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T-cell mediated responses against alpha-foetoprotein in hepatocellular carcinoma: Relationship with hepatitis C virus infection, tumour phenotype and patients' survival

David J. Pinato[1] | Petros Fessas[1] | Antonello Gibbin[2] | Giuseppa Occhino[2] | Elisa Boccato[2] | Carlo Smirne[2] | Rosalba Minisini[2] | Mario Pirisi[2,3]

[1]Division of Surgery and Cancer, Imperial College London, Hammersmith Hospital, London, UK

[2]Department of Translational Medicine, Università degli Studi del Piemonte Orientale "A. Avogadro", Novara, Italy

[3]Interdisciplinary Research Center of Autoimmune Diseases, Università degli Studi del Piemonte Orientale "A. Avogadro", Novara, Italy

Correspondence
Mario Pirisi, Full Professor in Internal Medicine, Università degli Studi del Piemonte Orientale "A. Avogadro", Via Solaroli, 17 - 27100, Novara, Italy.
Email: mario.pirisi@med.unipmn.it

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Abstract

Background: Alpha-foetoprotein (AFP) is a potential immunotherapeutic target in hepatocellular carcinoma (HCC). However, T-cell response (TR) to AFP is suppressed in HCC due to immune evasion. It is unknown whether HCV infection may precondition TR against AFP, or whether TR may influence the clinical course of HCC.

Methods: We prospectively enrolled 18 HCV+ treatment-naïve patients with cirrhosis (CC), 18 HCV+ HCC cases and 17 HCV- HCC cases. TR was quantified by ELISPOT using assays specific to interleukin (IL) 2, IL10 and granulocyte-monocyte colony stimulating factor (GM-CSF) on ex-vivo peripheral blood mononuclear cells (PBMC) stimulated in vitro with AFP peptides. Cytokine ratios were compared between groups and with clinicopathological features of HCC, including overall survival (OS).

Results: The proportion of AFP-specific responses was not different across the studied groups for any of the assayed cytokines. AFP-specific IL-2 responses were increased in larger ($P = .02$), multifocal tumours ($P = .01$) and correlated with advanced disease ($P = .01$). TRs did not correlate with other clinicopathological factors and did not predict for OS.

Conclusion: Tumour stage but not HCV infection is related to the emergence of anti-AFP TRs. These data enable formulation of a rationale for the further development of anti-AFP immunotherapy in HCC, facilitating optimal patient selection for future studies.

KEYWORDS

GM-CSF, HCC, HCV, IL-10, IL-2, T-cell response

David J. Pinato and Petros Fessas contributed equally to the manuscript.

1 | INTRODUCTION

Alpha-foetoprotein (AFP) is a functionally uncharacterized oncofetal protein whose expression is suppressed in adulthood,[1] being reactivated in chronic liver disease and hepatocarcinogenesis.[2] Serum AFP is a known biomarker in the prognostic assessment and therapeutic monitoring of hepatocellular carcinoma (HCC).[3-5] Lending further support to its biological significance in HCC, AFP is now an established stratification biomarker for the second line use of ramucirumab, a monoclonal antibody (mAb) against vascular endothelial growth factor (VEGF-R) receptor-2.[6,7]

The systemic therapy arsenal against HCC has significantly expanded in recent years. First line options sorafenib and lenvatinib, which are multi-targeted tyrosine kinase inhibitors,[8,9] have recently been joined by the combination of monoclonal antibodies against programmed cell death-1 protein (PD-1) and VEGF-A.[10] Other than ramucirumab, second line options include regorafenib and cabozantinib.[4,11] Immune checkpoint inhibition with mAbs against PD-1 and its ligand (PD-L1) and the cytotoxic T-cell lymphocyte associated antigen-4 (CTLA-4) is able to induce tumour rejection, although monotherapy has so far not demonstrated a significant survival benefit in HCC.[12-14]

