A gene expression signature associated with B cells predicts benefit from immune checkpoint blockade in lung adenocarcinoma

Jan Budcziesab##, Martina Kirchnerab##, Klaus Kluckab##, Daniel Kazdabc#, Julia Gladeb, Michael Allgäuerb, Mark Kriegsmannab, Claus-Peter Heußelahcde, Felix J. Herthcdef, Hauke Winterga, Michael Meisterch, Thomas Muleych, Stefan Fröhlingbij, Solange Petersi, Barbara Seligerck, Peter Schirmacherab, Michael Thomasj, Petros Christopoulosakl#, and Albrecht Stenzingerab##

*Institute of Pathology, Heidelberg University Hospital, Heidelberg, Germany; 2German Cancer Consortium (DKTK) and German Cancer Research Center (DKFZ), Heidelberg, Germany; 3Translational Lung Research Center Heidelberg (TLRC-H), Member of the German Center for Lung Research (DZL), Heidelberg, Germany; 4Department of Diagnostic and Interventional Radiology with Nuclear Medicine, Thoraxklinik at Heidelberg University Hospital, Heidelberg, Germany; 5Department of Diagnostic and Interventional Radiology, Heidelberg University Hospital, Heidelberg, Germany; 6Department of Pneumology, Thoraxklinik at Heidelberg University Hospital, Heidelberg, Germany; 7Department of Thoracic Surgery, Thoraxklinik at Heidelberg University Hospital, Heidelberg, Germany; 8Department of Translational Oncology, National Center for Tumor Diseases (NCT), Heidelberg, Germany; 9Department of Oncology, Centre Hospitalier Universitaire Vaudois (CHUV), Lausanne University, Lausanne, Switzerland; 10Institute for Medical Immunology, Martin Luther University Halle-Wittenberg, Halle, Germany; 11Department of Thoracic Oncology, Thoraxklinik and National Center for Tumor Diseases at Heidelberg University Hospital, Heidelberg, Germany

ABSTRACT

Immune checkpoint blockade (ICB) expands the therapeutic options for metastatic lung cancer nowadays representing a standard frontline strategy as monotherapy or combination therapy, as well as an option in oncogene-addicted NSCLC. Predictive markers are urgently needed, since only a minority of patients benefits from ICB, while serious adverse effects of immunotoxicity may occur. The study cohort included 43 ICB-treated metastatic lung adenocarcinoma showing long-term response (n=16), rapid progression (n=21) or intermediate patterns of response (n=6). Lung biopsies acquired before initiation of ICB were analyzed by targeted mRNA expression profiling of 770 genes. Level and proportions of 14 immune cell types were estimated using characteristic gene expression signatures. Abundance of B cells (HR=0.66, p=0.00074), CD45+ cells (HR=0.61, p=0.01) and total TILs (HR=0.62, p=0.025) were associated with prolonged progression-free survival after ICB treatment. In a ROC analysis, B cells (AUC=0.77, p=0.0055) and CD45+ cells (AUC=0.73, p=0.019) predicted benefit of ICB, which was not the case for PD-L1 mRNA (AUC=0.54, p=0.72) and PD-L1 protein expression (AUC=0.68, p=0.082). Clustering of 79 candidate predictive markers identified among 770 investigated genes revealed two distinct predictive clusters which included cytotoxic cell or macrophage markers, respectively. In summary, targeted gene expression profiling was feasible using routine diagnostics biopsies. This study proposes B cells and total TILs as complementary predictors of ICB benefit in NSCLC. While further preferably prospective validation is required, gene expression profiling could be integrated in the routine diagnostic work-up complementing existing NGS protocols.

Background

Immune checkpoint inhibitors targeting the programmed cell death protein 1 (PD-1) – programmed death-ligand 1 (PD-L1) axis1,2 and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4)3,4 have dramatically improved the treatment options for a growing number of tumor entities including advanced nonsmall cell lung cancer (NSCLC). However, only some patients respond to immune checkpoint blockade (ICB) and several mechanisms of de novo or acquired resistance are progressively being described,5–8 so that durable disease control is achieved only in a minority of ICB-treated patients.

Hence, predictive biomarkers are urgently needed for identification of the most likely responding patients and to offer better suited treatment alternatives for the others. While numerous biomarkers were evaluated, PD-L1 protein expression analyzed by immunohistochemistry (IHC)9,10 and microsatellite instability (MSI)/mismatch repair deficiency are the only ones approved so far.11 However, both of them are far away from being perfect: PD-L1 IHC is limited in both sensitivity and specificity – as well as its heterogeneity, while MSI will identify only a very restricted subset of all ICB responders. In addition, in retrospective molecular analyses complementing clinical trials, tumor mutational burden (TMB) showed
promising results as a predictive marker, \(^1\text{-}^4,^12\) but so far this has not been validated in prospective trials. Furthermore, we and others delineated biological, technological, and bioinformatical parameters impairing correct measurement of TMB in a clinical setting.\(^13\text{-}^15\) Hence, the suitability of TMB as a predictive marker is currently discussed controversially and remains practically challenging.

