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ORIGINAL ARTICLE

Early CTLA4 increase in CD45+ blood cells: an emerging biomarker of atezolizumab—bevacizumab resistance and worse survival in advanced hepatocarcinoma

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Background: Advanced hepatocellular carcinoma (HCC) has a dismal prognosis; however, the introduction of atezolizumab—bevacizumab combination has improved overall survival and novel immune checkpoint inhibitors are entering the clinics. Despite more therapeutic options being available, no biomarker guides treatment choice. Indeed, tissue-based analyses and complex analytical procedures hinder clinical translation. We explored the informativeness of a simple, non-invasive, repeatable cytofluorimetric assay on peripheral blood to predict response and survival in HCC patients treated with atezolizumab—bevacizumab.

Materials and methods: Twenty-five cirrhotic patients, 50 HCC patients undergoing atezolizumab—bevacizumab and an independent validation cohort of 25 HCC patients were subjected to a cytofluorimetric study of peripheral white blood cells (WBCs) to assess baseline programmed death-ligand 1-positive (PD-L1+) and cytotoxic T-lymphocyte antigen 4-positive (CTLA4+) cell percentage in the different populations and their early on-treatment variations. Immunophenotypes were evaluated against treatment response. RNA sequencing followed by RT—PCR validation were used to elucidate the molecular correlates of immunophenotypic observations.

Results: PD-L1+ cell percentage did not predict response either at baseline or when evaluating treatment-induced early changes. Conversely, the percentage of CTLA4+ lymphocytes at baseline showed a predictive significance (35.37 in responders versus 31.5 in non-responders, $P = 0.03$). More interestingly, the early CTLA4+ changes during treatment in lymphocytes (responders 0.95 versus non-responders 1.08, $P = 0.05$), monocytes (responders 0.95 versus non-responders 1.04, $P = 0.03$), granulocytes (responders 0.94 versus non-responders 1.14, $P = 0.001$) and, even stronger, the early CTLA4+ percentage change in the whole WBCs displayed a predictive significance in terms of time to progression (TTP) ($P < 0.0001$) and overall survival (OS) ($P = 0.005$). The immunophenotypic findings correlated with transcriptional modulation of CTLA4 target genes and genes involved in immune response.

Conclusions: A repeatable, easy, non-invasive blood test predicts response to immunotherapy in patients with HCC, both in terms of TTP and OS. CTLA4+ cell percentage increase in non-responders suggests a possible resistance mechanism which deserves attention as a druggable target.

Key words: immunotherapy, CTLA4, PD-L1, biomarkers

INTRODUCTION

Hepatocellular carcinoma (HCC) incidence and mortality are increasing, with an expectation of becoming the third leading cause of cancer-related deaths by 2030.[1] When diagnosed at advanced stages, treatments are represented by immune-based approaches and tyrosine kinase inhibitors (TKIs). Even though the combination of atezolizumab plus bevacizumab conveys an outstanding benefit, with ∼30%

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response rate and a disease stabilization as best response in another 40%-50%,[2,3] TKIs are still an option. Tissue lack and tumor heterogeneity are in part responsible for the absence of predictive biomarkers, which, in turn, hinders the personalization of treatments.[4]

Indeed, tumor tissue infiltrate offers a direct picture of the local antitumor immunity, yet these investigations suffer the heterogeneity of sampling and cannot be proposed to monitor treatment-induced changes. Moreover, repeated samplings during treatment, though informative, are very difficult, making a dynamic assessment hardly feasible in the clinics.

Recent studies have shown that circulating immune cells are representative of the immune response against tumors elicited by immune therapy, providing a longitudinal exploration of the immune response triggered by systemic stimuli.[5,6] Remarkably, a fraction of cytotoxic T cells, and particularly CD8+ T cells, recirculate and can be investigated in peripheral blood after treatment challenges.[7] Circulating monocytes and granulocytes also participate in the innate immune response and they are thought to play an important role in modulating the efficacy of anticancer immunotherapy.[8,9] Although they can be easily analyzed in peripheral blood, their investigation is still at an early stage when compared with circulating lymphocytes.

Cytotoxic T-lymphocyte antigen 4 (CTLA4) is one of the most relevant immune checkpoints. By competitively binding to CD80/CD86, it prevents its interaction with CD28, thus acting as a negative regulator of T-lymphocyte activation. CTLA4 plays a major role in regulatory T-cell function; however, the same events take place in the regulation of conventional T-cell response suggesting a similar mechanism of action in both T-cell populations.[10]

Mechanisms driving CTLA4 expression on cell membrane are still under investigation.[11] Intersection between CD80 (CTLA4 ligand) and programmed death-ligand 1 (PD-L1) is also being elucidated. Indeed, CTLA4-mediated transendocytosis depletes CD80 from the cell surface, resulting in the release of PD-L1 which can interact with programmed cell death protein 1 (PD-1).[12] In addition, PD-L1 and CTLA4 are regulated by common factors, such as the interferon-γ pathway, signal transducer and activator of transcription and epigenetic factors including histone deacetylase and DNA methylation.[13]

In HCC, higher CTLA4 expression by tumor-infiltrating mononuclear cells was observed in patients with a better survival,[14] while in the peripheral blood, it was associated with impaired T-cell functionality.[15]

Accordingly, CTLA4 inhibition has shown clinical activity in advanced HCC and its combination with PD-L1 blockade was recently approved.[16,17] On this basis, it can be argued that CTLA4 might contribute to impairment of immune response, due to either its constitutive activation or its upregulation during the course of other treatments.

Since dynamic changes triggered by immune checkpoint inhibitors (ICIs) in circulating immune cells were shown to be informative to predict response,[5,18] we have tested PD-L1 and CTLA4 expression in blood lymphocytes, granulocytes and monocytes to investigate their constitutive expression as well as any variation triggered by atezolizumab—bevacizumab in patients with advanced HCC with different outcomes. Results were complemented with molecular correlates of immune response activation. The percentages of CTLA4+ circulating immune cells, and their variation during PD-L1 blockade, show promise as a predictive biomarker helping to optimize treatment decisions and suggesting its future exploitation to identify patients who might benefit from combined immune checkpoint blockade.

MATERIALS AND METHODS

Patients

Fifty patients with intermediate/advanced HCC not amenable to curative treatments or locoregional treatments were consecutively enrolled in the study being referred to S. Orsola-Malpighi University Hospital of Bologna and to the Center for Liver Disease, Maggiore Hospital, University of Milan, for the diagnosis and cure of advanced HCC. All patients were treated by atezolizumab (1200 mg as an intravenous infusion) in association with bevacizumab (15 mg/kg intravenous infusion) on the same day, according to a 3-week infusion schedule between April 2022 and May 2023. Patients' characteristics are reported in Supplementary Table S1, available at https://doi.org/10.1016/j.esmoop.2025.104289. Patients in the three cohorts were tested immediately before treatment start and at 3 weeks after drug infusion by a cytofluorimetric analysis of PD-L1+ and CTLA4+ cell percentage in the different circulating populations (lymphocytes, granulocytes, monocytes), as well as among the whole white blood cells (WBCs), to assay their baseline expression and their early on-treatment variations. Immunophenotypes were evaluated with respect to treatment response. RNA sequencing (RNAseq) of peripheral blood mononuclear cells (PBMCs) followed by validation with RT—PCR was used to investigate the molecular correlates of the immunophenotypic observations. Study procedures, analytical methods and statistical analyses are described in the Supplementary Materials and Methods, available at https://doi.org/10.1016/j.esmoop.2025.104289.

