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ORIGINAL RESEARCH

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Dynamic of systemic immunity and its impact on tumor recurrence after radiofrequency ablation of hepatocellular carcinoma

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ABSTRACT

Background: Percutaneous radiofrequency ablation (RFA) is one of the main treatments of small hepatocellular carcinoma (HCC). However, it remains unclear whether this local treatment can induce systemic immune variations.

Methods: We conducted a prospective study in a tertiary center including consecutive cirrhotic patients with unifocal HCC<5 cm treated by a first RFA between 2010 and 2014. Peripheral blood mononuclear cells were isolated on the day before (D0), day after (D1) and month after RFA (M1). Frequencies and phenotypes of myeloid cells, T cells, and NK cells were compared between timepoints. Overall recurrence and associated variables were estimated using Kaplan-Meier, log-rank and Cox proportional-hazards models.

Results: 80 patients were included (69% male, median age: 67 years old). Main aetiologies of HCC were alcohol (51%), hepatitis C virus (45%), non-alcoholic steatohepatitis (36%) and hepatitis B virus (9%). Median overall survival was 55 months (M); median progression-free survival was 29.5M. Among innate immune populations, we observed variations between D0, D1 and M1 in NKp30+ NK cells ($p < .0001$) and in plasmacytoid dendritic cells (pDC, $p < .01$). Concerning adaptive immunity, we observed variations in CD8 Central Memory ($p < .05$) and CD28+ CD8 Central Memory ($p < .01$). An early dynamic (D0/D1) of activated $NKp30^{+}$ NK cells was associated with a decreased overall recurrence (log-rank, $p = .016$, median delay 25.1 vs 40.6 months). In contrast, a late dynamic (D1/M1) of immature NK cells ($CD56^{bright}$) and altered myeloid DC ($PDL1^{+}$) was associated with an increased overall recurrence (log-rank, $p = .011$ and $p = .0044$, respectively). In multivariate analysis, variation of immature NK cells predicts tumor recurrence independently of classical clinical prognostic features (HR = 2.41, 95% CI: 1.15–5.057), $p = .019$).

Conclusions: Percutaneous RFA of small HCC leads to systemic modifications of innate and adaptive immunity closely linked with overall tumor recurrence.

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KEYWORDS
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Background

Hepatocellular carcinoma (HCC) is the 2nd leading cause of cancer deaths worldwide.[1] The incidence of HCC is increasing from 14 million cases worldwide in 2012 to an expected 22 million cases in 2030.[1] Advanced stage diagnosis (locally advanced or metastatic) means that only palliative treatments can be offered, with a median overall survival of 10 months.[2] In addition, less than 30% of HCC diagnosed at an early stage can potentially be cured, thanks to three major curative treatments: transplantation, resection and radiofrequency ablation (RFA).[3]

RFA is a percutaneous technique, in which one or more needles are placed under radiological control, delivering alternating current (375 to 500 kHz) responsible for local heat (60–100°C) and thermic necrosis of tumor cells.[4] RFA has been proven more effective than ethanol injection,[5] and is currently considered one of the main curative treatments of small HCC on cirrhosis.[6,7] Although significant technical progress has been made in the field of ablation over the last 20 years (no-touch multipolar RFA, microwave ablation, irreversible electroporation), RFA and other emerging ablative techniques still see an overall tumor recurrence rate of approximately 70% at 5 years.[8] With only

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a limited number of plasmatic biomarkers available to predict prognosis after RFA (AFP, DCP, AFP-L3, VEGF) validated immune biomarkers could prove extremely useful.

With the recent success of a phase II clinical trial of anti-PD1 therapy (nivolumab), immuno-oncology is quickly emerging in the field of advanced hepatocellular carcinoma.[9] For small HCC, mice models suggest that RFA leads to the release of immunogenic dead cell-derived antigens which in turn induce an anti-tumor response.[10] Addition of immunotherapy to RFA has been proposed to boost the immunogenic cell death induced by RFA,[11] but supplementary data are required to better understand the immune response in patients treated by percutaneous ablation.

We are reporting here an exploratory prospective study of 80 patients undergoing immune monitoring before and after percutaneous RFA of small HCC developed on cirrhosis BCLC 0/A. The study had two main objectives: (i) describing the immunological systemic dynamic induced by RFA; (ii) testing clinical correlations between this immune dynamic and tumor recurrence.