The restriction of AFP expression to neoplastic hepatocytes in patients with AFP-producing HCC may render it a suitable target for tumour-selective toxicity in the evolving landscape of immunotherapy.[15] Previous studies have shown that stimulation of the immune system with AFP peptides may give rise to a coordinated T-helper 1 (Th1) response ex vivo.[16] However, the parallel emergence of a suppressive response following stimulation may limit the clinical efficacy of AFP-based immunotherapy.[17,18] Factors affecting the delicate balance between an active antitumour response and immune tolerance towards tumour-associated antigens (TAA) are yet to be fully elucidated. However, the generation of inducible regulatory T cells (T-reg), a specific CD4+ T lymphocyte subpopulation that secretes interleukin (IL) 10 and granulocyte-monocyte colony stimulating factor (GM-CSF),[19] represents a key immunological event underlying tumour cell escape from immune surveillance [20] and promoting tumour progression.[21]

Previous reports have shown that tumour-related features are not the sole determinants influencing the mounting of a specific immune response against AFP. The severity of the underlying chronic liver disease (CLD) has in fact been shown to shape T-cell responses (TR), with a predominant activation of the cytotoxic branch in advanced cirrhosis independent from tumour burden.[22] The progression of CLD to cirrhosis and HCC is intimately determined by the host immune response, which is differentially modulated in post-viral CLD, whether Hepatitis B (HBV) or C virus (HCV) related, compared to other etiologies.[23,24] In particular, HCV infection is known to directly impair dendritic cell maturation,[25] leading to less effective priming and activation of CD4+ T lymphocytes.[26,27] This lower basal level of immune activation may explain the observation that responses to immunotherapy and sorafenib are greater in HCV+ HCC.[28] Defining patient subpopulations with preserved immunocompetence against AFP epitopes is a necessary step to facilitate the clinical development of AFP-based immunotherapy as a potential treatment strategy in HCC. Following on from previous observations, we devised this study to ascertain whether HCV infection could represent a pre-conditioning factor influencing the deployment of an effective antitumour TR following stimulation with immunogenic AFP peptides. For this purpose, we comparatively tested ex vivo the emergence and clinicopathological significance of TR in matched cohorts of HCV+ and HCV- patients with superimposed HCC in comparison with HCV+ patients with cirrhosis recruited as controls.

2 | MATERIAL AND METHODS

2.1 | Patient recruitment

Following informed, written consent, we prospectively recruited 32 patients with histologically or radiologically confirmed HCC according to EASL guidelines.[29] Inclusion criteria were the presence of Child-Pugh (CP) class A, Eastern Cooperative Oncology Group (ECOG) performance status <1, age >18. Patients with HCC were stratified into two groups according to anti-HCV IgG status. As a control group, 18 patients with histologically proven HCV-related cirrhosis who had not received prior antiviral treatment and had no evidence of superimposed HCC (including negative imaging studies) were recruited. Known Human Immunodeficiency Virus (HIV) or Hepatitis B virus (HBV) co-infection, immunosuppression or other immunological comorbidities were exclusion criteria. Complete clinical and follow-up data including patient's demographics, stage and complete blood picture were recorded. Overall survival (OS) times were calculated from the time of study entry. The study was conducted in accordance with the principles of the Declaration of Helsinki after approval of the study protocol by the local Research Ethics Committee.

2.2 | Clinical samples and ex-vivo enzyme linked immunospot (ELISPOT) assay

Peripheral blood mononuclear cells (PBMCs) were isolated from 50 mL of heparinized blood by Ficoll gradient centrifugation (Biowest, Nuaillé France). PBMCs were immediately frozen in 90% fetal bovine serum (FBS) and 10% dimethyl sulphoxide (DMSO) and stored in liquid nitrogen until analysis. Interleukin 2 (IL-2), IL-10 and granulocyte-monocyte colony stimulating factor (GM-CSF) release in the presence of AFP peptides was quantified by ELISPOT assay using an A.EL.VIS reader (Tema Ricerca, Bologna, Italy). Defrosted PBMCs (1x106/well) were cultured in 96-well flat-bottomed precoated ELISPOT plates (Thermo-Fisher Scientific, Waltham, USA for IL-2 and U-CyTech Biosciences, Utrecht, Netherlands for IL-10 and GM-CSF) in RPMI-1640 medium with 10% FBS (Sigma Aldrich, St. Louis, USA). Major histocompatibility complex-I (MHC-I) restricted immunogenic (LATIFFAQFV, Invitrogen, Carlsbad, USA)

and non-immunogenic (QLAVSVILRV, Invitrogen, Carlsbad, USA) AFP peptides[5] were solubilized in DMSO and added to the medium (15 μg/mL). Negative controls with medium alone and positive control reactions with phorbol 12-myristate 13-acetate (PMA, 50 μg/mL) and ionomycin (1 μg/mL) were included. ELISPOT data acquisition and processing was performed using the A.EL.VIS software. Individual cytokine release was expressed, for each reaction, as a ratio between spot counts in response to the immunogenic against the non-immunogenic peptide. A valid response was identified by spot counts >10 in each reaction well. Specificity of response was indicated by ratios between immunogenic and non-immunogenic peptide >2, as described before.[16]