This study protocol was reviewed and approved by the local ethics committee (Comitato Etico Area Vasta Emilia Centro—AVEC) on 6 June 2021 (approval number 528/2021/Sper/AOUBo). The study was conducted in accordance with the 1964 Helsinki declaration and its later amendments. Written informed consent was obtained from all individual participants included in the study.

RESULTS

Differential expression of PD-L1 among circulating immune cell subsets

As a first step we have determined PD-L1+ circulating cell percentage in the different WBC populations in a cohort of patients with advanced HCC undergoing atezolizumab—

bevacizumab treatment. PD-L1+ percentages in lymphocytes, granulocytes and monocytes were assessed before PD-L1 blockade (T0). Their variations soon after the beginning of treatment were tested at 3 weeks after the first drug infusion (T1). Reproducibility of readings was previously verified in different blood samples.[19,20] At the baseline, circulating white cell populations expressed different PD-L1 levels on their surface with monocytes emerging as the ones with the highest expression followed by granulocytes and lymphocytes, respectively [analysis of variance (ANOVA) test $P < 0.001$; Figure 1A].

Baseline PD-L1 levels do not differentiate responder and non-responder patients

To investigate any possible role of PD-L1 as an upfront predictive biomarker in the setting of atezolizumab—bevacizumab treatment, we compared PD-L1+ circulating

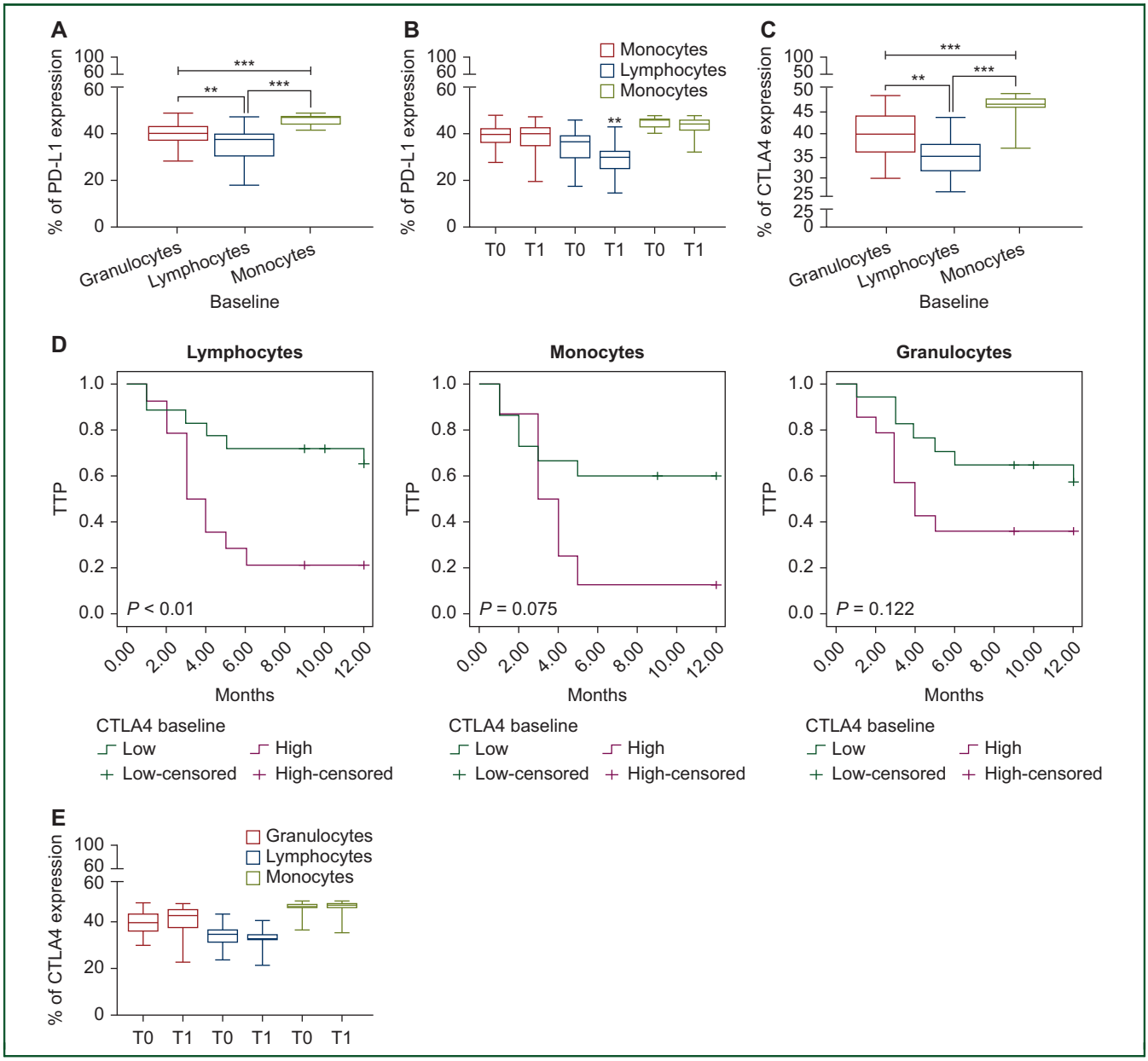

Figure 1. Baseline and early on-treatment variations of PD-L1 and CTLA4 in peripheral white cell populations. (A and B) Box-plot graphic representation of baseline and dynamic variations of PD-L1 on peripheral granulocytes, lymphocytes and monocytes in patients treated with atezolizumab—bevacizumab. (C) Box-plot graphic representation of baseline CTLA4 expression on peripheral white cell populations. (D) A higher percentage of CTLA4+ lymphocytes differentiate responders from non-responders at baseline and are associated with longer TTP as shown by Kaplan—Meier curves. Patients were categorized as high or low according to the mean value of CTLA4+ percentage on circulating lymphocytes, monocytes and granulocytes. Patients were censored at treatment stop due to HCC progression documented at imaging. Statistical *P* value was generated by the log-rank test. (E) Box-plot graphic representation of baseline and dynamic variations of CTLA4 on peripheral granulocytes, lymphocytes and monocytes in 50 HCC patients treated with atezolizumab—bevacizumab.

ANOVA, analysis of variance; CTLA4, cytotoxic T-lymphocyte antigen 4; HCC, hepatocellular carcinoma; PD-L1, programmed death-ligand 1; T0, baseline assessment; T1, 3-week assessment, before the second drug infusion; TTP, time to progression.

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ by ANOVA or unpaired *t*-test.

immune cell percentages in responder and non-responder patients at baseline. At the first imaging assessment, 37 of 50 patients (74%) showed disease control, including 30 with stable disease (60%) and 7 with partial response (14%) among which 1 had pseudoprogression, while the remaining 13 (26%) patients showed a disease progression. The mean time to progression (TTP) was 11.5 months in responders and 3.6 months in non-responders. Baseline PD-L1+ cell percentages did not differentiate responders from non-responders in any white cell population (Supplementary Figure S1A, available at https://doi.org/10.1016/j.esmoop.2025.104289).

PD-L1+ circulating lymphocyte variation during atezolizumab–bevacizumab treatment is independent of response

We next tested treatment-associated variations of PD-L1+ cell percentages in circulating immune cells in the whole patient cohort. After the first infusion, a significant reduction was detected in lymphocytes only (*t*-test, $P = 0.0013$, Figure 1B). This observation likely resulted from the persistent binding of atezolizumab to PD-L1 at 3 weeks in lymphocytes. Indeed, the half-life of lymphocytes is long enough to display the persistent binding of atezolizumab 3 weeks after drug infusion, differently from the short-lived granulocytes and monocytes. By separating responder and non-responder patients, neither PD-L1+ lymphocyte percentage reduction nor PD-L1 fold change (FC) was associated with response to atezolizumab–bevacizumab, ruling out its possible predictive value (Supplementary Figure S1B-D, available at https://doi.org/10.1016/j.esmoop.2025.104289).