Methods

Study population

This monocentric prospective study was performed in a French tertiary care university liver center (Jean Verdier Hospital) from 2010 to 2014 (Figure 1).

Adults aged 18 years or older fulfilling all of the following criteria were included prospectively: (1) a hepatocellular carcinoma diagnosed by histology or non-invasive criteria using contrast-enhanced CT scan or MRI according to EASL criteria,[12] (2) an unique HCC < 5 cm without extrahepatic metastasis or tumor portal thrombosis (BCLC 0/A), (3) an underlying cirrhosis, (4) a first treatment with RFA between 2010 and 2014, (5) no previous treatment for HCC.

Patient with infectious complications after RFA, liver failure (Child-Pugh C) or HIV infections were excluded.

Study design

RFA treatment was decided upon during a weekly multidisciplinary meeting that included a surgeon, a radiologist, oncologists, and hepatologists. Patients were treated under general anesthesia using no-touch multi-bipolar RFA with ultrasound guidance (Prosurge, Celon/Olympus, Berlin, Germany) (66%) or using intra-tumoral multipolar RFA (Prosurge, Celon/Olympus, Berlin, Germany) (34%).[13] At the imaging performed 1 month after RFA, HCC was considered completely ablated if no enhancement adjacent to the ablation zone was visible during the arterial phase. Otherwise, an additional RFA session was performed in order to achieve complete ablation. Treatment failure was defined as incomplete ablation of HCC despite an additional RFA procedure. Patients had blood draws of 50 ml (EDTA vials) at three different time points: immediately prior to RFA treatment (D0 = Day 0), 24-h post RFA treatment (D1 = Day 1), and 1month after RFA treatment (M1 = Month 1). Peripheral blood mononuclear cells (PBMC) were isolated, cryopreserved at –80°C, and transferred to Paoli Calmettes Immunomonitoring Platform for flow cytometry analysis. All patients signed an informed consent form. The study was approved by the Institutional Review Board of the hospital, and the designated Ethics Committee.

Flow cytometry analysis

After thawing, cells were incubated with specific surface and intracellular antibodies as previously described,[14] and flow cytometry was performed using a BD Fortessa ® cytometer (BD Biosciences). A pilot cohort of 43 patients was tested for a combination of population markers (Dendritic Cells, NK

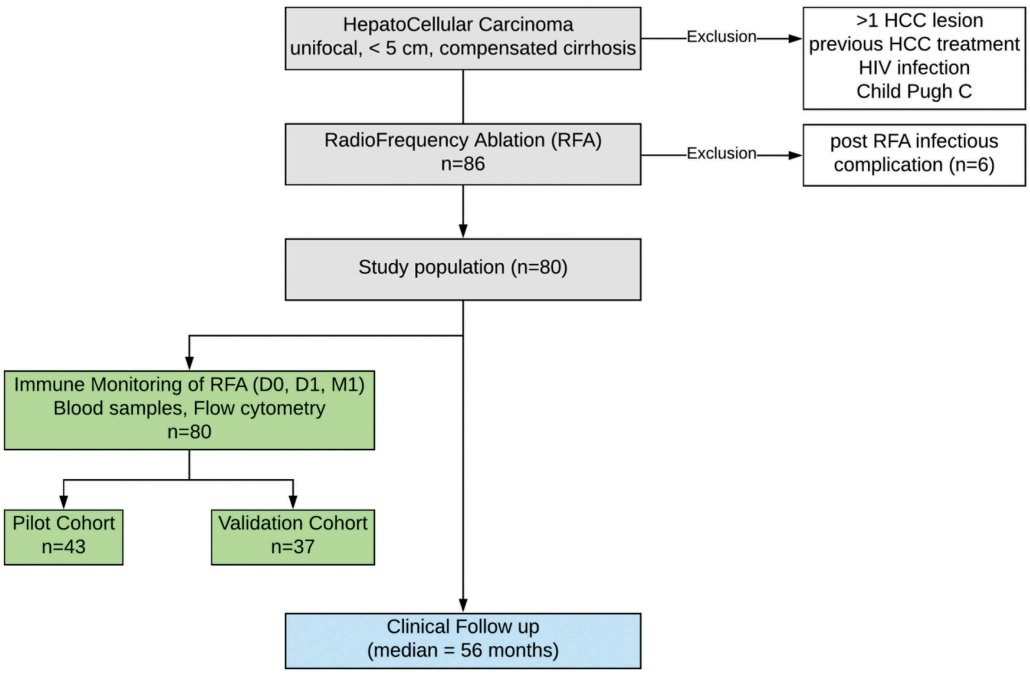

Figure 1. Flow chart of the study.