ICB-related mRNA expression profiling was advocated in several studies as a promising approach enabling comprehensive interrogation of the ICB effector compartment.\(^16,^17\) The characterization of tumor-infiltrating immune cell populations\(^18\) plays a key role in understanding potential anti-tumor effects and the response mediated by the tumor microenvironment (TME). In this context, gene signatures that were described to correlate with the occurrence of specific immune cell populations\(^19\) are of particular interest from a clinical perspective. In a growing number of clinical laboratories, mRNA analysis is performed in parallel to DNA analysis as part of the routine NGS diagnostic work-up of lung adenocarcinoma for the detection of oncogenic gene fusions.\(^20\) Gene expression based immune profiling could be easily integrated in this workflow without the need for additional tissue and would represent an excellent opportunity to incorporate comprehensive TME characterization into the clinical work-up.

In the present study, we demonstrate that targeted mRNA expression profiling in routine diagnostic biopsies of metastatic lung adenocarcinoma is feasible. We confirm the hypothesis that gene signatures related to specific immune cell types – in particular B cell and macrophages – can contribute to the prediction of long-term ICB benefit.

**Material and methods**

**Patient and public involvement**

Neither patients nor the public were involved in the design of the study. The results of the study will be disseminated in public patient Information events.

**Study cohort**

The study cohort included 43 patients diagnosed with stage IV lung adenocarcinoma according to the 8th TNM staging and the current WHO classification and treated with ICB at the Thoraxklinik of Heidelberg University Hospital (Supplement 1 and 2). To analyze the difference of the TME in cases with a significantly divergent clinical course, the study cohort was intentionally enriched for patients showing durable response (of at least 12 months) and showing early progress (within 2 months). Only patients with lung biopsies taken prior to initiation of ICB available and suitable for mRNA expression analysis were included. The majority of patients received ICB as monotherapy (n = 31, 72%), while the remaining patients received ICB together with a platinum doublet (n = 12, 28%). Patients were closely followed up for response with chest-abdomen CT and brain MRI at baseline and every 6–8 weeks (for patients without brain involvement, brain MRI was repeated every 6 months). Clinical data were systematically collected from the patients’ records, and the date of tumor progression for each case was verified by review of radiological images by the investigators. All patients provided signed informed consent for inclusion of their clinical data and specimens in our Lung Biobank and use in research projects, according to the recommendation of the Heidelberg University ethics committee (S-270/2001). The retrospective study presented here was approved separately by the ethics committee of Heidelberg University (S-145/2017).

**TCGA lung adenocarcinoma cohort**

Gene expression and clinical data of the TCGA were downloaded from the PanCanAtlas webpage at Genomic Data Commons repository ([https://gdac.broadinstitute.org/about-data/publications/pancanatlas](https://gdac.broadinstitute.org/about-data/publications/pancanatlas)). Samples diagnosed as lung adenocarcinoma (LUAD) and of the type “primary solid tumor” (code: 01) were included in the analysis. With the exception of two recurrent tumors, all LUAD tumors analyzed in the TCGA could be included (515/517 = 99.6%).

**Nucleic acid extraction and semiconductor sequencing**

Starting with formalin-fixed and paraffin-embedded (FFPE) biopsy samples, tumor tissue was macerated to achieve a histological tumor cell content of at least 30%. DNA and RNA extraction, library preparation, and semiconductor sequencing were performed as described previously.\(^21\) Data analysis was performed using the Ion Torrent Suite Software (Thermo Fisher Scientific Inc., Waltham, MA). Variant calling was performed with the variant caller plugin (version 5.0 up to 5.6) within the Torrent Suite Software and the IonReporter package using a corresponding bed-file containing the coordinates of the amplified regions.\(^22\)

**Targeted mRNA expression profiling**

RNA extracts passing the following steps of quality control were considered as suitable for gene expression analysis: RNA concentration of at least 10 ng/µl, sufficient RNA purity with a A260/A280 in the range 1.7–2.3 and sufficient RNA integrity with at least 90% of the fragments longer than 100 nucleotides. Targeted mRNA expression profiling was performed using the NanoString nCounter gene expression platform (NanoString Technologies, Seattle, WA) using a 770-gene panel (PanCancer Human IO360 Panel) focused on the complex interplay between the tumor, the tumor microenvironment, and the immune response in cancer. Per sample, 100 ng of total RNA in a final volume of 5 µl were mixed with a 3’ biotinylated capture probe and a 5’ reporter probe tagged with a fluorescent barcode from the PanCancer Human IO360 gene expression code set. Probes and target transcripts were hybridized at 65°C for 18 hours according to the manufacturer's recommendations. Hybridized samples were run on the NanoString nCounter preparation station using the high-sensitivity protocol, in which excess capture and reporter probes are removed and transcript-specific complexes are immobilized on a streptavidin-coated cartridge. The samples were scanned at maximum resolution on the nCounter Digital Analyzer.
**Immunohistochemistry**
For immunohistochemical staining of PD-L1, CD3, CD30, and CD21, 3 μm thick paraffin sections were prepared. Samples used for IHC and mRNA were consecutive sections from the same FFPE block minimizing a possible interference with tumor heterogeneity. Deparaffinization and tissue staining were performed using a Benchmark Ultra IHC Staining module according to standard protocols (Ventana PD-L1 assay, clone SP263; CONFIRM anti-CD3 Primary Antibody, clone 2G6; CONFIRM anti-CD20 Primary Antibody, clone L26; Rabbit Monoclonal Primary Antibody anti CD21, clone EP3093; all four Roche, Mannheim, Germany). Hematoxylin was used for counterstaining of cell nuclei. IHC stainings were evaluated by a specialist in pathology and scoring of PD-L1 was performed according to standardized criteria.\(^9\) CD20 expression was evaluated semiquantitatively and the number of tertiary lymphoid structures (TLS) was reported by a pathologist.

