Extracellular Vesicle-Associated PD-L1 as a Biomarker of Hepatocarcinogenesis in HIV/HCV Coinfection

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BACKGROUND

Despite achieving sustained virologic response (SVR) with direct-acting antivirals (DAA), individuals **HCV/HIV+** with **cirrhosis** remain at risk of developing **hepatocellular carcinoma (HCC)**. Identifying biomarkers that anticipate HCC onset is therefore crucial. We have previously reported that **plasma extracellular vesicles (pEV)** from individuals HCV/HIV+ who later developed HCC **downmodulate NK cell function**. The **aim of this study** was to define the underlying **mechanisms** contributing to **immune evasion** and **tumor development**, focusing on **PD-L1** carried by **pEV** as a potential target for early detection and prevention.

METHODS

Plasma samples were collected after SVR from HCV/HIV+ individuals with mild **(METAVIR F0/F1, n=4)** and advanced liver disease **(METAVIR F4, n=9)** following DAA therapy. Notably, four participants with cirrhosis who later developed HCC, a median of 7.00 years (IQR 4.67–7.96) after achieving viral clearance, had available plasma samples collected prior to their cancer diagnosis, providing a unique opportunity to identify early plasma signatures linked to HCC emergence. The **pEV** were isolated by **size exclusion chromatography** from 2 mL of plasma and captured overnight with anti-CD9–coupled beads and stained with anti-CD9 and anti–PD-L1 antibodies. Expression of markers on pEV was analyzed by **flow cytometry**.

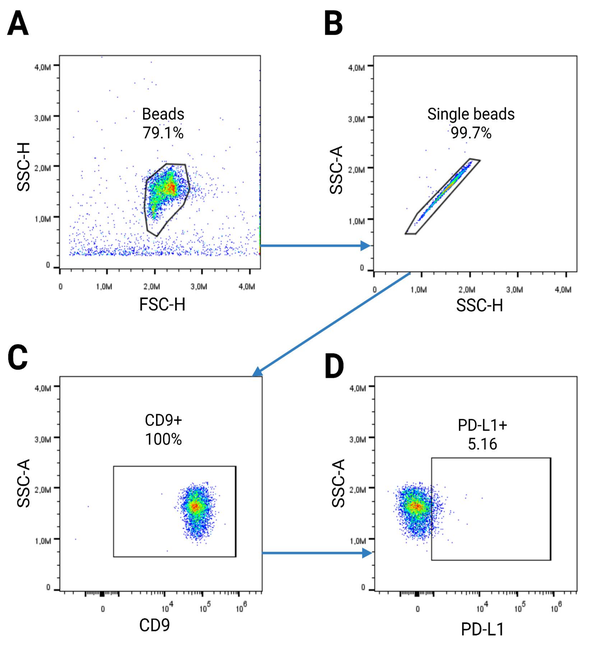


Figure 1. Flow cytometry gating strategy for EVs captured on anti-CD9 microbeads.
(A) Identification of microbeads by SSC-H vs FSC-H.(B) Singlet selection by SSC-A vs SSC-H.(C) Detection of CD9-positive microbeads confirming EV binding. (D) Identification of PD-L1–positive events.

Longitudinal analysis reveals that PD-L1 expression increases on plasma extracellular vesicles years before HCC diagnosis in individuals HCV/HIV+ with cirrhosis, supporting its potential as an early biomarker of hepatocarcinogenesis.

RESULTS

At 12 months post-SVR (12mpt), **PD-L1** expression on **pEV** was low across all groups: **3.17%** (IQR 1.59 - 3.96) in healthy controls, **6.53%** (IQR 6.00–8.70) in individuals with **METAVIR F0/F1**, **1.70%** in individuals with **METAVIR F4 without HCC**, and **3.15%** (IQR 1.38–4.92) in individuals with **METAVIR F4 with HCC**.

Importantly, longitudinal samples available from **F4** individuals who later developed **HCC** revealed a marked **rise** in **PD-L1** expression at later post-SVR intervals (40–90 months, >>12mpt), reaching **23.30%** (IQR 17.10–29.50) at least 5 months prior to HCC suspicions. This increase was **not observed** in individuals with cirrhosis who **did not develop HCC**, underscoring the potential of **pEV-associated PD-L1** as an **early indicator** of **hepatocarcinogenesis**.

