A Baseline Myeloid Immunosuppression Signature Associated with Resistance to Anti–PD-1 Plus Lenvatinib in Hepatocellular Carcinoma

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Abstract

Background. Combination anti–PD-1 immune checkpoint blockade with the anti-angiogenic multi-kinase inhibitor lenvatinib is a systemic treatment option for advanced hepatocellular carcinoma (HCC), yet most patients derive no durable benefit, and no validated pre-treatment biomarker of response exists. The myeloid compartment is increasingly recognised as a determinant of immunotherapy outcome, but how the baseline myeloid landscape differs between responders and non-responders, and whether such differences are therapeutically actionable, remains poorly defined.

Methods. Re-analysed a public single-cell RNA-sequencing dataset (GSE235863) of nine HBV-positive HCC patients treated with anti–PD-1 plus lenvatinib. Pre-treatment tumours were compared between Partial Response (PR; n = 3, 13,124 cells) and Progressive Disease (PD; n = 2, 8,741 cells) using a patient-level pseudobulk framework with DESeq2. Robustness was assessed by limma-voom, gene-set enrichment analysis (GSEA) across five MSigDB collections, a cell-type specificity control, and a complete-responder sensitivity analysis. Druggability and genetic co-dependency were evaluated using DGIdb and DepMap. Myeloid–T-cell communication was explored using CellChat (preliminary).

Results. Pseudobulk analysis identified 71 differentially expressed genes (adjusted $p < 0.05$), of which 49 were elevated in PD and 22 in PR. The PD-elevated genes formed a coherent myeloid/macrophage programme, including CD14, FCGR1A, FCGR3A, SYK, MERTK and ADORA3; PR-elevated genes were dominated by patient-specific immunoglobulin and T-cell-receptor clonotypes. The myeloid signal was recovered by five independent analyses, most strikingly a liver Kupffer-cell signature (normalised enrichment score [NES] −3.62; adjusted $p = 1.1 \times 10^{-58}$). Three druggable targets were nominated — SYK (fostamatinib), MERTK/FLT3 (gilteritinib) and ADORA3 (namodenoson) — each with an approved or clinical-stage agent.

Conclusions. A baseline myeloid/inflammatory signature is associated with resistance to anti–PD-1 plus lenvatinib in HCC and nominates druggable, repurposable therapeutic targets. The findings are hypothesis-generating, limited by cohort size, and require prospective validation.

Keywords: *hepatocellular carcinoma; immune checkpoint blockade; lenvatinib; tumour-associated macrophages; single-cell RNA-sequencing; pseudobulk differential expression; drug repurposing.*

1. Introduction

Hepatocellular carcinoma (HCC) is the most common primary liver cancer and the third leading cause of cancer-related death worldwide; the global burden is projected to rise, with liver cancer incidence expected to increase by approximately 55% by 2040 to an estimated 1.4 million new cases and 1.3 million deaths annually[1,2]. For advanced disease, combination anti–PD-1 immune checkpoint blockade with the anti-angiogenic multi-kinase inhibitor lenvatinib is a systemic treatment option; however, in the phase III LEAP-002 trial the objective response rate was approximately 26%, and the trial did not meet its primary survival endpoint, underscoring that the majority of patients derive no durable benefit[3]. No validated pre-treatment biomarker distinguishes likely responders from non-responders, and treatment selection therefore remains largely empirical[4].

Although anti-tumour immunity has historically centred on T cells, the myeloid compartment is increasingly recognised as a decisive determinant of immunotherapy outcome[4,5]. Tumour-associated macrophages (TAMs) and monocytes establish an immunosuppressive tumour microenvironment, restrain cytotoxic T-cell activity, and drive both primary and acquired resistance to checkpoint blockade[5]. In HCC specifically, intratumoural hypoxia promotes the recruitment and polarisation of immunosuppressive TAM populations; for example, hypoxia-induced TREM1-positive TAMs recruit

regulatory T cells and confer resistance to anti–PD-L1 therapy[6] — a mechanism of direct relevance to combined anti-angiogenic and immunotherapy regimens.