**Data processing**
Statistical analysis and graphics generation were performed using the programming language R. Expression data were preprocessed by background subtraction and subsequent sample normalization. For sample normalization, the 20 panel genes with the lowest coefficient of variation and an expression level of at least 100 in The Cancer Genome Atlas (TCGA) lung adenocarcinoma (LUAD) dataset were used as housekeepers (AKT1, API5, DNAJC14, EIF2B4, ELA, ERCC3, GLU1, HDAC3, HMGB1, IFNAR1, MLH1, OAZ1, PUM1, RIKP1, SF3A1, STAT3, TBC1D10B, TLK2, TMUB2, and UBB). The gene expression profile of each sample was scaled by the median expression level of these housekeeping genes. Gene expression data were log2-transformed prior to statistical analysis.

**Absolute and relative scores of immune cell infiltration**
The abundance of 14 immune cell populations (B cells, CD45+ cells, CD56dim, CD8+ T cells, cytotoxic cells, dendritic cells, exhausted CD8+ T cells, macrophages, mast cells, neutrophils, NK cells, T cells, Th1 cells, and Treg cells) was estimated from the mRNA expression of marker genes as described before.\(^19\) Fifty-four of the 60 genes described there (90%) were covered by the 770-gene panel. RNA markers for other immune cell types were not available or could not be validated.\(^19\) The expression of the marker genes in the study cohort was clustered and visualized in a heatmap (Supplement 3). The B cell expression signature included the eight marker genes BLK, CD19, FCRL2, MAA4A1, TNFRSF17, TCL1A, SP1B, and PNOC, the macrophage gene expression signature included the four marker genes CD68, CD84, CD163, and MS4A4A.\(^19\) A total score of tumor-infiltrating lymphocytes (total TILs) was calculated as the average of the scores of 11 immune cell populations (all populations, but excluding dendritic cells, mast cells, and Treg cells). In addition to the absolute scores of the 14 cell populations, relative scores of the 11 cell populations with respect to the total level of immune activity were calculated as residuals with respect to a linear regression fit against the total TIL score.

**Heatmaps and hierarchical clustering**
For heatmap displays, each of the cell populations (or genes) was centered (but not scaled) with respect to the mean abundance (or mean mRNA expression) over the samples. Abundances (or expression levels) above the mean appear in red, abundances below the mean in green. Hierarchical clustering was performed using Pearson correlations as similarity measure and the average linkage as measure of distances between clusters.

**Immune gene signatures**
The T cell-infiltrated gene expression profile (GEP) was calculated as average of the expression of the 16 genes of the 18 signature genes that were interrogated by the NanoString assay (IL2RG, CXCR6, CD3D, CD2, TAGAP, HLA-DRA, CCL5, NKG7, CD3E, HLA-E, GZMB, GZMK, CXCL13, CXCL10, IDO1, LAG3, STAT1, CITTA) as described.\(^23\) The T effector and IFNγ signature\(^24\) were calculated as average of the expression of the eight signature genes (CD8A, GZMA, GZMB, IFNG, EOMES, CXCL9, CXCL10, and TBX21) was calculated as average of granzyme A (GZMA) and perforin (PRF1) expression.

**Statistical analysis**
The suitability of cell populations as prognostic markers was analyzed using Cox regression with respect to progression-free survival (PFS) after ICB initiation. The suitability of cell populations as predictive markers was analyzed using logistic regression based on ICB benefit (long term response vs. rapid progress). Markers were considered as continuous variables and both analyses were performed without cutpoints. Hazard ratios (HRs) and odds ratios (ORs) per doubling of marker levels were reported including 95% confidence intervals (CIs). Multiple testing was accounted for by correcting \(p\)-values using the Benjamini–Hochberg method. Candidate markers were further analyzed by receiver operator characteristic (ROC) curves including significance assessment with the unpaired Wilcoxon test.

