox regression analysis was performed. $p < 0.05$ was considered statistically significant. Predictor variables with a significant difference between patients' groups with and without a therapy response were analyzed with receiver operating characteristics (ROC) curves to determine the overall strength of association (area under the ROC curve (AUC)), as well as the optimal cut-point for the prediction of therapy response. Youden's Index was used to calculate which cut-off point gives the best sensitivity and specificity (with J = sensitivity + specificity-1).

5. Conclusions

The observed heterogeneity in clinical responses to checkpoint blockade therapy in cancer patients has led to major efforts to define biomarkers predicting therapy responses. Using multicolor flow cytometry, we prospectively monitored blood immune cells from patients with advanced NSCLC undergoing therapy with PD1 inhibitors to investigate the consequences of a PD-L1/CD274 expression of monocytes and DC in peripheral blood. A high pretherapeutic PD-L1/CD274 expression has been detected as an adverse factor for PD1 inhibitor therapy in this study, with the highest PD-L1/CD274 expression found in $CD14^+HLA\text{-}DR^{++}CD16^+$ intermediate monocytes. Since PD-L1/CD274-expressing monocytes and blood DC may be of pathophysiological relevance, a better understanding of the underlying mechanisms of their regulation and the functional consequences of a PD-L1/CD274 expression on blood immune cells might help to generate novel hypotheses for immune evasion and might offer novel opportunities for the design and optimization of immunotherapies. Rather than assessing only the PD-L1/CD274 expression of tumor cells, the additional monitoring of PD-L1/CD274 expression of immune cells in the blood appears to be mandatory for predicting therapy responses in patients undergoing checkpoint blockade therapy.

Author Contributions: Patient recruitment, M.M.; Conceptualization, D.R. and M.M.; project administration, W.S.; funding acquisition, W.S.; methodology, S.T.; analysis, writing—original draft preparation, D.R.; writing—review and editing, B.S. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Mildred Scheel foundation, grant number 70113450.

Acknowledgments: We would like to thank Anja Müller and Katharina Biel for the performance of flow cytometry of clinical samples and Jens Wulfänger for help with illustrations.

Conflicts of Interest: The authors declare no conflict of interest.

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Blood Immune Cell Biomarkers in Lung Cancer Patients Undergoing Treatment with a Combination of Chemotherapy and Immune Checkpoint Blockade

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Simple Summary: Tumor cells can evade destruction via immune cells by expressing coinhibitory membrane molecules, which can suppress tumor-specific T cells. Immune checkpoint inhibitor therapies act by blocking these inhibitory pathways via monoclonal antibodies. Although this type of immunotherapy has shown promising results for advanced cancers of different entities during recent years, an important challenge is to identify the baseline characteristics of patients who will mostly benefit from such treatment. Blood biomarkers have limitations to reflect the tumor microenvironment but are easier to handle than markers in tumor lesions. The aim of our study was to identify blood cell parameters correlating with patients' survival in 90 patients with non-small cell lung cancer undergoing immune checkpoint inhibitor therapy combined with chemotherapy. We found that patients with a neutrophil-to-lymphocyte ratio ≥6.1, a percentage of HLA-DR^{low} monocytes ≥22%, a frequency of slan+ non-classical monocytes <0.25%, and/or of dendritic cells ≤0.14% of leukocytes had a poorer prognosis. Long-term survivors were patients without any of the risk factors investigated. Our results implicate that blood neutrophil counts, special types of monocytes, and the number of blood dendritic cells might be useful predictive biomarkers for cancer patients' survival.

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Citation: Möller, M.; Turzer, S.; Ganchev, G.; Wienke, A.; Schütte, W.; Seliger, B.; Riemann, D. Blood Immune Cell Biomarkers in Lung Cancer Patients Undergoing Treatment with a Combination of Chemotherapy and Immune Checkpoint Blockade. *Cancers* **2022**, *14*, 3690. https://doi.org/10.3390/cancers14153690

Academic Editor: Eric Tartour

Received: 24 June 2022
Accepted: 25 July 2022
Published: 28 July 2022

Abstract: Although immune checkpoint inhibitor (ICI) therapies have improved the treatment of patients with advanced non-small cell lung cancer (NSCLC), several patients do not achieve durable clinical responses. Biomarkers for the prediction of therapy responses are urgently needed. To identify blood cell parameters correlating with patients' survival, immune cells from 90 patients with NSCLC undergoing a combination of ICI and chemotherapy were prospectively monitored. At the time point of the first and third antibody administration, complete leukocyte blood count, the percentage of HLA-DR^{low} monocytes, the percentage of 6-Sulfo LacNAc (slan)+CD16+ non-classical monocytes, and the number of circulating dendritic cell (DC) subtypes, as well as T-, B-, and NK cells were determined by multi-color flow cytometry in peripheral blood. The prognostic value of the immune cell parameters investigated was evaluated by patients' survival analysis, with progression-free survival (PFS) as the main criterion. A total of 67 patients (74.4%) showed a partial remission or a stable disease, and 35% of patients even survived 12 months and longer. Patients with a neutrophil-to-lymphocyte ratio (NLR) ≥6.1, a frequency of HLA-DR^{low} monocytes ≥22%, of slan+ non-classical monocytes <0.25% of leukocytes, and/or a sum of myeloid DC (MDC) and plasmacytoid DC (PDC) ≤0.14% of leukocytes had a poorer prognosis. The hazard ratio for PFS was 2.097 (1.208–3.640) for the NLR, 1.964 (1.046–3.688) for HLA-DR^{low} monocytes, 3.202 (1.712–5.99) for slan+ non-classical monocytes, and 2.596 (1.478–4.56) for the MDC/PDC sum. Patients without any of the four risk factors showed the best PFS. Furthermore, low NK cell counts correlated with shorter PFS (cutoff 200 cells/µL). Female patients had lower baseline NK cell counts and a shorter PFS. Our

study confirms the usefulness of blood immune cells as biomarkers for clinical response and survival in NSCLC patients undergoing a combined ICI/chemotherapy.

Keywords: biomarker; immune checkpoint blockade; dendritic cells; immune monitoring; lung cancer; prognosis

1. Introduction

Lung cancer is one of the most common malignant tumors and a leading cause of cancer-related death worldwide [1]. Non-small cell lung cancer (NSCLC), accounting for about 83% of all patients with lung cancer, is subdivided into adenocarcinoma (AC) (50–70%), squamous cell carcinoma (SqC) (20–30%), and other subtypes (<10%) [2]. At the time point of diagnosis, about 75% of NSCLC patients have an advanced disease associated with a bad prognosis implying low survival rates [3]. Platinum-based chemotherapy has been the standard therapy despite modest responses to these agents and short intervals until disease progression [4,5]. Recently, immune checkpoint inhibitors (ICI) targeting the PD-L1/PD-1 signaling axis have emerged as a treatment option for these patients, although only a limited proportion of patients benefit [6,7]. The identification of this target population remains challenging, which denotes an unmet need to develop accurate biomarkers predictive of response to immune checkpoint inhibition for patients' selection.

In cancer patients, tumor-specific immune responses are inhibited, and in patients with advanced lung cancer, a systemic immune suppression has been observed [8]. Different cells and factors have been implicated in this process, including regulatory T cells, myeloid-derived suppressor cells (MDSC), various soluble factors, and cytokines as well as inhibitory receptor molecules expressed by tumor cells [9]. Despite blood cell biomarkers' difficulty in reflecting the tumor microenvironment, immune cell profiling in peripheral blood is an attractive alternative tool for biomarker identification. The aim of the current study was to investigate blood immune cells as putative biomarkers to select patients who could benefit from immune/chemotherapy. We enrolled patients with histologically confirmed unresectable locally advanced or metastatic NSCLC lung cancer prior to PD-1 or PD-L1 blockade treatment combined with chemotherapy. Since a high neutrophil/lymphocyte ratio (NLR), a high number of HLA-DR^{low} monocytes, and low DC levels correlated with a bad patients' survival in a recent study with lung cancer patients undergoing ICI monotherapy [10], we mainly focused on these three risk factors in our study. We complemented the blood biomarkers by 6-Sulfo LacNAc (slan)+CD16+ non-classical monocytes, since these cells have been shown to be involved in anti-tumoral activity [11].

2. Materials and Methods

The study was approved by the institutional review board of the Ärztekammer Sachsen-Anhalt (No. 69/18). EDTA peripheral blood samples were obtained from patients with advanced lung cancer of NSCLC histology. From June 2019 to June 2021, 90 patients with histologically confirmed unresectable locally advanced or metastatic lung cancer prior to PD-1 or PD-L1 blockade treatment in combination with chemotherapy were prospectively enrolled (convenience sample). Patients met the following criteria: age >18 years, histologically confirmed diagnosis of advanced lung cancer, 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. Furthermore, patients with a previous history of active autoimmune disease were excluded. All patients gave written informed consent for the study proposal and procedures. The cutoff date of the study was January 2022.

Patients with SqC received a combined immunochemotherapy with nab-paclitaxel, carboplatin, and pembrolizumab, according to KEYNOTE-407 trial [12]. The same regimen was also given to patients with thyroid transcription factor (TTF)-1-negative AC, as pemetrexed

might be less effective in these patients [13]. Since, according to the IMpower150 study, the combination has a clear advantage in the presence of liver metastases [14], patients with AC and liver metastases received a combined immune/chemotherapy with atezolizumab, bevacizumab, carboplatin, and nab-paclitaxel. All other patients with AC received a combined immunochemotherapy with pemetrexed, carboplatin, and pembrolizumab according to the data from the KEYNOTE-189 trial [15]. Furthermore, four patients received thorax radiation before or during the initiation of the systemic therapy due to a high tumor burden with existing or threatening superior vena cava syndrome. With ongoing maintenance therapy, some patients also received additional radiation therapy either due to particularly good tumor response and oligometastasis to improve the prognosis according to the study by Gomez et al. [16,17] (7 patients), or in terms of palliative radiation, for example, in the case of pain or symptomatic tumor progress (10 patients).

Patients' responses were determined according to the Response Evaluation Criteria in Solid Tumors (RECIST 1.1). Patients underwent CT scans at baseline and after 10 weeks. Subsequent assessments of disease extent by CT scan were scheduled every 12 weeks or earlier if clinically indicated. In the case of progressive disease, patients were allowed to continue the treatment if clinical improvement was maintained, and CT was repeated after 8 weeks to confirm progression. Besides RECIST-defined objective response, we assembled complete and partial clinical remission with stable disease to obtain the disease control benefit group, which was compared to the patients' group without durable clinical benefit. Primary endpoint was the progression-free survival (PFS) of patients. PFS was defined as the time elapsed from initiation of ICI/chemotherapy until the first observation of progressive disease or death from any cause. Overall survival (OS) was defined as the time from initiation of ICI/chemotherapy until death from any cause. Patients who did not die or progress and those lost to follow-up were censored.

Peripheral blood samples were collected within 7 days before initiation of ICI/chemotherapy (time point 1, baseline) and prior to the third cycle of ICI therapy (time point 2). In case of early treatment drop-out before the expected time point 2, a peripheral blood sample was drawn if possible before the first assessment of disease response. The leukocyte count and the complete blood count were determined using a CELL-Dyn Ruby (Abbott Lab., Wiesbaden, Germany). Circulating DC subpopulations were identified with the "Blood DC Enumeration Kit" (Miltenyi, Bergisch Gladbach, Germany) supplemented for gating reasons with CD45 APC-H7 and an HLA-DR V500 monoclonal antibody (mAb) (BD Biosciences, Heidelberg, Germany). Briefly, 300µL whole blood was incubated with a cocktail of mAbs including anti-CD1c PE as a marker for myeloid DC (cDC2), CD141/BDCA-3 APC (myeloid cDC1), and CD303/BDCA-2 FITC for plasmacytoid DC (pDC) [18]. The test kit contained an anti-CD14 mAb and CD19 PE-Cy5 to exclude monocytes and B cells from the analysis and a dead-cell discriminator. After antibody incubation, erythrocyte lysis, and two washing steps, blood cells were fixed according to the manufacturer's instructions. At least 1 million blood leukocytes were analyzed. The gating strategy is illustrated in Figure S1. HLA-DR expression on monocytes was quantified using a mAb labeled on a protein/fluorophore ratio of 1/1 (clone L243; QuantiBRITE™ reagent; BD Biosciences). A total of 50µL of blood was stained according to the manufacturer's instruction. A standard curve for antigen quantification was established using multi-level calibrated QuantiBRITE beads (BD Biosciences). The measured geometric mean fluorescence intensity (MFI) of the gated population was converted into "antibody molecules bound per cell" (ABC) using a Microsoft Excel™ spreadsheet (version 2016, Microsoft Corporation, Redmond, WA, U.S.). HLA-DR MFI values of $\leq$5000 ABC for the whole monocyte population have been designated as "immunoparalysis" in former studies, since the patients are at high risk of infectious diseases [19]. Taking an MFI of 5000 ABC as a borderline value for a low HLA-DR intensity, the number of HLA-DR^{low} monocytes was estimated as a percentage of monocytes, as described in [20]. A lysed whole blood technique with 8-color staining of blood cells was used for the immune cell labeling of lymphocytes and monocytes. A sample of 300 µL of EDTA-treated blood was

subjected to staining with mAbs specific to slan (M-DC8) FITC (Miltenyi Biotec); CD56 PE from Beckmann Coulter (Hamburg, Germany); CD16-PE-Cy7 from Biolegend (San Diego, CA, U.S.); and CD19 PerCP-Cy5.5 from InVitrogen (Thermo Fisher, Waltham, MA, U.S.); all other mAbs (CD14 APC, CD45 APC-H7, CD3 V450, HLA-DR V500) were from BD Biosciences. The blood–mAbs mixture was incubated for 15 min at room temperature in the dark before 4 mL of 1:10 FACS erythrocytes lysing solution (BD Biosciences) was added. After 10 min of incubation and two washing steps, cells were analyzed in the flow cytometer. Gating strategy for slan+ non-classical monocytes has been provided in Figure S2. Blood cell samples were measured on a FACS CANTO II Flow Cytometer (BD Biosciences, Heidelberg, Germany). Data analysis was performed using the BD FacsDIVA^{TM} software. Since standardized procedures are essential to allow for inter-individual comparisons in the context of studies persisting several months, Cytometer Setup and Tracking (CST) Beads (BD Biosciences) were used daily to set standardized geometric MFI ranges in the fluorescence channels used.

The statistical analyses were performed with the commercial software SPSS 28.0 (SPSS Inc., Munich, Germany). Medians with interquartile ranges (IQRs) are given for most data. Differences in the immune cell parameters between patient groups or between different time points were analyzed using non-parametric tests for unpaired or paired samples, as appropriate. Accordingly, the comparison between different patient groups was based on the Mann–Whitney U test or the Chi-Square test. Survival analysis comprised a descriptive presentation of the cumulative survival functions according to Kaplan-Meier, and differences among the curves were evaluated using the log-rank test. Univariable and multivariable analyses were performed using the Cox proportional hazards model. Correlations among quantitative variables were based on the non-parametric Spearman rank correlation coefficient. For the primary outcome, a p-value of less than 0.05 was considered statistically significant; p-values of secondary outcomes were interpreted exploratorily.

3. Results

3.1. Patient Characteristics and General Outcome

The general baseline characteristics of NSCLC patients of this study are summarized in Table 1. The median age was 65 years (range, 31–87 years); most patients were male (67%) and smokers (90%). There was no relevant difference in survival found between 18 patients with tumor recurrence and 72 patients with primary advanced state; therefore, they were analyzed together. All patients received at least two cycles of anti-PD-1/PD-L1 therapy. Patients treated with pembrolizumab underwent a mean of 9 cycles (range 2–31) and those patients treated with atezolizumab underwent a mean of 10 cycles (range 8–12). The median follow up was 13 $\pm$ 2.2 months. As shown in Table 1, most of the patients responded to therapy, but often only for a few months. The rate of confirmed objective response was 74.4% for all patients (75% for AC, 70.4% for SqC). Ten patients stopped treatment before the third antibody application, in most cases due to clinical worsening. Patients without a disease control had a median OS of 4 months (95% CI: 2.8–5.2). At the censoring date, 23 patients (25.6%) were still on treatment. The global median PFS was 14 $\pm$ 2.1 months (95% CI: 9.8–18.2) and the median OS 18 $\pm$ 1.6 months (95% CI: 14.9–21.1).

Table 1. Patient characteristics and therapy response of patients. Patients were grouped by cancer histotype to show different therapy strategies.

