European Journal of Immunology Clinical·Basic·Translational

Tumor immunology

Clinical

Research Article

# Circulating CD8 lymphocytes predict response to atezolizumab–bevacizumab in hepatocellular carcinoma

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**Due to the lack of biomarkers predictive of response to atezolizumab–bevacizumab, the standard of care for advanced HCC, we analyzed baseline and early on-treatment variation of peripheral lymphocyte populations of 37 prospective patients treated by atezolizumab–bevacizumab and in 15 prospective patients treated by sorafenib or lenvatinib (TKIs). RNAseq analysis followed by RT-PCR validation on patients-derived PBMC was also performed. At first imaging, re-evaluation 13 patients receiving atezolizumab–bevacizumab, showed an objective response, 17 stable disease, while 7 were nonresponders. Baseline CD8+ and CD8+PD-L1+ peripheral lymphocytes were lower in responders versus non-responders (*T*-test, $p = 0.012$ and 0.004, respectively). At 3 weeks, 28 of 30 responders displayed a rise of CD8+PD1+ lymphocytes with a positive mean fold change of 4.35 (±5.6 SD), whereas 6 of 7 nonresponders displayed a negative fold change of 0.89 (±0.84 SD). These changes were not observed in patients treated by TKIs. TRIM56, TRIM16, TRIM64, and Ki67 mRNAs were validated as upregulated in responders versus nonresponders after 3 weeks after treatment start, providing possible evidence of immune activation. Baseline CD8+ and CD8+PD-L1+ peripheral lymphocytes and early changes in CD8+PD1+ lymphocytes predict response to atezolizumab–bevacizumab providing noninvasive markers to complement clinical practice in the very early phases of treatment of HCC patients.**

**Keywords:** HCC · PD-L1 · PD1 · Biomarker

Additional supporting information may be found online in the Supporting Information section at the end of the article.

## Introduction

Immune checkpoint inhibitors (ICIs) lead to long-lasting responses in subgroups of patients across different tumor types including hepatocellular carcinoma (HCC) [1]. Very roughly, combination treatments induce a radiological response in about 30% of patients and achieve a disease stabilization as best response in another 40–50%, with 20–30% remaining nonresponders [1, 2].

Differently from tyrosin kinase inhibitors (TKIs), an additional ICIs specific condition exists at the first radiological

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assessment, the "pseudoprogression" [3], being estimated in up to 5% of apparent progressing HCC [4]. Indeed, tumor infiltration by inflammatory cells may increase tumor size at the first radiologic reassessment, which converts into response at later re-evaluation. No definitive radiological feature distinguishes true progressors from pseudoprogressors, entailing the risk of a premature treatment interruption, in patients who would otherwise benefit from treatment.

Despite the active search, any upfront or early on-treatment biomarker [5, 6] predicts individual HCC response to any of the available drugs [2]. Concerning ICIs, mutational burden, PD-L1 tissue expression, and tumor infiltrate did not show consistent performance in HCC [7]. Moreover, such biomarkers require tissue sampling, which raises the issues of the questionable representativity of a single biopsy in advanced, multinodular, or metastatic HCCs and the difficulty, up to the ethical unfeasibility, of repeated bioptic sampling to assess treatment-induced variations. Accordingly, biomarkers able to early predict response to any therapy for HCC remain an unmet clinical need. Predictive biomarkers might help to early move patients to alternative treatments avoiding losing precious time and deleterious liver function deterioration [8, 9], skip adverse events caused by useless drugs [7], and maintain pseudoprogressors on treatment. Testing circulating immune cells is minimally invasive, repeatable, readily available, and may reflect response to immunotherapy [10–12]. Mechanistically, PD-L1 aberrantly expressed by cancer and tumor-infiltrating cells binds to PD1 expressed on lymphocytes, thus activating resting programs and inhibiting their antitumor cytotoxicity [13]. Blockade of the PD-L1-PD1 axis restores T-cell activity and function against viruses and malignant cells via mechanisms that are only partially understood [14, 15]. Based on this background, we assessed the predictive accuracy of a very simple, cheap, and ready immunophenotypic test by assessing whether the baseline and early on-treatment variation of CD8+, PD1+, PD-L1+, CD8+PD1+, and CD8+PD-L1+ peripheral lymphocytes might predict the response to the combination treatment with atezolizumab (anti-PD-L1) and bevacizumab (anti-VEGF) in advanced HCC.

To identify whether immunophenotypic changes observed on PBMCs were really associated with an immune response, an RNAseq based approach was adopted to explore gene expression variation in responder versus nonresponder patients.

## Results

### Intersample and interday reproducibility

We verified the reproducibility of our readings in different blood samples from the same patients taken on the same day as well as in different blood samples from the same patients taken on different days in close proximity. Three patients were assessed on the same day by testing two different blood samplings. Two more patients were tested before immunotherapy started on two different days (the day of drug infusion and 5 days before). Table S1 shows very high reproducibility across these assays.

### Baseline immunophenotype and patient outcome

Among the 37 HCC patients receiving atezolizumab–bevacizumab, 30 (81.1%) were responders at the 8–10 weeks imaging assessment, including 13 (35.1%) partial response and 17 (45.9%) stable disease, whereas the remaining 7 (18.9%) experienced a disease progression.