Correlation of the mRNA marker signature for B cells with the level of CD20-positive immune cell infiltrates was assessed using the Jonckheere–Terpstra test for trends as implemented in the R package clinfun. Correlation the of mRNA marker signature for B cells with the presence of TLS was assessed using the Wilcoxon test.

A list of candidate marker genes was compiled using the threshold \(p < .05\) for uncorrected \(p\)-values.

**Results**
The retrospective study cohort included 43 metastatic lung adenocarcinoma patients that were treated with immune checkpoint inhibitors either as monotherapy or combination therapy at the Heidelberg University Hospital, most of them
between 2016 and 2020 (Figure 1). There were 16 long-term responders (LTR; durable response of 12 months or more), 21 rapid progressors (RP; disease progression within 2 months) and 6 patients with an intermediate duration of response (IR). The study cohort was intentionally enriched for LTR and RP by inclusion of all patients that met the predefined inclusion criteria (see Material and Methods). Gene expression profiles including 770 genes were generated from lung biopsies using the NanoString nCounter technology (Supplement 4). The focus of the gene expression assay on immune biology was reflected by annotation of 430 of the investigated genes (55.8%) to the Gene Ontology (GO) category “immune system process” (GO: 0009987).

**Absolute and relative quantification of immune cell populations**

The abundance of 14 specific immune cell populations (“absolute scores”) was estimated from the mRNA expression profiles as recently described. Hierarchical clustering separated samples into immunological “hot” tumors versus immunological “cold” tumors (Figure 2a). Response to ICB was significantly different ($p = .046$) with a higher percentage of LTR within the group of “hot” tumors (52%) compared to “cold” tumors (20%). Pairwise correlations between the different immune cell types were either significantly positive or non-significant. Highly positive pairwise correlations were detected between cytotoxic cells, T cells, CD8+ T cells and exhausted CD8+ T cells (all $R > 0.71$) as well as between macrophages and CD45+ cells ($R = 0.75$).

In a second analysis, the TME was characterized by a total TIL score plus relative immune cell scores instead of absolute immune cell scores. Following a concept similar to “partial correlations,” we calculated relative scores of each immune cell population as residual in the linear regression of the absolute scores against total TILs. Figure 2b shows the contribution of the different immune cell types to total TILs in each of the tumors.

**Immune cell populations as predictive markers for response to immunotherapy**

Immune cell populations were correlated with PFS after ICB using univariate Cox proportional hazard models and with ICB response using univariate logistic regression. In general, high absolute immune scores were associated with a lower risk of

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**Figure 1.** Study cohort, routine molecular pathology work-up and mRNA expression profiling of immune-related genes.
progression (Figure 3a). Controlling the false discovery rate (FDR < 10%) two of the absolute scores showed a significant association: B cells with HR = 0.66 (0.52–0.84, p = .00074) and CD45+ cells with HR = 0.61 (0.42–0.89, p = .01). Furthermore, high absolute scores of B cells, CD45+ cells and macrophages were associated with better ICB response, OR = 2.1 (1.2–4.0, p = .012), OR = 2.3 (1.2–5.4, p = .029) and OR = 2.6 (1.1–7.5, p = .046), respectively.

Next, we correlated relative immune cell and total TIL scores with PFS and response to therapy (Figure 3b). High relative scores of B cells and high total TILs were associated with significantly longer PFS with HR = 0.6 (0.43–0.84, p = .0033) and HR = 0.62 (0.41–0.94, p = .025), respectively. Furthermore, high relative scores of B cells were associated with significantly better response with OR = 2.0 (1.1–4.5, p = .048), while high total TILs were associated with better response without reaching significance with OR = 2.4 (1.1–6.2, p = .052).

Up-regulation of the B cell signature was associated with favorable clinical outcome after ICB treatment, an observation that could be either therapy-independent or ICB-specific. To differentiate between the two possibilities, we additionally investigated immune cell populations as prognostic markers in a large cohort of conventionally treated lung adenocarcinoma patients (TCGA LUAD, n = 506, Supplement 5). In the TCGA cohort, high absolute scores of B cells and high relative scores were associated with only slightly better PFS, HR = 0.91 (0.85–0.98, p = .0098) and HR = 0.90 (0.81–1.01, p = .067), respectively. By contrast, the association in ICB-treated patients was much stronger,
Figure 3. Immune cell scores as positive predictive markers for ICB benefit. Absolute quantification (a,c-e,h) and relative quantification of immune cell populations (b) from mRNA expression data. (a,b) Odds ratios (OR) of long-term responders (LTR, n = 16) versus rapid progressors (RP, n = 21) and hazard ratios (HR) of PFS (n = 43). ORs and HRs relate to a doubling of the immune cell abundance. (c-e) ROC analysis of B cells, CD45+ cells and macrophages. (f) ROC analysis of PD-L1 mRNA. (g) ROC analysis of PD-L1 protein including cutpoints relating to positivity of 50% and 1% of tumor cells (black dots). (h) Correlation analysis of B cells and PD-L1 protein. An increase of one on the y-axis corresponds to doubling of the B cell abundance. HR = hazard ratio per doubling of abundance, OR = odds ratio per doubling of abundance.
supporting a role of B cells as predictive marker specifically linked to ICB treatment.