cells, CD8 T cells, CD4 T cells, T_{regs}, δγ T cells) and phenotypic markers (NKp30, NKp46, NKG2A). In a second cohort of 37 patients (validation cohort), together with the previously cited populations and phenotypic markers, we added more population markers (Monocytes, Myeloid-Derived Suppressor Cells, CD4 and CD8 T cell subpopulations) and phenotypic markers (PDL1, PD1, CTLA4, ICOS, HLA-DR, CD28, etc.). Two populations could not be analyzed: granulocytes because of sensitivity to cryopreservation, and B cells because CD19 marker was used in an exclusion channel with other antibodies. The detailed list of antibodies, the detailed list of phenotypes, the gating strategy for lymphoid cells and the gating strategy for myeloid cells is presented in Supplementary Table 1, Supplementary Table 2, Supplementary Figure 1, and Supplementary Figure 2, respectively.

Outcomes

The variations of immune populations were studied between time points if there were more than 100 cells by gate, and the markers of interest were studied with percentage of positivity and median fluorescence intensity (MFI). The dynamics of immune cell populations were defined as the absolute difference of populations' percentage between time points. The following data were recorded before treatment: medical history, cause of underlying chronic liver disease, liver biology including serum AFP and liver function according to Child-Pugh classification.

Patients treated by RFA had a clinical, biological and radiological follow-up (CT scan or hepatic MRI) to monitor for tumor recurrence every 3 months during the first 2 years and then every 6 months until follow-up ended in December 2016. Local recurrence was defined as a recurrence of HCC in the vicinity of the ablation zone or treatment failure as previously described, and distant recurrence as new HCC in other segments or in the same segment distant to the treated area. Overall recurrence was defined as either a local or a distant recurrence. Progression-free survival (PFS) was defined as the time between RFA and recurrence (local or distant), lost follow-up, or death by any causes. Data in patients who underwent liver transplantation were censored from the study at the date of transplantation. Overall survival (OS) was defined as the time between RFA and lost follow-up or death by any cause.

Statistical analysis

Cytometry data were analyzed using Flowjo® software version 10.0 (TreeStar, Ashland, OR). Statistical analysis was performed with GraphPad® PRISM version 5.01 (La Jolla, CA) and R software version 3.4.4; package ggplot 2, survival and survminer (https://www.r-project.org/). The patients' features were reported as medians [range] for continuous variables, and as numbers (percentages) for categorical data. Frequencies were compared using a one-way ANOVA analysis with a Bonferroni post-hoc test comparing all pairs of time points (D0, D1, M1). Unsupervised clustering of patients was performed using the MeV software (http://mev.tm4.org; HClust algorithm, Pearson correlation). Data were normalized, zero-centered and reduced prior to clustering. To avoid the potential effect of values below the 5th percentile and above the 95th percentile, we used the following formula: fnorm = (((f-centile5%)/(centile 95%-centile5%))-0.05x6 where f is the frequency and fnorm is the normalized frequency. Patients were clustered according to percentages of CD16+ monocytes, monocytes, GrMDSC, moMDSC, mDC, pDC, as well as the percentages of expression of PDL1 and HLA-DR (the two expression makers of our myeloid panel) in monocytes, pDC, mDC. The clustering used one minus Pearson correlation as distance and average linkage as clustering method.

The primary endpoint of the study was overall recurrence. Times to events were estimated from the last procedure and the incidence of events was assessed by Kaplan–Meier method and compared using the log-rank test. In the uni/multivariate analysis, the Cox proportional-hazards model was used to estimate the variables associated with tumor recurrence, using hazard ratio and its associated 95% confidence interval. All variables with a P-value <0.05 were included in a multivariate Cox regression model computing the estimate of the hazard ratio (HR) along with their 95% CI. A P-value <0.05 was considered as statistically significant.