2.3 | Statistical analysis

Statistical analysis was performed using SPSS statistical package 19.0 (SPSS Inc, Chicago, USA). Continuous variables were tested for normality of distribution using the D'Agostino-Pearson test. Kruskal-Wallis or Mann-Whitney's tests were used to verify differences in sample medians as appropriate. Categorical variables were presented as frequencies (%) and Pearson's Chi-square or Fisher's exact test were used to test for associations as appropriate. Kaplan-Meier statistics and Log-rank test were used to study the impact of the different clinical factors associated with OS on univariate analysis. A *P* value <.05 was taken to be significant.

3 | RESULTS

A total number of 50 patients, whose baseline characteristics are listed in Table 1, were prospectively recruited from 06/2009 to 11/2011 following informed written consent. Eighteen were HCV infected, treatment naïve patients with cirrhosis (CC). HCC patients were selected according to the etiology of underlying liver disease, with 15 being HCV+ and 17 HCV-. The prevailing etiology of

TABLE 1 General characteristics of the patient population

Baseline Characteristic, mean ± SD	HCV+Cirrhosis (n = 18)	HCV+HCC (n = 15)	HCV- HCC (n = 17)	*P*-value
Gender, M/F	9/9	13/2	12/5	.08
Age, years	70 ± 12	67 ± 10	71 ± 10	.61
AST, U/L	51 ± 27	39 ± 15	114 ± 119	**.009**
ALT, U/L	48 ± 23	37 ± 22	77 ± 56	**.01**
ALP, U/L	178 ± 82	348 ± 112	262 ± 83	**<.001**
Albumin, g/L	39 ± 6	38 ± 2	35 ± 5	.10
Bilirubin, mg/dL	1 ± 0.6	1.2 ± 0.5	1.1 ± 0.5	.66
INR, units	1.1 ± 0.14	1.25 ± 0.25	1.15 ± 0.21	.20
AFP, ng/ml	7.4 ± 5.7	162 ± 492	1645 ± 3867	.80
Child-Pugh Score, 5/6	13/5	12/3	7/10	.23
Alcohol consumption, absent/present	16/2	4/11	11/6	**<.001**
Tumour size, <3cm/>3cm	NA	9/6	11/6	.78
Tumour morphology, Uninodular <50% Multinodular <50% Massive >50%	NA	4/9/2	5/10/2	.98
Number of nodules, <3/>3	NA	9/6	8/9	.40
Portal vein thrombosis, absent/present	NA	10/5	11/6	.90
Extrahepatic diffusion, absent/present	NA	10/5	16/1	.08
BCLC stage, A/B/C	NA	2/4/9	6/5/6	.27
CLIP Stage, 0-1/≥2	NA	9/6	9/8	.68
Presentation, primary/ recurrent	NA	9/6	13/4	.45

Note: Bold indicates significant values.

HCV- HCC was alcoholic cirrhosis (n = 11, 65%) followed by HBV infection (n = 2, 11%). The three groups did not differ in terms of gender distribution, age and liver functional reserve. The two HCC sub-cohorts were homogeneous in terms of tumour stage. Ten patients had received prior treatment for HCC: one surgical resection (3%), six radiofrequency ablation (22%) and a further six trans-arterial chemoembolization (18%). Overall survival, calculated from the time of recruitment, was 8.3 months for the entire HCC subgroup (range 2-40). All patients were followed up until death or database closure for a median of 14.3 months (95% CI 9.6-19.0), with 16 patients (50%) having died at the end of observation.