Baseline (T0) CD8+ lymphocytes were lower in responders versus nonresponders (mean ± SD: 70 ± 28.7 vs. 95 ± 5; *T*-test, $p = 0.012$, Fig. 1A). Similarly, responders patients showed baseline CD8+PD-L1+ lymphocytes lower than nonresponders (mean ± SD: 31.4 ± 13.1 vs. 41 ± 2.3; *T*-test: $p = 0.01$, Fig. 1B). ROC curve analysis showed a sensitivity and specificity, respectively, of 0.55 and 1 (cut-off 81.8% and AUC 0.77) for CD8+ lymphocytes and 0.74 and 1 (cut-off 37.37% and AUC 0.77) for CD8+PD-L1+ lymphocytes. No other baseline parameter including PD1+ (Fig. 1C), CD8+PD1+ (Fig. 1D), and PD-L1+ (Fig. 1E) lymphocytes, AFP, NLR, eosinophil count, associated with response. A negative correlation was observed between baseline AFP and CD8+ or CD8+PD-L1+ lymphocytes (Pearson's correlation $R = -0.45$; $p = 0.024$ and $R = -0.63$; $p = 0.006$, respectively) and between eosinophil count and NLR or CD8+PD-L1+(Pearson's correlation $R = -0.52$; $p = 0.01$ and $R = -0.61$; $p = 0.016$, respectively).

### Early on treatment change in immunophenotype and patient's outcome

To confirm that atezolizumab binds to the PD-L1 epitope identified by the antibody used in our assays, one patient was tested before and 24 h after treatment displaying a reduction by 50%. The percentage of PD-L1+ lymphocytes in all patients showed a reduction at the 3-week (T1) evaluation (mean ± SD from T0 to T1 38.7 ± 4.7 to 32.5 ± 5.5; *T*-test, $p<0.0001$, Fig. 2A), that did not predict the response to treatment (Fig. 2B).

No variation of CD8+ and PD1+ peripheral lymphocytes was found in the whole group (Fig. 2C,D). Conversely, at 3-week, a rise of CD8+PD1+ lymphocytes was observed in the whole cohort (Fig. 2E). Remarkably, responders displayed an increase, with a positive fold change (FC) in 28 of 30, while nonresponders showed a decrease with a negative FC in 6 of 7 patients (mean FC ± SD in responders versus nonresponders: 4.35 ± 5.6 vs. 0.89 ± 0.84; *T*-test, $p = 0.003$; Chi-square test 16.52, $p < 0.00001$, Fig. 2F,G). Moreover, CD8+PD1+ FC differed in nonresponders, responders, and stable disease (Fig. 2H). The patient with a pseudoprogressive disease followed by a partial response displayed a CD8+PD1+ FC of 7.5, among the highest observed. The sensitivity and specificity for CD8+PD1+ lymphocytes FC resulted to be, respectively, 0.78 and 0.93, with a

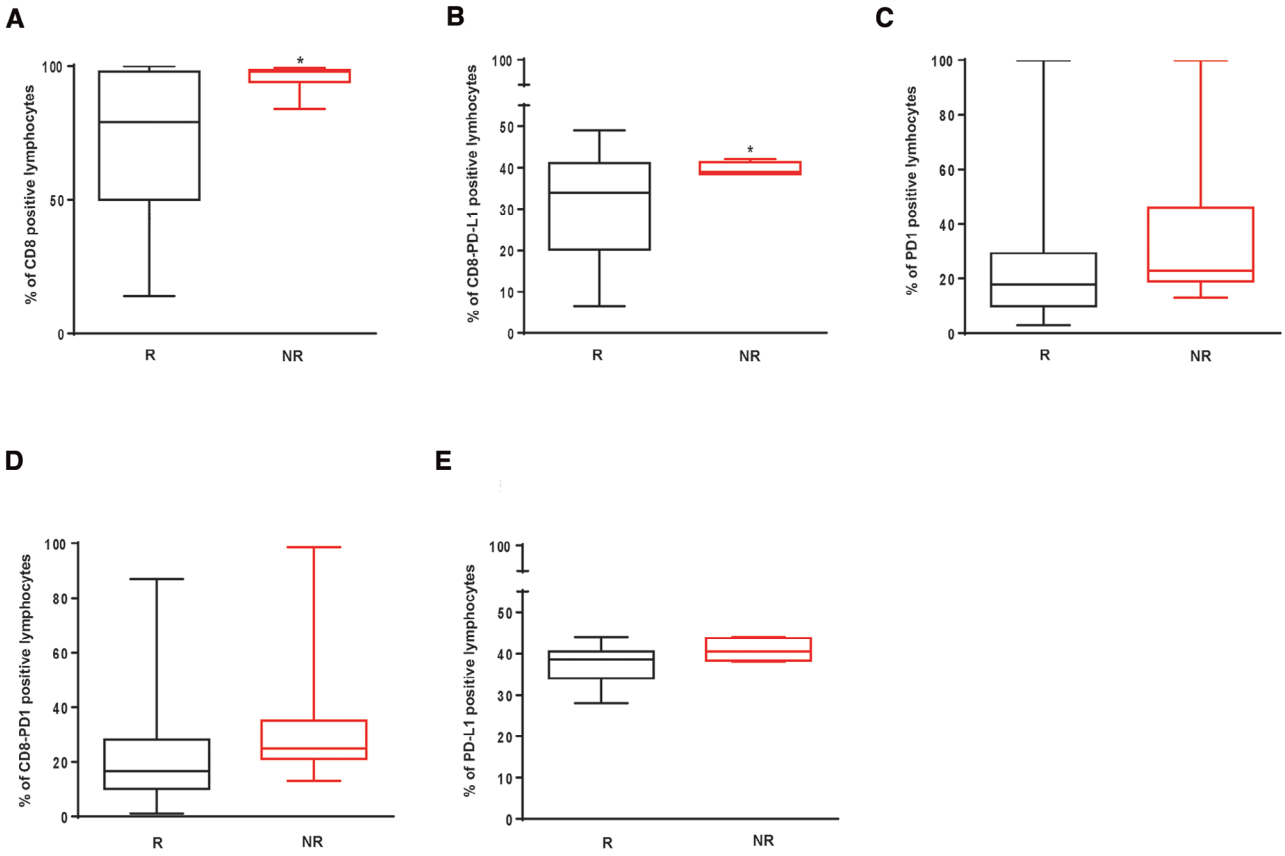


**Figure 1.** Baseline lymphocyte populations in responders versus nonresponders.
Box-plot graphic representation of CD8+ (A), CD8+PD-L1+ (B), PD1+ (C), CD8+PD1+ (D), PD-L1+ (E) baseline lymphocytes in responder (R) and nonresponder (NR) patients subsequently treated with atezolizumab–bevacizumab. Responders include patients with partial response and patients with stable disease. $^{*}p < 0.05$ by unpaired *t*-test.