Results

Characteristics of the study population

Between 2010 and 2014, 86 consecutive patients were prospectively included in this study. As six patients were excluded for mild infectious complications after RFA, the total population included in the immune monitoring was 80 patients. Table 1 summarises the main characteristics of the studied population. 68.7% of patients were male with 75% over 60 years old (with a median age of 67). The main aetiologies of chronic liver disease underlying HCC were alcohol intake (51%), hepatitis C virus (45%), non-alcoholic steatohepatitis (36%) and hepatitis B virus (9%) and 42.5% of patients had mixed aetiologies. 90% of patients were classified as Child-Pugh A. The majority of patients had an HCC size <3 cm (67.5%) with a median AFP of 8 ng/ml. All patients were treated successfully by RFA, and post-treatment complications occurred in 20 patients with mostly minor adverse events such as pain and pleural effusion.

Clinical outcomes of the study population

After a median follow-up of 56 months, 34 patients died and 67 had tumor recurrence (local: n = 17/80; distant: n = 41/80

Table 1. Characteristics of the study population (n = 80).

Baseline features	Available data	Total n = 80. Median (range or %)
Sex (Male)	80	55 (68.7%)
Age (years old)	80	67 (46–83)
Child-Pugh score A	80	72 (90%)
Total bilirubin (µmol/l)	79	13 (4–65)
Albumin (g/L)	55	38 (24–54)
Platelet count (mm/^{3})	80	132 (36–296)
Prothrombin time (%)	80	80 (40–100)
High alcohol intake	79	41 (51.3%)
NASH	79	29 (36.3%)
Chronic hepatitis B	79	7 (8.8%)
Chronic hepatitis C	79	36 (45%)
Mixed etiologies	79	34 (42.5%)
HCC size ≥ 3 cm	80	26 (32.5%)
Serum AFP (ng/ml)	78	8 (1–2185)

and both: n = 9/80). Median progression-free survival (PFS) was 29.5 months and median overall survival (OS) was 55.0 months (Supplementary Figure 3: A, B). The survival rates at 1, 3,and 5 years after RFA were 90%, 70%, and 45%, respectively. The rates of overall tumor recurrence at 1, 3,and 5years after RFA were 19%, 59%, and 78%, respectively (Supplementary Figure 3: C-E).

Dynamic of innate immunity after radiofrequency ablation

In both pilot and validation cohorts (Figure 2), the percentage of NK cells expressing the activation marker NKp30 (Figure 2a) decreased at D1 (pilot and validation P-value <0.0001) and increased at M1 (pilot P-value <0.05/validation P-value <0.0001). The same dynamic was observed for NKp30 median fluorescence