Baseline CTLA4 immune checkpoint expression and patients' outcome

Searching for a predictive biomarker of treatment response, we focused on CTLA4 that is gaining more and more attention as an interesting therapeutic target.[21] This immune checkpoint plays a relevant role in immune resting programs; it relates to PD-L1 pathway, and its assessment is not masked by atezolizumab. Thus, we moved toward investigating the percentage of circulating WBCs expressing CTLA4 on their surface before treatment start. In line with PD-L1 baseline data, all circulating cell populations expressed CLTA4 on their surface with a stronger expression on monocytes and granulocytes and moderate on lymphocytes (ANOVA test $P < 0.0001$; Figure 1C). Accordingly, a direct correlation was found between baseline percentages of CTLA4+ and PD-L1+ granulocytes (Pearson's correlation $R = 0.604$, $P = 0.001$), lymphocytes (Pearson's correlation $R = 0.561$, $P = 0.001$) and monocytes (Pearson's correlation $R = 0.611$, $P = 0.003$). Moving to the investigation of a possible predictive role of CTLA4 on circulating WBCs, baseline percentage of CTLA4+ cells differentiated responders from non-responders to atezolizumab–bevacizumab in the lymphocyte population only [mean ± standard error (SE) in responders versus non-responders: 35.37 ± 0.87 versus 31.5 ± 1.68 CTLA4+ lymphocyte percentage; *t*-test $P = 0.03$] and associated with a longer TTP (mean ± SE in CTLA4+ high versus CTLA4+ low: 9.44 ± 1.08 months versus 5.14 ± 1.00 months, log-rank test 6.905; $P = 0.009$). Conversely, the baseline percentage of CTLA4+ granulocytes and monocytes did not show any association with TTP (Figure 1D).

CTLA4 early dynamic changes in circulating immune cells predict response to atezolizumab–bevacizumab

When all patients were considered, CTLA4+ cell percentage on granulocytes, lymphocytes and monocytes did not display any variation occurring early on atezolizumab–bevacizumab treatment (Figure 1E). Conversely, when comparing non-responders with responders, the former showed a significant early on-treatment increase of CTLA4+ percentage on monocytes and granulocytes (Figure 2A). Accordingly, the FC of CTLA4+ cell percentages assessed at 3 weeks after treatment start showed that responders were characterized by a negative FC while non-responder patients displayed a positive FC in all white cell populations (Figure 2B). Early dynamic changes of CTLA4+ granulocyte percentage strongly differentiated responders from non-responders (median values ± SE: 0.94 ± 0.03 in responders versus 1.13 ± 0.03 in non-responders; Mann–Whitney test $P < 0.0001$). The same trend was observed for FC in CTLA4+ monocytes (mean ± SE: responders 0.95 ± 0.04 versus non-responders 1.04 ± 0.12; *t*-test $P = 0.03$) and lymphocytes (mean ± SE: responders 0.95 ± 0.03 versus non-responders 1.08 ± 0.07; *t*-test $P = 0.05$; Figure 2B). To sum up, while PD-L1+ cell percentage was not informative in the setting of atezolizumab–bevacizumab treatment, a higher baseline CTLA4+ lymphocyte percentage as well as an early increase of the CTLA4+ granulocyte, monocyte and lymphocyte percentage predicted non-response.

Receiver operating characteristic (ROC) curve analysis confirmed the impressive performance of CTLA4+ percentage early variation on granulocytes, with an area under the curve (AUC) of 0.82 [confidence interval (95% CI) 0.77-0.91] (Figure 3A). The variation of CTLA4+ percentage on monocytes displayed an AUC of 0.74 (95% CI 0.62-0.85), while CTLA4+ percentage variation on lymphocytes showed an AUC of 0.68 (95% CI 0.74-0.90) (Figure 3A). These immunophenotypic markers performed better than baseline α-fetoprotein (AFP) which, in our series, displayed an AUC of 0.568 (95% CI 0.29-0.84). According to clinical practice, we did not carry out AFP determination at 3 weeks in most of our patients; thus, the 3-week AFP FC could not be compared with the immunophenotypic parameters.

ROC curves were also used to assess the best cut-offs, which were adopted to calculate Kaplan–Meier curves. The early increase of CTLA4+ granulocyte percentage, assessed as an FC, displayed the strongest association with a shorter TTP (mean TTP ± SE in CTLA4 FC high versus CTLA4 FC low: 4.46 ± 0.73 months versus 9.99 ± 1.06 months, log-rank test 10.93; $P < 0.001$, Figure 3B),

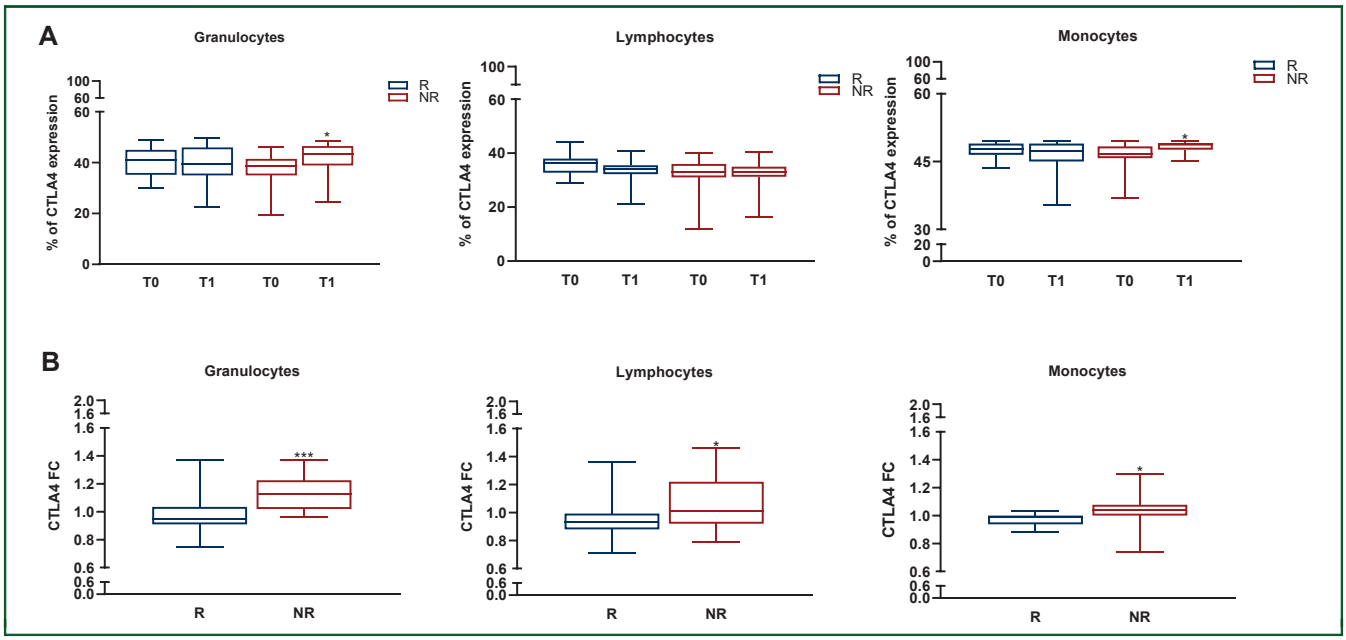

Figure 2. CTLA4 analyses in white cell populations of responder and non-responder patients to atezolizumab—bevacizumab. (A) Box-plot graphic representation of dynamic variations of CTLA4+ granulocytes, lymphocytes and monocytes in responder and non-responder patients to atezolizumab—bevacizumab. (B) CTLA4+ FC (T1/T0) at 3 weeks after treatment start in responders and non-responders to atezolizumab—bevacizumab.
CTLA4, cytotoxic T-lymphocyte antigen 4; FC, fold change; NR, non-responders; R, responders; T0, baseline; T1, 3 weeks after first drug infusion.
*$P < 0.05$; ***$P < 0.001$; by unpaired *t*-test.