Parameters	AC	SqC	NSCLC Other Than AC and SqC
Number	56	27	7
Age, median (IQR)	64 (15)	67 (8)	59 (17)
Sex			
Male, *n* (%)	35 (62.5)	25 (92.6)	4
Female, *n* (%)	21 (37.5)	2 (7.4)	3
ECOG			
0	34 (61.4)	14 (51.85)	3
1	22 (38.6)	13 (48.15)	4
2	0	0	0
PD-L1 expression, *n* (%)			
<1%	25 (44.6)	12 (44.4)	3 (42.9)
1–49%	18 (32.1)	9 (33.3)	2 (28.6)
≥50	11 (19.6)	6 (22.2)	2 (28.6)
missing	2		
Smoker status			
- Never-smoker	11 (19.6)	1 (3.7)	0
- Smoker	45 (80.4)	26 (96.3)	7 (100)
Metastases			
<3, *n* (%)	26 (46.4)	17 (63)	1 (14.3)
≥3, *n* (%)	30 (53.6)	10 (37)	6 (85.7)
Brain and/or liver metastases	16 (28.6)	6 (22.2)	3 (42.9)
Therapy setting: Chemotherapy	Carboplatin + pemetrexed (TTF-1-pos.) or + nab-Paditaxel (TTF-1neg.)	Carboplatin + nab-Paclitaxel	Carboplatin + nab-Paclitaxel
Therapy setting: ICI + others	Pembrolizumab or (if liver metastasis) Atezolizumab + Bevacizumab	Pembrolizumab	Pembrolizumab
Radiatio before ICI, *n* (%)	6	3	1
Radiatio after ICI, *n* (%)	9	7	1
Patients with tumor recurrence, *n* (%)	10 (17.8)	7 (25.9)	1
Patients with primary advanced state, *n* (%)	46 (82.1)	20 (74.1)	6
Clinical response, *n* (%)			
- Progression/Discontinuation	14 (25)	8 (29.6)	1
- Disease stabilization	10 (17.9)	2 (7.4)	0
- Partial/complete remission	32 (57.1)	17 (63)	6 (85.7)

3.2. Blood Cells and Therapy Response

In order to determine blood biomarkers, which predict patients' clinical response, the composition of blood immune cells in the patient group "progressive disease/therapy discontinuation" and the group "clinical response to therapy" was investigated (Table 2). In comparison to baseline values, the differences observed between the groups were more pronounced at the third cycle of ICI therapy. At baseline, patients with a clinical response to therapy had lower neutrophil counts and higher numbers of MDC. At the third cycle of ICI therapy, the clinical response group had significantly lower neutrophil counts, a lower NLR, and lower frequencies of HLA-DR^{low} monocytes. Furthermore, higher numbers of slan+ non-classical monocytes and higher frequencies of MDC and PDC were associated with clinical response.

Table 2. Blood immune cell parameters at baseline and at third cycle of ICI/chemotherapy. The patients were grouped into progress/discontinuation and clinical response (stabilization of disease, or partial/complete remission). Median and interquartile range (IQR) are given.

Parameters	Baseline Values			Third Cycle Values		
	Progressive Disease/ Discontinuation	Clinical Response	*p*-Value	Progressive Disease/ Discontinuation	Clinical Response	*p*-Value
n	23	67		16	66	
Leukocyte counts (cells/µL)	11,000 (4600)	8910 (5550)		10,250 (7620)	7535 (4780)	0.004
Neutrophil counts (cells/µL)	8170 (5560)	6120 (4960)	0.016	7770 (7930)	4845 (3805)	0.002
Lymphocyte counts (cells/µL)	1890 (1250)	1630 (690)		1445 (1074)	1460 (1160)	
T cells (cells/µL)	1142 (695)	1086 (646)		1043 (826)	1109 (878)	
B cells (cells/µL)	209 (239)	109 (103)		160 (212)	75 (58)	
NK cells (cells/µL)	188 (396)	268 (276)		202 (340)	235 (196)	
NLR	4.54 (5.26)	3.88 (4.19)		6.77 (5.74)	3.46 (3.12)	0.006
Monocytes (cells/µL)	840 (340)	660 (280)		807 (352)	710 (423)	
CD16+ monocytes (% of monocytes)	9.3 (8.6)	13 (7.3)		10.2 (6.8)	14.6 (8.9)	
Slan+ monocytes (% leukocytes)	0.16 (0.32)	0.26 (0.54)		0.13 (0.21)	0.32 (0.52)	0.014
HLA-DR^{low} MDSC (% of monocytes)	7.9 (22.1)	6.9 (13.4)		11.4 (17.1)	6.65 (8.7)	0.026
CD1c^{+} MDC (% of leukocytes)	0.062 (0.074)	0.105 (0.091)	0.04	0.070 (0.063)	0.162 (0.143)	<0.001
CD141^{+} MDC (% of leukocytes)	0.004 (0.005)	0.007 (0.006)	0.022	0.004 (0.004)	0.008 (0.006)	0.001
PDC (% of leukocytes)	0.067 (0.068)	0.093 (0.092)		0.051 (0.047)	0.116 (0.134)	0.011
Sum of MDC/PDC (% of leukocytes)	0.142 (0.167)	0.198 (0.162)	0.043	0.149 (0.142)	0.313 (0.268)	0.001

Predictor variables with a significant difference between the patients' groups with and without a PFS of ≥12 months were analyzed with ROC curves to determine the overall strength of association (area under the ROC curve [AUC]) and the optimal cutoff point for the prediction of therapy response (maximizing the sum of sensitivity and specificity). The consideration of the single parameters NLR and HLA-DR^{low} monocytes, evaluated at baseline, resulted in unsatisfactory AUC values <0.7. The best AUC values of ROC curves were observed both for the baseline parameters slan+ non-classical monocytes (AUC 0.725; $p = 0.001$) and for the sum of MDC/PDC (AUC 0.734; $p = 0.001$) (Table S1). At the time point of cycle 3 of ICI therapy, better AUC values were observed; a lower NLR correlated with long term PFS (AUC 0.749) as did a lower amount of HLA-DR^{low} monocytes (AUC 0.676). Otherwise, higher frequencies of slan+ non-classical monocytes (AUC 0.804) and of the sum of MDC/PDC (AUC 0.817) correlated with long term PFS (Table S1). The cutoff values of the risk factors were >6.1 for the NLR, >22% for the HLA-DR^{low} monocytes, <0.25% of leukocytes for slan+ non-classical monocytes, and <0.14% of leukocytes for the sum of MDC/PDC. Of the 56 patients with the AC histotype, 25 patients had no risk factor (45%), 17 patients had one or two risk factors (30%) and 12 patients had three or four factors (21%). Out of the 27 SqC patients, 11 had no risk factor (41%), 8 patients had one or two factors (30%), and 7 patients had three or four (26 %). At cycle 3, 27 out of 50 AC patients had no risk factor (54%), as had 10 out of 25 SqC patients (40%).

3.3. Comparison of Baseline and Third-Cycle Blood Cell Markers

Investigating immune cell composition overtime, an increase in neutrophil counts could be observed in patients with progress/therapy discontinuation. Otherwise, a decreased neutrophil count was found in patients with a clinical response to therapy (Figure 1). In addition, HLA-DR^{low} monocytes increased with disease progress, whereas a decrease was detected in patients with a PFS ≥12 months. This decrease was associated with lower baseline values in the patients' group with the best clinical benefit. Both for slan+ non-classical monocytes and the sum of MDC/PDC, no obvious differences were found between patients with progression and short-term PFS. Only patients with a PFS ≥12 months showed an increase of these parameters, although starting from higher baseline values (Figure 1).

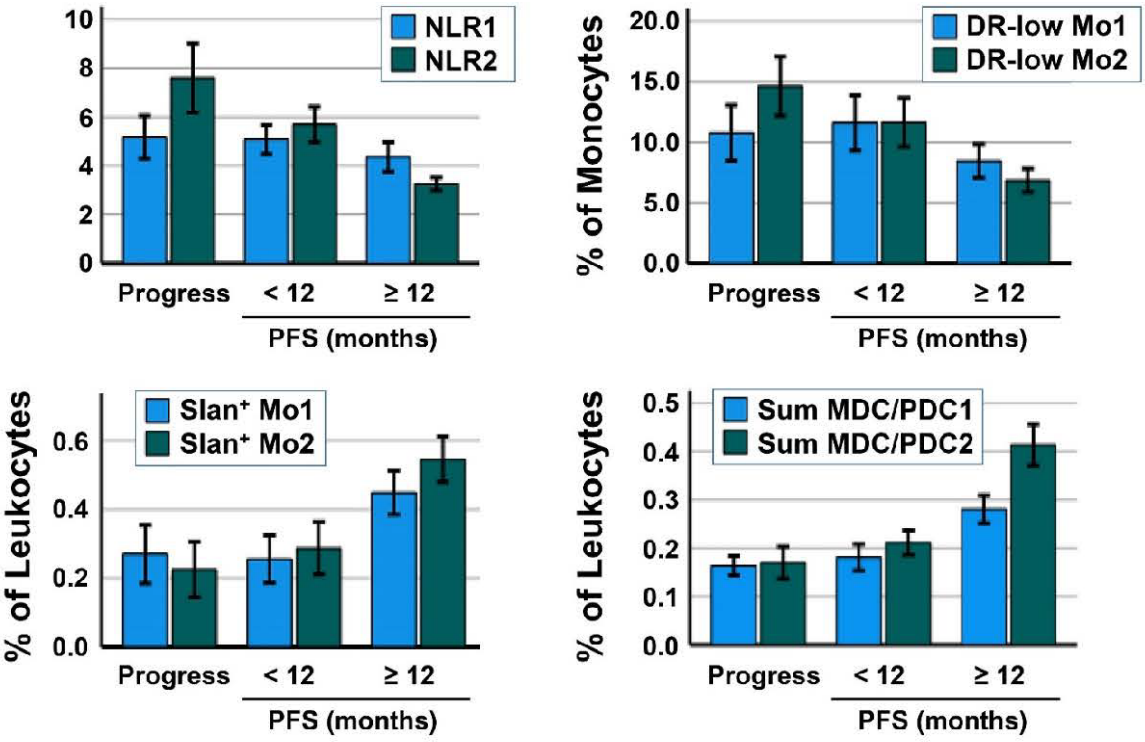

Figure 1. Correlation of blood immune cell markers in the patients' groups progression/therapy discontinuation, PFS <12 months und PFS ≥12 months overtime (baseline values as 1, ICI-third cycle values as 2). Mean values and error bars are displayed regarding the NLR, the amount of HLA-DR^{low} MDSC (% of monocytes), the percentage of slan+ non-classical monocytes (% of leukocytes), and the sum of MDC/PDC (% of leukocytes).

3.4. Survival Analyses

Kaplan–Meier analyses were performed in order to determine whether there were survival differences based on several risk factors, including sex and the immune cell repertoire. Lung cancer patients of the two main NSCLC histotypes AC and SqC had a comparable survival. Patients with ≥50% PD-L1 staining in tumor lesions had a longer PFS ($p = 0.034$, Figure 2) and a tendency towards a better OS ($p = 0.062$). Furthermore, female patients had a shorter PFS ($p = 0.029$) and a tendency towards a worse OS in our study ($p = 0.078$). No relevant difference was found for the smoker status, for patients' age (<75 and ≥75 years), or for the number of metastases. With respect to the baseline immune cell parameters, 61 patients with a NLR <6.1, 74 patients with a frequency of HLA-DR^{low} <22% of monocytes, 40 patients with ≥0.25% slan+ non-classical monocytes (as % of leukocytes), and 59 patients with a sum of MDC/PDC ≥0.14% of leukocytes showed better PFS compared to the respective reference group, as illustrated in Figure 2. The 21 patients

with three or four immune cell risk factors had a worse PFS than the 29 patients with one or two risk factors, which had a worse PFS than patients without any risk factor (Figure 2). No relevant survival differences were found for total lymphocyte counts or for the numbers of T and B cells. With respect to NK cells, 34 patients with <200 NK cells/µL blood had a worse PFS than patients with higher NK cell numbers (Figure 2). Results of univariable prognostic factor analysis (Kaplan–Meier and Cox regression) are provided in Table 3. In a multivariable Cox regression analysis of PFS considering the covariates sex and PD-L1 expression of tumor lesions, the baseline values of NLR, HLA-DR^{low} monocytes, slan+ non-classical monocytes, and the sum of MDC/PDC were independent prognostic factors.

Table 3. Relationship between baseline blood immune cell parameters with patients' survival (3A PFS; 3B OS). Data of univariate prognostic factor analysis is provided, with estimated mean of survival ± standard error, hazard ratios (HR) with 95% confidence interval (CI) and *p* values.

3A	Cutoff	*n*	Kaplan–Meier PFS			Cox Regression, PFS		
			% Cen-sored	PFS (months)	*p*-Value	HR	95% CI	*p*-Value
Neutrophil counts(cells/µL)	≤10,000	67	50.7	16.7 ± 1.6	0.013	1	1.11–3.48	0.019
	>10,000	23	17.4	8.7 ± 1.6		1.98		
NLR	<6.1	61	52.5	16.9 ± 1.6	0.005	1	1.21–3.64	0.009
	≥6.1	29	20.7	9.3 ± 1.7		2.10		
NK cells (cells/µL)	<200	34	29.4	11.4 ± 2.05	0.030	1	0.32–0.97	0.038
	≥200	56	50.0	16.1 ± 1.67		0.56		
HLA-DR^{low} MDSC (% of monocytes)	<22	74	47.3	16.0 ± 1.52	0.027	1	1.05–3.69	0.036
	≥22	16	18.8	8.4 ± 1.98		1.96		
CD16+ monocytes (% of monocytes)	<10	28	28.6	10.2 ± 2.1	0.024	1	0.30–0.95	0.031
	≥10	60	48.3	16.4 ± 1.67		0.54		
Slan+ monocytes (% of leukocytes)	<0.25	35	17.1	6.97 ± 0.87	<0.001	1	0.18–0.58	<0.001
	≥0.25	52	59.6	19.3 ± 1.78		0.32		
Sum of MDC/PDC (% of leukocytes)	<0.14	31	19.4	7.1 ± 0.88	<0.001	1		
	≥0.14	59	54.2	17.9 ± 1.70		0.38	0.22-0.68	<0.001
3B	Cutoff	*n*	Kaplan-Meier OS			Cox Regression, OS		
			% censored	OS (months)	*p*-Value	HR	95% CI	*p*-Value
Neutrophil counts(cells/µL)	≤10,000	67	50.7	17.9 ± 1.47	0.012	1	1.14–3.53	0.016
	>10,000	23	17.4	10.5 ± 1.49		2.00		
NLR	<6.1	61	52.5	17.9 ± 1.49	0.008	1	1.18–3.53	0.011
	≥6.1	29	20.7	11.3 ± 1.55		2.03		
NK cells (cells/µL)	<200	34	29.4	13.1 ± 1.87	0.044	1	0.34–1.01	0.053
	≥200	56	50.0	17.4 ± 1.5		0.58		
HLA-DR^{low} MDSC (% of monocytes)	<22	74	47.3	17.2 ± 1.39	0.033	1	1.03–3.61	0.041
	≥22	16	18.8	10.3 ± 1.96		1.93		
CD16+ monocytes (% of monocytes)	<10	28	28.6	11.3 ± 1.72	0.030	1	0.31–0.97	0.038
	≥10	60	48.3	17.6 ± 1.52		0.55		
Slan+ monocytes (% of leukocytes)	<0.25	35	17.1	11.8 ± 1.38	<0.001	1	0.19–0.66	<0.001
	≥0.25	52	59.6	20.6 ± 1.8		0.35		
Sum of MDC/PDC (% of leukocytes)	<0.14	31	19.4	8.95 ± 0.81	<0.001	1	0.20–0.64	<0.001
	≥0.14	59	54.2	18.9 ± 1.55		0.36		

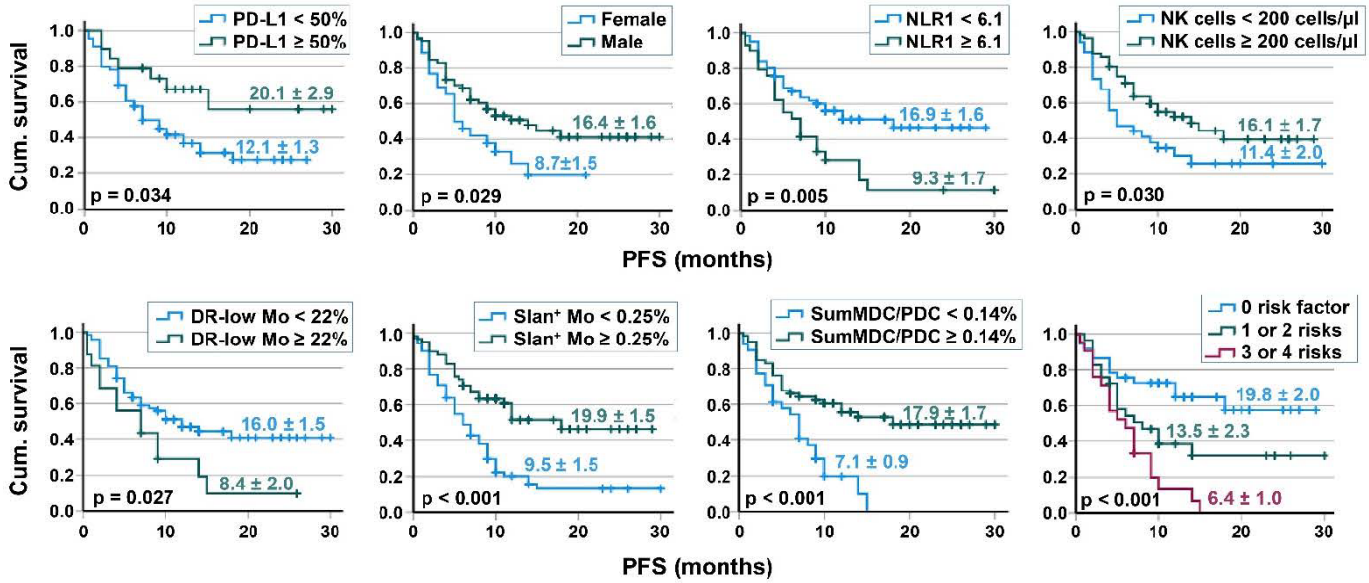

Figure 2. Relationship between risk factors/baseline immune cell parameters and patients' PFS. Kaplan–Meier curves are shown for PD-L1 expression of tumor lesions, the sex, NLR, NK cells (cells/µL blood), HLA-DR^{low} MDSC (% of monocytes), slan+CD16+ non-classical monocytes (% of leukocytes), the sum of MDC/PDC (% of leukocytes), and a 'Risk Score' of the 4 risks 'high NLR, high HLA-DR^{low} MDSC, low slan+CD16+ non-classical monocytes and low MDC/PDC sum', with mean survival time and *p* value of the log rank test.