ELISPOT-measured cytokine release in cirrhotic patients and in patients with HCC is summarized in Figure 1A. In the CC subgroup, valid and specific responses to IL-2, IL-10 and GM-CSF were seen in 6 (33%) 11 (61%) and 7 (39%) of patients respectively. In the HCC subgroup, the proportion of valid and specific responses to IL-2, IL-10 and GM-CSF were 3 (17%), 10 (58%) and 7 (41%) for HCV+ patients and 7 (46%), 11 (73%), 9 (60%) for HCV- patients. There was no significant difference in the distribution of IL-2, IL-10 and GM-CSF production in the pairwise comparison of each studied cohort. In order to study the effect of the secretion of each cytokine on common clinicopathological parameters of HCC, we categorized patients as "responders" and "non-responders" based on the presence of a valid and specific response to AFP peptides, as previously described [17] (Table 2). We found that IL-2 release correlated with larger tumour size ($P = .02$), more extensive intrahepatic spread ($P = .01$) and the presence of multifocal disease ($P = .02$). Similarly, a higher proportion of IL-2 responses was typical of more advanced BCLC stage tumours ($P = .01$) (Figure 1B). The release of IL-10 or GM-CSF was not influenced by any of the studied clinicopathological factors.

For survival analysis, we correlated the presence of a specific response to AFP peptides in terms of IL-2, IL-10 and GM-CSF secretion with patients' OS (Figure 2). GM-CSF responders had better OS compared to non-responders (median OS not reached vs 7.0 months, 95% CI 2.2-11.7), although this difference failed to reach statistical significance ($P = .07$).

4 | DISCUSSION

The paucity of systemic treatments makes the management of HCC patients who progress after standard therapies a particularly challenging clinical scenario. Active immunotherapy against TAAs in HCC is felt to be a viable alternative to cytotoxic or molecularly targeted agents, with initial evidence being corroborated by clinical studies confirming a therapeutic role for immune-checkpoint inhibitors including Programmed-Death ligand 1 (PD-L1) and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) targeted therapies.[12-14] Several studies have identified AFP as a potential tumour rejection antigen in HCC, with the stimulation of AFP peptides being able to