suggesting a possible role of CTLA4 high FC in granulocytes as a predictive biomarker of non-response. CTLA4+ monocytes' early increase was associated with a shorter TTP too (mean TTP ± SE in CTLA4 FC high versus low: 4.11 ± 0.99 months versus 8.57 ± 1.25 months, log-rank test 5.05; $P = 0.025$, Figure 3B). Even though less significant, the same observation applied to the increase of circulating CTLA-4+ lymphocyte FC, which predicted non-response to treatment in terms of a shorter TTP too (mean TTP ± SE in CTLA4 FC high versus CTLA4 FC low: 5.41 ± 1.14 months versus 8.85 ± 1.05 months, log-rank test 4.055; $P = 0.044$, Figure 3B).

Early variation of CTLA4+ percentage on total WBC predicts response to atezolizumab—bevacizumab

The strong predictive role of granulocyte immunophenotype early variation prompted us to explore the same assay in the whole CD45+ WBC population. Indeed, granulocytes account for the majority of CD45+ circulating cells, and the analysis of the total CD45+ cells is easier to carry out and interpret. As observed for each separate cell population, baseline percentages of CTLA4+ and PD-L1+ CD45+ WBC displayed a direct correlation (Pearson's correlation $R = 0.520$, $P = 0.003$). Conversely, no correlation with baseline AFP was found for both these tests.

At the baseline, neither the PD-L1+ nor the CTLA4+ cell percentage on the total CD45+ WBC differentiated responders from non-responders even though a higher baseline CTLA4+ cell percentage on all WBC population displayed a weak association with TTP (mean TTP ± SE in CTLA4+ high versus CTLA4+ low: 8.70 ± 1.13 months versus 6.26 ± 1.10 months, log-rank test 3.60; $P = 0.057$, Supplementary Figure S2A, available at https://doi.org/10.1016/j.esmoop.2025.104289).

Treatment-associated variations of CTLA4+ cell percentage in the total CD45+ WBC were not significant in the whole cohort of HCC patients (Supplementary Figure S2B, available at https://doi.org/10.1016/j.esmoop.2025.104289), while the FC of CTLA4+ cell percentage at 3 weeks from treatment start clearly differentiated responders from non-responders (mean ± SE CTLA4 FC: 0.91 ± 0.02 versus 1.27 ± 0.07; *t*-test $P < 0.001$, Figure 4A) as previously described for granulocytes, confirming the informativeness of early dynamic changes.

ROC curve analysis confirmed the performance of CTLA4+ percentage early variation on the total CD45+ WBC population which displayed an AUC of 0.927 (95% CI 0.84-1.00) and identified a Youden index of 0.73 corresponding to a CTLA4+ FC best cut-off of 1.02 (Figure 4B).

The early on-treatment reduction of CTLA4+ cell percentage on the total CD45+ WBC strongly predicted the response and associated with a longer TTP (mean ± SE in CTLA4+ FC high versus low: 11.03 ± 0.54 months versus 4.46 ± 0.852 months; log-rank test 34.32; $P < 0.0001$, Figure 4C); conversely, its increase was associated with lack of response. Hence, while PD-L1+ variation on all CD45+ WBC was not informative to predict response (chi-square with Yates correction 1.55; $P = 0.21$), the increase of CTLA4+ percentage on all CD45+ WBC allowed the identification of 12 of 13 non-responder patients and its decrease identified 31 of 37 responder patients (chi-square with Yates correction 20.99; $P < 0.0001$, Figure 4D).

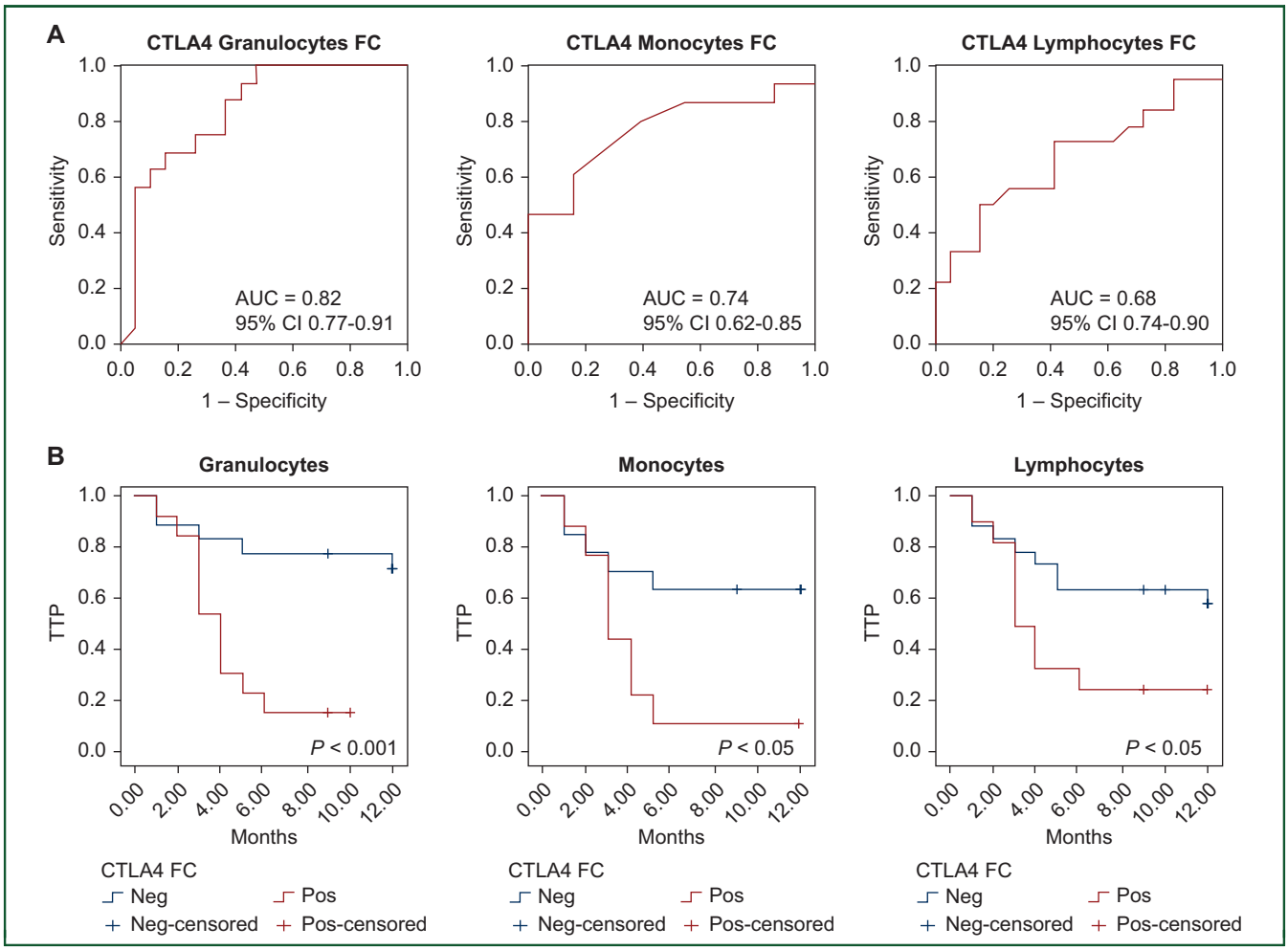

Figure 3. CTLA4 FC in circulating white cells as biomarker of treatment response to atezolizumab—bevacizumab. (A) ROC curve indicating the performance of CTLA4 FC in circulating white cell populations as biomarker to predict treatment response to atezolizumab—bevacizumab. (B) Kaplan—Meier survival curves comparing TTP in 50 patients treated with atezolizumab—bevacizumab. Patients were categorized according to a positive or negative CTLA4 FC in circulating white cell populations. Significant events were the HCC progression documented at imaging. Statistical *P* value was generated by the log-rank test.
AUC, area under the curve; CI, confidence interval; CTLA4, cytotoxic T-lymphocyte antigen 4; FC, fold change; HCC, hepatocellular carcinoma; ROC, receiver operating characteristic; TTP, time to progression.