3.5. Correlation of Immune Cell Subpopulations

The baseline neutrophil counts directly correlated with the monocyte counts, and were correlated even stronger with the percentages of HLA-DR^{low} MDSC (Table 4). Neutrophil numbers did not correlate with lymphocyte counts). The neutrophil count indirectly correlated with the percentage of CD16+ monocytes, especially with the percentage of slan+ non-classical monocytes and with the sum of MDC/PDC. HLA-DR^{low} MDSC indirectly correlated with CD16+ monocytes, including slan+ non-classical monocytes. Furthermore, an indirect correlation between the frequency of HLA-DR^{low} MDSC and the sum of MDC/PDC was detected (Table 4).

Table 4. Association of baseline blood immune cell parameters analyzed by Spearman's rank correlation.

Baseline Blood Immune Cells	Correlation Coefficient	*p*-Value
Neutrophil number with monocyte count	0.420	<0.001
Neutrophil number with the frequency of HLA-DR^{low} MDSC	0.598	<0.001
Neutrophil number with the frequency of CD16+ monocytes	−0.477	<0.001
Neutrophil number with the frequency of slan+CD16+ monocytes	−0.599	<0.001
Neutrophil number with the frequency of MDC/PDC	−0.662	<0.001
HLA-DR^{low} MDSC with the frequency of MDC/PDC	−0.600	<0.001
HLA-DR^{low} MDSC with the frequency of CD16+ monocytes	−0.548	<0.001
HLA-DR^{low} MDSC with the frequency of slan+CD16+ monocytes	−0.440	<0.001

3.6. Comparison of Female and Male Patients

To obtain insight into the observed sex-specific differences in PFS, clinical parameters and immune monitoring results were compared in female and male patients. The frequency of female never-smokers was 23.1%, higher than that in male patients (9.4%). The Sqc

histology was rare in female (7.7%) compared to male patients (39.1%). Interestingly, 65.4% of female patients had ≥3 metastases, whereas only 45.3% of male patients were in this risk group. Comparing PD-L1 expression of tumor tissues, only 3/24 female patients (12.5%) expressed ≥50% PD-L1 in the tumor lesions, whereas 16/64 male patients (25%) were in this group associated with a better PFS. No difference was found regarding the age of patients as well as for most of the blood parameters investigated, including the NLR and HLA-DR^{low} MDSC (Table S2). However, whereas female patients had higher baseline amounts of B cells, male patients had higher NK cell numbers, as illustrated in Figure 3. DC subpopulations showed a tendency towards higher values in male patients, significant only with respect to the amount of CD141+ MDC (Table S2).

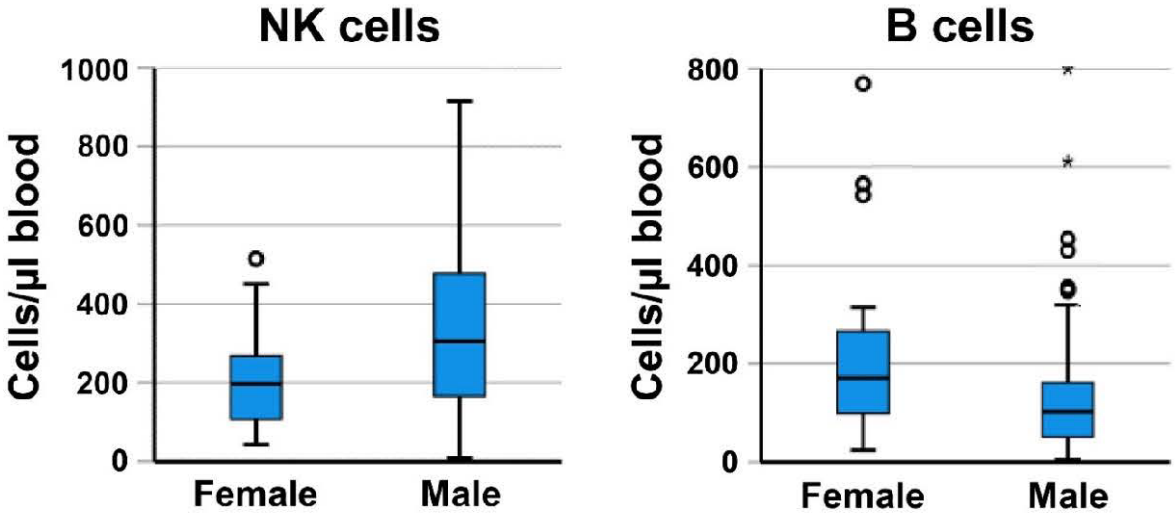

Figure 3. Comparison between baseline values of NK cells and B cells, respectively. Boxplots show a significant difference ($p < 0.05$) between female and male NSCLC patients, with lower NK cell counts and higher B cell counts in female patients. The whiskers indicate the largest/lowest points inside the range defined by 1st or 3rd quartile plus 1.5 times interquartile range (IQR). The circles represent outliers, the stars are extreme outliers (outside triple IQR).

4. Discussion

Lung cancer therapy has been revolutionized by the implementation of ICI therapy. Since not all patients with advanced or metastatic disease can benefit from ICI therapy, predictive biomarkers are an urgent issue [21]. The optimal predictive biomarker should be easily applicable in clinical settings, cost-effective, and provide an accurate prediction of a patient's clinical response. Currently, the only FDA-approved biomarkers for ICI therapy are PD-L1 expression of tumor tissue, tumor mutational burden, and DNA mismatch repair deficiency/microsatellite instability (for review, see [22]). Blood biomarkers have difficulties reflecting the tumor microenvironment but are easier to handle. Blood-based cellular immune biomarkers are promising in predicting responses to ICI therapy due to specimen accessibility, opportunity for serial monitoring, quantitative measurement, and the availability of the unique analytic platforms [23]. High absolute neutrophil counts as well as a high NLR can identify non-responders to immune checkpoint inhibition; a meta-analysis reported that a high NLR resulted in a worse PFS and OS in NSCLC, melanoma, and genitourinary cancer treated with ICI therapy [24]. First results have been reported also for monocytes; using CyTOF mass cytometry, Olingy et al. described a link of CD33-high classical monocytes to the effectiveness of ICI therapy in NSCLC patients [25]. Using multi-color flow cytometry of blood from NSCLC patients undergoing ICI monotherapy, a high NLR, a high frequency of HLA-DR^{low} monocytes, and low DC percentages were defined as adverse factors for clinical response and patients' survival [10]. In the current study with 90 NSCLC patients undergoing a combination of ICI and chemotherapy, we confirm the

immunomonitoring data of ICI monotherapy and show that the NLR, the frequency of HLA-DR^{low} monocytes, and the sum of MDC/PDC might be useful predictive biomarkers both for the clinical response to therapy and for patients' survival. We complemented the three biomarkers by slan+CD16+ non-classical monocytes, which show a behavior comparable to DC regarding patients' survival (PFS and OS), which was significantly improved with higher amounts of slan+ monocytes (cutoff 0.25% of leukocytes) and with a higher sum of MDC/PDC (cutoff 0.14% of leukocytes). Patients without any of the four risk factors (high NLR, high number of HLA-DR^{low} monocytes, low frequency of slan+ non-classical monocytes, and a low MDC/PDC sum) had the best outcome, while patients with three or four risk factors had the worst PFS in this study.

The therapeutic activity of ICI is the result of a complex interplay between multiple factors in the tumor microenvironment and the immune system. Mechanistically, ICI could either compete for the ligands of co-inhibitory receptors or control the surface expression of these receptors. The efficacy of ICI treatment depends on the tumor mutational burden [26], the intratumoral heterogeneity (which is associated with patterns of immune suppression) [27], and the tumor cell expression of immune checkpoint molecules (for review see [22]). Different mechanisms of immune suppression are known to prevent effective anti-tumor immunity, including increased secretion of immunosuppressive cytokines, enhanced differentiation of immune effector cells to a regulatory phenotype, and an influx of MDSC [28]. Currently, considerable efforts are performed to elucidate the mechanisms controlling the development of primary and acquired resistance to ICI therapy [29]. By deciphering the resistance mechanisms involved, strategies might be developed to overcome resistance and treatment failure. ICI combination with chemotherapy improved the therapy response in our patients' cohort compared to ICI monotherapy in the previous study; 74.4% of the 90 NSCLC patients showed a clinical response compared to 40% of the 35 NSCLC patients undergoing ICI monotherapy [10]. First-line ICI therapy combined with chemotherapy is among the current standard therapies for advanced NSCLC, compensating for the disadvantage of early treatment failure with ICI monotherapy [15]. Several studies have reported that a combination therapy of ICI plus other approaches, such as chemotherapy [30,31] or radiotherapy [32,33], can improve the prognosis of patients. Platinum agents, the backbone of chemotherapy for metastatic NSCLC, can increase antigen presentation by cancer cells, promote T cell trafficking into the tumor microenvironment, and diminish MDSC [34,35]. Chemotherapy has been shown to induce immunogenic cell death, enhance tumor antigenicity, disrupt immune suppressive pathways, and enhance effector T-cell response (for review, see [36]). Intriguingly, expression of immune checkpoint molecules such as PD1 and PD-L1 in the tumor lesions has been linked to patients' responses to chemotherapy in NSCLC [37].

Neutrophils, representing the most abundant myeloid cells in human blood, are emerging as important regulators of cancer. Neutrophils have been discussed to contain a subpopulation that facilitates tumorigenesis, promotes tumor growth and metastasis, stimulates angiogenesis, and mediates immunosuppression [38]. The NLR combining neutrophils and lymphocytes is an established marker for the prognosis of lung cancer patients in therapy [22,24,39]. Within this ratio, neutrophils seem to be more important than lymphocytes in our study, because for lymphocyte numbers, no significant differences could be detected in patients with and without a response to therapy. With respect to lymphocytic subpopulations, only NK cell numbers had an influence on patients' prognosis: A lower NK cell number (cutoff 200 cells/µL) correlated with shorter PFS in this study, an observation shared by other authors [40,41]. In our study, high neutrophil numbers positively correlated with monocyte counts and with the percentage of HLA-DR^{low} MDSC, as described earlier in NSCLC patients of different tumor stages [20]. HLA-DR^{low} monocytes are known to suppress the functions of lymphocytes in cancer patients [42,43], similar to the situation described in sepsis [44] and major trauma [45]. We investigated HLA-DR^{low} MDSC and slan+ non-classical monocytes as two monocytic subpopulations with contrasting properties. Both types of monocytes even showed an inverse correlation

in our study. Blood monocytes can be divided into classical (CD14highCD16−), intermediate (CD14highCD16+), and non-classical (CD14low/negCD16+) subpopulations. These subsets show transcriptomic differences that translate into specialization and different functions [46,47]. CD16+ monocytes can be further divided into slan-negative and slan+ subpopulations, the latter representing non-classical monocytes [48,49]. While being of monocytic origin, slan+ cells may either rapidly acquire DC functions or differentiate into macrophages [11]. Non-classical monocytes have been regarded as a pro-inflammatory population exhibiting tumor-killing properties [50]. In the context of malignant melanoma, CD16+ non-classical monocytes were shown to be crucial for ICI therapy, since they mediated the killing of regulatory T cells via a CTLA-4 (cytotoxic T lymphocyte-associated antigen 4)-specific mAb [51]. Slan+ monocytes can activate NK cells via IL-12, and the crosstalk between slan+ cells and NK cells improves differentiation of naïve CD4+ T lymphocytes into interferon (IFN)-gamma-producing Th1 cells [52]. In the current study, no obvious differences were found between patients with progression and short-term PFS, both for the baseline numbers of slan+ non-classical monocytes and the MDC/PDC sum. However, slan+ non-classical monocytes and the MDC/PDC sum better correlated with long-term survival (PFS $\geq$ 12months) than the NLR and could therefore be useful predictive markers. Interestingly, an inverse correlation could be observed between neutrophils and slan+ non-classical monocytes, and neutrophils and DC in this study: the higher the neutrophil counts, the lower were the amounts of both slan+ non-classical monocytes as well as the MDC/PDC sum, respectively. Some of the NSCLC patients had very low amounts of blood DC, which might contribute to their disturbed immune functions and poor prognosis. NSCLC patients have a significantly lower percentage of blood DCs than healthy donors [20,53]. The paucity of activated CD103+ DC in melanoma lesions has been discussed to limit ICI therapy efficacy [54]. Otherwise, a DC gene signature was strongly associated with improved patients' OS in NSCLC patients undergoing Atezolizumab therapy (PD-L1 blockade) [55]. DC counts and their expression of coinhibitory molecules, such as PD-L1, can affect therapy response and patients' survival; patients undergoing ICI monotherapy and exhibiting a higher PD-L1/CD274 expression of DC subtypes and monocytes, respectively, showed a significantly poorer survival [56]. Understanding and modulating DC counts and functional activity might help to improve the efficacy of T cell-centric immunotherapies in tumor patients [57].

In metastatic NSCLC, PD-L1 expression in tumor tissues is associated with a benefit from ICI therapy (KEYNOTE-024 trial) [58]. This observation could be confirmed in this study regarding the tumor lesions with high PD-L1 expression ($\geq$50%). Furthermore, we found female sex to be an independent risk factor for a poor response to ICI therapy, an observation shared by Conforti et al. in a meta-analysis with patients of different tumor histotypes [59]. Male and female cancer patients have been discussed to respond in a different way to immunotherapies, regardless of the tumor histological type, the type of treatment, or the setting of therapy [59]. In animal studies, PD-1/PD-L1 expression might even be modulated by sex hormones [60]. In female study participants, a higher rate of hyperprogression was observed by Kanjanapan and coworkers [61]. Olingy et al. described a lower frequency of CD33high monocytes in the blood of female NSCLC patients and discussed a link to a reduced responsiveness to ICI therapy [25]. Comparing female and male patients in our cohort, female patients had a lower PD-L1 tumor expression, were more often never-smokers, and had more metastases. With respect to blood immune cells, female patients had lower baseline NK cell numbers, as already described earlier for an age-matched control group and NSCLC patients of different tumor stages [20]. Intriguingly, lower NK cell numbers correlated with worse PFS in this study.

Despite notable clinical responses, basic and clinical studies are still required to investigate the exact mechanism of immune checkpoint inhibitor immunotherapy and to improve the appropriate selection of patients. Identifying low percentages of both slan+ non-classical monocytes and DC as well as a high NLR and high percentages of HLA-DR^{low} monocytes as risk factors for patients' response to a combined immune/chemotherapy,

this study extends our knowledge on biomarkers and pathophysiological causes of a therapy resistance.

5. Conclusions

Adverse factors, which highlight NSCLC patients with primary resistance to a combination therapy of ICI and chemotherapy, are low baseline frequency of slan+ non-classical monocytes, a low sum of MDC/PDC, a high NLR, or high amounts of HLA-DR^{low} MDSC. Patients without any of the four risk factors had the best outcome, while patients with three or four risk factors had the shortest PFS in this study. A longer PFS could also be found for patients with $\geq$50% PD-L1 expression in tumor lesions, for male patients, and for those with baseline NK cell numbers $\geq$200 cells/µL blood. Understanding tumor-induced systemic immune cell abnormalities will lead to an improved risk evaluation of cancer patients and provide the rational for novel therapeutic options.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/cancers14153690/s1, Figure S1: Gating strategy for DC subpopulations; Figure S2: Gating stra-tegy for slan+ non-classical monocytes; Table S1: Receiver operating characteristic curve analysis for the prediction of long-term survival (PFS $\geq$ 12 months) by several single immune cell parameters; Table S2: Comparison of blood immune cells in female and male NSCLC patients.