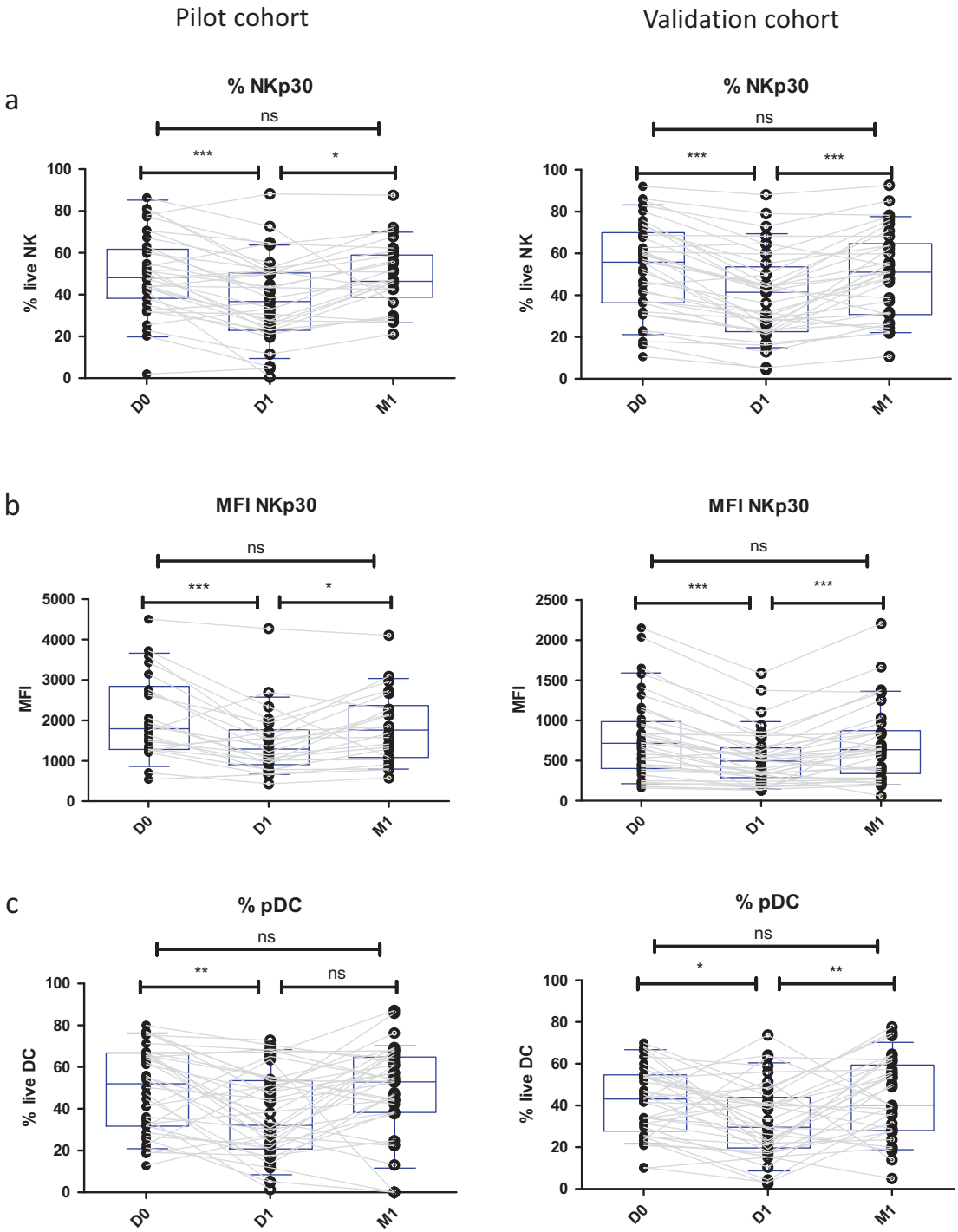

Figure 2. Variation of innate immunity after radiofrequency ablation. (a) Percentage of natural killer cell expressing 30 p marker, (b) median of Fluorescence Intensity (MFI) of natural killer cell expressing 30 p marker (the absolute MFI differ between the two cohorts due to different antibodies), (c) percentages of plasmacytoid Dendritic Cells in the pilot and validation cohort between day 0 (D0) before RFA, day 1 (D1) and month 1 (M1) after RFA. * $P < .05$, ** $P < .01$, *** $P < .0001$, ns = non-significant. Frequencies were compared using one-way ANOVA analysis with Bonferroni post-hoc test.

intensity in NK cells (Figure 2b, between D0/D1: pilot and validation P-value <0.0001). Similarly, in both cohorts (Figure 2c) the percentage of plasmacytoid dendritic cells decreased at D1 (pilot P-value <0.01/validation P-value <0.05) and increased at M1 in the validation cohort (P-value <0.01). Additionally, in the validation cohort (Supplementary Figure 4), we observed an increase of monocytes from D0 to D1 (50.9% to 60.2% of lineage negative PBMC, P-value <0.01) followed by a decrease at M1 (48.5%, P-value <0.0001). We did not find statistically significant variations for MDSC or Tγδ populations, and expression of NKp46 or NKG2A.

Dynamic of adaptive immunity after radiofrequency ablation

As shown in Figure 3, RFA led to a transient increase of HLA-DR MFI at D1 in $CD4^+$ T cells and especially in $CD4^+$ Naïve T cells (Figure 3-b, P-value <0.01). The frequency of total $CD8^+$ T cells