cut-off value of 0.93 and an AUC of 0.9. Despite this value, a positive or negative FC is a simpler and more informative marker predicting response. The most relevant results are summarized in Fig. 2I.

We also assessed changes in absolute numbers of informative lymphocyte populations. Despite total lymphocyte number does not vary early on treatment, informativeness of dynamic changes observed for PD-L1+, CD8+PD1+, and CD8+PD1+ FC was confirmed also by analyzing absolute cell numbers (Fig. S1).

A correlation was observed between baseline AFP and CD8+PD1+ and CD8+PD-L1+ lymphocytes FC at 3-week (Pearson's correlation $R = 0.82$; $p < 0.001$ and $R = 0.97$; $p < 0.001$, respectively) suggesting that atezolizumab–bevacizumab in AFP producing HCCs determines a stronger trigger on these lymphocyte populations.

Our findings suggest baseline CD8+ and CD8+PD-L1+ lymphocytes and early on-treatment rise of CD8+PD1+ lymphocytes as putative predictive noninvasive biomarkers of response to atezolizumab–bevacizumab. The early rise or reduction of CD8+PD1+ lymphocytes, which results in a positive or negative FC, provides an easy and unequivocal parameter.

## Validation of baseline immunophenotype and early on-treatment changes of CD8+PD1+ peripheral lymphocytes and patients' outcome

To reinforce our observation, a further prospective cohort of 18 patients (described in Table S2) prospectively followed by the two enrolling centers was investigated, aiming to validate the putative predictive role of baseline CD8+ and CD8+PD-L1+ peripheral lymphocytes and the early on-treatment changes of CD8+PD1+ peripheral lymphocytes. Among these novel 18 patients, all assessed with the first imaging evaluation after treatment start, 13 patients were responders (7 stable disease and 6 partial response) and 5 patients displayed a progression subsequently confirmed 4–5 weeks later. Baseline CD8+PD-L1+ peripheral lymphocytes were confirmed as lower in responder patients than in nonresponders (mean ± SD: 29.07 ± 12.1 vs. 45.4 ± 4.6; *T*-test: $p = 0.01$; Fig. 2A). Concerning fold changes of CD8+PD1+ lymphocytes early on-treatment, an increase in responder patients was confirmed (mean FC ± SD in responders versus nonresponders: (4.05 ± 2.49 vs. 0.92 ± 0.91; *T*-test, $p = 0.001$; Fig. S2B).

As observed in the previous cohort, the dynamic changes of CD8+PD1+ peripheral lymphocytes, predicted all but one patient