Sensitivity, specificity, positive and negative predictive values of CTLA4+ early on-treatment FC on all circulating WBC were determined based on response to treatment assessed by RECIST 1.1 criteria, to differentiate responders versus non-responders. In detail, CTLA4+ FC in CD45+ WBC shows a sensibility of 0.97, a specificity of 0.92, a positive predictive value of 0.84 and a negative predictive value of 0.66, with an accuracy of 0.86. Among the six responders without a decrease of CTLA4+ percentage on all CD45+ WBC, five had received treatments [transarterial radioembolization (TARE) in three cases and transarterial chemoembolization (TACE) in two cases] in the previous 30-40 days. Among the patients correctly predicted by CTLA4 immunophenotyping, only two had undergone TACE nearly 2 months before atezolizumab—bevacizumab start.

The early variation of CTLA4+ WBC percentage was also evaluated to distinguish patients based on overall survival (OS) from treatment start to death from any cause. At data cut-off (24 months, mean follow-up of 15.70 months), 24 patients had died, with a median OS ± standard deviation (SD) of 10.66 ± 5.37. Twenty-six patients were still alive, with a median survival ± SD of 20.18 ± 3.34 months. The early on-treatment CTLA4+ cell percentage FC on the total CD45+ WBC (based on the best cut-off emerged from ROC curve) was associated with a longer OS (mean ± SE in CTLA4+ FC low versus high: 20.09 ± 1.05 months versus 13.42 ± 1.75 months; log-rank test 8.036; $P = 0.005$, Figure 4E).

Summing up, patients with a longer TTP and a longer OS displayed an early on-treatment reduction of CTLA4+ cell percentage on all CD45+ WBC, after the first drug infusion. Conversely, an early on-treatment increase of CTLA4+ cell percentage on all CD45+ WBC predicted non-response.

To determine the role of HCC in the modulation of CTLA4+ and PD-L1+ percentage of circulating WBC, we compared HCC patients with cirrhotic patients without HCC (Supplementary Table S2, available at https://doi.org/10.1016/j.esmoop.2025.104289). A higher baseline percentage of both PD-L1+ (mean ± SE in HCC versus cirrhotic patients 36.6 ± 6.4 versus 30.4 ± 6.9, *t*-test, $P = 0.015$) and CTLA4+ (mean ± SE in HCC versus cirrhotic patients: 33.2 ± 7.6 versus 20.7 ± 7.8, *t*-test, $P < 0.0001$) cell percentage

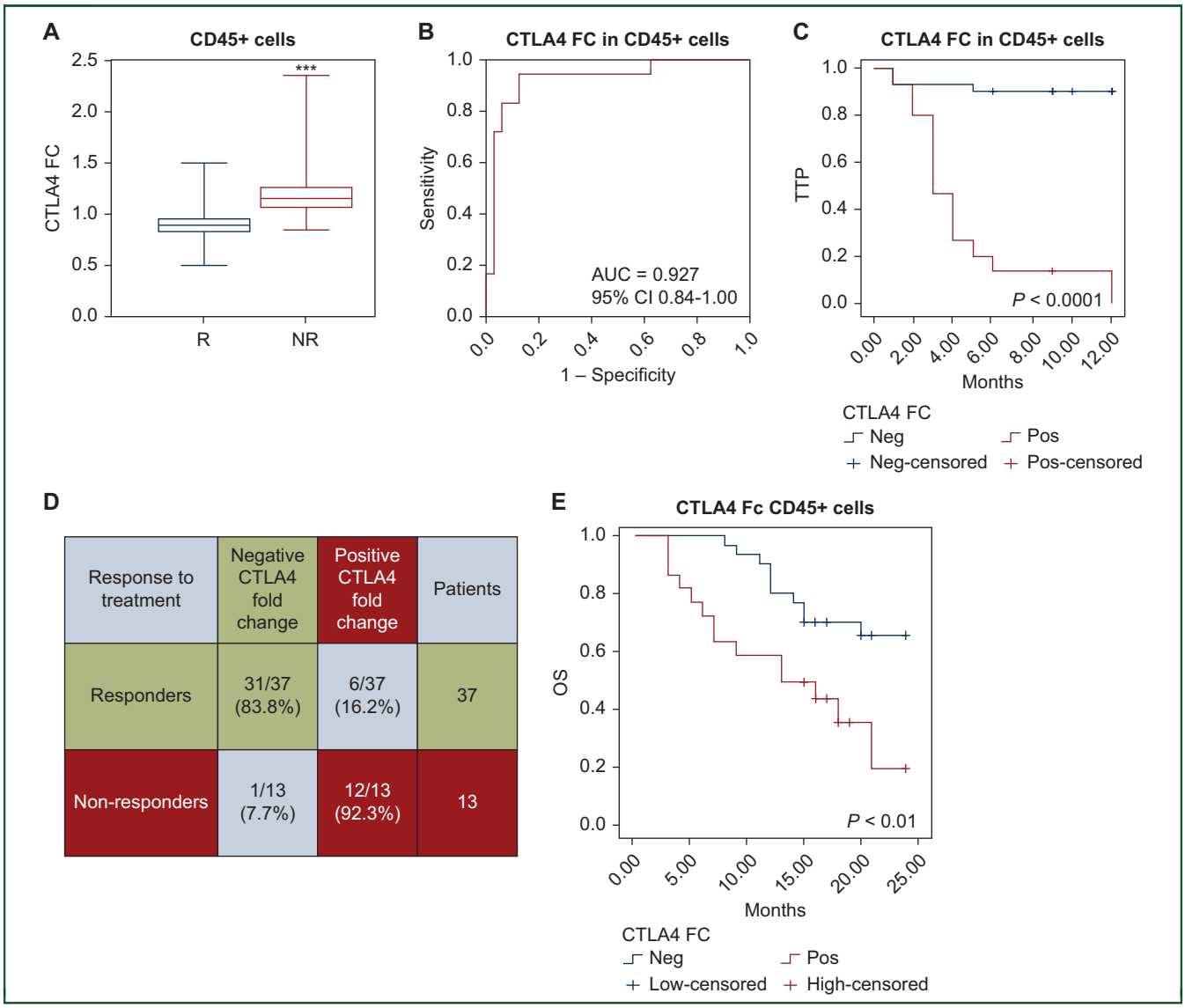

Figure 4. CTLA4 early FC in CD45+ circulating immune cells predicts response to atezolizumab–bevacizumab. (A) Box-plot graphic representation of dynamic variations of CTLA4 percentage in CD45+ circulating cells. (B) ROC curve indicating the performance of CTLA4 FC in CD45+ circulating cells as biomarker to predict treatment response to atezolizumab–bevacizumab. (C) Kaplan–Meier survival curves comparing TTP in 50 patients treated with atezolizumab–bevacizumab. Patients were categorized as positive or negative for CTLA4 FC in circulating CD45+ cells. (D) Schematic view of CTLA4 FC (T1/T0) in CD45+ circulating cells in HCC patients showing disease control or non-response to atezolizumab–bevacizumab. (E) Kaplan–Meier curves of OS in 50 HCC patients. Patients with negative CTLA4 FC in CD45 circulating white cells ($n = 36$) had a significantly higher OS as compared with patients with CTLA4+ FC.
CTLA4, cytotoxic T-lymphocyte antigen 4; FC, fold change; HCC, hepatocellular carcinoma; NR, non-responders; OS, overall survival; R, responders; ROC, receiver operating characteristic; TTP, time to progression.

on the total CD45+ WBC confirmed the association of a resting immunophenotype with the presence of HCC (Supplementary Figure S3A and B, available at https://doi.org/10.1016/j.esmoop.2025.104289).