These myeloid states are functionally heterogeneous: distinct macrophage and dendritic-cell populations differ in their inflammatory, angiogenic and regulatory programmes, and their balance can either support or suppress an effective anti-tumour response[5]. How this baseline myeloid landscape differs between patients who respond and those who progress remains poorly defined, and whether such differences are therapeutically actionable is unknown. Here, we performed a single-cell transcriptomic re-analysis of pre-treatment HCC tumours to (i) define differential gene expression between Partial Response (PR) and Progressive Disease (PD), (ii) resolve the myeloid compartment at single-cell resolution, and (iii) nominate druggable therapeutic targets from any validated resistance signature.

2. Methods

2.1 Data source and cohort

Publicly available single-cell RNA-sequencing data were obtained from Gene Expression Omnibus accession GSE235863[7]. The dataset comprises nine HBV-positive HCC patients treated with anti–PD-1 plus lenvatinib, with paired pre- and post-treatment CD45-positive tumour biopsies where available (86,252 cells in total). Patients were stratified by Response Evaluation Criteria in Solid Tumours (RECIST) version 1.1 into Partial Response (PR; n = 3), Progressive Disease (PD; n = 2), Stable Disease (n = 3) and Complete Response (n = 1). The single complete responder and the stable-disease patients were excluded from formal differential testing to provide an unambiguous contrast, leaving five evaluable patients (three PR, two PD) for the primary comparison.

2.2 Preprocessing and dimensionality reduction

Deposited per-cell quality-control metrics (mitochondrial content, doublet calls and cell-cycle phase) were used directly. Raw counts were normalised using SCTransform (version 2) with 5,000 variable features and regression of mitochondrial percentage, followed by principal component analysis (50 components) within a single shared embedding.

2.3 Clustering and cluster-structure evaluation

A shared-nearest-neighbour graph (k = 30) and Leiden clustering (resolution 0.3) were computed. To determine whether the resulting clusters represented discrete cell states, silhouette width was evaluated across 110 tested combinations of neighbourhood size and clustering resolution (k = 10–100; resolution 0.1–1.0). All silhouette values were below 0.081, indicating a continuous rather than discretely partitioned transcriptional landscape. This finding motivated a patient-level pseudobulk framework for differential expression, in preference to cluster-based testing.

2.4 Cell-type annotation and cross-validation

Whole-tumour cell-type annotation was performed with CellTypist using majority voting, yielding seven major cell types. Annotations were cross-validated against three independent reference-based methods (SingleR, scType and SCINA) without manual gene curation; concordance in the myeloid compartment was 99.9% precision and 95.6% recall (13,159 of 13,168 cells). The myeloid compartment was sub-clustered at resolution 0.4 into nine functional states, whose identities were confirmed by canonical marker expression.

2.5 Pseudobulk differential expression

For each patient, raw RNA counts were summed across all cells to generate a single pseudobulk profile, collapsing 21,865 pre-treatment cells into five patient-level samples. Differential expression between PR and PD was tested with DESeq2 using a negative-binomial generalised linear model and Wald test,

with PD as the reference level (contrast: PR versus PD); accordingly, negative log2 fold-changes denote genes elevated in PD. Genes with a total count below 10 across all samples were removed prior to testing (19,321 genes retained). Multiple testing was controlled by the Benjamini–Hochberg procedure, with significance defined as adjusted $p < 0.05$.

2.6 Robustness and validation

The differential expression result was assessed by four additional analyses: (i) an independent precision-weighted linear-model framework (limma-voom); (ii) gene-set enrichment analysis (GSEA; fgsea) across five MSigDB collections (Hallmark [H], curated pathways [C2], oncogenic signatures [C6], immunologic signatures [C7] and cell-type signatures [C8]), with genes ranked by the DESeq2 Wald statistic; (iii) a cell-type specificity control comprising separate pseudobulk analyses of natural killer/natural killer T (NK/NKT) and regulatory T (Treg) cells; and (iv) a complete-responder sensitivity analysis.

2.7 Druggability and genetic co-dependency

Significant genes were queried against the Drug Gene Interaction Database (DGIdb) to identify druggable targets with approved or clinical-stage agents. Genetic co-dependency for the nominated target MERTK was assessed using genome-wide CRISPR knockout essentiality data from the Cancer Dependency Map (DepMap; approximately 1,005 cancer cell lines) by Spearman correlation of essentiality profiles.