Author Contributions: Patient recruitment, M.M. and G.G.; conceptualization, D.R. and M.M.; project administration, W.S. and M.M.; funding acquisition, W.S.; methodology, S.T.; statistical help, A.W.; analysis, writing—original draft preparation, D.R.; writing—review and editing, B.S. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and approved by the Ethics Committee of the Ärztekammer Sachsen-Anhalt (69/18, 1 January 2019).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data well be available after publication on request.

Acknowledgments: We would like to thank Anja Müller and Katharina Biel for the performance of flow cytometry of clinical samples and Jens Wulfänger for help with illustrations.

Conflicts of Interest: The authors declare no conflict of interest.

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Monitoring Blood Immune Cells in Patients with Advanced Small Cell Lung Cancer Undergoing a Combined Immune Checkpoint Inhibitor/Chemotherapy

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Abstract: In this exploratory prospective observational study on 40 small cell lung cancer (SCLC) patients treated with a combination of chemotherapy and immune checkpoint inhibitors, blood immune cells were characterized by multi-color flow cytometry at the baseline and at the third therapy cycle. The numbers of neutrophils and of T-, B-, and NK cells, as well as the frequency of HLA-DR^{low} monocytes, 6-SulfoLacNAc (slan)+ non-classical monocytes and circulating dendritic cell (DC) subtypes were determined. The prognostic value of the parameters was evaluated by the patient's survival analysis with overall survival (OS) as the primary endpoint. In addition, blood cell parameters from SCLC patients were compared to those from non-SCLC (NSCLC). The global median OS of patients was 10.4 ± 1.1 months. Disease progression (15% of patients) correlated with a higher baseline neutrophil/lymphocyte ratio (NLR), more HLA-DR^{low} monocytes, and lower NK cell and DC numbers. The risk factors for poor OS were the presence of brain/liver metastases, a baseline NLR ≥ 6.1, HLA-DR^{low} monocytes ≥ 21% of monocytes, slan+ non-classical monocytes < 0.12%, and/or CD1c+ myeloid DC < 0.05% of leukocytes. Lymphocytic subpopulations did not correlate with OS. When comparing biomarkers in SCLC versus NSCLC, SCLC had a higher frequency of brain/liver metastases, a higher NLR, the lowest DC frequencies, and lower NK cell numbers. Brain/liver metastases had a substantial impact on the survival of SCLC patients. At the baseline, 45% of SCLC patients, but only 24% of NSCLC patients, had between three and five risk factors. A high basal NLR, a high frequency of HLA-DR^{low} monocytes, and low levels of slan+ non-classical monocytes were associated with poor survival in all lung cancer histotypes. Thus, the blood immune cell signature might contribute to a better prediction of SCLC patient outcomes and may uncover the pathophysiological peculiarities of this tumor entity.

Keywords: biomarker; dendritic cells; HLA-DR^{low} monocytes; immune monitoring; immune checkpoint inhibitor; neutrophil/lymphocyte ratio; overall survival; slan+ non-classical monocytes; small-cell lung cancer

Citation: Riemann, D.; Turzer, S.; Ganchev, G.; Schütte, W.; Seliger, B.; Möller, M. Monitoring Blood Immune Cells in Patients with Advanced Small Cell Lung Cancer Undergoing a Combined Immune Checkpoint Inhibitor/Chemotherapy. *Biomolecules* **2023**, *13*, 190. https://doi.org/10.3390/biom13020190

Academic Editors: Jelena Skuljec and Cornelia Deeg

Received: 6 December 2022
Revised: 11 January 2023
Accepted: 13 January 2023
Published: 17 January 2023

1. Introduction

Small-cell lung cancer (SCLC) is an aggressive neuroendocrine carcinoma that constitutes about 13–15% of all lung cancers [1]; two-thirds of cases occur in an advanced stage. Despite chemotherapy sensitivity, patients often rapidly progress, and overall survival (OS) is poor. In recent years, the addition of immune checkpoint inhibitor (ICI) therapy to front-line platinum-based chemotherapy has modestly improved the median survival of patients with extended-stage SCLC, and this combination is approved as a standard of care [2–5].

The identification of the baseline characteristics of patients, who will most benefit from treatment with chemo/immunotherapy, remains an important challenge. The biomarker-driven categorization of therapy responders and non-responders would minimize unnecessary exposure of patients to potentially permanent immune-related toxicities and reduce

the financial burden of health systems due to these expensive treatments [6]. In non-SCLC (NSCLC), the suggested biomarkers for ICI therapy are PD-L1 expression of tumor tissue, tumor mutational burden, and DNA mismatch repair deficiency/microsatellite instability (for review, see [7]). PD-L1 expression is less prevalent in SCLC than in NSCLC [8]. In randomized studies, PD-L1 has not been shown to be predictive of the response to ICI therapy [9,10]. In addition, the CheckMate 331 trial demonstrated that the tumor mutational burden did not predict clinical outcomes [10]. On the other hand, the OS of patients with baseline neutrophilia is poor both in NSCLC and in SCLC [11,12]. Despite limitations on reflecting the tumor microenvironment, blood-based cellular biomarkers are easier to handle than tumor tissues and have a great advantage because of specimen accessibility, quantitative measurement, the opportunity for serial monitoring, and the availability of unique analytic platforms [13].

Tumor defense can be regarded as a fine-tuned equilibrium between the destruction of cells recognized as "non-self" and the tolerance of healthy cells in the body [14]. Tolerance is maintained by multiple mechanisms, including regulatory immune cells, such as regulatory T cells or myeloid-derived suppressor cells (MDSC), immunosuppressive cytokines, and cellular ligand/receptor pairs, named immune checkpoints, which are known to down-modulate immune effector functions. By competing for the ligands or by controlling the surface expression of inhibitory immune checkpoint molecules, ICI therapy can shift the immune balance toward tumor destruction. Putative biomarkers for immunotherapy could be the number and/or the products of tolerance-inducing regulatory immune cells, which should be down-regulated with an ongoing anti-tumor immune response. On the other hand, the cells and cellular receptors involved in priming, trafficking, and target recognition of tumor-specific T cells might represent opportunities for biomarkers [14], including the number of antigen-presenting cells, such as dendritic cells (DC).

In the present study, blood immune cells were analyzed in SCLC patients undergoing combined chemo/immunotherapy. Since a high neutrophil/lymphocyte ratio (NLR), a high amount of HLA-DR^{low} MDSC, and low frequencies of 6-Sulfo LacNAc (slan)+ non-classical monocytes and DC have correlated with poor patient survival in a recent study with NSCLC patients undergoing ICI/chemotherapy [15]; we mainly focused on the analysis of these four immune cell markers. Furthermore, we compared blood immune cell parameters in SCLC and NSCLC patients to uncover the peculiarities of SCLC as a very aggressive variant of lung cancer.

2. Materials and Methods

2.1. Patient Characteristics and General Outcome

The study was approved by the institutional review board of the Aerztekammer Sachsen-Anhalt (69/18). EDTA peripheral blood samples were obtained from patients with advanced lung cancer of SCLC histology. From February 2020 to September 2021, 40 patients with histologically confirmed locally advanced or metastatic lung cancer prior to ICI treatment with an anti-PD-L1 antibody in combination with chemotherapy were prospectively enrolled. Patients met the following criteria: age > 18 years, histologically confirmed diagnosis of advanced lung cancer, adequate organ function, and the capacity to make an informed decision. Patients with a previous history of active autoimmune disease were excluded. All patients gave written informed consent for the study proposal and procedures. The cut-off date of the study was February 2022. Patients received combined chemo/immunotherapy with carboplatin, etoposid, and atezolizumab, according to the IMpower133 trial [16]. The primary endpoint of the study was the OS of patients. The minimum follow-up for the OS (from the inclusion of the last patient to the patient's last visit date) was 9 months. Patient's responses were determined according to the Response Evaluation Criteria in Solid Tumors (RECIST 1.1). Patients underwent CT scans at the baseline and after 10 weeks. Subsequent assessments of the disease extent by CT scan were scheduled every 12 weeks or earlier if clinically indicated. In the case of progressive disease, patients were allowed to continue the treatment if clinical improvement was maintained,

2.3. Statistical Analysis

The statistical analyses were performed with the commercial software SPSS 28.0 (SPSS Inc., Munich, Germany). The median with interquartile range (IQR) was given for most data. Differences in the immune cell parameters between patient groups or between different time points were analyzed using non-parametric tests for the unpaired or paired samples, as appropriate. Accordingly, the comparison between different patient groups was based on the Mann–Whitney U test or the Chi-Square test. Survival analysis comprised a descriptive presentation of the cumulative survival functions according to Kaplan–Meier, and differences among the curves were evaluated using the log-rank test. Univariable and multivariable analyses were performed using the Cox proportional hazards model. Correlations among quantitative variables were based on the non-parametric Spearman rank correlation coefficient. For the primary outcome, a p-value of less than 0.05 was considered statistically significant, and the p-values of secondary outcomes were interpreted as exploratory.

3. Results

3.1. Patient Characteristics and General Outcome

The general baseline characteristics of the 40 SCLC patients of this study and 84 NSCLC patients of the control group are summarized in Table 1. The median age of the SCLC patients was 65 years (range, 50–87 years); most patients were male (58%) and smokers (98%). Patients underwent a mean of eight cycles of atezolizumab therapy (range 1–26). As shown in Table 1, most of the patients responded to therapy, though often only for a few months. The rate of the confirmed objective response was 85%. With a median follow-up of 21 months [95% CI 13.8–28.2], the median PFS versus OS for all the patients was 6 months [95% CI, 4.4–7.6] versus 10 months [95% CI, 8.8–11.2], respectively. Six patients (15%) stopped treatment before the third antibody application, in most cases due to clinical worsening. Patients without disease control had a mean OS of 3.2 ± 2.0 months. At the censoring date, seven patients were still on treatment.

Table 1. Patient characteristics and clinical response to therapy.

	SCLC	NSCLC (AC)	NSCLC (SqC)
Number	40	57	27
Age, median (IQR)	65 (9)	64 (15)	67 (8)
Sex Male, n (%) Female, n (%)	 23 (57.5) 17 (42.5)	 36 (63.2) 21 (36.8)	 25 (92.6) 2 (7.4)
ECOG, n (%) 0 1 2	 10 (25) 26 (65) 4 (10)	 35 (61.4) 22 (38.6) 0	 14 (51.85) 13 (48.15) 0
Smoker status -Never-smoker -Smoker	 1 (2.5) 39 (97.5)	 11 (19.3) 46 (80.7)	 1 (3.7) 26 (96.3)
Metastases, n (%) <3 ≥3	 17 (42.5) 23 (57.5)	 26 (45.6) 31 (54.4)	 17 (63) 10 (37)
Brain and/or liver metastases n (%)	22 (55)	16 (28.6)	6 (22.2)
Therapy setting: Chemotherapy	Carboplatin + Etoposid	Carboplatin + pemetrexed (TTF-1+) or + nab-Paclitaxel (TTF-1neg.)	Carboplatin + nab-Paclitaxel

Table 1. *Cont.*

	SCLC	NSCLC (AC)	NSCLC (SqC)
Therapy setting: ICI + others	Atezolizumab	Pembrolizumab or (if liver metastasis) Atezolizumab + Bevacizumab	Pembrolizumab
Radiation before ICI, n (%)	4 (10)	6 (10.5)	3 (11.1)
Radiation after ICI, n (%)	15 (37.5)	9 (15.8)	7 (25.9)
Clinical response, n (%)			
-Progression/Discontinuation	6 (15)	14 (25)	8 (29.6)
-Disease stabilization	3 (7.5)	10 (18)	2 (7.4)
-Partial/complete response	31 (77.5)	32 (59)	17 (63)

AC—adenocarcinoma; SqC—squamous cell carcinoma; ICI—immune checkpoint inhibitor; IQR—interquartile range; TTF—thyroid transcription factor.

3.2. Blood Cells and Therapy Response

In order to determine blood biomarkers, which predict the patient's response to therapy, baseline blood immune cells were investigated in the patient group "progressive disease/therapy discontinuation" and the group "clinical response to therapy" (Table 2). Patients without a clinical response to therapy had a higher NLR, higher amounts of HLA-DR^{low} MDSC, a lower frequency of DC, both of MDC and PDC, as well as a lower NK cell count. No differences were found for slan+ non-classical monocytes. Predictor variables that had a significant difference between the patients' groups with or without progress were analyzed with ROC (receiver operating characteristics) curves to determine the overall strength of association (area under the ROC curve [AUC]) and the optimal cut-off point for the prediction of therapy resistance (maximizing the sum of sensitivity and specificity). The AUC values of the ROC curves for the NLR, HLA-DR^{low} monocytes, and DC subtypes were between 0.79 and 0.89 (Table 3). The cut-off values of the risk factors were >6.1 for NLR, >21% of monocytes for HLA-DR^{low} MDSC, and < 0.05% of the leukocytes for the CD1c+ MDC. With 750 monocytes /µL blood in the mean, 21% HLA-DR^{low} monocytes correspond to 158 cells/µL blood. With 9200 leukocytes/µL blood, 0.05% DC correspond to 5 cells/µL blood. Baseline counts of leukocytes, eosinophils, erythrocytes, and platelets had no association with the patient's response to immune/chemotherapy.

Table 2. Baseline blood immune cell parameters. Patients with SCLC were grouped into progress/therapy discontinuation and clinical response (stabilization of disease, or partial/complete remission). Median and IQR are given as well as significant differences of Mann-Whitney U-test.

Parameters	Progressive Disease/ Therapy Discontinuation	Clinical Response	*p* Value
n	6	34	
Neutrophil counts (cells/µL)	12,950 (9530)	7540 (3560)	
Lymphocyte counts (cells/µL)	983 (1168)	1625 (1073)	
NLR	9.3 (6.5)	5.0 (6.5)	0.024
CD3+ T cells	672 (854)	1075 (1108)	
CD19+ B cells	194 (176)	178 (181)	
NK cells	67.5 (95)	237.5 (237)	0.010
Monocytes (cells/µL)	748 (478)	840 (340)	
HLA-DR^{low} MDSC (% of monocytes)	30.5 (16.1)	7.9 (22.1)	0.008
Slan+ non-classical monocytes (% of leukocytes)	0.17 (0.30)	0.16 (0.32)	
CD1c+ MDC (% of leukocytes)	0.013 (0.033)	0.062 (0.074)	0.019
CD141+ MDC (% of leukocytes)	0.001 (0.001)	0.004 (0.005)	0.001
CD303+ PDC (% of leukocytes)	0.0095 (0.019)	0.067 (0.068)	0.021

Table 3. Receiver operating characteristic curve analysis for the prediction of therapy failure/progress by baseline immune cell parameters.

Prediction Variable at Baseline	Cutoff Point	AUC	95% CI	*p* Value
NLR	6.1	0.789	0.645–0.934	0.025
NK cells (cells/µL)	150	0.824	0.671–0.976	0.012
HLA-DR^{low} MDSC (% of monocytes)	21	0.831	0.649–1.000	0.011
Slan+ non-classical monocytes (% of leukocytes)	0.12	0.576		
CD1c+ MDC (% of leukocytes)	0.05	0.799	0.644–0.954	0.021
CD141+ MDC (% of leukocytes)	0.0015	0.887	0.782–0.992	0.003
CD303+ PDC (% of leukocytes)	0.014	0.792	0.628–0.956	0.024

AUC—area under the ROC curve; CI—confidence interval.

3.3. Comparison of Baseline and Third-Cycle Blood Cell Markers

Most of the patients with disease progression did not obtain a second blood sampling. Therefore, only the 33 patients with clinical response and with a second blood collection were chosen for the comparison of baseline and third-cycle parameters (Supplementary Table S1). An increase in neutrophils from 7600 (IQR 3500) to 9090 (6825) cells/µL ($p = 0.043$) and of lymphocytes from 1690 (1110) to 2070 (1255) cells/µL blood ($p = 0.051$) resulted in constant NLR values. The number of monocytes increased ($p = 0.032$). Other significant differences observed between the basal and third-cycle values were an increase in CD3 + T cells from 1098 (1156) to 1560 (985) cells/µL ($p = 0.019$) and an elevation of CD1c+ MDC from 0.070 (0.068) to 0.109 (0.155) percent of leukocytes ($p = 0.004$)

3.4. Survival Analyses

The global median OS of patients was 10.4 ± 1.1 months (95% confidence interval (CI): 8.85–11.15), with ten patients (25%) demonstrating an OS of at least 12 months. Kaplan–Meier analyses were performed to analyze survival differences based on several risk factors, including the presence of brain/liver metastases and the baseline immune cell repertoire. Compared to the respective reference group, a better OS was found for 17 patients without any brain/liver metastases, 19 patients with an NLR < 6.1, 30 patients with a frequency of HLA-DR^{low} MDSC < 21% of monocytes, 27 patients with ≥ 0.12% slan+ non-classical monocytes (as % of leukocytes), and 21 patients with CD1c+ MDC ≥ 0.05% of leukocytes. The hazard ratio for OS was 3.04 (1.45–6.99) for the NLR, 2.48 (1.15–5.33) for HLA-DR^{low} MDSC, 2.51 (1.20–5.24) for slan+ non-classical monocytes and 2.08 (1.03–4.2) for MDC (Table 4). Patients with zero–two risk factors had a significantly better OS compared to patients with three–five risk factors. No relevant survival differences were found for basal PDC frequencies, as well as for the numbers of T-, B-, and NK cells. The results of univariable prognostic factor analysis (Kaplan–Meier and Cox regression) are provided in Table 4. Kaplan–Meier pictures are shown in Figure 1.