**ROC curve analysis and comparison with established gene expression signatures and PD-L1 expression**

Absolute scores of B cells, CD45+ cells and macrophages were significantly predictive with AUC = 0.77 (95% CI 0.61–0.93, \( p = .0055 \)), AUC = 0.73 (0.56–0.89, \( p = .019 \)) and AUC = 0.72 (0.55–0.89, \( p = .026 \)), respectively (Figure 3c-e). Immune gene signatures such as the T cell-inflamed GEP\(^{25} \) and T-effector/INF\( \gamma \) signature\(^{24} \) were developed to predict the response to ICB but neither of them nor cytolytic activity showed a significant association with long-term ICB benefit in the study cohort (Supplement 6).

PD-L1 protein expression evaluated by IHC represents an approved companion diagnostic test for pembrolizumab monotherapy in NSCLC.\(^{10} \) However, neither PD-L1 mRNA nor PD-L1 protein expression predicted ICB response in the study cohort (Figure 3f-g). In a stratified analysis for therapy type, PD-L1 protein expression was significantly associated with response in the subgroup of patients receiving combination therapy, but not in the subgroup of patients receiving monotherapy (Supplement 7). The latter negative result can potentially be explained by a selection bias concerning PD-L1 expression and a very low number of PD-L1-negative tumors in the monotherapy subcohort as a consequence. Of the 30 tumors treated with monotherapy and with PD-L1 staining available, 22 (73%) had 50% or more PD-L1-positive tumors cells, while 29 (97%) had 1% or more PD-L1-positive tumor cells. Interestingly, correlation between B cells and PD-L1 protein was absent in the study cohort (Figure 3h) supporting a role of B cells as independent biomarker for the prediction of immune therapy response complementary to PD-L1.

**Validation of B-cell infiltration using immunohistochemistry**

To validate the B cell infiltrates detected by the gene expression analysis, all cases from the study cohort with sufficient tissue available (\( n = 26 \)) were stained for CD20 (Figure 4). Both, the presence of TLS as well as levels of CD20-positive cells correlated significantly with the mRNA marker of B cell infiltration (\( p = 9.6e-05 \) and \( p = .0013 \)). Two example cases (one LTR and one RP) with very high and very low levels of the mRNA marker were additional stained for CD3 and CD21. The patterns of immune cell populations observed in the first case included CD21-positive networks of follicular dendritic cells and were indicative of TLS, while CD20-positive B cells were low in the second case (Supplement 8).

**Bivariate analysis of specific immune cell types and total TILs**

Relative B cell scores and total TILs were combined to separate LTR from RP. To this end both cell scores were included as independent variables in a bivariate logistic regression model to predict the response status (LTR or RP). Separation by the model was significant (\( p = .012 \)) with OR = 2.0 (1.0–4.7) for

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**Figure 4.** Validation of the B cell mRNA signature by CD20 IHC. (a) Significant correlation of the mRNA signature with the presence of tertiary lymphoid structures (TLS). (b) Significant correlation of the mRNA signature with the abundance of CD20-positive immune cells. (c) Example cases showing high, intermediate and low infiltration of CD20-positive B cells.
Figure 5. Prediction of ICB benefit by combining B cells or macrophages with total TILs. Bivariate logistic regression to separate LTR from RP combining total TILs and the relative B cell score or the relative macrophage score. (a,c) Separating LTR from RP using the logistic regression model. An increase of one on the x-axis or y-axis corresponds to a double abundance of the corresponding cell population. (b,d) ROC curve showing the performance of the logistic model when varying the cutpoint.

Table 1. Multivariate analysis of absolute B cell scores. Cox regression was used to analyze PFS after immunotherapy. Logistic regression to analyze response to immunotherapy (LTR vs. RP).

| Variable                        | Cox regression | Logistic regression |
|---------------------------------|----------------|---------------------|
|                                 | HR             | p                   | OR             | p       |
| B cells                         | 0.64 (0.47–0.87) | 0.0046               | 2.3 (1.3–5.2)  | 0.016   |
| Sex (female vs. male)           | 2.2 (0.91–5.1) | 0.082               | 0.18 (0.019–1.2) | 0.092   |
| Age (per year)                  | 0.95 (0.9–1)   | 0.033               | 1.1 (1–1.3)    | 0.029   |
| Prior therapy (treated vs. naive) | 0.34 (0.12–0.98) | 0.045               | 4.6 (0.61–54)  | 0.17    |
| Therapy type (combination vs. mono) | 0.9 (0.36–2.2) | 0.81                | 0.35 (0.027–3) | 0.37    |
B cells and OR = 2.3 (1.0–6.3) for total TILs. Also, prediction of PFS was significant (p = .0031) in bivariate Cox regression with HR = 0.63 (0.44–0.89) for B cells and HR = 0.68 (0.44–1) for total TILs. The performance of the model in the study cohort (which also served as training set) is shown in Figure 5ab. Prediction sensitivity was 75% at a specificity of 71% for the cutpoint suggested by the logistic model. Varying of the cutpoint (corresponding to a parallel shift of the decision horizon in Figure 4a) resulted in an AUC of 0.78 (0.62–0.93) in an ROC analysis. A model combining total TILs and relative macrophagescores instead of B cells performed slightly inferior (Figure 5c,d). While the results of the bivariate analyses support the feasibility of prediction models including a specific immune cell marker (such as B cells or macrophages) together with a marker of overall immune reaction (such as total TILs), we are aware that the sample size in the study cohort is too small for fine tuning of a cutpoint and for valid estimation of prediction sensitivity and specificity.