2.8 Myeloid–T-cell communication (preliminary)

Intercellular communication between myeloid and lymphoid subtypes was explored using CellChat on the pre-treatment PR and PD cohorts (21,763 cells; PR = 13,084, PD = 8,679). Subpopulations with fewer than 10 cells in a given arm (mregDC, perivascular-TAM and S100A8-monocyte in the PR arm) were handled using CellChat's liftCellChat alignment before merging. Pathways detected in only one group produced mathematically undefined contribution ratios; these were classified and excluded, and only pathways with genuine graded differences were retained. This analysis is reported as preliminary.

2.9 Software and reproducibility

All analyses were performed in R version 4.5.0 with a fixed random seed (42) on the KAUST Ibex high-performance computing cluster. Primary packages included Seurat, SCTransform, CellTypist, SingleR, scType, SCINA, DESeq2, limma, fgsea and CellChat.

3. Results

3.1 A baseline myeloid signature distinguishes progressive disease

Patient-level pseudobulk comparison of pre-treatment tumours (PR, n = 3, 13,124 cells; PD, n = 2, 8,741 cells; 19,321 genes tested) identified 71 differentially expressed genes at adjusted $p < 0.05$, of which 49 were elevated in Progressive Disease and 22 in Partial Response. The cohort composition is summarised in Table 1. The PD-elevated genes formed a coherent myeloid/macrophage programme; the most statistically robust and druggable members are listed in Table 2, and include CD14 (log2 fold-change −2.06; adjusted $p = 2.2 \times 10^{-5}$), FCGR3A, FCGR1A, SYK, MERTK and ADORA3. In contrast, the PR-elevated genes were dominated by patient-specific (private) immunoglobulin and T-cell-receptor clonotypes and did not constitute a coherent transcriptional programme.

Table 1. *Cohort composition and evaluable groups for the primary pre-treatment comparison.*

RECIST group	Patients (n)	Cells	Use in analysis
Partial Response (PR)	3	13,124	Primary comparison

RECIST group	Patients (n)	Cells	Use in analysis
Progressive Disease (PD)	2	8,741	Primary comparison
Stable Disease (SD)	3	—	Excluded
Complete Response (CR)	1	—	Excluded; sensitivity analysis
Total (evaluable)	**5**	**21,865**	**3 PR vs 2 PD**

Table 2. *Druggable, high-confidence genes elevated in Progressive Disease. Negative log2 fold-changes denote elevation in PD (contrast: PR versus PD). Adjusted p-values are Benjamini–Hochberg corrected.*

Gene	log2 fold-change	Adjusted p	Function / rationale
CD14	−2.06	2.2×10^{-5}	Monocyte/macrophage co-receptor; innate immune activation
FCGR3A	−1.67	2.4×10^{-5}	Fc-γ receptor IIIa; antibody-mediated myeloid signalling
FCGR1A	−2.03	4.1×10^{-5}	Fc-γ receptor Ia; upstream of SYK signalling
SYK	−1.22	7.7×10^{-4}	Spleen tyrosine kinase; Fc-receptor signal transduction
MERTK	−2.06	2.8×10^{-3}	TAM-family kinase; efferocytosis-driven immunosuppression
FLT3	−1.96	3.7×10^{-2}	Receptor tyrosine kinase; myeloid/dendritic differentiation
ADORA3	−3.40	1.7×10^{-2}	A3 adenosine receptor; adenosine-mediated immunosuppression

3.2 Independent annotation methods converge on the same myeloid landscape

Whole-tumour annotation by CellTypist (seven cell types) and independent SingleR-fine annotation (15 clusters) recovered the same major lineages. Concordance in the myeloid compartment was 99.9% precision and 95.6% recall (13,159 of 13,168 cells), confirming a robust cellular atlas prior to differential testing. Sub-clustering of the myeloid compartment resolved nine functional states, including an angiogenesis-associated perivascular-TAM population (NRP1, STAB1, FOLR2) and a mature regulatory dendritic-cell (mregDC) population (LAMP3, CCR7, IDO1); each state was defined by its canonical markers.