In a multivariable Cox regression analysis of OS, considering the covariate status of brain/liver metastases, only the NLR baseline values were an independent prognostic factor ($p = 0.042$). Comparing blood parameters in the 17 patients without versus the 23 patients with brain/liver metastases, no significant differences were detected for the number of neutrophils, lymphocytes, slan+ non-classical monocytes, and the DC subtypes. The metastasis group had a higher frequency of HLA-DR^{low} MDSC (12.2% versus 5.3%, $p = 0.034$) and a tendency to both a higher NLR (6.9 versus 4.9, $p = 0.066$) and lower MDC (0.03% versus 0.07% of leukocytes), as illustrated in Figure 2. Data are provided in Supplementary Table S2.

Table 4. Relationship between baseline blood immune cell parameters with patient's survival for 40 SCLC patients (A PFS; B OS). Data of univariate prognostic factor analysis are provided, with estimated mean of survival ± standard error, hazard ratios (HR) with 95% confidence interval (CI), and *p*-values.

A	Cut-Off	n	Kaplan–Meier PFS			Cox Regression, PFS		
			% Censored	PFS (Months)	*p* Value	HR	95% CI	*p* Value
Neutrophil counts (cells/μL)	≤10,000	30	23.3	10.2 ± 1.4	0.002	3.14	1.44–6.88	0.004
	>10,000	10	0	4.0 ± 1.1				
NLR	<6.1	19	31.6	12.3 ± 1.8	0.001	3.04	1.46–6.54	0.003
	≥6.1	21	4.8	5.1 ± 0.77				
HLA-DR^{low} MDSC (% of monocytes)	<21	30	23.3	10.0 ±1.4	0.012	2.44	1.14–5.19	0.021
	≥21	10	0	4.4 ± 1.3				
Slan+ monocytes (% of leukocytes)	<0.12	13	0	5.6 ± 0.93	0.038	2.03	0.99–4.16	0.053
	≥0.12	27	25.9	10.2 ± 1.6				
CD1c+ MDC (% of leukocytes)	<0.05	19	9.5	5.4 ± 0.8	0.017	2.24	1.09–4.57	0.028
	≥0.05	21	26.3	11.4 ± 1.7				
Baseline risk score	0–2 risk factors	22	31.8	12.1 ± 1.7	<0.001	3.43	1.67–7.06	<0.001
	3–5 risk factors	18	0	4.6 ± 0.8				
B	**Cut-off**	**n**	**Kaplan–Meier OS**			**Cox Regression, OS**		
			% Censored	OS (Months)	*p* Value	HR	95% CI	*p* Value
Neutrophil counts (cells/μL)	≤10,000	30	23.3	12.2 ± 1.2	<0.001	3.95	1.75–8.89	<0.001
	>10,000	10	0	5.2 ± 1.3				
NLR	<6.1	19	31.6	14.0 ± 1.7	0.001	3.18	1.45–6.99	0.004
	≥6.1	21	4.8	7.1 ± 1.0				
HLA-DR^{low} MDSC (% of monocytes)	<21	30	23.3	11.9 ± 1.3	0.010	2.48	1.15–5.33	0.020
	≥21	10	0.0	6.0 ± 1.5				
Slan+ monocytes (% of leukocytes)	<0.12	13	0	6.7 ± 1.1	0.007	2.51	1.20–5.24	0.014
	≥0.12	27	25.9	12.3 ± 1.4				
CD1c+ MDC (% of leukocytes)	<0.05	19	9.5	7.6 ± 1.2	0.027	2.08	1.03–4.20	0.041
	≥0.05	21	26.3	13.2 ± 1.6				
Baseline risk score	0–2 risk factors	22	31.8	13.8 ± 1.5	<0.001	3.23	1.54–6.76	0.002
	3–5 risk factors	18	0	6.5 ± 1.04				

To obtain a better insight into whether the baseline immune cell parameters—irrespective of primary therapy resistance—correlate with survival, we repeated survival analyses in the 34 patients who responded to therapy (Supplementary Table S3). Again, the patients with brain/liver metastases or a baseline NLR ≥ 6.1 had a significantly worse OS with <0.12% slan+ non-classical monocytes. No significant differences were found for HLA-DR^{low} MDSC and the different DC subtypes. Our results suggest that the basal abundance of HLA-DR^{low} MDSC might be involved in primary therapy resistance to chemo/immunotherapy in SCLC patients. By contrast, slan+ non-classical monocytes could represent a factor that is important for long-lasting therapy response and survival. Additional factors, such as the presence of brain/liver metastases, also had an impact. The 11 SCLC patients without any brain/liver metastases and with an NLR < 6.1 had a mean OS of 16.9 (13.4–20.3) months. Already one of both risk factors resulted in a significantly shorter OS (8.2 months) ($p < 0.001$).

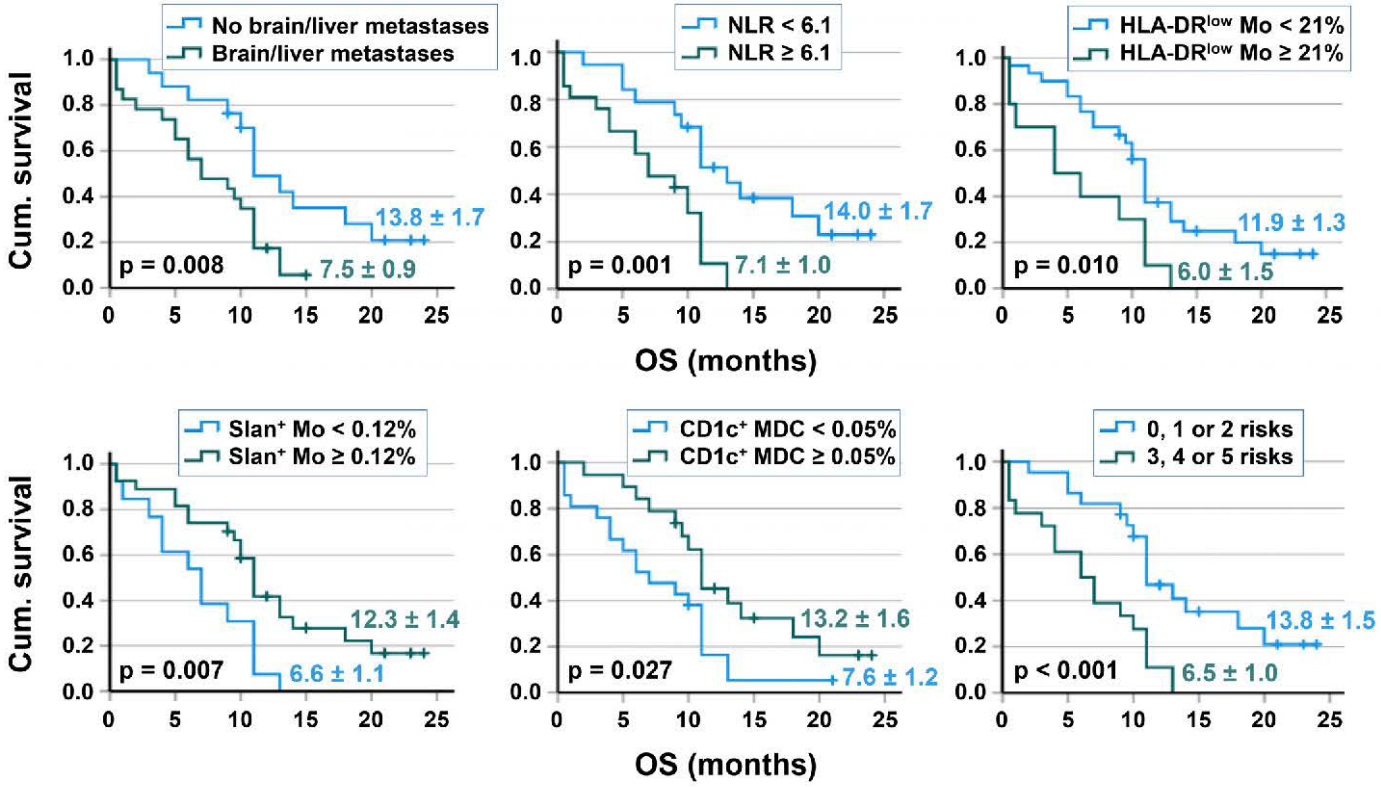

Figure 1. Relationship between risk factors/baseline immune cell parameters and patient's OS. Kaplan–Meier curves are shown for the presence of brain/liver metastases, the NLR, HLA-DR^{low} MDSC (% of monocytes), slan+ non-classical monocytes (% of leukocytes), CD1c+ MDC (% of leukocytes), and a "Risk Score" of the five risks "presence of brain/liver metastases, high NLR, high amount of HLA-DR^{low} MDSC, low frequency of slan+ non-classical monocytes, and low CD1c+ MDC". Mean survival time and *p*-value of the log-rank test are given.

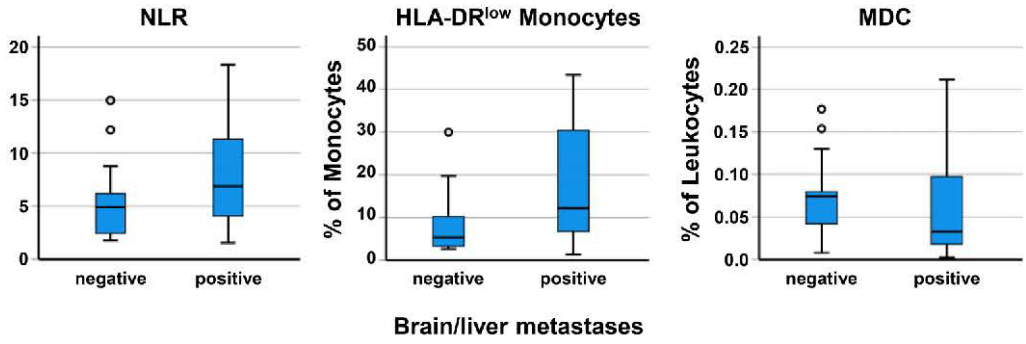

Figure 2. Comparison of baseline immune parameters in SCLC patients with or without brain/liver metastases. The observed differences are significant in the case of HLA-DR^{low} monocytes ($p = 0.034$). The whiskers of box plots indicate the largest/lowest points inside the range defined by first or third quartile plus 1.5 times interquartile range (IQR). The circles represent outliers.

To investigate whether immune cell parameters at the third cycle of therapy better correlated with the patient's OS, we repeated survival analyses with the blood parameters of the third cycle (n = 35). As shown in Supplementary Table S4, neutrophil counts and the NLR lost their impact on OS, whereas DC subtypes (PDC and MDC) became more strongly correlated with OS.

3.5. Correlation of Immune Cell Subpopulations

The baseline neutrophil counts directly correlated with the monocyte counts. Neutrophil numbers correlated even more strongly with the percentages of HLA-DR^{low} MDSC (Table 5). Neutrophil numbers did not correlate with lymphocyte counts. The neutrophil counts indirectly correlated with the percentages of slan+ non-classical monocytes and with DC, whereby the correlation with CD1c+ MDC was stronger than the correlation with PDC. Correlations between the immune cell subpopulations were also found during the third therapy cycle and were often even stronger (Table 5).

Table 5. Association of blood immune cell parameters analyzed by Spearman's rank correlation.

Baseline Blood Immune Cells	Correlation Coefficient	*p* Value
Neutrophil number with monocyte count	0.560	<0.001
Neutrophil number with percentage of HLA-DR^{low} MDSC	0.571	<0.001
Neutrophil number with frequency of slan+ non-classical monocytes	−0.629	<0.001
Neutrophil number with frequency of CD1c+ MDC	−0.610	<0.001
Neutrophil number with frequency of CD303+ PDC	−0.463	0.003
HLA-DR^{low} MDSC with frequency of slan+ non-classical monocytes	−0.527	<0.001
HLA-DR^{low} MDSC with frequency of CD1c+ MDC	−0.655	<0.001
HLA-DR^{low} MDSC with frequency of CD303+ PDC	−0.629	<0.001
slan+ non-classical monocytes with frequency of CD1c+ MDC	0.506	<0.001
Blood immune cells at third cycle of therapy		
Neutrophil number with percentage of HLA-DR^{low} MDSC	0.553	<0.001
Neutrophil number with frequency of slan+ non-classical monocytes	−0.691	<0.001
Neutrophil number with frequency of CD1c+ MDC	−0.727	<0.001
HLA-DR^{low} MDSC with frequency of slan+ non-classical monocytes	−0.767	<0.001
HLA-DR^{low} MDSC with frequency of CD1c+ MDC	−0.663	<0.001
slan+ non-classical monocytes with frequency of CD1c+ MDC	0.721	<0.001

3.6. Comparison of Immune Cell Parameters in Patients with SCLC and NSCLC

Despite the patient's ages being comparable in SCLC and NSCLC (Table 1), SCLC patients had a significantly higher frequency of brain/liver metastases (55% in SCLC, 26% in NSCLC). Comparing the baseline blood immune cell parameters, a significantly lower amount of MDC and PDC was detected in SCLC (Supplementary Table S5). Furthermore, SCLC patients tended to have a higher NLR. No significant differences could be found for HLA-DR^{low} MDSC, slan+ non-classical monocytes, and T-, B-, and NK cells. The number of risk factors differed significantly between the patients of the two histotypes. Figure 3 illustrates that 46% of NSCLC patients, compared to 22.5% of SCLC had no basal risk factor with respect to the five risk factors "brain/liver metastasis, high NLR, high amount of HLA-DR^{low} MDSC, low frequency of slan+ non-classical monocytes and of CD1c+ MDC (cut-off values of SCLC). Otherwise, 45% of SCLC patients, compared to only 24% of NSCLC patients, had three–five risk factors. Both in SCLC and NSCLC, the neutrophil numbers were directly correlated with monocyte counts, especially with the frequency of HLA-DR^{low} MDSC. Furthermore, neutrophils and the NLR were indirectly correlated with slan+ non-classical monocytes and with DC subpopulations in both histotypes.

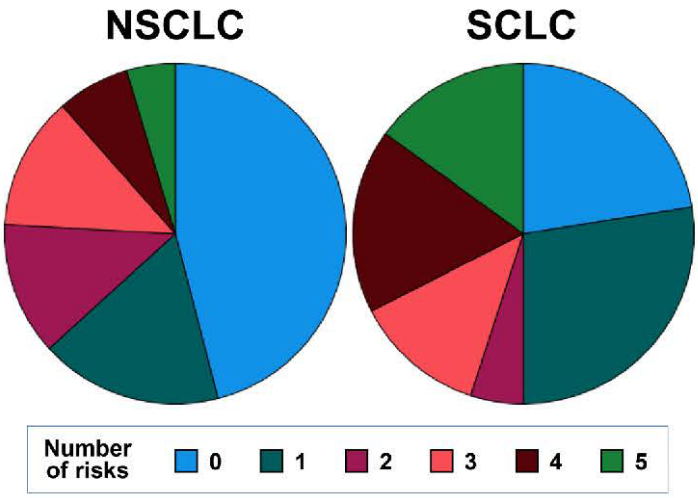

Figure 3. Comparison of the basal number of risk factors (presence of brain/liver metastases, high NLR, high amount of HLA-DR^{low} MDSC, low frequency of slan+ non-classical monocytes, and low CD1c+ MDC) between NSCLC and SCLC.

With respect to therapy response, higher neutrophil counts were associated with a lack of therapy response and progressive disease in the case of NSCLC patients [15]. The baseline neutrophil counts of SCLC patients did not differ significantly between therapy responders and non-responders, but the NLR of SCLC patients was significantly higher in the non-responder group (Table 2). HLA-DR^{low} MDSC did not differ between therapy responders and non-responders in the case of NSCLC patients [15] but were significantly higher in SCLC patients with progress. Those patients also had the lowest NK cell numbers. A significantly lower MDC/PDC sum in patients with progress could be observed both for patients with SCLC and NSCLC.