Validation of CTLA4+ early variation in CD45+ circulating immune cells as a predictive biomarker

The predictive performance of treatment-associated variation of the CTLA4+ percentage on CD45+ WBC population was validated in an independent prospective population of 25 HCC patients among which 7 had progressive disease and 18 had disease control (3 partial response and 15 stable disease) at the first imaging evaluation. In this second cohort, CTLA4+ CD45 WBC FC was 0.83 ± 0.12 in responders versus 1.15 ± 0.19 in non-responders (*t*-test: $P = 0.0021$, Supplementary Figure S3C, available at https://doi.org/10.1016/j.esmoop.2025.104289). Results are presented in detail in Supplementary Material, available at https://doi.org/10.1016/j.esmoop.2025.104289.

RNAseq analysis and gene validation by RT–PCR

To explore molecular mechanisms associated with CTLA4 variation, we interrogated our RNAseq analysis carried out on PBMCs in a subgroup of patients.[20] After filtrating genes according to their involvement in immune response, we identified and validated gene families differentially

regulated at 3 weeks after immunotherapy start in responders versus non-responders (Supplementary Tables S3—S5, available at https://doi.org/10.1016/j.esmoop.2025.104289). CTLA4 did not emerge among modulated transcripts; indeed, it is not regulated by transcriptional mechanisms but through intracellular trafficking.[11]

Among the CTLA4 target genes, the early on-treatment variation of TRIM53, TRIM61 and CREB1 messenger RNA (mRNA) expression was validated by RT—PCR by assessing the baseline versus 3-week expression in a subgroup of 20 patients not assessed by RNAseq, categorized according to a positive or negative CTLA4+ FC as well as according to response versus non-response to treatment. Due to the predictive role of early variations of CTLA4+ granulocyte percentage, this cell population was isolated to further assay gene expression by RT—PCR in this population too. TRIM53, TRIM61 and CREB1 mRNA expression was up-regulated in patients displaying a CTLA4+ FC early on treatment, as well as in most non-responder patients both in isolated granulocytes and in PBMCs (Figure 5A and B).

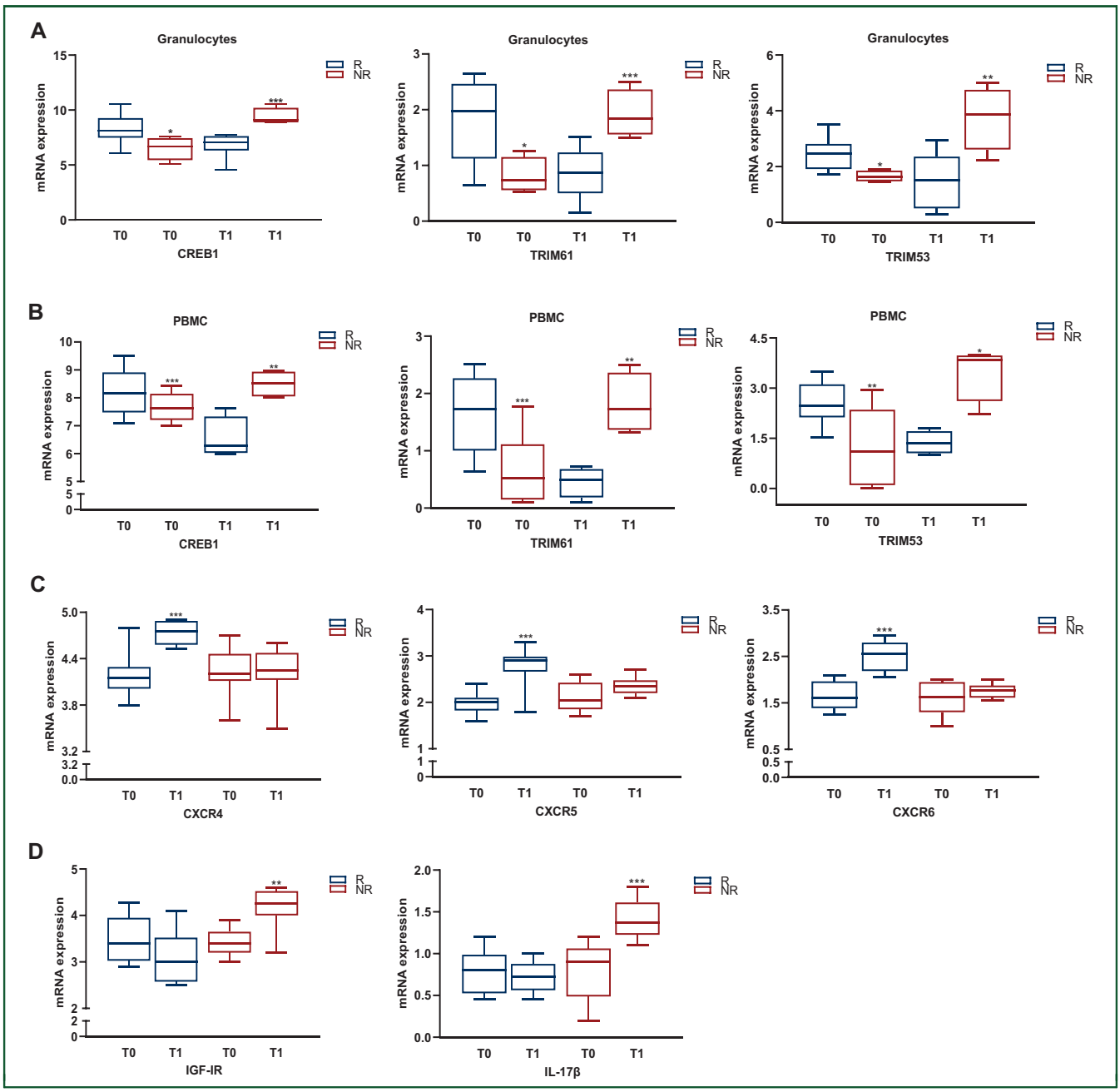

Figure 5. RNAseq validation. (A and B) Box-plot graphs of CREB1, TRIM61 and TRIM53 mRNA levels tested by real-time RT—PCR in granulocytes and in PBMCs from 20 patients treated by atezolizumab—bevacizumab. (C and D) Box-plot graphs of CXCR4, CXCR5, CXCR6, IGF-IR and mRNA levels tested by real-time RT—PCR in PBMCs from 20 patients treated by atezolizumab—bevacizumab. *Y*-axes report gene mRNA expression normalized to control represented by a pool of five HCC tissues. Real-time PCR was run in triplicate. Difference in expression levels was confirmed as statistically significant (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$) by two-tailed Student's *t*-test. IGF-IR, insulin-like growth factor-I receptor; IL-17β, interleukin 17β; NR, non-responders; PBMC, peripheral blood mononuclear cell; PCR, polymerase chain reaction; R, responders; RNAseq, RNA sequencing.