3.3 Five independent analyses converge on myeloid and inflammatory biology

The myeloid/inflammatory signal in PD was recovered by five independent analyses. First, GSEA across all five MSigDB collections independently converged on myeloid and inflammatory biology (Table 3), most strikingly a liver Kupffer-cell signature in the cell-type collection (NES −3.62; adjusted $p = 1.1 \times 10^{-58}$). Second, an independent precision-weighted framework (limma-voom) preserved 65 of 71 genes (92%) in a concordant direction. Third, a cell-type specificity control demonstrated that NK/NKT and Treg populations yielded only dissociation-stress and private-clonotype artefacts, confirming that the signal was specifically myeloid. Fourth, a complete-responder sensitivity analysis confirmed that the principal components were stable with and without the excluded complete responder (patient-level correlation $r < 0.19$). Fifth, an on-treatment analysis identified a distinct inflammatory-monocyte programme in responders (151 genes; S100A8/9/12, CD14, FCN1) with no overlap with the baseline signature, confirming the temporal specificity of the pre-treatment signature.

Table 3. *Top Progressive-Disease-enriched gene set per MSigDB collection (GSEA; genes ranked by the DESeq2 Wald statistic). Negative NES denotes enrichment in PD.*

Collection	Top PD-enriched gene set	NES	Adjusted p	Theme
H (Hallmark)	*TNFα signalling via NF-κB*	−2.37	4.9×10^{-12}	Inflammation
C2 (pathways)	*Myeloid / AML programme (RUTELLA CSF2RB–IL4)*	−2.88	1.9×10^{-29}	Myeloid
C6 (oncogenic)	*RPS14 myeloid axis (RPS14_DN)*	−2.56	1.1×10^{-13}	Myeloid
C7 (immunologic)	*Myeloid-over-lymphoid signatures*	−3.56	1.8×10^{-48}	Myeloid skew
C8 (cell-type)	*Liver Kupffer cells (AIZARANI)*	−3.62	1.1×10^{-58}	Liver macrophage

Note: *The single most-enriched C7 set is a natural killer-cell contrast; C7 is therefore summarised as a myeloid-over-lymphoid skew. In C8, fetal-myeloid signatures rank marginally above Kupffer cells by NES; the liver-resident Kupffer-cell signature is highlighted as the top disease-relevant hit.*

3.4 Genetic co-dependency links MERTK to a Wnt/β-catenin subtype

To place the nominated target MERTK within established cancer biology, genome-wide CRISPR essentiality data from DepMap (approximately 1,005 cancer cell lines) were examined. MERTK's most strongly co-dependent gene, genome-wide, was CTNNB1 (β-catenin; Spearman $r = -0.18$; adjusted $p = 7.2 \times 10^{-8}$). As DepMap is pan-cancer, this represents a general functional association rather than HCC-specific evidence; nonetheless, because CTNNB1 activation defines a major molecular subtype of HCC, the co-dependency provides suggestive corroboration that MERTK lies within HCC-relevant Wnt/β-catenin biology.

3.5 Three druggable, repurposable targets

Querying the significant genes against DGIdb nominated three targets, each with an approved or clinical-stage agent and a distinct immunosuppressive mechanism. The Fc-receptor–SYK axis (FCGR1A, FCGR3A, SYK) is targetable by fostamatinib, an oral prodrug whose active metabolite inhibits spleen tyrosine kinase; MERTK and FLT3 are jointly targetable by gilteritinib, a single approved FLT3/AXL inhibitor with activity against MER kinase; and ADORA3 is the direct target of namodenoson, an A3-adenosine-receptor agonist in phase III development specifically for HCC. Fostamatinib and gilteritinib are approved for other indications (chronic immune thrombocytopenia and FLT3-mutated acute myeloid leukaemia, respectively), positioning all three as drug-repurposing hypotheses.

4. Discussion

This study identifies a baseline myeloid/inflammatory transcriptional signature associated with resistance to anti–PD-1 plus lenvatinib in HCC, present before treatment, recovered by five independent analyses, and connected to druggable therapeutic targets. The result reframes immunotherapy resistance in this setting as, in part, a myeloid phenomenon, and demonstrates that the pre-treatment myeloid state already encodes information distinguishing responders from non-responders.