To compare the survival data of SCLC and NSCLC patients, the risk factors of SCLC patients were applied to the OS of NSCLC patients. Brain/liver metastasis had a weak effect on the OS of NSCLC patients, and Kaplan–Meier curves showed separate lines only after 14 months, as illustrated in Supplementary Figure S1. Both in SCLC and NSCLC patients, the NLR and the frequency of slan+ non-classical monocytes, and CD1c+ MDC significantly correlated with OS. Patients with <21% HLA-DR^{low} MDSC showed a tendency for better OS ($p = 0.055$) in NSCLC. A total of 61 NSCLC patients with zero–two risks (of the five risks: brain/liver metastases, high NLR, high level of HLA-DR^{low} MDSC, low amounts of slan+ non-classical monocytes and low CD1c+ MDC) had a significantly better OS than 19 patients with three–five risks (Supplementary Figure S1).

4. Discussion

SCLC is a lung cancer subtype with a particularly poor prognosis because of a strong predilection for early metastasis and therapeutic resistance. SCLC has one of the highest rates of mutational burden, suggesting that this cancer type is particularly susceptible to immune-based therapeutic approaches [19]. Prior findings that have associated a higher number of tumor-infiltrating immune cells with improved SCLC outcomes support this view [20,21]. Platinum-based chemotherapy with or without ICI is currently a first-line therapy for SCLC patients, despite the heterogeneous outcome [2–5,9]. The application of ICI therapy in SCLC appears to be less effective when compared to NSCLC, and only a minority of SCLC patients benefit [22]. Efforts to obtain a more comprehensive knowledge of how different cell types interact with each other during ICI therapy and ultimately affect clinical response is still an ongoing task. The greatest obstacles to the optimal success of immunotherapy remain a large percentage of partial responders (primary resistance) and the high rate of resistance acquisition. The mechanisms of immunotherapy resistance remain poorly understood. Both tumor cell-intrinsic (lack of tumor antigens, disturbed antigen

presentation, genetic T cell exclusion) and tumor cell-extrinsic (absence of T cells, inhibitory immune checkpoints, immunosuppressive cells) factors contribute to immunotherapy resistance (for review, see [23]). A combination of agents with different mechanisms of action is one major strategy to overcome resistance mechanisms and to maximize the benefits of immunotherapy (for review, see [24]).

On the other hand, biomarkers have to be developed to select potential responders or to exclude potential non-responders. In NSCLC, several papers exploring predictive biomarkers for a response to ICI have been published [7,14,25–28]. NSCLC patients responding to ICI/chemotherapy have already at the baseline a favorable immune profile with a low baseline NLR, a low number of HLA-DR^{low} MDSC, and higher levels of slan+ non-classical monocytes and DC, correlating with longer survival [15]. A similar picture could be observed in the SCLC patients of the current study, though with some peculiarities. A high NLR was strongly associated both with primary resistance to therapy and with poor OS. The presence of brain/liver metastases correlated with poor OS in SCLC but scarcely in NSCLC patients. All SCLC patients without a therapy response had brain/liver metastases associated with a higher frequency of HLA-DR^{low} MDSC. HLA-DR^{low} MDSC seemed to be associated more with primary than with a late-acquired therapy resistance since the impact of these blood parameters on the OS of a subgroup of patients responding to therapy was limited. A lower NLR was associated with a higher frequency of slan+ non-classical monocytes and correlated with better OS. In patients responding to therapy, the combined chemo/immunotherapy resulted in an increase in CD3+ T cells at the time point of the third therapy cycle. Nevertheless, no correlation was found between the number of lymphocytic subpopulations and survival. Compared to NSCLC, SCLC patients had a more suppressed state of blood DC (both of MDC and PDC) and more risk factors at the baseline, such as the presence of brain/liver metastases, high NLR, a high amount of HLA-DR^{low} MDSC, low amounts of slan+ non-classical monocytes, and low amounts of CD1c+ MDC. While the frequency of basal PDC correlated with survival in NSCLC, only the third cycle and not the baseline frequencies of PDC did this in SCLC.

The OS of SCLC patients with baseline neutrophilia has been shown to be poor [11,29]. During chronic inflammatory processes, such as malignancy, there is a persistent signal to recruit neutrophils and monocytes from the bone marrow. Tumor cells produce the granulocyte colony-stimulating factor (G-CSF), which skews the neutrophil retention/release balance in the bone marrow, leading to increased neutrophils in the blood [30]. Suppressive neutrophils, which are the granulocytic arm of MDSC, promote tumor progression by contributing to genetic instability, tumor cell proliferation, and angiogenesis (for review, see [31]). Furthermore, neutrophils can dampen anti-tumor immunity by suppressing T-cell proliferation, cytokine secretion, and the cytotoxic activity of activated T cells and natural killer cells [32]. Targeting MDSC via all-trans-retinoic acid can improve the induction of immune responses by a cancer vaccine in SCLC [33]. Neutrophils might represent an escape mechanism that is linked to the resistance against ICI therapy and to poor patient outcomes (for review, see [34]). Blood neutrophils and the derived NLR are established biomarkers of therapy response. A pretreatment NLR < 5 was associated with longer OS in patients who had several advanced cancers undergoing therapy with different ICI variants [35]. Our result of a correlation of a high NLR with a poor prognosis in SCLC patients corroborates the data of other investigators (for meta-analysis, see [36]). SCLC patients even had higher NLR values than those observed for NSCLC. Rice and Belani compared blood-based biomarkers and indicators for systemic inflammation in NSCLC and SCLC patients, but these authors described higher levels of systemic inflammation in NSCLC, including a higher NLR [37]. The cause of this discrepancy with our data remains unclear.

In the current study, neutrophil numbers positively correlated with monocyte counts and with the percentage of HLA-DR^{low} MDSC, as earlier described for NSCLC patients of different tumor stages [38]. Circulating monocytes are the precursors of essential myeloid cells, such as tumor-associated macrophages, MDSC, and DC. HLA-DR^{low} monocytes are known to suppress the functions of lymphocytes in cancer patients [39,40], similar to the

situation described in sepsis [41] and poly-trauma [42]. HLA-DR is one of three MHC class II glycoproteins expressed on antigen-presenting cells whose function is to present tumor peptide antigens to the T-cell receptors on CD4+ T cells resulting in cellular activation. As such, HLA-DR^{low} monocytes have a diminished capacity to present antigens to T cells. Furthermore, MDSC suppress immune cells by expressing PD-L1 [43], by secreting IL-10 and transforming growth factor (TGF)β [44,45], or by showing a deficient generation of mature DC [46]. Monocytic MDSC is known to impede the treatment response to ICI and, compared to polymorphonuclear MDSC, might even have a stronger prognostic value in NSCLC patients [47]. In the current study, primary therapy resistance was associated with the highest frequencies of HLA-DR^{low} MDSC, and especially patients with brain/liver metastases expressed huge amounts of this MDSC type. While an age-matched control group has 2.6 ± 2.5% of HLA-DR^{low} monocytes [38], tenfold higher amounts could be found in patients with progressive SCLC. The high levels of HLA-DR^{low} MDSC might suggest that those cancer patients have reached a point of immunoparalysis prior to treatment and thus may not be responsive to immunotherapeutic approaches. Interestingly, platinum agents as the backbone of chemotherapy for metastatic lung cancer can not only increase antigen presentation by cancer cells and promote T cell trafficking into the tumor microenvironment but could also diminish HLA-DR^{low} MDSC [48,49]. Comparing baseline and third cycle values of HLA-DR^{low} monocytes, we could not observe a decline in these MDSC in SCLC patients undergoing therapy, though the further time course has not been considered. The detailed mechanisms of monocytic reprogramming by cancer therapy still have to be elucidated [50].

In the current study, HLA-DR^{low} MDSC and slan+ non-classical monocytes were investigated as two monocytic subpopulations with contrasting properties. Both types of monocytes even showed an inverse correlation in SCLC, similar to that earlier described for NSCLC [15]. CD16+ non-classical monocytes can be further divided into slan+ and slan-negative populations [51,52]. While being of monocyte origin, slan+ cells either rapidly acquire DC functions or differentiate into macrophages [53]. Slan+ non-classical monocytes have been shown to be involved in anti-tumoral activity [53] since they can activate NK cells via IL-12. The crosstalk between slan+ cells and NK cells improves the differentiation of naïve CD4+ T lymphocytes into interferon (IFN)-gamma-producing TH1 cells [54]. The median number of slan+ cells in SCLC patients fits the amount of slan+ cells reported in the literature for a control group (21 cells/µL, [55]). The frequency of baseline slan+ monocytes was not useful as a marker of treatment failure but correlated with the survival both of SCLC and NSCLC patients in the current study.

Although the frequency of slan+ non-classical monocytes directly correlated with DC subtypes in both SCLC (current study) and NSCLC [15], several differences were noticed between DC and slan+ non-classical monocytes. From the baseline to the third cycle, the frequency of all blood DC subpopulations rose in patients with a clinical response, but slan+ non-classical monocytes stayed stable over time. SCLC compared to NSCLC patients, had similar levels of slan+ non-classical monocytes (median 0.23% of leukocytes) but significantly lower DC concentrations. The MDC/PDC median was 0.10% of leukocytes in SCLC; however, it was 0.20% in advanced NSCLC. The observation of significantly lower blood DC in SCLC confirms the data published by Afifi and coauthor [56]. In SCLC therapy responders, basal slan+ non-classical monocytes but not DC levels correlated with patients' OS. In particular, basal PDC frequencies were not associated with OS in our study. However, in the third cycle of ICI administration, all DC subpopulations were significantly correlated with survival. The impact of these observations remains unclear. Some of the SCLC patients had very low basal amounts of blood DC, which might contribute to their disturbed immune functions and poor prognosis, suggesting that a critical minimum number of DC is not reached in the case of basal PDC. In a recent study, an age-matched control group had 7.5 ± 4.2 PDC/µL blood [38]; in the current study, the baseline PDC of SCLC patients was 0.3–5.0 in the progression group, and 0–17.2 PDC/µL of blood in the clinical response, with a median of 4.9 PDC/µL blood. Although DC constitutes a

rare immune cell population, these cells are central for the initiation of tumor antigen-specific immunity [57]. Systemic effects induced by lung cancer cells upon circulating DC may result in diminished and functionally handicapped cells [58]. DC in NSCLC tissues upregulates the co-inhibitory receptor B7-H3/CD276, thus failing to stimulate T lymphocytes [59]. DC dictates responses to ICI treatment since a DC gene signature was strongly associated with the improved OS of NSCLC patients [60]. Until now, data on DC in SCLC patients have been scarce. Understanding the possibilities to augment DC functions could offer new approaches to enhance the efficacy of immunotherapy.

In addition to the combination of ICI with other therapies, several new treatment strategies for SCLC are under investigation with promising results [61]. Despite notable clinical responses, basic and clinical studies are still required to investigate the exact mechanism of response to ICI therapy and to improve the appropriate selection of patients. Identifying low percentages of both slan+ non-classical monocytes and CD1c+ MDC as well as a high NLR and high percentages of HLA-DR^{low} MDSC as risk factors for the patient's response to a combined chemo/immunotherapy, this study extends our knowledge of biomarkers and the pathophysiological causes of therapy resistance. If validated in larger studies, biomarker analysis in blood samples could help to select SCLC patients for a higher benefit from immunotherapy.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/biom13020190/s1, **Figure S1**: Relationship between risk factors/baseline immune cell parameters and OS in 84 NSCLC patients based on cutoffs of the SCLC group. Mean survival time and *p*-value of the log-rank test are given; **Table S1**: Comparison of baseline and third cycle values of blood immune cells in 33 SCLC patients with therapy response to immune/chemotherapy. The median and interquartile ranges are given. Bold values highlight significant differences in the Wilcoxon test; **Table S2**: Comparison of blood immune cells in SCLC patients without and with brain/liver metastases. The median and interquartile ranges are given. Bold values highlight significant differences in Mann–Whitney U test; **Table S3**: Relationship between baseline immune-cell parameters with patients' OS for 34 SCLC patients responding to chemo/immunotherapy. Data of univariate prognostic factor analysis are provided with estimated mean of survival ± standard error, hazard ratios (HR) with 95% confidence interval (CI), and *p*-values; **Table S4**: Relationship between cycle 3 blood immune cell parameters with patients' OS for 35 SCLC patients. Data of univariate prognostic factor analysis is provided, with estimated mean of survival ± standard error, hazard ratios (HR) with 95% confidence interval (CI), and *p*-values; **Table S5**: Baseline blood immune cell parameters of SCLC compared to NSCLC histotypes. Data represent the median and interquartile range (IQR). The results of the Mann–Whitney U-test are shown.

Author Contributions: Patient recruitment, M.M. and G.G.; conceptualization, D.R. and M.M.; project administration, W.S.; funding acquisition, W.S.; methodology, S.T.; analysis, writing—original draft preparation, D.R.; writing—review and editing, B.S. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and approved by the Ethics Committee of the "Ärztekammer Sachsen-Anhalt" (69/18, 20 January 2020).

Informed Consent Statement: Written informed consent was obtained from the patients to publish this paper.

Data Availability Statement: The data will be available after publication on request.

Acknowledgments: We would like to thank Anja Müller and Katharina Biehl for the performance of the flow cytometry analyses of clinical samples and Jens Wulfänger for help with illustrations.

Conflicts of Interest: The authors declare no conflict of interest.

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Blood Immune Cells as Biomarkers in Long-Term Surviving Patients with Advanced Non-Small-Cell Lung Cancer Undergoing a Combined Immune/Chemotherapy

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Simple Summary: Tumor cells can evade recognition and killing via immune cells by expressing co-inhibitory membrane molecules, which suppress the activity of tumor-specific T cells. Immune checkpoint inhibitor (ICI) therapies act by blocking these inhibitory pathways via monoclonal antibodies. Due to the limited efficacy of ICI therapy, biomarkers have to be detected, a process that can identify patients who benefit from this long-term treatment. In this pilot study, immune monitoring of four blood cell markers was performed over time in advanced non-small cell lung cancer (NSCLC) patients undergoing combined immune/chemotherapy and surviving ≥12 months. We demonstrate that a low neutrophil/lymphocyte ratio (NLR), a low percentage of suppressive HLA-DR^{low} monocytes, and/or clearly detectable numbers of slan+ non-classical monocytes and of dendritic cells can be predictive markers for therapy responses and treatment outcomes. These markers might have an impact on the treatment decisions for NSCLC patients, but need to be validated in larger cohorts.

Citation: Möller, M.; Schütte, W.; Turzer, S.; Seliger, B.; Riemann, D. Blood Immune Cells as Biomarkers in Long-Term Surviving Patients with Advanced Non-Small-Cell Lung Cancer Undergoing a Combined Immune/Chemotherapy. *Cancers* **2023**, *15*, 4873. https://doi.org/10.3390/cancers15194873

Academic Editor: Akiteru Goto

Received: 23 August 2023
Revised: 28 September 2023
Accepted: 5 October 2023
Published: 6 October 2023

Abstract: An important challenge remains in identifying the baseline characteristics of cancer patients who will mostly benefit from immune checkpoint inhibitor (ICI) therapies. Furthermore, biomarkers could help in the choice of an optimal therapy duration after a primary therapy response. In this pilot study, the time courses of four different immune cell parameters were followed in 12 patients with advanced non-small-cell lung cancer (NSCLC) undergoing ICI therapy combined with chemotherapy and surviving at least 12 months. Blood was collected at the time point of the first and third antibody administration, as well as after 12 months of patients' survival. Using multi-color flow cytometry, two suppressive markers (neutrophil/lymphocyte ratio (NLR) and the frequency of circulating HLA-DR^{low} monocytes), as well as two markers of an ongoing immune response (6-Sulfo LacNAc (slan)+ non-classical monocytes and dendritic cell (DC) subtypes), were determined. In most of those who survived > 12 months, a low NLR and a low number of HLA-DR^{low} monocytes combined with clearly detectable numbers of slan+ non-classical monocytes and of DC subtypes were seen. Two of the patients had an increase in the suppressive markers paired with a decrease in slan+ non-classical monocytes and in DC subtypes, which, in at least one patient, was the correlate of an ongoing clinical progression. Our results implicate that the NLR, specific subtypes of monocytes, and the number of blood DCs might be useful predictive biomarkers for cancer patients during long-term treatment with ICI/chemotherapy.