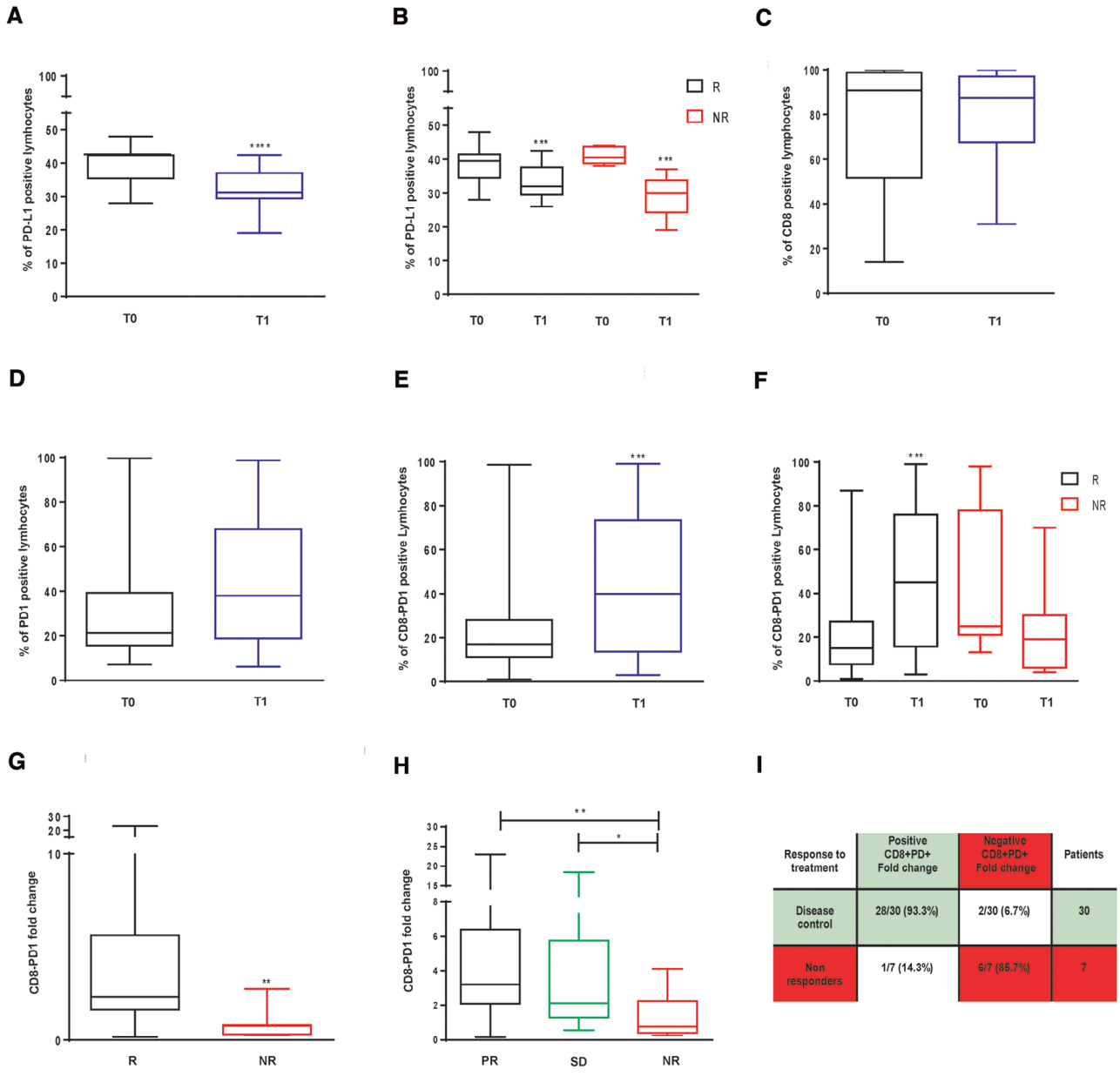


**Figure 2.** Dynamic changes in peripheral lymphocyte populations in the early treatment phase.
Box-plot graphic representation of dynamic variations of: (A) PD-L1+ lymphocytes in all patients and (B) in responders (R) and nonresponders (NR); (C) CD8+ lymphocytes in all patients; (D): PD1+lymphocytes in all patients; (E) CD8+PD1+ lymphocytes in all patients and (F) CD8+PD1+ lymphocytes in responders and nonresponders; (G) CD8+PD1+ lymphocyte FC at 3 weeks after treatment start in responders and nonresponders and (H) in patients with partial response (PR), stable disease (S) nonresponse (NR). (I) Schematic view of CD8+PD1+ lymphocyte FC (T1/T0) in HCC patients showing disease control or nonresponse to atezolizumab–bevacizumab. T0: baseline assessment. T1: 3-week assessment, before the second drug infusion. (H) $^{*}p < 0.05$; $^{**}p < 0.01$ by ANOVA test, $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$ by unpaired *t*-test.

among responders and nonresponders. In detail, the CD8+PD1+ peripheral lymphocyte population showed an increase in 13 of 13 responders, with a positive fold change. Conversely, a decrease of CD8+PD1+ peripheral lymphocytes was observed in 4 of 5 nonresponders (Fig. S2A,C). Remarkably, the only nonresponder patient missed by our test showed lymphopenia across all his blood examinations before and while on treatment, which may account for the uncertain potential of our text.

## TKI treatments and circulating CD8+ lymphocyte variations

To evaluate a possible role of VEGF inhibition in the immunophenotypic changes observed in patients undergoing atezolizumab–bevacizumab, we assessed six prospective patients treated with sorafenib and nine patients treated with lenvatinib. At the 2 months imaging assessment, 2 of 6 in the sorafenib group and 5 of

9 in the lenvatinib group showed disease control. Any difference was observed in basal levels and early on-treatment variation of CD8+, CD8+PD-L1+ lymphocytes, and CD8+PD1+ lymphocytes FC when responder and nonresponder patients were compared (Fig. S3). Even though sorafenib and lenvatinib do not target VEGF only, these data do not support a causative role of VEGF inhibition as a driver of CD8+ lymphocyte immunophenotypic changes.

### RNAseq analysis and gene validation by RT-PCR

To get an insight into molecular mechanisms associated with the CD8+PD1+ increase observed in responder patients after the first treatment cycle, an RNAseq-based approach was performed to explore gene expression variation in responder versus nonresponder patients A subgroup of transcripts emerged as differentially regulated at the 3 weeks assay according to response to treatment (Fig. 3A,B).

Among these genes, TRIM56, TRIM16, and TRIM64 were validated by RT-PCR due to their role in the immune response. Ki67 mRNA expression was validated as well to confirm previous findings obtained in lung cancer patients where Ki67 induction was used to identify patients with CD8 T-cell responses elicited by PD1 targeted therapy [10]. The 27 patients not assessed by RNAseq were thus tested by RT-PCR on RNA extracted from PBMCs. TRIM56, TRIM16, TRIM64, and Ki67 turned out to be upregulated in responders and downregulated in nonresponder patients at the 3 weeks assay, confirming their involvement in the immune response elicited by atezolizumab–bevacizumab (Fig. 3C–F).

## Discussion

Atezolizumab–bevacizumab is the standard of care in advanced HCC. By targeting PD-L1, atezolizumab counteracts the immune tolerance induced by PD-L1 binding to PD1. The identification of predictive markers of response to ICIs represents an unmet need in patients with advanced HCC since more options are available in the front line and tissue sampling is not easy and of questionable representativity in advanced and multifocal cases. By using a simple and reproducible cytofluorimetric assay, we showed that lower baseline CD8+ and CD8+PD-L1+ peripheral lymphocytes might predict the response to atezolizumab–bevacizumab in HCC. Then, the investigation of on-treatment longitudinal dynamic changes of peripheral lymphocytes showed informative variations of CD8+PD1+ lymphocytes. A positive CD8+PD1+ lymphocyte FC was observed at 3 weeks after the first atezolizumab–bevacizumab infusion in 28 of 30 HCC patients with disease control, suggesting a reinvigoration of CD8+PD1+ cells after PD-L1 blockade. Conversely, all but one patient with disease progression showed a negative FC due to a decrease of CD8+PD1+ lymphocytes. Interestingly, the baseline CD8+ and CD8+PD-L1+ lymphocytes correctly identified the two responder patients missed by CD8+PD1+ early changes. The only patient experiencing a pseudoprogression displayed a positive CD8+PD1+ lymphocytes FC.