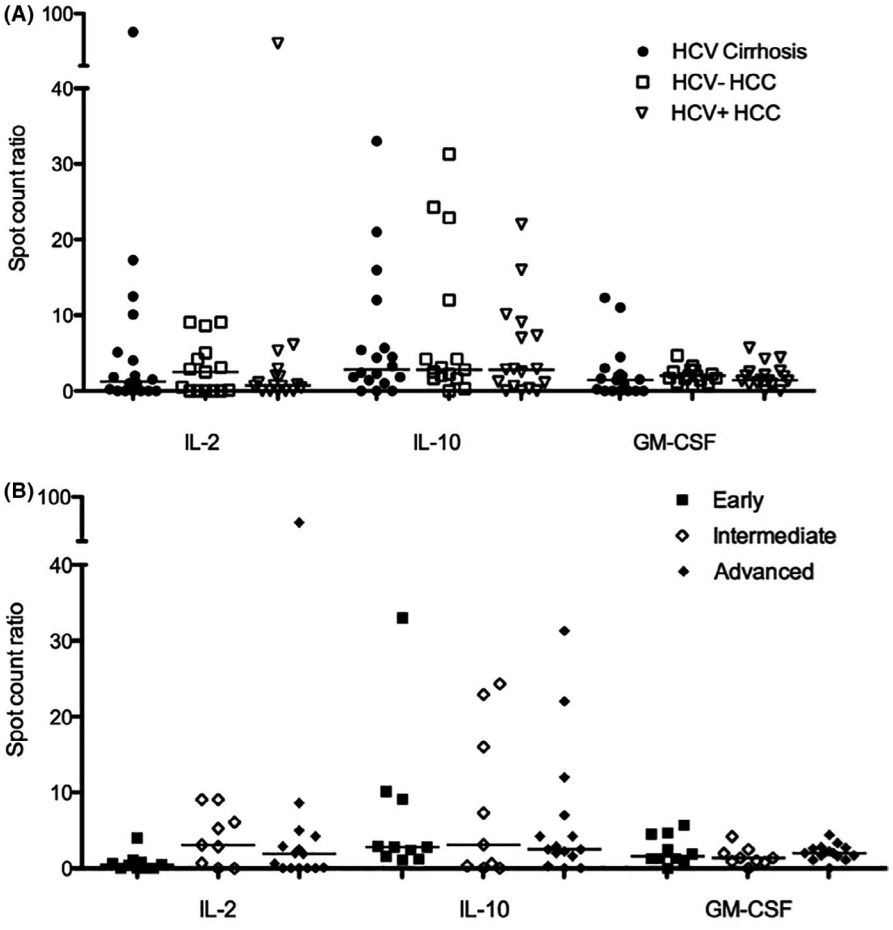

FIGURE 1 Panel A shows anti-AFP T cell responses grouped by cytokine secretion in patients with HCV-related cirrhosis (black spots, n = 18), HCV- HCC (white squares, n = 17) and HCV-related HCC (white triangles, n = 15). Panel B shows anti-AFP T cell responses grouped by cytokine secretion across the different BCLC stages of HCC

TABLE 2 The relationship between T-cell responses measured by IL-2, IL-10 and GM-CSF secretion in PBMCs and the clinico-pathological features of patients with HCC

Variable	IL-2 non responders	IL-2 responders	*P*	IL-10 non responders	IL-10 responders	*P*	GM-CSF non responders	GM-CSF responders	*P*
Tumour Size, <3/≥3 cm	15/4	5/8	**.02**	5/6	15/6	**.15**	11/6	9/6	**.68**
AFP, <400/ ≥400 ng/ml	15/4	12/1	**.62**	9/2	18/3	**.58**	14/3	13/2	**.73**
PVT, absent/present	12/7	9/4	**.74**	8/3	13/8	**.70**	13/4	8/7	**.26**
Intrahepatic spread, Uninodular <50% Multinodular <50%, Massive ≥50%	8/11/0	1/8/4	**.01**	2/6/3	7/13/1	**.17**	5/8/4	4/11/0	**.10**
Number of tumours, 1/2/≥3	7/5/7	2/0/11	**.02**	3/3/5	6/2/13	**.20**	5/2/10	4/3/8	**.81**
Extrahepatic spread, absent/present	17/2	9/4	**.15**	9/2	17/4	**.99**	13/4	13/2	**.66**
BCLC: A/B/C	8/3/8	0/6/7	**.01**	3/4/4	5/5/11	**.66**	5/6/6	3/3/9	**.37**
CLIP: 0-1/2-6	12/7	6/7	**.34**	6/5	12/9	**.88**	9/8	9/6	**.68**
Presentation: primary/recurrent	12/7	10/3	**.40**	9/2	13/8	**.24**	14/3	8/7	**.12**

Note: Associations reaching statistical significance ($P < .05$) are marked in bold.

Abbreviations: AFP, Alpha-fetoprotein; BCLC, Barcelona Clinic Liver Cancer Score; CLIP, Cancer of the Liver Italian Program Score; PVT, Portal Vein Thrombosis.

lead to a coordinated Th1 response.[16] However, amongst the factors that influence the quality and efficacy of such a response in each individual patient, the pro-inflammatory milieu of cirrhosis, which represents the natural pre-cancerous stage for at least 80% of the incident cases of HCC, is known to play a relevant modulatory role.[22]