**Multivariate analysis of B cells**

Absolute B cell scores were analyzed in a multivariate analysis including sex, age, prior therapy and therapy type (Table 1). B cells remained a significant prognostic factor in both multivariate Cox regression and multivariate logistic regression, HR = 0.64 (0.47–0.87, p = .0046) and OR = 2.9 (1.3–5.2, p = .016). Also, relative B cells scores remained a prognostic factor in both multivariate Cox regression and multivariate logistic regression (data not shown).

**Potentially confounding factors**

The association of B cells and total TILs with ICB benefit was investigated in a subgroup analysis (Figure 6). Both markers were predictive in patients treated by ICB monotherapy (OR = 2.3, p = .017 and OR = 3.2, p = .042), while no significant association was observed in patients treated with combination therapy (OR = 1.5, p = .52 and OR = 0.9, p = .9). Neither ICB benefit (Supplement 9A) nor the abundance of specific immune cell types (data not shown) was associated with prior therapies. Furthermore, ICB benefit was not associated with therapy type (Supplement 9B). Status of driver mutations had been determined during routine molecular diagnostics using targeted NGS: Twenty-three tumors of the study cohort had activating KRAS mutations, 19 had deleterious or probably deleterious TP53 mutations, 9 had non-synonymous KEAP1 mutations. Neither ICB benefit (Supplement 9C-E) nor the abundance of specific immune cell types (data not shown) was associated with mutation status. Thus, neither prior therapies nor driver mutation status interfered with the association between ICB benefit and the immune cell markers.

**Genes associated with ICB benefit**

We analyzed the suitability of the 770 genes as predictive markers using ROC curves and significance assessment with the unpaired Wilcoxon test. A list of 79 significant (p < .05) candidate markers emerged and was analyzed in a heatmap (Figure 7, AUCs between 0.69 and 0.81). The majority of these genes (n = 76) correlated positively with ICB benefit, while three genes (SOX2, HDAC4, and G6PD) correlated negatively. The gene list partitioned the tumors into three clusters, T1 including 17% LTR, T2a including 25% LRT and T2b including 65% LTR. The expression patterns in these tumor clusters were different in the two gene clusters G1 and G2: The cluster T2b with the highest proportion of LTR was characterized by high expression of cytotoxic cell
markers (cluster G1) and macrophage markers (cluster G2). The cluster T2a with a moderate number of LTR was characterized by high expression of cytotoxic cell markers, while the cluster T1 with the lowest number of LTR was characterized by low expression of cytotoxic cell and macrophage markers. The highest prediction accuracy was reached for the genes IGF2R (AUC = 0.8, 95% CI 0.65--0.95), IL7R (AUC = 0.77, 95% CI 0.61--0.92) and HCK (AUC = 0.76, 95% CI 0.6--0.92), see Supplement 10. The confidence intervals of the genes included the AUC values for B cells, macrophages, and total TILs. Thus, there was no evidence that a gene-based stratification could outperform an immune cell-type-based stratification.

**Discussion**

Investigating the immune TME of NSCLC from LTR and RP we observed that the total level of immune cell infiltration defined a group of immunological "hot" tumors and was associated with a higher probability of response to ICB. Cytotoxic cell including CD8+ T cells and CD8+ exhausted T cells contributed with about 30% to total TILs (Figure 2b), in line with previous reports which assign a major role to these cell types in biological concepts and clinical response prediction. Additionally, we observed that macrophages and specifically the abundance of B cells were associated with improved PFS of patients. The identification of macrophages fits well into the
current concept that macrophages appear to mediate T cell recruitment and ICB response. B cells remained an independent predictor of PFS when analyzed in a bivariate model together with total TILs and when analyzed in a multivariate model together with key clinico-pathological characteristics of the patients.