Even though our focus is not to provide a mechanistic inside we are in line with previous data obtained in lung cancer patients receiving anti-PD1 who displayed increased CD8+PD1+ proliferation in 70% of cases. Most importantly, these proliferating CD8+ T cells had an effector-like phenotype and were present in 80% of responders. On the other hand, 70% of nonresponders showed a delayed or absent PD1+CD8+ response [10]. The increase of CD8+PD1+ lymphocytes in responder patients is in line with the known dual role of PD1 [19]. Constitutive PD1 expression occurs in immune adaptation to chronic stimulation and contributes to impairing immune response. Conversely, several lines of evidence identify PD1 as a marker of T-cell activation and CD8+PD1+ cells as tumor-reactive elements in patients with melanoma. At the tissue level, seminal studies in metastatic melanoma showed that clonally-expanded tumor-reactive cells can be found among PD1+CD8+ TILs [20] and PD1 expression on CD8+ melanoma TILs accurately identifies clonally expanded tumor-reactive mutation-specific lymphocytes [20]. Accordingly, PD1 expression on CD8 TILs was suggested as a potential predictive biomarker of antitumor efficacy for ICI-based treatments [20, 21]. Further on, Gros et al. [22] confirmed that also in the circulating neoantigen-specific lymphocytes with anticancer reactivity can also be identified among CD8+PD1+ cells in patients with melanoma.

Remarkably, patients treated with TKI did not show either a difference in CD8+ and CD8+PD-L1+ baseline levels or did CD8+PD1+ lymphocytes increase, irrespective of response. Despite not being conclusive, this points to the more likely role of PD-L1 inhibition instead of VEGF inhibition as a trigger of CD8+PD1+ lymphocyte raise.

To summarize, baseline CD8+ and CD8+PDL1+ lymphocytes, and the early variation of CD8+PD1+ lymphocytes resulting in a negative or positive FC, provide widely feasible predictive markers of response to atezolizumab–bevacizumab treatment in HCC.

The molecular pathways triggered by atezolizumab–bevacizumab to activate the immune response are still poorly understood. By using an RNAseq approach we have assessed differentially modulated genes in PBMC of responder and nonresponder patients treated with atezolizuamb–bevacizumab. Among the possible mechanisms sustaining an early activation of the immune response, an upregulation of TRIM56 gene expression was observed in PBMC at the 3-week assay when compared with the baseline levels in responder patients only. Accordingly, TRIM56 modulates the innate immune response by regulating the TLR pathway [16] and its upregulation was observed in human primary lymphocytes in response to interferons [17]. TRIM56 antitumor activity was recently reported in HCC associated with downregulation of RBM24 and inactivation of the Wnt/β-catenin pathway [18]. TRIM16 and TRIM64 showed a similar pattern of expression, suggesting the TRIM family as a crucial player in the immune response elicited by atezolizumab–bevacizumab.