Keywords: biomarkers; immune checkpoint blockade; non-small-cell lung cancer; long-term survival; neutrophil/lymphocyte ratio; HLA-DR^{low} monocytes; slan+ non-classical monocytes; dendritic cells

1. Introduction

Despite modest responses to platinum-based chemotherapy and short intervals until disease progression [1,2], these agents were used as standard forms of therapy for patients with advanced non-small-cell lung cancer (NSCLC) for many years. In the past decade, immune checkpoint inhibitors (ICIs) targeting the PD-L1/PD-1 signaling axis have emerged as a novel treatment option for lung cancer patients, though only a limited proportion of patients can benefit from them [3,4]. The therapeutic activity of ICIs is the result of a complex interplay between multiple cells and soluble factors in the tumor microenvironment (TME) and the immune system (for review, see [5]). Based on the ICI which was employed, two distinct activities have been reported: (i) competition for the ligands of co-inhibitory receptors and (ii) regulation of the surface expression of these receptors. ICIs' efficacy depends on the tumor mutational burden [6], the cellular heterogeneity associated with patterns of immune suppression [7], and the tumor cell expression of immune checkpoint molecules (for review see [8]). With respect to patients' prognosis, the immunotherapy response patterns, immune-related adverse events, and tumor stages are associated with the diversity of the continuation of ICI therapy in previous studies, but there exists no clear consensus on the optimal duration of ICI therapy [9]. Biomarkers of ongoing immune response/disease progression could help to guide the long-term therapeutic regimen and could optimize ICI-based combination therapies. Blood biomarkers have difficulty in reflecting the TME, including the spatial distribution of tumor and immune cells, but are easier to handle than tumor tissues/biopsies. Blood-based cellular immune biomarkers have shown great promise in predicting responses to ICI therapies due to easy specimen accessibility, the opportunity for serial monitoring, quantitative measurement, and the availability of unique analytic high-throughput (single cell) platforms [10]. In a recent study [11], we found that NSCLC patients with a baseline neutrophil-to-lymphocyte ratio (NLR) $\geq$ 6.1, $\geq$ 22% HLA-DR^{low} monocytes, a frequency of slan+ non-classical monocytes < 0.25% of leukocytes, and/or amounts of dendritic cells (DC) $\leq$ 0.14% of leukocytes showed poor progression-free survival (PFS). In this study, the four blood cell biomarkers were monitored in patients with advanced NSCLC surviving at least 12 months of a combined ICI/chemotherapy to investigate whether these markers are useful to predict the treatment outcomes of patients.

2. Materials and Methods

Patients' cohort. The study was approved by the institutional review board of the Aerztekammer Sachsen-Anhalt (69/18). All patients gave written informed consent for the study's proposal and procedures. In a former study, 90 patients with histologically confirmed unresectable locally advanced or metastatic lung cancer, prior to PD-1 or PD-L1 blockade treatment in combination with chemotherapy, were prospectively enrolled from June 2019 to June 2021 (Table 1 in [11]). In total, 12 of the 36 patients surviving $\geq$ 12 months consented to a third blood draw and were included in the current pilot study. All patients were >18 years old and had a histologically confirmed diagnosis of advanced lung cancer, adequate organ function, and the capacity to make an informed decision. Patients with an active autoimmune disease were excluded from the study, as were patients with treatable oncogenic driver alterations. A combined immune/chemotherapy with nab-paclitaxel, carboplatin, and pembrolizumab was given to patients with squamous cell carcinoma (SqC), according to the KEYNOTE-407 trial [12], but also to patients with thyroid transcription factor (TTF)-1-negative adenocarcinoma (AC), as pemetrexed might have been less effective in these patients [13]. Patients with AC and liver metastases received a combined immune/chemotherapy with atezolizumab, bevacizumab, carboplatin, and nab-paclitaxel, since this combination had a clear advantage in terms of the presence of liver metastases, according to the IMpower150 study [14]. All other AC patients received a combined immune/chemotherapy with pemetrexed, carboplatin, and pembrolizumab (KEYNOTE-189 trial [15]). Therapy response rates were determined according to RECIST 1.1 criteria. PFS was defined as the time from the initiation of immune/chemotherapy to the first obser-

vation of cancer progression or death from any cause, whereas overall survival (OS) was defined as the time from the initiation of therapy until death from any cause.

Blood samples, antibody staining, and flow cytometry. Blood samples of a former study [11] were collected before the initiation of immune/chemotherapy (time point 0, baseline) and prior to the third therapy cycle (t_1). For the current pilot study, 12 patients with an OS of at least 12 months consented to a third blood draw (t_2). Leukocyte count and complete blood count were measured with a routine hematology analyzer. Circulating DC subpopulations were determined with the "Blood DC Enumeration Kit" (Miltenyi, Bergisch Gladbach, Germany), which was supplemented for gating reasons with CD45-APC-H7 and a HLA-DR-V500 monoclonal antibody (mAb). Briefly, 300 µL of blood was incubated with mAb CD141/BDCA-3-APC as a marker for myeloid conventional DC (cDC1), anti-CD1 c-PE (cDC2), and CD303/BDCA-2-FITC for plasmacytoid DC (pDC) [16]. At least 1×10^6 leukocytes were analyzed on a FACS CANTO II Flow Cytometer (BD Biosciences, Heidelberg, Germany) using a recently published gating strategy [11]. Monocytic HLA-DR intensity was quantified using a mAb labeled on a protein/fluorophore ratio of 1/1 (clone L243; QuantiBRITE™ reagent; BD Biosciences), according to the manufacturer's instructions. A standard curve for antigen quantification was established with QuantiBRITE beads (BD Biosciences) to convert the measured geometric mean fluorescence intensity (MFI) of the gated monocytes into "antibody molecules bound per cell" (ABC) values. Monocytic HLA-DR MFI values of $\leq$5000 ABC were designated as "immunoparalysis", since the patients were at high risk of infectious diseases [17]. The percentage of HLA-DR^{low} monocytes was estimated by taking a MFI of 5000 ABC as the cut-off value for a low HLA-DR intensity [18].

For the labeling of monocytes/lymphocytes, 300 µL blood was stained with mAbs specific to slan (M-DC8)-FITC (Miltenyi Biotec, Bergisch Gladbach, Germany); CD16-PE-Cy7 (Biolegend, San Diego, CA, USA); and CD19-PerCP-Cy5.5 (InVitrogen/Thermo Fisher, Waltham, MA, USA). All other mAbs (CD14-APC, CD45-APC-H7, CD3-V450, HLA-DR-V500) were obtained from BD Biosciences (Heidelberg, Germany). After two washing steps, the cells were analyzed with the FACS CANTO II (BD Biosciences, Heidelberg, Germany). The gating strategy for slan+ non-classical monocytes is provided in [11]. Data analysis was performed using the BD FACSDiva^{TM} software V8.0.1. Cytometer Setup and Tracking (CST) Beads (BD Biosciences) were used daily to set standardized geometric MFI ranges in the fluorescence channels which were used.

3. Results

3.1. Patient Characteristics

The general baseline characteristics of the 12 NSCLC patients surviving at least 12 months and who were available for a third blood draw are summarized in Table 1. Concerning the cohort of patients, 10/12 patients had AC histology, and 11/12 patients were male. Patients were between 48 and 84 years old. PD-L1 tumor expression ranged between zero and 100%, and complete remission was associated both with 0% and with 100% PD-L1 expression. Patients received 15–34 cycles of ICI therapy. Of the 12 patients, 5 patients were still living at the time of the evaluation of this study in January 2023, with complete remission in four patients. The other patients survived 5–18 months after the third blood draw (shown as OS3 in Table 1).

3.2. The Time Course of Blood Cell Markers

Blood samples from the 12 NSCLC patients were monitored over time. Table 2 summarizes the four blood cell markers measured at baseline (t_0), both at the third ICI application (t_1) and after surviving at least one year (t_2). The baseline values associated with poor PFS were described in a recent study [11] to be a NLR $\geq$ 6.1, HLA-DR^{low} myeloid-derived suppressor cells (MDSC) $\geq$ 22% of monocytes, slan+ non-classical monocytes < 0.25% of leukocytes, and/or DC levels $\leq$ 0.14% of leukocytes. With two exceptions (patients #6 and #11), most of the 12 patients had a baseline NLR below the critical value of 6.1

(mean 4.3). At the time point of the third ICI application, the NLR values of all patients dropped below 6.1. This result can be interpreted as an initial therapy response, since patients with tumor progress showed an NLR increase at t_1 in the former study [11]. Similar results were obtained for HLA-DR^{low} MDSC: Only 2/12 patients (patients #3 and #11) exhibited baseline levels $\geq$ 22% of monocytes, which had declined by the time of the third ICI cycle. Interestingly, the high baseline levels of HLA-DR^{low} monocytes were associated with low percentages of slan+ monocytes (<0.25% of leukocytes and <2% of monocytes), and additionally, in patient #11, with low DC levels. Patient #11 had an OS of 15 months despite poor baseline markers and the presence of brain/liver metastases (Table 1). Our results show that although high baseline values of immunosuppressive neutrophils and monocytes are a risk factor for a lacking therapy response and a poor PFS [11], patients can survive > 12 months if they have ameliorated their blood cell markers by the third ICI cycle.

Table 1. Patients' characteristics. Tumor histology, sex, age, metastases of brain/liver, PD-L1 status, ECOG performance status, smoker status, ICI cycle numbers until the end of the study, best therapy response (CR = complete remission; PR= partial remission; SD = stable disease; PD= progressive disease), overall survival (OS; in months), and survival time after third blood draw (OS3; in months) are shown. Patients still alive at the end of the study are highlighted in yellow; patients with complete remission in red.

	Histo	Sex	Age	Meta. Brain/Liver	PD-L1	ECOG	Smo	ICI Cycles	Response	OS	OS3
1	AC	M	67	0	0%	0	yes	33	CR	>38	>16
2	AC	M	62	yes	100%	0	yes	31	CR	>38	>16
3	AC	M	73	0	90%	1	yes	30	PR (PD 05/22)	34	12
4	AC	M	76	0	85%	1	yes	15	PR (PD 07/21)	25	5
5	AC	M	69	0	0%	1	yes	31	SD	29	11
6	SqC	M	84	0	0%	0	yes	29	CR	>35	>18
7	AC	M	71	0	10%	0	yes	27	SD	34	18
8	AC	F	56	0	0%	0	yes	34	PR	>32	>12
9	AC	M	79	0	0%	0	yes	17	PR	23	11
10	AC	M	48	0	0%	0	yes	19	PR	22	11
11	SqC	M	74	yes	70%	0	no	13	PR	15	5
12	AC	M	55	0	80%	1	yes	21	CR	>38	>18

Table 2. Cellular blood biomarkers in 12 NSCLC patients surviving at least 12 months over time. The biomarkers NLR, HLA-DR^{low} MDSC (as % of monocytes), slan+ non-classical monocytes (as % of monocytes), and sum of MDC/PDC (in % of leukocytes) were monitored over time. The time points given are the baseline values (t_0), the third antibody application (t_1), and after at least 12 months OS (t_2). D shows the dynamics between t_1 and t_2. Values above/below the cut-off point for poor survival are marked in bold. Patients still alive at the end of the study are highlighted in yellow.

Pat. No.	Response	NLR				HLA-DR^{low} Monocytes				Slan+ Monocytes				MDC/PDC Sum			
		t_0	t_1	t_2	D	t_0	t_1	t_2	D	t_0	t_1	t_2	D	t_0	t_1	t_2	D
1	CR	3.4	5.3	4.6	↔	0.8	3.2	6.8	↔	3.3	4.1	2.9	↔	0.37	0.34	0.36	↔
2	CR	3.6	2.3	5.5	↔	5.2	4.2	8.9	↔	4.9	5.5	4.3	↔	0.48	0.57	0.24	↔
3	PR/ PD	5.7	2.9	**10.3**	↑	**29.1**	17.7	**38.7**	↑	**1.9**	6.7	2.5	↔	0.27	0.17	**0.08**	↓

Table 2. *Cont.*

Pat. No.	Response	NLR				HLA-DR^{low} Monocytes				Slan+ Monocytes				MDC/PDC Sum			
		t_0	t_1	t_2	D	t_0	t_1	t_2	D	t_0	t_1	t_2	D	t_0	t_1	t_2	D
4	PR/ PD	3.4	3.1	**15.3**	↑	4.4	2.1	7.9	↔	2.7	3.7	**0.7**	↓	0.31	0.31	**0.01**	↓
5	SD	3.4	5.4	3.0	↔	17.5	12	9.1	↔	3.7	2.6	7.5	↔	0.15	0.28	0.32	↔
6	CR	**7.2**	2.6	3.5	↔	6.9	1.1	1.3	↔	10	13.5	8.7	↔	**0.05**	0.17	0.16	↔
7	SD	5.0	3.3	2.6	↔	4.4	5.6	0.9	↔	3.2	4.6	5.8	↔	0.175	0.39	0.24	↔
8	PR	2.0	2.9	1.2	↔	8.2	7	3.4	↔	4.9	2.7	5.5	↔	0.36	0.46	0.46	↔
9	PR	2.8	4.4	2.6	↔	11	5.7	13.1	(↑)	10.5	7.3	8.4	↔	0.24	0.31	0.22	↔
10	PR	3.9	3.2	4.5	↔	9.7	6.4	8.2	↔	4.8	5.4	5.1	↔	0.22	0.27	0.20	↔
11	PR	**8.7**	4.4	2.7	↔	**22.3**	11	6.9	↔	**0.3**	2.2	3.5	↔	**0.03**	0.13	0.19	↔
12	CR	1.9	2.3	3.3	↔	6.3	7.4	1.7	↔	11.8	11.8	11.8	↔	0.22	0.15	0.16	↔

In total, 4 of the 12 patients with a third blood collection went into complete remission. After at least 12 months of the patients' survival (t_2), these patients showed low blood cell counts associated with immune suppression (NLR < 6.1 and/or <10% of monocytes with a HLA-DR^{low} phenotype) and clearly detectable markers of an ongoing immune response (2.8–11.8% slan+ non-classical monocytes and/or DC levels of ≥0.16% of leukocytes) (Table 2). The marker values of the third cycle (t_1) and after 12 months of patients' survival (t_2) were stable and comparable in most cases, as displayed by arrows in Table 2. Figure 1A illustrates the time course of the four blood markers in patient #1 as an example of a long-term survivor with complete remission, demonstrating relatively stable blood cell values.

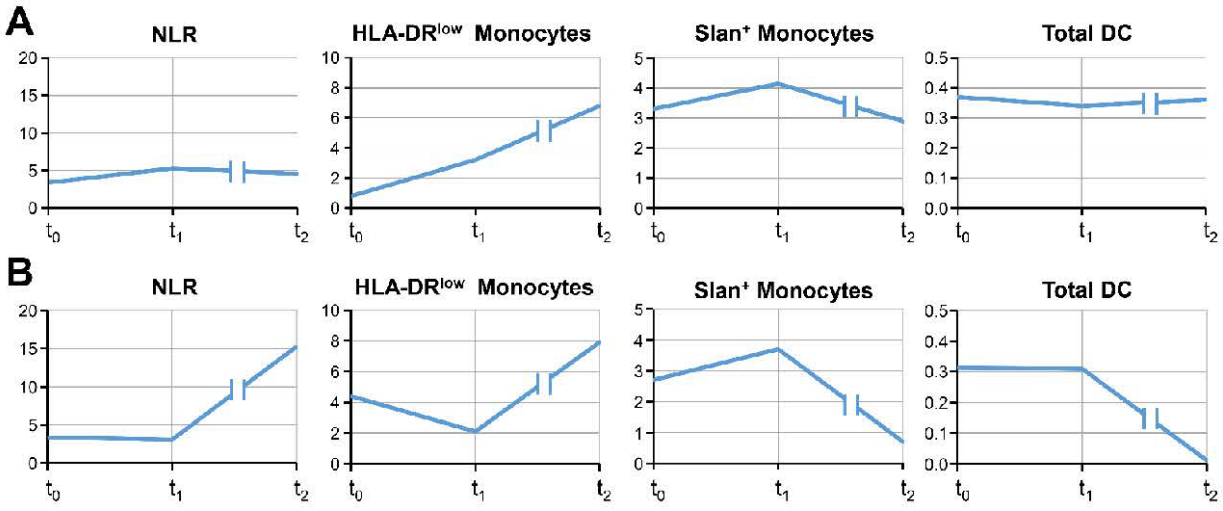

Figure 1. Time course of the four biomarkers NLR, HLA-DR^{low} monocytes, slan+ non-classical monocytes, and total DC in one patient with complete remission (**A**) and in one patient with disease progression (**B**). The time points given at the X-axis are the baseline values (t_0), the time of the third ICI application (t_1), and a time point after at least 12 months OS (t_2). HLA-DR^{low} and slan+ non-classical monocytes are given as % of monocytes; the number of DC is given as % of leukocytes.