The nominated targets converge on complementary immunosuppressive mechanisms. The Fc-γ receptor–SYK axis integrates antibody-mediated signalling in TAMs; its inhibition by fostamatinib reduces macrophage phagocytic and inflammatory signalling. MERTK drives efferocytosis — the clearance of apoptotic cells — which delivers a tolerogenic, immunosuppressive signal that dampens anti-tumour immunity; gilteritinib inhibits MERTK alongside FLT3, addressing two signature genes

with a single agent. ADORA3 mediates adenosine-driven immunosuppression, and its agonist namodenoson acts through downregulation of TNFα/NF-κB inflammatory signalling — notably, the same programme independently identified as the top Hallmark enrichment in progressive disease. This convergence between an independent enrichment result and a candidate drug mechanism strengthens the biological plausibility of the nomination.

From a clinical perspective, unselected anti–PD-1 plus lenvatinib benefits only a minority of patients. A validated baseline myeloid signature could enable patient selection — sparing likely non-responders from ineffective, toxic therapy — and could indicate which myeloid-targeted agent to combine with checkpoint blockade for tumours carrying the resistance programme. Because all three nominated agents are already approved or in late-stage trials, the translational path from this signature to a biomarker-guided combination trial is unusually short.

Several limitations must be emphasised. The cohort is small (three responders, two progressors), and with two progressors the response group and individual patients are partly confounded; the findings are therefore hypothesis-generating rather than a validated biomarker. The analysis is computational and correlative: it prioritises targets but does not demonstrate functional efficacy. The DepMap co-dependency is modest in magnitude and pan-cancer in origin, and is presented as supporting rather than primary evidence. Finally, the results are specific to the anti–PD-1 plus lenvatinib regimen represented in the dataset and may not generalise to other first-line combinations such as atezolizumab plus bevacizumab. The principal mitigations against over-interpretation are the convergence of five methodologically independent analyses and, where applicable, internal replication across analytical schemes.

4.1 Future directions

Three priorities follow. First, prospective validation in an independent, adequately powered cohort is essential to test whether the signature predicts response and to separate patient-level variation from the response effect. Second, mechanistic characterisation of myeloid–T-cell communication (see Supplementary analysis) in a larger cohort would define how these myeloid populations suppress the T-cell response. Third, functional validation — testing whether SYK or MERTK inhibition re-sensitises resistant tumours to checkpoint blockade in co-culture or animal models — would establish causality and move the nominated targets from association toward therapeutic strategy.

5. Conclusion

A baseline myeloid/inflammatory signature is associated with resistance to anti–PD-1 plus lenvatinib in hepatocellular carcinoma and nominates three druggable, repurposable targets — SYK, MERTK/FLT3 and ADORA3. Validated across five independent analyses and connected to approved or clinical-stage agents, the signature offers a concrete, testable hypothesis for pre-treatment patient stratification in a disease where treatment selection is currently empirical. Prospective and functional validation are the necessary next steps.

Supplementary Analysis: Myeloid–T-cell Communication (Preliminary)

To explore how the myeloid compartment signals to T cells, intercellular communication was inferred using CellChat on pre-treatment PR and PD tumours (21,763 cells; PR = 13,084, PD = 8,679), including myeloid and lymphoid subtypes together. Three rare myeloid subpopulations fell below the 10-cell threshold in the PR arm (mregDC, 4 cells; perivascular-TAM, 1 cell; S100A8-monocyte, 9 cells) and were handled by CellChat's liftCellChat alignment prior to merging, which succeeded. On differential pathway testing, 20 pathways were detected in only one group and produced mathematically undefined contribution ratios; these degenerate results were classified and excluded, along with near-null and non-

significant pathways. Twenty-seven pathways showed genuine, graded differences between groups, of which 26 were elevated in PD and one (MHC-I) in PR. The strongest PD-elevated signalling pathways included LIGHT (TNFSF14), PECAM1, FN1, SPP1 and SEMA7, and ITGB2. Two pathways — ITGB2 and LIGHT — independently replicated the direction of a separate whole-tumour CellChat comparison, and the single PR-elevated pathway (MHC-I) likewise echoed the antigen-presentation signal seen in responders in that comparison. This analysis is preliminary, is limited by the same cohort size as the primary comparison, and is reported to indicate a direction for mechanistic follow-up rather than as a definitive result. CellChat infers communication from ligand–receptor co-expression and does not measure interaction directly; the pathways are therefore prioritised hypotheses.

Acknowledgements

The authors thank the KAUST Supercomputing Core Laboratory for access to the Ibex high-performance computing cluster, on which all analyses were performed, and the KAUST Academy for programme support.

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