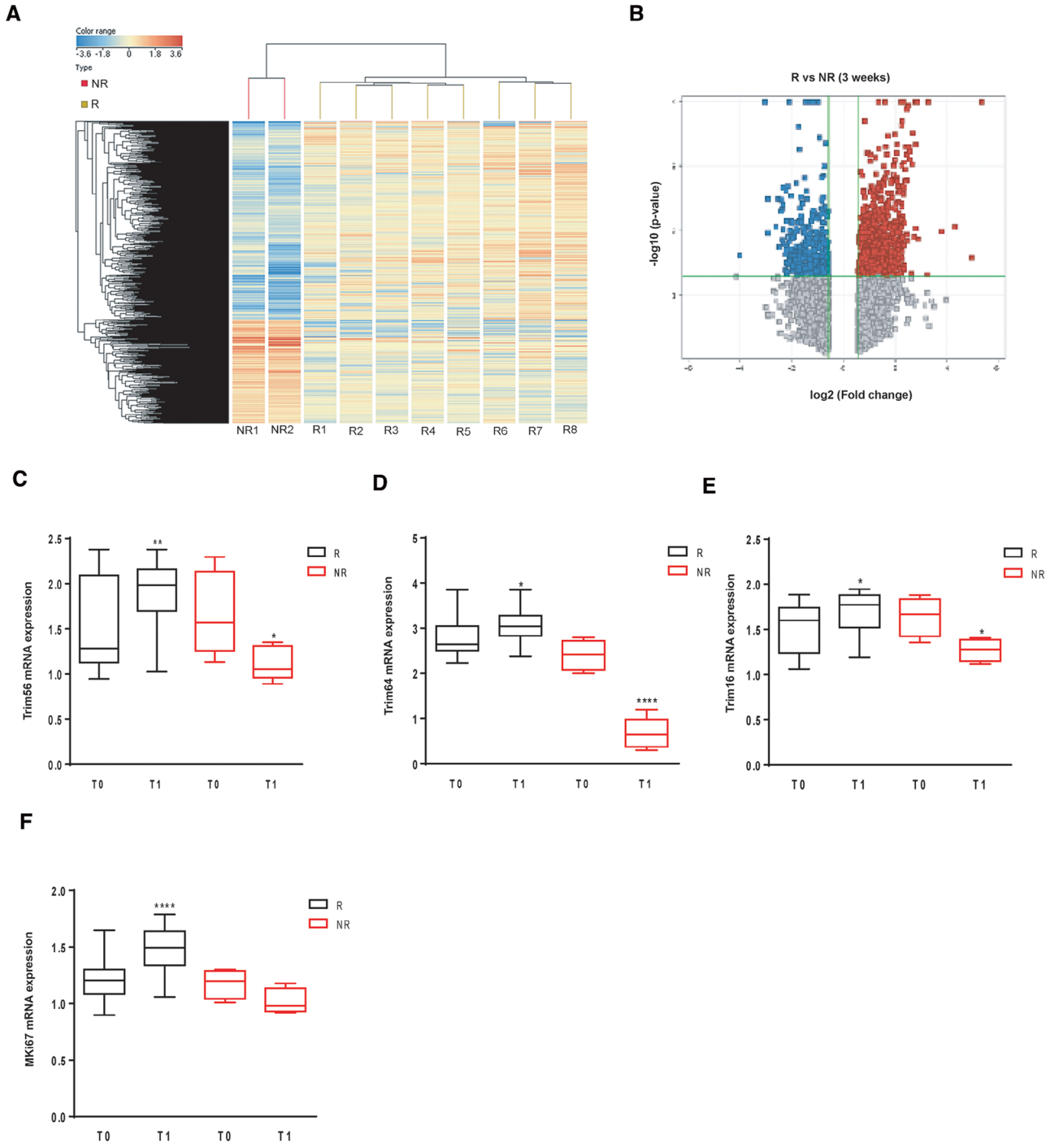


**Figure 3.** RNAseq analyses and validation. (A) Heatmap visualization of the subgroup of transcripts emerged as differentially regulated, with hierarchical clustering analysis. The nonresponder (NR) samples are colored in red, the responder (R) samples are colored in yellow. In the figure, the genes expression is normalized on the mean value of each gene across the samples and ranges from blue (representing a lower expression) to red (representing a higher expression). (B) Volcano plot showing the differentially expressed genes. The red squares on the right side of the figure represent the upregulated genes; "the blue squares on the left side of the figure represent the downregulated genes; grey squares represent genes with no significant difference. The *x*-axis corresponds to log2 (fold change) and the *y*-axis corresponds to –log10 (*p*-value)." (C–F) Box-plot graphs of TRIM56, TRIM64, TRIM16, and MKI67 mRNA levels tested by real-time RT-PCR in PBMC from 27 patients treated with atezolizumab–bevacizumab. *y*-axes report genes mRNA expression normalized to control represented by a pool of five HCC tissue. Real-time PCR was run in triplicate. Difference in expression levels were confirmed as statistically significant ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{****}p < 0.0001$) by two-tailed student's *t*-test.

### Data limitations and perspectives

Limitations of this study include the investigation of few markers and the small cohorts. However, findings are very consistent across the responder and nonresponder groups., The PBMC immunophenotype is easy, quick, repeatable, cheap, reproducible, and widely available and it might provide a valuable marker to complement clinical and imaging evaluations in the very early phases of atezolizumab–bevacizumab treatment of HCC patients, allowing a more precise and timing assessment. It is very difficult to propose appropriate cut-off values for baseline immune cell percentages to predict response due to the limited number of patients and the variability in the responder group. The number of patients is a critical element to draw reliable findings and a validation in a larger series is warranted before assessing cut-off values to be suggested in the clinics. Etiology-based analyses still need to be performed to rule out any relationship between immunophenotype and etiology. In addition, different antibodies used in the cytofluorimetric analysis and experimental procedures might give different results in terms of quantitative assessment. Thus, before giving cut-offs for clinical validation, different experimental settings should be compared and larger series should be analyzed.

The dynamic changes of CD8+PD1+ lymphocytes and their positive or negative FC at the second drug infusion are predictive for response to atezolizumab–bevacizumab. This determination is very simple and largely applicable. Patients' follow-up will assess whether higher fold-changes are associated with clinical characteristics and a different time to progression.

## Materials and methods

### Patients

A prospective cohort of 37 patients was referred to the Division of Hepatobiliary and Immunoallergic Diseases, IRCCS S.Orsola-Malpighi Hospital of Bologna and to the Division of Gastroenterology and Hepatology, Fondazione IRCCS Ca' Granda of Milan, treated by atezolizumab–bevacizumab in the front line for advanced HCC was enrolled in this study. An independent, prospective validation cohort of 18 patients was studied to validate preliminary findings (Table S2). Fifteen HCC patients treated by TKIs (sorafenib in six and lenvatinib in nine cases) were tested as controls. The baseline and on-treatment variation of the percentage of CD8+, PD1+, PD-L1+, CD8+PD1+, and CD8+PD-L1+ were assayed in peripheral lymphocytes. Study procedures, patients' characteristics (Table 1), and analytical methods are described in the Supporting Information section.

### RNAseq analysis on PBMC and RT-PCR validation

RNA sequencing was carried out according to the Illumina pipeline on a NextSeq 500 Instrument (Illumina) using the NextSeq High Output kit v2 (150 cycles) (Illumina).

Obtained sequences were mapped to the human genome (GRCh38) using the algorithm HISAT2 [https://pubmed.ncbi.nlm.nih.gov/25751142/] and a prebuilt genome index downloadable from the HISAT2 website. Then, StringTie [https://pubmed.ncbi.nlm.nih.gov/25690850/] was used to assemble and quantify the transcripts in each sample. Finally, expressed transcripts have been normalized using the DeSeq2 [https://pubmed.ncbi.nlm.nih.gov/25516281/] package for R, low abundance features filtered, and differential gene expression analysis performed with Agilent Genespring GX software (Agilent Technologies). The data that support the findings of this study are available from the corresponding author upon request.