CXCR4, CXCR5 and CXCR6, belonging to the chemokine family, emerged by the RNAseq study as up-regulated in responder patients and were confirmed by RT—PCR. Instead, their variation was negligible in non-responders (Figure 5C). This observation might be the epiphenomena of a reinvigorated immune response triggered by immunotherapy; however, the mechanistic significance needs deeper investigations.

Among molecules inhibiting the immune response, we validated insulin-like growth factor-I receptor (IGF-IR) and interleukin 17β (IL-17β) expression, which turned out to be up-regulated in non-responders and not significantly modulated in responders, both in the RNAseq study and in the subsequent RT—PCR validation, as shown in Figure 5D.

The correlation between CTLA4+ percentage variation and its target genes suggested that CTLA4 might be functionally active and involved in immunotherapy response; however, it might also be the epiphenomenon of activating or resting programs.

DISCUSSION

Atezolizumab—bevacizumab is the standard of care for advanced HCC and novel ICIs have entered the clinical practice.[3,17,21] Despite more options being available for these patients, including TKIs, no predictive biomarker guides the personalization of treatments. Searching for a putative companion biomarker, with a prompt translational potential into the clinics, we investigated whether the baseline percentage of PD-L1+ and CTLA4+ circulating lymphocytes, monocytes and granulocytes and their early on-treatment variations might help the stratification of patients in terms of response.

We first focused on PD-L1, the target of atezolizumab. Baseline and early on-treatment variations of PD-L1+ CD45+ WBC populations did not display any predictive power. Then, CTLA4 was analyzed due to its intersection with PD-L1 pathway, the common regulatory factors triggering CTLA4 and PD-L1 expression[12,13] and its druggability.[21] A direct interaction between CD80 (one of the two CTLA4 ligands) and PD-L1 occurs in *cis*, in the same cell,[22,23] with a high affinity,[24,25] finally resulting in the significant interplay of the two pathways and in the regulation of PD-L1—PD-1 interactions by CD80 itself.[12] In line with this, we found a direct correlation between CTLA4+ and PD-L1+ cell percentages among granulocytes, lymphocytes and monocytes.

In our cohort of patients with HCC treated by atezolizumab—bevacizumab, a higher baseline percentage of CTLA4+ lymphocytes predicted response to treatment suggesting that a more pronounced immune resting phenotype might represent the ideal background for this kind of treatment.

Early on-treatment longitudinal dynamic changes of peripheral WBC immunophenotype turned out to be even more informative. An early reduction of CTLA4+ cell percentage on lymphocytes, granulocytes, monocytes and even more relevantly, gating together all CD45+ cells, was associated with response to treatment and predicted a longer TTP and OS. Conversely, the early increase of CTLA4+ cell percentage on lymphocytes, granulocytes, monocytes and, even more impressively, on all CD45+ cells predicted non-response, a shorter TTP and OS.

As a molecular correlate of CTLA4 variation, we confirmed the transcriptional modulation of CREB1, TRIM53 and TRIM61. These genes are known downstream targets of CTLA4 and regulators of cancer growth and immune-related pathways, correlating with expression of immune checkpoints and immune cell infiltration and predicting the response to immune therapy.[26-28] TRIM53, TRIM61 and CREB1 mRNA expression was up-regulated both in granulocytes and in PBMCs from patients displaying a CTLA4+ FC early on treatment, as well as in most non-responder patients.

We also validated the effects of atezolizumab—bevacizumab treatment in the modulation of CXCR4, CXCR5 and CXCR6. Being involved in cancer immune resistance and angiogenesis, some CXCR members are crucial in cell proliferation, survival, migration, angiogenesis and cross-talk between cancer and immune cells, contributing to modulation of immunotherapy.[29] CXCR4, CXCR5 and CXCR6 turned out to be up-regulated in responder patients, in line with their role in the activation of immune response.[30] Among factors involved in the inhibition of the immune response, IGF-IR and IL-17β expression was confirmed as up-regulated in non-responder patients. The coherence between variations of CTLA4+ cell percentage and CTLA4 molecular targets, together with the clinical correlates, suggests its effective participation in the modulation of response to treatment; however, this needs to be further confirmed.

Recently, AFP early drop was suggested as an early biomarker of non-response to atezolizumab—bevacizumab in HCC.[31] We cannot compare our findings with these data; however, 30% of our patients showed basal AFP levels $<$20 ng/ml. An interesting issue that emerged in our study was the relevance of granulocyte immunophenotype as a predictor of response to atezolizumab—bevacizumab in HCC. Although changes in CTLA4+ lymphocytes and monocytes were informative, those observed in granulocytes were the most relevant. Since granulocytes account for the majority of circulating CD45+ WBCs, we carried out analyses gating all white cell populations together. As expected, total CD45+ WBC immunophenotype changes displayed a higher accuracy than individual populations in the prediction of response to treatment in terms of both TTP and OS. On a translational perspective, this provides a very easy, cheap and feasible non-invasive test. On a mechanistic perspective, it outlines the relevance of the innate immunity component, which has been less investigated so far. Even though our data do not allow to generate mechanistic insights connecting CTLA4 modulation and immune system activation by atezolizumab—bevacizumab, yet they suggest CTLA4 inhibition as a possible approach to be evaluated, especially in non-responders. Notably, CTLA4 blocking agents have recently been introduced in the clinical practice with promising results[16,21] still

without companion biomarkers. Our findings might provide a rationale for investigating their predictive relevance to complement and eventually anticipate imaging assessment and to accelerate treatment adjustments and associations. This study has some limitations, including the small cohorts, still very well annotated, which are related to the disease and to the need to process fresh blood samples. In addition, besides the role of atezolizumab in the determination of immunophenotypic changes, we cannot rule out that of bevacizumab. Our previous investigations did not identify significant immunophenotypic changes resulting from TKIs[20]; however, the contribution of vascular endothelial growth factor inhibition cannot be excluded. We also need to remark the poor performance of our test in the few patients treated by locoregional approaches in the month before immunotherapy start. This is not unexpected since TARE or TACE might interfere with local immune system reactivity. This finding is in line with immune cell trapping in fibrous or damaged tissues surrounding treated tumor nodules or with a negative effect on immune response soon after a treatment such as TACE (which uses chemotherapy- and ischemia-induced damage) or TARE with radioactive compounds. Among patients missed by our test, five of six had been recently subjected to these treatments and larger cohorts are being enrolled to validate these findings.

Finally, it should be acknowledged that flow cytometry is an operator-dependent technique even though its use is widespread in the clinical practice.[32] Here, we limited the investigation to CTLA4 and PD-L1. Remarkably, other immune checkpoints, which include LAG-3, TIM-3, TIGIT and VISTA, have been investigated as possible targets for immunotherapy and might be revealed as informative biomarkers.[33] In the same line, within granulocytes and monocytes, we must consider the presence of myeloid-derived suppressor cells and other populations with crucial roles in cancer progression and immune response inhibition. Remarkably, our focus was to identify a non-invasive biomarker with a high accuracy to predict response to immunotherapy in HCC, to be rapidly translated into the clinics. Future studies will be directed to the deepening of underlying mechanistic events.

In conclusion, a higher baseline CTLA4+ lymphocyte percentage and the early dynamic changes of CTLA4+ cell percentage on total CD45+WBC provide an easy, quick and cheap putative predictive biomarker of response to atezolizumab—bevacizumab. The increase in the CTLA4+ total CD45+ WBC population, mainly sustained by an increased CTLA4+ granulocyte percentage, outlines the role of innate immunity and suggests its further validation as a biomarker to avoid delaying potential more effective treatments. Moreover, our results present a rationale for exploring CTLA4 inhibition as a drug combo to break down cancer resistance to atezolizumab.