Based on previous evidence demonstrating the immune-modulating effects of chronic HCV infection, which, unlike HBV, invariably leads to HCC through cirrhosis,[30] we sought to determine whether HCV-related HCC patients displayed a different pattern of TR compared to non-HCV-related HCC and matched HCV+ cirrhotic controls. Interestingly, our study shows that TR to two previously validated AFP immunogenic peptides,[16] measured by standardized indicators of T cell proliferation (IL-2) and T-reg subdifferentiation (IL-10 and GM-CSF) [19] is not qualitatively or quantitatively influenced by HCV infection status in patients with preserved liver function (CP class A). Similarly, no difference could be found in the comparison between cirrhosis and HCC, suggesting that the emergence of a specific response to immunogenic AFP peptides may predate the onset of HCC, with a significant proportion of patients with HCV-related cirrhosis displaying evidence of an ongoing suppressive response to AFP. IL-2 secretion following antigen stimulation is a known surrogate indicator of T cell activation, common to both CD4+ and CD8+ lymphocytes. Although we did not fully dissect the phenotype of the IL-2-secreting T cell clone we identified, our data seem to overlap with the findings by Behboudi and colleagues, where anti-AFP CD8+ cells were isolated from a smaller group of eight cirrhotic controls of mixed etiology.[22] The existence of an increased proportion of inducible T-reg in HCV-related CLD, cirrhosis and HCC compared to healthy controls is not a novel concept in the understanding of the immunological features underlying the pathogenesis and progression of HCC.[31] However, our data further substantiate this by showing that AFP is one of the TAA against which such previously uncharacterized suppressive TR is mounted in both CC and HCC patients, independently from HCV infection status. When correlated with common clinicopathological variables of HCC, TRs were generally independent from each individual factor including AFP circulating levels, portal vein thrombosis and prior treatment for HCC. However, when TRs were studied across different stages of HCC, we found a higher proportion of AFP-specific IL-2-producing cells in advanced disease, in keeping with previously published reports.[32] Such increased T-cell responsiveness may relate to the invasion of microvascular spaces and lymphatic tissue, a typical feature of advanced HCC that may facilitate sustained interaction between TAA and the immune system.[32]