Differentially expressed genes were selected to have a 1.5-fold expression difference between their geometrical mean in two or more groups of interest and a statistically significant $p$-value ($<0.05$), using the moderated $T$-test and Benjamini–Hochberg multiple testing correction for reduction of false-positive values. Differentially expressed genes were employed for cluster analysis of samples using the Manhattan correlation as a measure of similarity. Ten patients were analyzed at baseline, before treatment started, and at 3-week, before the second drug infusion. The remaining 27 patients were assessed in the validation step, by Syber-green RT-PCR, using β-Actin as a housekeeping gene. RT-PCR experiments were run in triplicate. The expression of mRNAs relative to β-actin gene expression was determined using the $2^{-\Delta\Delta Ct}$ method corresponding to TRIM56, TRIM64, TRIM16, and KI67 mRNA levels normalized to a pool of HCC RNA tissues as control. PCR primers were as follow: TRIM56 (Fw 5'GAGTCATCACCACTCCCTAATC-3'; Rev 5'GAGGGAAGGTCAGCAGATAAA-3'), TRIM64 (Fw 5'TTCCTCCTTCCTCCTTCTCTT-3'; Rev 5' GCTCTCGCACATAGTGTCTTAG-3'),

TRIM16 (Fw 5'CTGGTCTCCTTGGTAGGATTTC-3'; Rev 5' CTGTGTACCGCTGCTTCTT-3'), MKI67 (Fw 5' GACCTCAAACTGGCTCCTAATC-3'; Rev 5' GCTGCCAGATAGAGTCAGAAAG-3'), β-ACTIN (Fw 5' GGACCTGACTGACTACCTCAT-3'; Rev 5'CGTAGCACAGCTTCTCCTTAAT-3').

### Statistical analysis

Comparisons between the percentage of baseline PD1+, CD8+, PD-L1+, CD8+PD1+, and CD8+PD-L1+ peripheral lymphocyte populations in patients displaying partial response, stable, and progressive disease were performed by ANOVA and unpaired $t$-test. To evaluate the early on-treatment variation of the PD1+, CD8+, PD-L1+, CD8+PD1+, and CD8+PD-L1+ lymphocyte populations, their FC was assessed by calculating the ratio between the three weeks (T1) and the baseline (T0) determinations. Fold changes were then compared by unpaired $T$-test in responders and nonresponders. TRIM56, TRIM16, TRIM64, and KI67 mRNA expression (by RT-PCR) was compared in responder and nonresponder patients at baseline and after the first treatment cycle by unpaired $t$-test.

Pearson's correlation was used to explore the relationships between CD8+, PD1+, PD-L1+, CD8+PD1+, CD8+PD-L1+

**Table 1.** Baseline demographics and clinical characteristics of patients treated with atezolizumab/bevacizumab and TKIs

| Patient's characteristics | | Atezolizumab bevacizumab 37 pts | Response to atezolizumab-bevacizumab PR/SD/PD | *sorafenib (6 pts) lenvatinib (9 pts) |
|---|---|---|---|---|
| Age (years old) | <65 | 11 (29.7%) | 3/5/3 | 3 (20%) |
| | ≥65 | 26 (70.3%) | 10/12/4 | 12 (80%) |
| Gender | M | 32 | 12/13/7 | 12 (80%) |
| | F | 5 | 1/4/0 | 3 (20%) |
| ECOG PS | 0 | 30 (81.1%) | 11/12/7 | 10 (66.7%) |
| | 1 | 7 (18.9%) | 2/5/0 | 5 (33.3%) |
| Child–Pugh class | A | 36 (97.3%) | 12/17/7 | 15 (100%) |
| | B | 1 (2.7%) | 1/0/0 | 0 |
| | C | 0 | 0 | 0 |
| ALBI grade | 1 | 22 (59.5%) | 7/12/3 | 9 (60%) |
| | 2 | 15 (40.5%) | 6/5/4 | 6 (40%) |
| | 3 | 0 | 0 | 0 |
| BCLC stage | A | 0 | 0 | 0 |
| | B | 15 (40.5%) | 5/7/3 | 3 (20%) |
| | C | 22 (59.5%) | 8/10/4 | 12 (80%) |
| CLD etiology | HBV | 3 (8.1%) | 0/1/2 | 0 |
| | Active HCV | 3 (8.1%) | 2/1/0 | 3 (20%) |
| | Cured HCV | 11 (29.7%) | 6/3/2 | 6 (40%) |
| | NASH/NAFLD | 16 (43.2%) | 3/10/3 | 5 (33.3%) |
| | Alcohol | 4 (10.8%) | 2/2/0 | 1 (6.7%) |
| Nodularity (diameter, cm) | Uninodular <5 | 3 (8.1%) | 2/1/0 | 1 (6.7%) |
| | Uninodular >5 | 6 (16.2%) | 2/2/2 | 1 (6.7%) |
| | Multinodular ≤3 | 5 (13.5%) | 1/3/1 | 6 (40%) |
| | Multinodular >3 | 12 (32.4%) | 4/6/2 | 3 (20%) |
| | Infiltrating | 11 (29.7%) | 4/5/2 | 4 (26.7%) |
| Size (main lesion in multinodular) | ≤3 cm | 7 (18.9%) | 4/2/1 | 1 (6.7%) |
| | 3–5 cm | 8 (21.6%) | 4/3/1 | 5 (33.3%) |
| | 5–10 cm | 12 (32.4%) | 3/7/2 | 7 (46.7%) |
| | >10 cm | 8 (21.6%) | 2/4/2 | 2 (13.3%) |
| | Poorly defined | 2 (5.5%) | 0/1/1 | 0 |
| Portal vein invasion | Yes | 16 (43.2%) | 6/6/4 | 4 (26.7%) |
| | No | 21 (56.8%) | 7/11/3 | 11 (73.3%) |
| AFP (ng/mL) | ≤20 | 15 (40.5%) | 6/7/2 | 5 (33.3%) |
| | 21–400 | 15 (40.5%) | 6/6/3 | 7 (46.7%) |
| | ≥401 | 7 (18.9%) | 1/4/2 | 3 (20%) |
| Extrahepatic spread | Yes | 13 (35.1%) | 5/5/3 | 3 (20%) |
| | No | 24 (64.9%) | 8/12/4 | 12 (80%) |
| Response to treatment 1st imaging | Partial response (R) | 13 (35.1%) | | 2 (13.3%) |
| | Stable disease (SD) | 17 (45.9%) | | 5 (33.3%) |
| | Progression (NR) | 7 (18.9%) | | 8 (53.3%) |
| Response to treatment 2nd imaging | Partial response (R) | 12 (32.4%) | | 2 (13.3%) |
| | Stable disease (SD) | 13 (35.1%) | | 5 (33.3%) |
| | Progression (NR) | 11 (29.7%) | | 8 (53.3%) |
| | Lost to follow-up | 1 (2.7%) | | |