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DISCLOSURE

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DATA SHARING

The data of this study is available from the corresponding author upon reasonable request (10.5281/zenodo.14725172).

REFERENCES

1. Llovet JM, Kelley RK, Villanueva A, et al. Hepatocellular carcinoma. *Nat Rev Dis Primers*. 2021;7(1):6.
2. Finn RS, Qin S, Ikeda M, et al. Atezolizumab plus bevacizumab in unresectable hepatocellular carcinoma. *N Engl J Med*. 2020;382(20):1894-1905.
3. Yau T, Kang YK, Kim TY, et al. Efficacy and safety of nivolumab plus ipilimumab in patients with advanced hepatocellular carcinoma previously treated with sorafenib: the CheckMate 040 randomized clinical trial. *JAMA Oncol*. 2020;6(11):e204564.
4. Lee CK, Chan SL, Chon HJ. Could we predict the response of immune checkpoint inhibitor treatment in hepatocellular carcinoma? *Cancers (Basel)*. 2022;14(13):3213.
5. Griffiths JI, Wallet P, Pflieger LT, et al. Circulating immune cell phenotype dynamics reflect the strength of tumor-immune cell interactions in patients during immunotherapy. *Proc Natl Acad Sci U S A*. 2020;117(27):16072-16082.
6. Suh KJ, Kim JW, Kim JE, et al. Correlation between tumor infiltrating immune cells and peripheral regulatory T cell determined using methylation analyses and its prognostic significance in resected gastric cancer. *PLoS One*. 2021;16(6):e0252480.
7. Gitto S, Natalini A, Antonangeli F, et al. The emerging interplay between recirculating and tissue-resident memory T cells in cancer immunity: lessons learned from PD-1/PD-L1 blockade therapy and remaining gaps. *Front Immunol*. 2021;12:755304.
8. Gungabeesoon J, Gort-Freitas NA, Kiss M, et al. A neutrophil response linked to tumor control in immunotherapy. *Cell*. 2023;186(7):1448-1464.e20.
9. Arvanitakis K, Mitroulis I, Germanidis G. Tumor-associated neutrophils in hepatocellular carcinoma pathogenesis, prognosis, and therapy. *Cancers (Basel)*. 2021;13(12):2899.
10. Wang CJ, Kenefeck R, Wardzinski L, et al. Cutting edge: cell-extrinsic immune regulation by CTLA-4 expressed on conventional T cells. *J Immunol*. 2012;189(3):1118-1122.
11. Rowshanravan B, Halliday N, Sansom DM. CTLA-4: a moving target in immunotherapy. *Blood*. 2018;131(1):58-67.

12. Kennedy A, Robinson MA, Hinze C, et al. The CTLA-4 immune checkpoint protein regulates PD-L1:PD-1 interaction via transendocytosis of its ligand CD80. *EMBO J*. 2023;42(5):e111556.
13. Zhang H, Dai Z, Wu W, et al. Regulatory mechanisms of immune checkpoints PD-L1 and CTLA-4 in cancer. *J Exp Clin Cancer Res*. 2021; 40(1):184.
14. An J, Kang HJ, Yu E, et al. The effects of immune checkpoint modulators on the clinical course of patients with resectable hepatocellular carcinoma. *J Liver Cancer*. 2022;22(1):40-50.
15. Kalathil S, Lugade AA, Miller A, et al. Higher frequencies of GARP(+) CTLA-4(+)Foxp3(+) T regulatory cells and myeloid-derived suppressor cells in hepatocellular carcinoma patients are associated with impaired T-cell functionality. *Cancer Res*. 2013;73(8):2435-2444.
16. Yau T, Zagonel V, Santoro A, et al. Nivolumab plus cabozantinib with or without ipilimumab for advanced hepatocellular carcinoma: results from cohort 6 of the CheckMate 040 trial. *J Clin Oncol*. 2023;41(9): 1747-1757.
17. Sangro B, Chan SL, Kelley RK, et al. Four-year overall survival update from the phase III HIMALAYA study of tremelimumab plus durvalumab in unresectable hepatocellular carcinoma. *Ann Oncol*. 2024;35(5): 448-457.
18. Kamphorst AO, Pillai RN, Yang S, et al. Proliferation of PD-1+ CD8 T cells in peripheral blood after PD-1-targeted therapy in lung cancer patients. *Proc Natl Acad Sci U S A*. 2017;114(19):4993-4998.
19. Giovannini C, Suzzi F, Tovoli F, et al. Low-baseline PD1+ granulocytes predict responses to atezolizumab-bevacizumab in hepatocellular carcinoma. *Cancers (Basel)*. 2023;15(6):1661.
20. Gramantieri L, Suzzi F, Bassi C, et al. Circulating CD8 lymphocytes predict response to atezolizumab-bevacizumab in hepatocellular carcinoma. *Eur J Immunol*. 2024;54(2):e2350637.
21. Abou-Alfa GK, Lau G, Kudo M, et al. Tremelimumab plus durvalumab in unresectable hepatocellular carcinoma. *NEJM Evid*. 2022;1(8):EVIDoa 2100070.
22. Chaudhri A, Xiao Y, Klee AN, et al. PD-L1 binds to B7-1 only in cis on the same cell surface. *Cancer Immunol Res*. 2018;6(8):921-929.
23. Sugiura D, Maruhashi T, Okazaki IM, et al. Restriction of PD-1 function by cis-PD-L1/CD80 interactions is required for optimal T cell responses. *Science*. 2019;364(6440):558-566.
24. Butte MJ, Keir ME, Phamduy TB, et al. Programmed death-1 ligand 1 interacts specifically with the B7-1 costimulatory molecule to inhibit T cell responses. *Immunity*. 2007;27(1):111-122.
25. Butte MJ, Pena-Cruz V, Kim MJ, et al. Interaction of human PD-L1 and B7-1. *Mol Immunol*. 2008;45(13):3567-3572.
26. Cao J, Su B, Peng R, et al. Bioinformatics analysis of immune infiltrates and tripartite motif (TRIM) family genes in hepatocellular carcinoma. *J Gastrointest Oncol*. 2022;13(4):1942-1958.
27. Gao Y, Pan T, Xu G, et al. Pan-cancer illumination of TRIM gene family reveals immunology regulation and potential therapeutic implications. *Hum Genomics*. 2022;16(1):65.
28. Shen H, Gu X, Li H, et al. Exploring prognosis, tumor microenvironment and tumor immune infiltration in hepatocellular carcinoma based on ATF/CREB transcription factor family gene-related model. *J Hepatocell Carcinoma*. 2023;10:327-345.
29. Santagata S, Ierano C, Trotta AM, et al. CXCR4 and CXCR7 signaling pathways: a focus on the cross-talk between cancer cells and tumor microenvironment. *Front Oncol*. 2021;11:591386.
30. Chen J, Bai Y, Xue K, et al. CREB1-driven CXCR4(hi) neutrophils promote skin inflammation in mouse models and human patients. *Nat Commun*. 2023;14(1):5894.
31. Zhu AX, Dayyani F, Yen CJ, et al. Alpha-fetoprotein as a potential surrogate biomarker for atezolizumab + bevacizumab treatment of hepatocellular carcinoma. *Clin Cancer Res*. 2022;28(16):3537-3545.
32. Cai Q, Lan H, Yi D, et al. Flow cytometry in acute myeloid leukemia and detection of minimal residual disease. *Clin Chim Acta*. 2025;564:119945.
33. Sharma P, Goswami S, Raychaudhuri D, et al. Immune checkpoint therapy-current perspective and future directions. *Cell*. 2023;13:1652-1659.