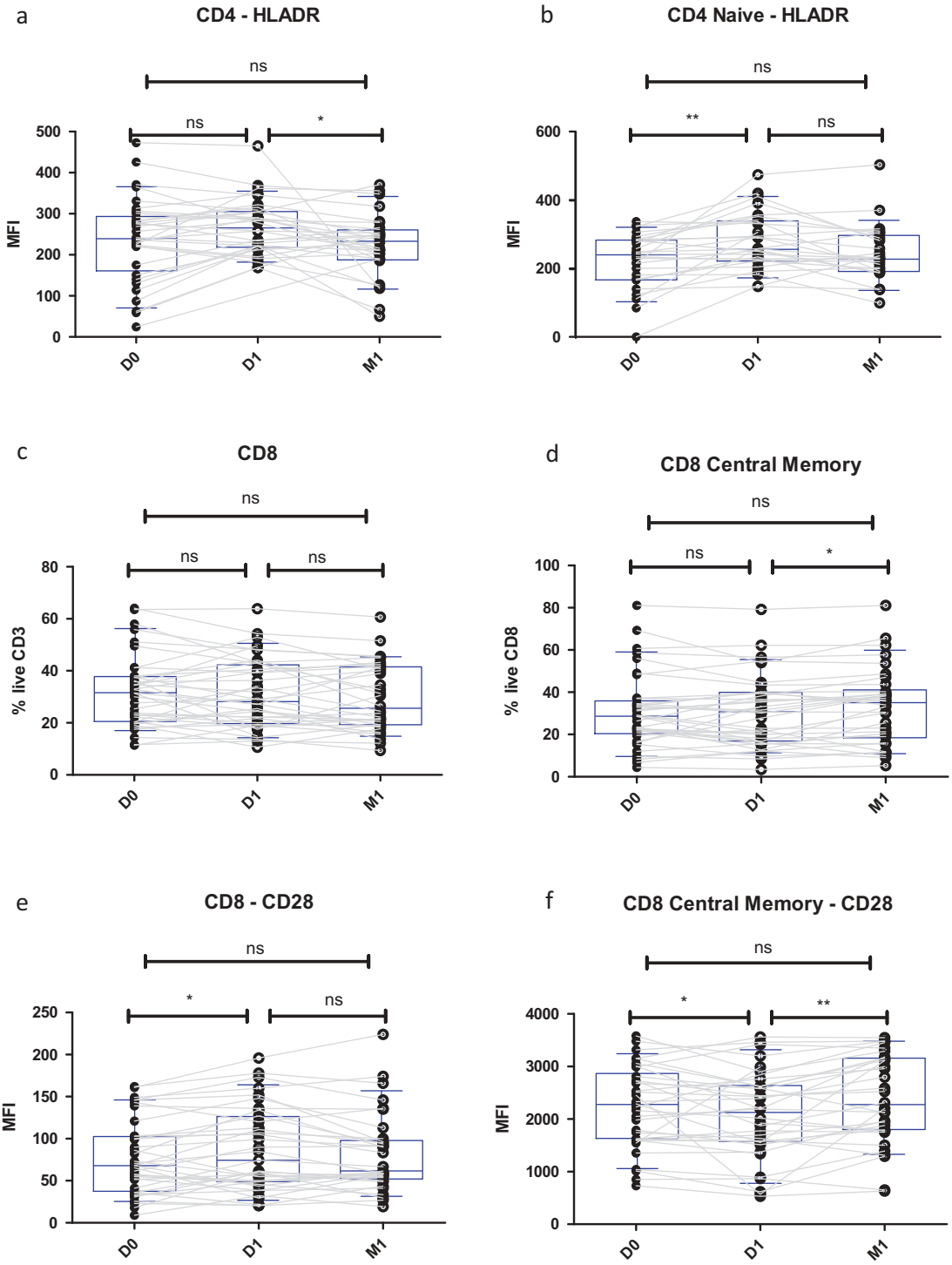

Figure 3. Variation of adaptive immunity after radiofrequency ablation. (a) Median of Fluorescence Intensity (MFI) of HLA-DR in CD4 T cells, (b) MFI of HLA-DR in CD4 Naive T cells, (c) percentages of CD8 T cells, (d) percentages of CD8 Central Memory T cells, (e) MFI of CD28 marker in CD8 T cells, and (e) MFI of CD28 marker in CD8 Central Memory T cells in the validation cohort between day 0 (D0) before RFA, day 1 (D1) and month 1 (M1) after RFA. * $P < .05$, ** $P < .01$, *** $P < .0001$, ns = non-significant. Frequencies were compared using one-way ANOVA analysis with Bonferroni post-hoc test.

did not statistically change between the 3 times points (Figure 3c). Interestingly, among total $CD8^+$ T cells, the percentage of CD8 Central Memory (CD 27^+ $CD45RA^-$) increased from 28.5% (D0) to 30.7% (D1) and 34.9% in M1 (Figure 3d, P-value <0.05). After hepatic RFA, expression of the activation marker CD28 (Figure 3e–f) increased at D1 for $CD8^+$ T cells (P-value <0.05) and at M1 for Central Memory $CD8^+$ T cells (P-value <0.01). We did not find statistically significant variations for T_{regs} or other CD4/CD8 subpopulations and expression of ICOS, PD1, or CTLA4.