Since inflammation [33] and cell-mediated immunity [20] are well-defined prognostic domains in HCC, we explored whether anti-AFP TRs could predict for patient's overall survival. The overall survival we observed is comparable to rates reported for the period during which patients were recruited, with sorafenib being the mainstay of therapy.[8] None of the studied cytokine responses were found to play a significant role in dictating patients' prognosis in our case series, although we acknowledge the major limitations in our evaluation, including the small sample size and heterogeneity in treatment received by patients. As our cohort was recruited between 2009 and 2011, a variable in the HCV therapeutic landscape that we were unable to examine is the impact of direct-acting antiviral therapy, which

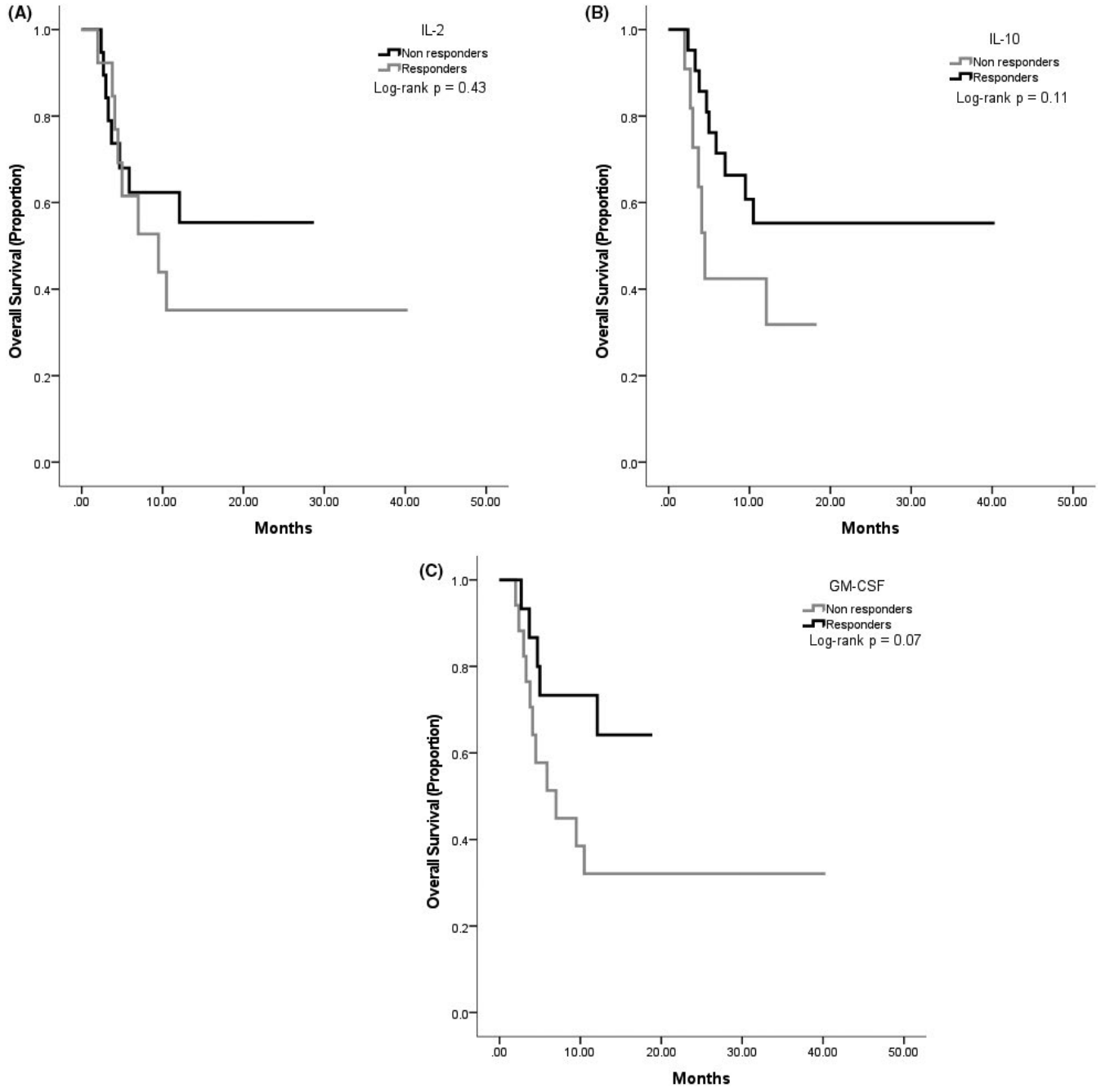

FIGURE 2 Kaplan-Meier curve analysis showing the difference in median OS for HCC patients categorized according to the presence of a valid and specific secretion of IL-2 (Panel A), IL-10 (Panel B) and GM-CSF (Panel C) in PBMC

is posited to favour an immunosuppressive milieu in the liver, with increased frequency and activity of Treg cells and myeloid-derived suppressor cells.[34]

Our data, taken together with the finding that serum levels of AFP increase more than 10 years before HCC detection[35] suggest that immune tolerance against AFP is a phenomenon that accompanies HCC since its earliest stages, potentially pre-dating its clinical onset. As a result, the aim of breaking such tolerance, which is mediated at least in part by the presence of antigen-specific T-reg clones, stands as a crucial step influencing the success of AFP-based immunotherapy as a fully developed treatment strategy for HCC. Recent results from AFP-based vaccination studies showing the emergence of mainly dysfunctional CD8+ responses in the absence of spontaneous AFP-specific CD4+ T cell expansion following therapeutic vaccination further reinforce this point.[36] Based on our study, HCV infection, in the context of preserved liver function, cannot be regarded as a factor influencing immune tolerance towards AFP. This is of major clinical consequence in the development of AFP-based immunotherapies, since it suggests the potential usefulness of this strategy in patients with HCC irrespective of the etiology of

the underlying CLD. On the other hand, the association between the AFP-specific IL-2 response and tumour burden suggests advanced stage HCC as an immunologically privileged patient subpopulation where therapeutic AFP-based vaccines may be a preferential strategy.

Our study is limited by the approach of investigating the secretion of three individual cytokines that, however central to the pathophysiology of T cell activation and Treg differentiation, do not provide a complete phenotypic dissection of the TR we observed. Notably, we were unable to clarify the relative contribution of lymphocyte subsets or of locally infiltrating lymphocytes compared to circulating lymphocytes. A detailed clinicopathological study on the differential activation of CD4+ vs CD8+ responses against AFP has already been reported elsewhere.[22] It would be perhaps more important in future studies to further characterize the potential for reversibility of the suppressive response against AFP using clinically available strategies such as CTLA-4 or PD1/PD-L1 inhibition.[37]

In conclusion, we have demonstrated a predominance of anti-AFP IL-2 secreting T cells in advanced HCC and that anti-AFP TRs are otherwise independent from common clinicopathological variables of HCC including patients' survival. More importantly, we have shown that HCV infection does not influence anti-AFP TRs, therefore contributing to provide a rationale to the selection of patients potentially suitable for anti-AFP immunotherapy.

ORCID

Petros Fessas https://orcid.org/0000-0002-7472-8913
Carlo Smirne https://orcid.org/0000-0002-3338-5416
Rosalba Minisini https://orcid.org/0000-0002-7548-9731
Mario Pirisi https://orcid.org/0000-0001-9740-0155

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