*Notes*: M: male; F: female; ECOG PS: Eastern Cooperative Oncology Group Performance Status (0–5). ALBI: albumin–bilirubin grade for HCC. PR/SD/PD: partial responder/stable disease/progressive disease according to RECIST 1.1 criteria; BCLC: Barcelona Clinic Liver Cancer staging system; CLD etiology: etiology of the underlying Chronic Liver Disease (CLD). In cases of more etiologies were recognized in the same patient, the most relevant was considered; HBV: Hepatitis B virus; HCV: Hepatitis C virus (active infection or previously cured infection); NASH/NAFLD: nonalcoholic steatohepatitis/nonalcoholic fatty liver disease; AFP: alfa-feto-protein in ng/mL; Extrahepatic spread: extrahepatic HCC localization include lung in six cases (associated with cutaneous metastasis in one case), lymph nodes in four cases (associated with peritoneal metastasis in one case and with adrenal gland metastasis in another case), bone in two cases (associated with peritoneal metastasis in one case), brain in one case.

* Patients treated with sorafenib or lenvatinib were tested as a control group and are shown to illustrate their demographics. Their CD8+PD1+ lymphocyte fold change at the first month after treatment started was 1.1 in two cases and ≤1 in all other cases, regardless of response to treatments.

peripheral lymphocyte populations and AFP, NLR (neutrophil to lymphocyte ratio) and eosinophil count. Categorical variables were compared using the chi-square test.

Reported P values were two-sided and considered significant when <0.05. Statistical calculations were carried out using SPSS version 20.0 (SPSS inc) ($p < 0.05$; $p < 0.01$; $p < 0.001$; $p < 0.0001$).

ROC curve analysis was used to determine the sensitivity and specificity for CD8+, CD8+PD-L1+, and CD8+PD1+ fold change.

**Acknowledgements:** This research was funded by the Italian Ministry of Health (RC-2023-2778942). It was also supported by Programma di Ricerca Regione-Università, Regione Emilia Romagna, Bando "Ricerca Innovativa" (innovative approaches to the diagnosis and pharmacogenetics-based therapies of primary hepatic tumors, peripheral B and T-cell lymphomas and lymphoblastic leukemias) to L.G; Bando "Alma Idea 2022" University of Bologna "Immunotherapy: the new frontier for the fight against advanced hepatocellular carcinoma (LIFE)" to C.G. PRIN 2022 (Enhancement of therapeutic response to standard-of-care drugs employed against hepatocellular carcinoma by hitting metabolic dependencies) to F.P. and M.N.
Open access funding provided by BIBLIOSAN.

**Conflict of interest:** Consulting or lecture fees in the last 2 years from Astrazeneca, Bayer, Bracco, EISAI, ESAOTE, Exact Sciences, IPSEN; MSD; Roche, Samsung to F.P.

**Author contributions:** The manuscript was conceived, designed, and drafted by Laura Gramantieri, Fabio Piscaglia, and Catia Giovannini. Data acquisition was performed by Catia Giovannini, Fabrizia Suzzi, Mariarosaria Marseglia, Mariangela Bruccoleri, Eleonora Alimenti, Francesca Fornari, Lucilla D'Abundo, and Francesco Tovoli. Data were analyzed and interpreted by Laura Gramantieri, Catia Giovannini, Fabrizia Suzzi, Fabio Piscaglia, Cristian Bassi, Massimo Negrini, and Massimo Iavarone. All the authors critically revised and approved the manuscript.

**Data availability statement:** All relevant data were reported within the manuscript. Further supporting data will be provided upon written request addressed to the corresponding authors.

**Ethics approval statement:** This study protocol was reviewed and approved by the local ethic committee (Comitato Etico Area Vasta Emilia Centro – AVEC) on June 6, 2021; approval number 528/2021/Sper/AOUBo. The study was conducted in accordance with the 1964 Helsinki Declaration and its later amendments.

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*Abbreviations:* **AFP**: alpha-feto-protein · **FC**: fold change · **HCC**: hepatocellular carcinoma · **ICIs**: immune check point inhibitors · **NLR**: neutrophil to lymphocyte ratio · **PD1**: programmed cell death protein 1 · **PD-L1**: programmed cell death ligand 1 · **T0**: baseline – before treatment start · **T1**: 3-weeks evaluation · **TKIs**: tyrosine kinase inhibitors · **VEGF**: vascular endothelial growth factor

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Received: 30/6/2023
Revised: 20/11/2023
Accepted: 21/11/2023
Accepted article online: 22/11/2023