Impact of nkp30 dynamics (day 0/day 1) on overall recurrence

The dynamics of $NKp30^+$ cells 24-h post RFA treatment (defined as the percentage difference of $NKp30^+$ NK cells between D1 and D0) had an impact on overall tumor recurrence (Figure 4a). Patients with an increased percentage of $NKp30^+$ NK cells over the median had less tumor recurrence (median time to recurrence: 40.6 months) compared to patients with a dynamic of $NKp30^+$ NK

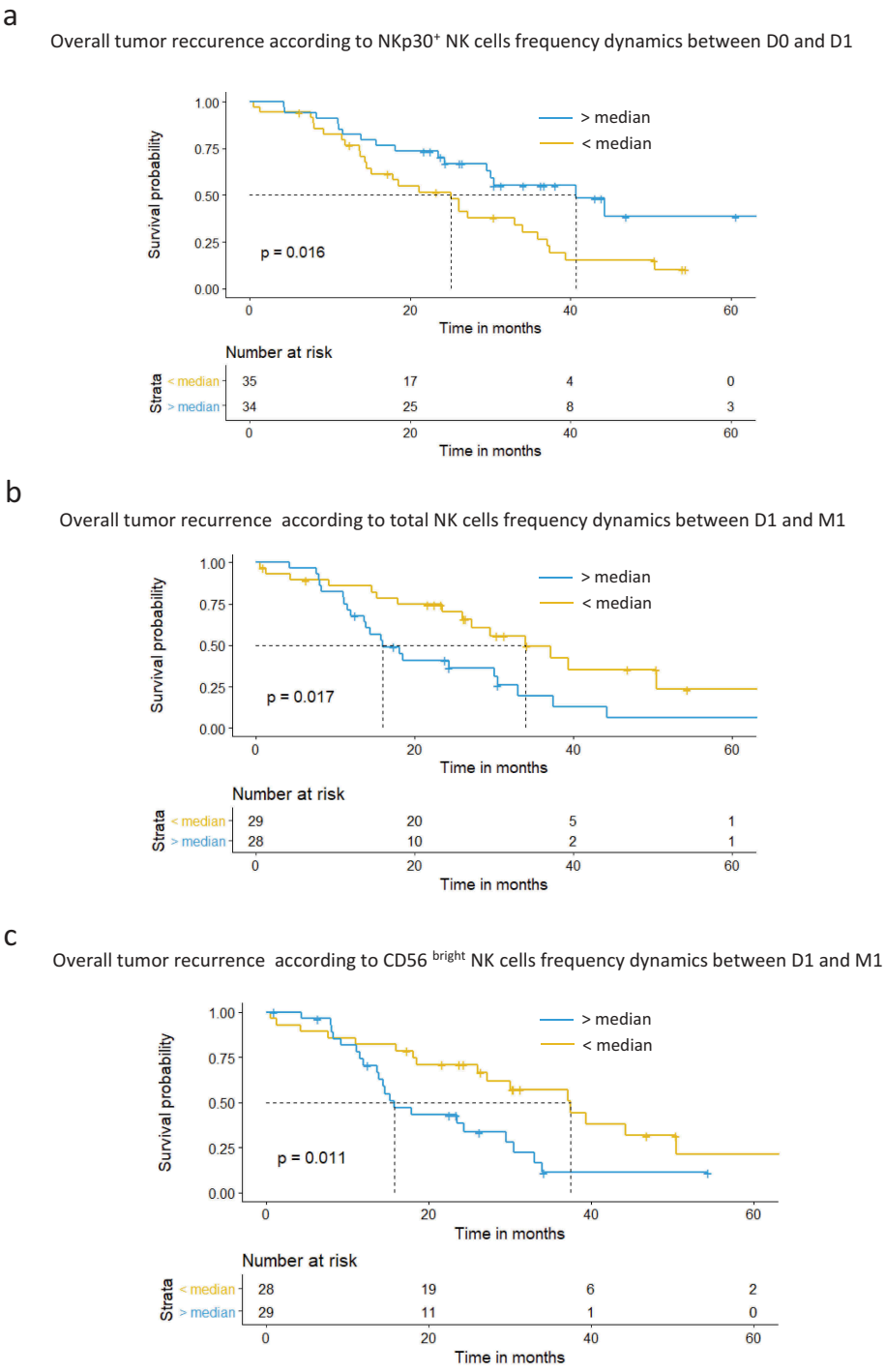

Figure 4. Overall tumor recurrence according to variations of NK cells after radiofrequency ablation. (a) Overall tumor recurrence according to $NKp30^+$ NK cells frequency dynamic between the day of the radiofrequency (D0) and the day after (D1), (b) according to total NK cells frequency dynamics between day 1(D1) and month 1(M1), (c) and according to $CD56^{bright}$ NK cells frequency dynamics between day 1(D1) and month 1(M1) in the overall cohort of patients. Results were represented using the Kaplan–Meier Method and compared using the log-rank test with the numbers at risk under the *X*-axis.

cells under the median (25.1 months of median time to recurrence, log-rank test: p = 0.016).

Impact of total NK cells and cd56^{bright} NK cells dynamics (day 1/month 1) on overall recurrence

We then studied the dynamics of NK cells between the day after (D1) and 1month after (M1) RFA treatment. Patients with an absolute percentage change of total NK cells over the median had earlier tumor recurrence (median time to recurrence: 16.0 months) than patients with an absolute percentage change of total NK cells below the median (median time to recurrence: 33.9 months) (Figure 4b, log-rank test: p = 0.017). This difference in overall tumor recurrence was also remarkable for the absolute percentage change of immature CD56^{bright} NK cells (over the median: median time to recurrence of 15.7 months/below the median: median time to recurrence of 37.4 months; Figure 4c, log-rank test: p = 0.011).