While B cells are known to be associated with autoimmune events during ICB treatment, they were only very recently reported to play a role in ICB response prediction and data on NSCLC patients have not yet been published until now. After antigen exposure, B cells can be broadly subdivided into those that contribute to antibody-mediated immune response and those that regulate immune response similar to the concept of regulatory T cells. These regulatory B cells (Breg) characterized by IL10 production were shown to interfere with immune responses in several diseases including cancer. IGF2 whose receptor IGF2R was upregulated and predictive in our study, appears to be one of the factors that can influence Breg function. By contrast, there are also data attributing an antigen-presenting and pro-inflammatory role to B cells. This is in line with a recent report of Petitprez and coauthors that B cells, partially located in TLS, were strong positive predictors of ICB response in sarcoma patients receiving pembrolizumab in a phase 2 clinical trial. Similar observations were made by Griss et al. and Cabrita et al. who investigated the role of B cells in melanoma. Griss and coauthors identified a specific B cell population with similarities to Bregs, which they termed TIPB and which appears to recruit CD8-positive T cells in melanomas. Their exploratory analysis in a small cohort of patients who received anti-CD20 antibodies showed that reduction of B cells in melanoma was associated with attenuated immune response and reduced TLS. Cabrita and coauthors investigating samples of metastatic melanoma noted that the spatial distribution of co-occurring CD8-positive T cells and CD20-positive B cells resembled TLS and was associated with improved PFS. Of note, the majority of their samples in this study were derived from lymph node metastases which might influence results. Nevertheless, consistent with our data, they also observed B cells and a TLS-related signature in non-lymph node samples.

Up-regulation of the B cell signature was associated with a higher probability of long-term response and prolonged progression-free survival after ICB treatment. Since two previous studies identified a prognostic impact of B-cells and TLS in small cohorts of lung cancer patients of various disease stages, we employed the TCGA dataset of lung adenocarcinomas for an independent analysis. In the TCGA LUAD cohort, which was sequenced at times when ICB treatment was still in development and not standard of care, the influence of B cells on PFS was weak (HR per doubling of score = 0.91). By contrast, the influence of B cells on PFS after ICB treatment was much stronger (HR per doubling of score = 0.66) supporting a possible role of B cells as predictive biomarker for ICB efficacy beyond a prognostic value only. However, differences in the clinico-pathological characteristics – advanced tumors in the study cohort, but surgically resected, mostly early-stage tumors in the TCGA cohort – represent a limitation for a comparison. Thus, further investigation of biomarkers for B cell infiltration, optimally in the setting of randomized clinical trials and including an interaction analysis, is recommended.

A limitation of our study is its single-center character and the limited number of samples available for analysis which was due to careful selection of patients and the associated samples. Analyzed cases had to adhere to clinical and technical inclusion criteria (see Material and Methods) and we did not include samples with lymph node metastases as they may influence data derived from profiling of the tumor immune microenvironment. In addition, a careful analysis of potential confounding factors was performed. We did not note an influence of any mutated genes analyzed in our sample set including mutated KEAP1, a genomic marker that was reported to influence ICB treatment outcome. B cells remained a significant prognostic factor in a multivariate analysis including sex, age, prior therapy, and therapy type.

In summary, our data suggest that, in addition to the molecular profile of the tumor cell compartment, analysis of the effector compartment, i.e. the tumor immune microenvironment, is important to identify NSCLC patients who likely benefit from ICB treatment. According to our results, B cells appear to influence clinical outcome and further studies are warranted to validate these findings. Since our study used routine diagnostic FFPE material from lung biopsies and did not require a specific clinical and molecular work-up beyond already established workflows, implementation in routine diagnostic procedures would be feasible if confirmed clinically relevant in the future.

**Abbreviations**

| AUC                        | area under the curve |
|----------------------------|----------------------|
| CI                         | confidence interval  |
| FFPE                       | formalin-fixed and paraffin-embedded |
| GEP                        | gene expression profile |
| HR                         | hazard ratio         |
| ICB                        | immune checkpoint blockade |
| IR                         | intermediate responder |
| LTR                        | long-term responder  |
| LUAD                       | the lung adenocarcinoma data set of TCGA |
| MSI                        | microsatellite instability |
| NSCLC                      | non small cell lung cancer |
| OR                         | odds ratio           |
| ROC                        | receiver operator characteristics |
| RP                         | rapid progressor     |
| TCGA                       | the cancer genome atlas |
| TILs                       | tumor infiltrating lymphocytes |
| TLS                        | tertiary lymphoid structure |
| TME                        | tumor microenvironment |

**Authors’ contributions**

Jan Budczies: Conceptualization, Methodology, Software, Formal analysis, Writing – Original Draft, Writing – Review & Editing, Visualization, Supervision, Project administration Martina Kirchner: Investigation, Writing – Original Draft, Writing – Review & Editing Klaus Kluck: Software, Formal analysis, Writing – Review & Editing Daniel Kazda: Writing – Original Draft, Writing – Review & Editing, Visualization Julia Glade: Investigation, Writing – Review & Editing Michael Allgäuer: Writing – Original Draft, Writing – Review & Editing Mark Kriegsmann: Investigation, Writing – Review & Editing Claus-Peter Heußel: Investigation, Writing – Review & Editing Felix J. Herth: Resources, Writing – Review & Editing Hauke Winter: Resources, Writing – Review & Editing Michael Meister: Resources, Writing – Review & Editing Thomas Muley: Resources, Writing – Review & Editing Stefan Fröhling: Conceptualization, Writing – Review & Editing Solange Peters: Conceptualization, Writing – Review & Editing Barbara Seliger: Conceptualization, Writing – Review & Editing Peter
Disclosure of interests

Dr. Budczies has nothing to disclose.