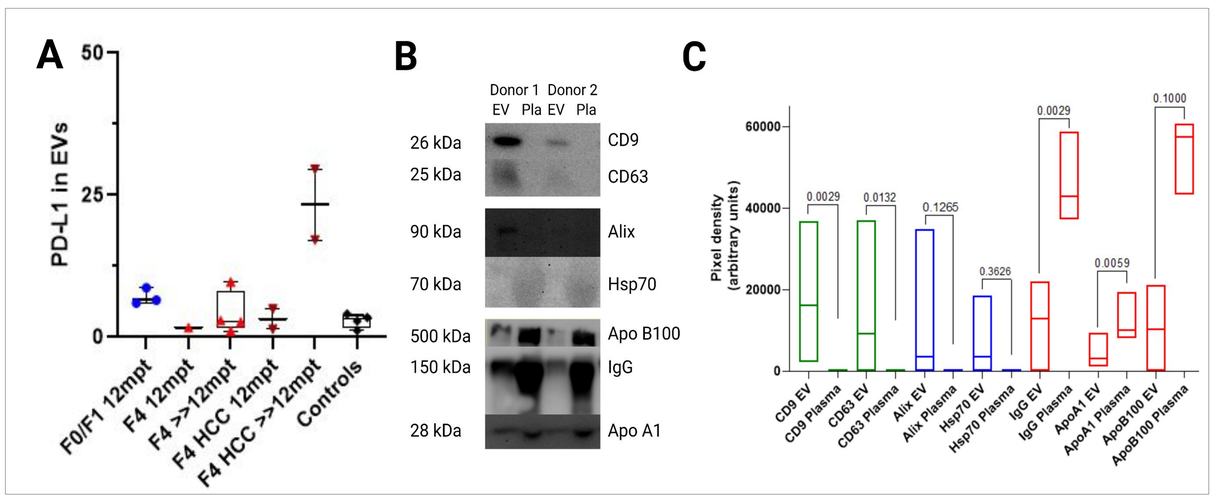


Figure 2. PD-L1 expression in plasma-derived extracellular vesicles (pEVs) and Western blot characterization.

(A) Percentage of PD-L1–positive pEVs across fibrosis stages and follow-up times.

(B) Representative Western blot of EV markers in EV fractions and plasma.

(C) Quantification of band intensity by pixel density analysis: **Tetraspanins**, **Endosomic-origin markers**, **Plasma-contamination markers**.

Characteristics	F4 without HCC (n = 12)				F4 with HCC (n = 6)				p value
	BSL	EoT	12mpt	>>12mpt	BSL	EoT	12mpt	>>12mpt	
Age (years)	49 (45-52)	–	–	–	52 (48-60)	–	–	–	0.24
Male sex (n, %)	8 (66.7)	–	–	–	5 (83.3)	–	–	–	0.46
T CD4 (cel/µL)	384 (244-682)	547 (379-741)	593 (273-946)	584[a] (513-829)	486 (248-842)	262 (156-616)	ND	245[a] (36-593)	0.08[a]
T CD8 (cel/µL)	917 (743-1134)	1023 (911-1247)	1146 (524-1710)	963[a] (800-1346)	431 (284-1064)	391 (158-575)	ND	403[a] (113-849)	0.04[a]
CD4/CD8 ratio	0.51 (0.23-0.80)	0.51 (0.37-0.73)	0.30 (0.20-1.30)	0.70[a] (0.50-1.00)	0.59 (0.45-1.83)	0.97 (0.57-2.07)	ND	0.68[a] (0.31-1.90)	0.93[a]

Table 1. Clinical characteristics of the total cohort. BSL: Baseline, before starting DAA. EoT: End of treatment. 12mpt: 12 months post-treatment. >>12mpt: more than 12 months post-treatment.

CONCLUSIONS

PD-L1 expression on **pEV** is low at early post-SVR time points but **increases** over time in individuals with cirrhosis who subsequently **develop HCC**, suggesting its potential value as **early biomarker** of **hepatocarcinogenesis**. The availability of longitudinal plasma samples obtained years before HCC diagnosis provides unique evidence supporting the role of PD-L1 as a predictive marker. Monitoring PD-L1 on pEVs may therefore support **risk stratification** for **HCC development** and inform **differential surveillance strategies** focused on high-risk individuals.

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