Another dynamic pattern was found in patient #4 (Figure 1B), who underwent the third blood draw at a time at which progressive disease had been proven. This patient had a lymphopenia (590 lymphocytes/µL blood), resulting in a high NLR of 15.2. Furthermore, the numbers of DC were close to zero, and the slan+ non-classical monocytes decreased below baseline levels, while HLA-DR^{low} MDSC increased, but remained <10% of monocytes. This patient died 5 months after the third blood collection (OS 25 months). A similar dy-

namic could be observed in patient #3, who had relatively high levels of HLA-DR^{low} MDSC over time (Table 2). At t_2, an increase in both the NLR and in HLA-DR^{low} MDSC above baseline levels was noticed. Although slan+ non-classical monocytes showed stable values, DC counts decreased. Nine months after the third blood collection, tumor progression was recognized in this patient, who died 3 months later (OS 34 months). The examples of patients #3 and #4 show that a deterioration of blood cell markers could indicate worsening of the tumor disease.

Interestingly, patient #12 (complete remission) had a high number of slan+ non-classical monocytes over the time course of all blood collections (Table 2). The patient's slan+ non-classical monocytes, known to exhibit anti-tumor activity, surpassed the number of HLA-DR^{low} MDSC in the blood. In three of the five patients still living at the end of our study, the quotient of slan+ non-classical monocytes/HLA-DR^{low} monocytes had values > 1 (Table 3). However, for most of the patients of this study, the monocytic quotient showed values < 1, suggesting a preponderance of monocytic MDSC in blood, even in patients with complete remission, such as in patient #1 and #2. Based on our data, it could be assumed that there exists an individual equilibrium of the different monocytic subpopulations in the blood. Tumor progression was associated with a very low quotient of slan+ non-classical monocytes/HLA-DR^{low} monocytes, as shown in patients #3 and #4. The ratio of slan+ non-classical monocytes/HLA-DR^{low} monocytes could be a hopeful new marker for the monitoring of tumor patients.

Table 3. Alterations in the ratio of slan+/HLA-DR^{low} monocytes over time. The quotients of slan+ non-classical monocytes and of HLA-DR^{low} MDSC (both as % of monocytes) were determined in the 12 patients at baseline (t_0), the time point of third ICI application (t_1), and after at least 12 months of OS (t_2). Patients still alive at the end of the study are highlighted in yellow. Patients with a very low quotient, e.g., a preponderance of HLA-DR^{low} MDSC, are marked in grey.

Patient No.	Slan+/HLA-DR^{low} Mono t_0	slan+/HLA-DR^{low} Mono t_1	slan+/HLA-DR^{low} Mono t_2
1	4.13	1.29	0.43
2	0.94	1.31	0.48
3	0.07	0.38	0.06
4	0.61	1.76	0.09
5	0.21	0.22	0.82
6	1.45	12.27	6.69
7	0.73	0.82	6.44
8	0.60	0.39	1.62
9	0.95	1.28	0.64
10	0.72	0.63	0.76
11	0.01	0.20	0.51
12	1.87	1.59	6.94

Taken together, our results implicate that the NLR, specific subtypes of monocytes, and the DC counts might be useful biomarkers for the long-time monitoring of NSCLC patients' survival. As illustrated in Figure 2, an ongoing and effective anti-tumor response is associated with both a detectable proportion of slan+ non-classical monocytes and of DC, resulting in better patient survival. In contrast, a high NLR (neutrophils dominate over lymphocytes) and a high percentage of HLA-DR^{low} MDSC are signs of immune tolerance, tumor progression, and poor patient survival.

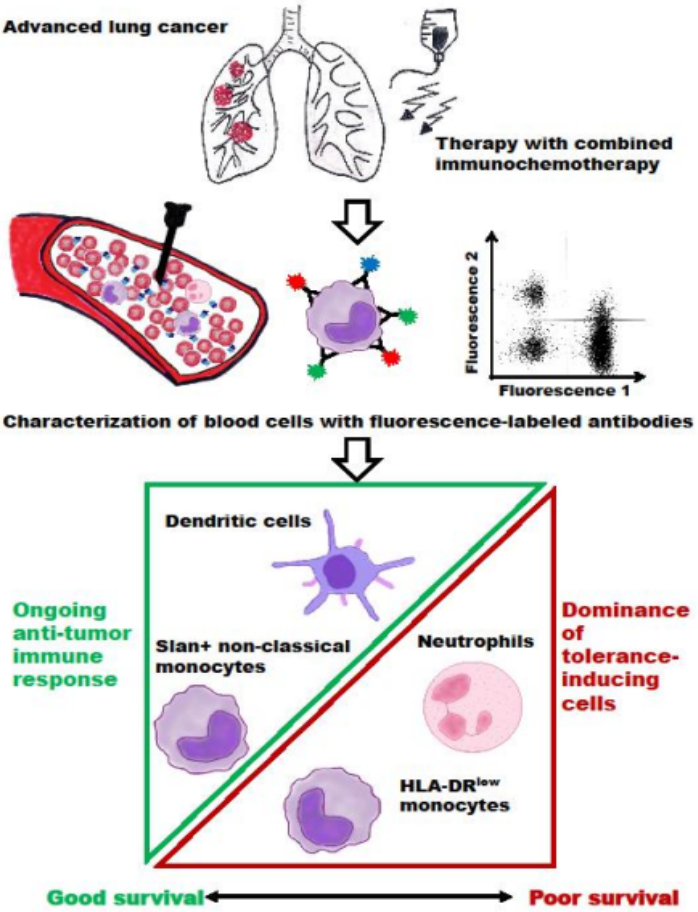

Figure 2. Schematic diagram of the immune monitoring process of this study. In patients with advanced NSCLC undergoing immune/chemotherapy, blood cells were stained using fluorescence-labeled mAb. For the monitoring of therapy-induced changes in immunogenicity, DC and slan+ non-classical monocytes, as well as neutrophil/lymphocyte ratio and HLA-DR^{low} monocytes, were quantified. A good patient survival rate was associated with an ongoing anti-tumor response with detectable amounts of DC and slan+ non-classical monocytes. The dominance of tolerance-inducing neutrophils and HLA-DR^{low} monocytes reflects the resistance to immune/chemotherapy and poor survival.

4. Discussion

The implementation of ICI therapy has revolutionized lung cancer therapy, with significant survival benefits for patients with advanced wild-type NSCLC. However, since not all patients with advanced and/or metastatic disease can benefit from ICI therapy, predictive biomarkers are urgently needed [19]. In ICI monotherapy, a good ECOG score, PD-L1 tumor expression of $\geq$50%, an absence of bone metastasis, and the presence of skin toxicity have been correlated with a good PFS and OS [20]. Since the response to ICI therapy is complex, single biomarkers are rarely able to precisely predict therapy responses. Thus, a combination of multiple biomarkers could be helpful, as demonstrated by the increased performance using a combination of PD-L1 immunohistochemistry, T-cell infiltration, and assessment of tumor mutational burden when compared with the three parameters alone [21].

Immune-related therapies can prolong the median OS of advanced wild-type NSCLC to 17–22 months, and a pooled OS of 16.2 months could be determined as the long-term survival standard (for review, see [22]). One of the major issues with ICI therapy is

the determination of the optimal treatment duration [9]. Immune response characteristics are the basis of the unprecedented long-term survival of lung cancer patients with ICI/chemotherapy. Therefore, biomarkers of an ongoing immune response could help to guide long-term therapeutic regimens and could optimize ICI-based combination therapies. Our present pilot study confirms the usefulness of selected blood immune cells as biomarkers in NSCLC patients with advanced tumor stages and/or metastasis surviving at least 12 months. Comparable to the baseline marker expression, the increases in NLR and HLA-DR^{low} MDSC, as well as the decreases in tumor-fighting slan+ non-classical monocytes and DC, could be found during the time course of therapy, and were associated with a declining immune response and ongoing tumor progression. In contrast, all four patients with complete remission exhibited a NLR < 6, HLA-DR^{low} MDSC <10% of monocytes, slan+ non-classical monocytes of >2% of monocytes, and DC numbers ≥0.16% of leukocytes.

In the resistance to immunotherapy and the tumor-driven down-regulation of the immune response, several cell types are involved and can be monitored. The NLR combining neutrophils and lymphocytes is an established marker for the prognosis of lung cancer patients [8,23,24]. Neutrophils contain a subpopulation that promotes tumor growth and metastasis, stimulates angiogenesis, and mediates immunosuppression [25]. An enrichment in neutrophil-related proteins was observed in NSCLC patients with lacking responses to ICI therapy [26]. Similarly, HLA-DR^{low} monocytes are known to suppress lymphocytic functions in cancer patients [27–29]: in patients with hepatocellular carcinoma, HLA-DR^{low} MDSC inhibits NK cell cytotoxicity and cytokine secretion [30]. Several mediators and cytokines can reduce monocytic HLA-DR intensity, including IL-10, which increases the intracellular sequestration of MHC class II molecules via ubiquitination [31], or TGF-ß, which down-regulates the transcription of HLA-DR through the class II transactivator (CIITA) [32]. TGF-ß might be involved in the resistance to chemotherapy and/or ICI therapy [33], as a continuation of the TGF-β inhibitor with immunotherapy showed promising results in pre-clinical studies [34].

Monocytic subpopulations, such as classical (CD14^{high}, CD16^{-}), intermediate (CD14^{high}, CD16^{+}), and non-classical (CD14$^{low/-}$, CD16^{+}) monocytes, show transcriptomic differences that translate into specialization and different functions [35,36]. The CD16+ non-classical monocytes especially have been regarded as a pro-inflammatory population exhibiting tumor-killing properties [37]. Non-classical monocytes are crucial for ICI therapy of patients with malignant melanoma, since they are involved in the killing of regulatory T cells via the CTLA-4 mAb [38]. CD16^{+} non-classical monocytes can be further divided into slan+ and slan− populations [39,40]. Although they are of monocytic origin, slan+ cells may either rapidly acquire DC functions or differentiate into macrophages [41]. Slan+ monocytes can activate NK cells via IL-12, and the crosstalk between slan+ cells and NK cells improves the differentiation of naïve CD4^{+} T lymphocytes into T-helper type 1 (Th1) cells [42]. In patients with diffuse large B-cell lymphoma, slan+CD16+ non-classical monocytes, but not CD14^{+} monocytes, increased and displayed highly efficient, rituximab-mediated, antibody-dependent cellular cytotoxicity, almost equivalent to that exerted by NK cells [43].

DCs form a functional bridge between the innate and adaptive immune systems, and are important regulators of immune reactions. DCs are also important players in immunotherapy approaches. Strategies to harness the T-cell stimulatory function of DC for cancer immunotherapy aim at inducing antigen-specific T-cell responses of a Th1 phenotype accompanied by priming of cytotoxic T lymphocytes with the ability to eradicate tumor cells [44,45]. In the context of cancer, several alterations in the frequency and function of DCs have been reported [46–48]. The efficacy of ICI therapy was limited by a paucity of activated CD103+ DC in melanoma lesions [49], and a DC gene signature was strongly associated with improved patient OS in NSCLC patients undergoing atezolizumab therapy [50]. Blood DC can be divided into at least three distinct main subsets: conventional DC type I or II (cDC1 or cDC2, respectively) and pDC (for review see [51]). pDC is known for its ability to produce high amounts of type-I interferons upon stimulation via viral patterns, and has limited antigen-presenting potential. CD141^{+} cDC1 has superior antigen presentation activity on

MHC class I molecules to $CD8^+$ T cells, whereas $CD1c^+$ cDC2 present antigens via MHC class II molecules to $CD4^+$ T cells. A vaccine of cDC2 and pDC pulsed with tumor antigens was tested in metastatic melanoma and prostate cancer [52,53], leading to immunological responses and, in some patients, also to long-term survival. Furthermore, vaccination with naturally occurring cDC1s loaded with immunogenic cell-death-derived whole-tumor antigens could synergize with anti-PD1 treatment [44]. In a former study [11], we found that NSCLC patients undergoing combined immune/chemotherapy and possessing a DC baseline frequency of $\leq$0.14% of leukocytes had poor PFS. Age-matched healthy persons had DC levels of 0.32 $\pm$ 0.03% of leukocytes [47].

5. Conclusions

Despite notable clinical responses, basic and clinical studies are still required in order to investigate the exact mechanism of ICI immunotherapy and to improve the appropriate selection of patients. In a pilot study, we demonstrated the value of a longitudinal monitoring of the immune surveillance in 12 NSCLC patients with an advanced tumor disease surviving at least 12 months while receiving immune/chemotherapy. Our results imply that the NLR, specific subtypes of monocytes, and blood DC counts might be useful biomarkers for the monitoring of NSCLC patients. An ongoing and effective anti-tumor response is associated with both a detectable proportion of slan+ non-classical monocytes and of DC subtypes, resulting in the better survival of patients. In contrast, dominating neutrophils and a high NLR, as well as a high percentage of $HLA\text{-}DR^{low}$ MDSC, are signs of immune tolerance and tumor progression. The cellular blood biomarkers which we investigated might improve the prediction of the clinical and durable benefit of patients, and might provide insights into the underlying mechanisms of therapy resistance associated with reduced patient survival. It is mandatory that these blood cellular markers are further investigated and validated in larger multicenter patient cohorts as soon as possible.

Author Contributions: Patient recruitment, M.M.; conceptualization, D.R. and M.M.; project administration, W.S. and M.M.; funding acquisition, W.S.; methodology, S.T.; analysis, writing—original draft preparation, D.R.; writing—review and editing, B.S. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Review Board (or Ethics Committee) of the Medical Faculty (protocol code 69/18; 1 January 2019) for studies involving humans.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: All the data of this study are available upon request.

Acknowledgments: We would like to thank Anja Müller and Katharina Biehl for the performance of flow cytometry of the clinical samples and Jens Wulfänger for help with illustrations.

Conflicts of Interest: The authors declare no conflict of interest.

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5. Thesen

1. Immuntherapien sind im metastasierten Stadium beim NSCLC ohne therapierbare Treiberalterationen, entweder allein oder in Kombination mit einer Chemotherapie sowie beim metastasierten SCLC in Kombination mit einer Chemotherapie der Standard in der Erstlinienbehandlung.
2. Der aktuell einzige im klinischen Alltag etablierte prädiktive Biomarker beim NSCLC ist PD-L1.
3. Beim SCLC existieren aktuell keine prädiktiven Biomarker in Bezug auf die Immuncheckpointinhibition.
4. Beim NSCLC dient die PD-L1 Testung lediglich dazu, Patienten zu selektionieren, welche eine Monoimmuntherapie erhalten können und nicht dazu, Patienten zu bestimmen, welche geeignet für eine Therapie mit Immuncheckpointinhibitoren sind.
5. Obwohl der überwiegende Teil der Patienten mit Lungenkarzinom eine Immuntherapie erhält, profitieren viele Patienten nicht von dieser.
6. Die nahezu unselektionierte Gabe der Immuntherapien ist mit hohen Therapiekosten und potenziellen Toxizitäten verbunden.
7. Neue potenzielle prädiktive Marker sollten leicht bestimmbar sein, kostengünstig und hoch spezifisch sowie sensitiv sein.
8. Die NLR, die HLA-DR^{low} MDSC, die DC und die Slan+ nicht klassischen Monozyten sind geeignete prädiktive Biomarker, die eine primäre Therapieresistenz in Bezug auf eine Immuntherapie vorhersagen können.
9. Eine schlechte Prognose haben Patienten mit einer hohen NLR und HLA-DR^{low} MDSC sowie niedrigen DC und Slan + nicht klassischen Monozyten.
10. Die NLR, die HLA-DR^{low} MDSC, die DC und die Slan+ nicht klassischen Monozyten sind ebenfalls für ein Blutimmunzellmonitoring geeignet, um im Therapieverlauf einen Progress zu erkennen.

Selbstständigkeitserklärung

Hiermit erkläre ich an Eides statt, dass ich die hier vorliegende Habilitationsschrift selbständig und nur unter Verwendung der angegebenen Literatur und Hilfsmittel angefertigt habe.

Ein Habilitationsverfahren wurde an keiner anderen Universität eröffnet oder beantragt.

Frühere Habilitationsversuche sind nicht unternommen worden.

Halle, den 14.12.2023 Dr. med. Miriam Möller

Danksagung

Herzlich möchte ich mich bei Prof. Wolfgang Schütte bedanken für die seit vielen Jahren gute und enge Zusammenarbeit. Für das Vertrauen, was er mir entgegenbringt und das Potential was er von Beginn an in mir gesehen hat.

Des Weiteren möchte ich mich sehr bei Frau PD Riemann und dem gesamten Team der Immunologie der Uni Halle bedanken für die perfekte Zusammenarbeit in Forschung und Lehre, die meine Habilitation erst möglich gemacht haben.

Ein großer Dank geht auch an meine gesamte Klinik sowie das MVZ inkl. Studiensekreteriat und das Team des Lungenkrebszentrums, die mich immer in allem unterstützt haben, wo wir so viele Studien durchgeführt und so viele Patienten erfolgreich behandelt haben.

Zuletzt möchte ich meiner Familie danken, die mich immer unendlich unterstützt haben, sodass ich trotz unserer drei Kinder meine Karriere weiterverfolgen konnte. Vor allem meinem Mann Andreas sowie meinen Eltern und Schwiegereltern möchte ich in besonderem Maße Dank sagen.