Dr. Kirchner reports personal fees from QuIP, outside the submitted work.

Mr. Kluck has nothing to disclose.

Dr. Kazdal reports personal fees from AstraZeneca, personal fees from Bristol-Myers Squibb GmbH, personal fees from Pfizer Pharma GmbH, outside the submitted work.

Dr. Glade has nothing to disclose.

Dr. Allgäuer has nothing to disclose.

Dr. Kriegsmann has nothing to disclose.

Dr. Heußel reports personal fees from Schering-Plough, personal fees from Pfizer, personal fees from Basilea, personal fees from Boehringer Ingelheim, personal fees from Novartis, personal fees from Roche, personal fees from Astellas, personal fees from Gilead, personal fees from MSD, personal fees from Lilly, personal fees from Internune, personal fees from Fresenius, personal fees from Essex, personal fees from AstraZeneca, personal fees from Bracco, personal fees from MEDA Pharma, personal fees from Chiesi, personal fees from Siemens, personal fees from Covidien, personal fees from Pierre Fabre, personal fees from Grifols, personal fees from Bayer, personal fees from Siemens, personal fees from Pfizer, personal fees from MeVis, personal fees from Boehringer Ingelheim, personal fees from German Cancer for Lung Research, outside the submitted work.

Dr. Herth has nothing to disclose.

Dr. Winter has nothing to disclose.

Dr. Meister reports grants from German Center for Lung Research, during the conduct of the study.

Dr. Muley reports grants, personal fees and non-financial support from Roche Diagnostics, grants from Chugai, grants from Hummingbird Diagnostics GmbH, outside the submitted work; In addition, Dr. Muley has patents Roche Diagnostics pending.

Dr. Fröhling reports personal fees from Amgen, grants from AstraZeneca, personal fees from Bayer, personal fees from Eli Lilly, grants and personal fees from PharmaMar, grants and personal fees from Roche, outside the submitted work.

Dr. Peters has received education grants, provided consultation, attended advisory boards, and/or provided lectures for: Abbvie, Amgen, AstraZeneca, Bayer, Biocartis, Bioinvent, Blueprint Medicines, Boehringer-Ingelheim, Bristol-Myers Squibb, Clovis, Daiichi Sankyo, Debiopharm, Eli Lilly, F. Hoffmann-La Roche, Foundation Medicine, Illumina, Janssen, Merck Sharp and Dohme, Merck Serono, Merrimack, Novartis, Pharma Mar, Pfizer, Regeneron, Sanofi, Seattle Genetics, Takeda and Vaccibody, from whom she has received honoraria (all fees to institution).

Dr. Seliger has nothing to disclose.

Dr. Schirmacher has nothing to disclose.

Dr. Thomas reports grants, personal fees and non-financial support from AstraZeneca, grants, personal fees and non-financial support from Bristol-Myers Squibb, personal fees and non-financial support from Boehringer Ingelheim, personal fees and non-financial support from Celgene, personal fees and non-financial support from Chugai, personal fees and non-financial support from Lilly, personal fees and non-financial support from MSD, personal fees and non-financial support from Novartis, personal fees and non-financial support from Pfizer, grants, personal fees and non-financial support from Roche, grants, personal fees and non-financial support from Takeda, outside the submitted work.

Dr. Christopoulos reports grants and personal fees from AstraZeneca, grants and personal fees from Novartis, grants and personal fees from Roche, grants and personal fees from Takeda, personal fees from Boehringer, personal fees from Chugai, personal fees from Pfizer, outside the submitted work.

Dr. Stenzinger reports personal fees from AstraZeneca, personal fees from Bayer, personal fees from BMS, personal fees from Illumina, personal fees from Novartis, personal fees from Seattle Genetics, personal fees from Takeda, personal fees from ThermoFisher, personal fees from AstraZeneca, personal fees from Bayer, personal fees from BMS, personal fees from Illumina, personal fees from MSD, personal fees from Novartis, personal fees from Roche, personal fees from Seattle Genetics, personal fees from Takeda, personal fees from ThermoFisher, personal fees from Chugai, personal fees from BMS, personal fees from Bayer, personal fees from Ventana Roche, personal fees from Lilly, during the conduct of the study.

Funding

This work was supported by the German Center for Lung Research (Deutsches Zentrum für Lungenforschung, DZL) and by the German Cancer Consortium (Deutsches Konsortium für Translationale Krebsforschung, DKTK) and partly by a research grant from Bristol-Myers-Squibb (CA209-74M).

ORCID

Barbara Seliger http://orcid.org/0000-0002-5544-4958

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