In a Cox regression analysis of variables potentially associated with HCC recurrence (Table 2), serum AFP level (HR = 1.001 (95% CI: 1.00–1.002)), NKp30^{+} NK cells dynamic between D0 and D1 (HR = 0.47 (95% CI: 0.25–0.88)), total NK cells dynamic between D1 and M1 (HR = 2.21 (95% CI: 1.13–4.29)) and CD56^{bright} NK cells dynamics between D1 and M1 (HR = 2.36 (95% CI %: 1.19–4.65)) were significantly associated with overall tumor recurrence in univariate analysis. In multivariate analysis, variation of the frequencies of total NK cells (HR = 2.35 (95% CI: 1.15–4.81, p = 0.018)) and CD56^{bright} NK cells ((HR = 2.41 (95% CI: 1.15–5.07, p = 0.019)) were independently associated with overall tumor recurrence.

Impact of other myeloid cells dynamics (day 1/month 1) on overall recurrence

In order to better understand the role of other innate immune cells, we conducted an exploratory analysis using unsupervised hierarchical clustering of myeloid cells dynamic in a subset of the population (n = 32). We identified two clusters of patients according to variations in myeloid cells between D1 and M1 (Figure 5a). The first cluster was characterized by a decreased number of myeloid dendritic cells (mDC), plasmacytoid dendritic cells (pDC) and an increased number of monocytes, pDC with overexpression of PDL1. In contrast, cluster 2 was defined by patients with enrichment of myeloid mDC, of pDC as well as with an increased expression of HLA-DR in pDC and expression of PDL1 in mDC. Patients classified in cluster 2 had a trend towards more tumor recurrence (Figure 5b, log-rank test: p = 0.066). Similarly, patients with a mDC dynamic over the median also had a trend towards a higher rate of tumor recurrence (Figure 5c, log-rank test, p = 0.052). Finally, patients with increased frequencies of mDC positive for PDL1 between D1 and M1 had significantly more tumor recurrence (median time to recurrence of 16.9 months versus 33.9 months, Figure 5d, log-rank test: p = 0.0044).

Table 2. Multivariate Cox regression analysis of variables potentially associated with HCC overall recurrence.

	Univariate analysis			Multivariate analysis		
Variables	HR	95%CI	*p*-value	HR	95%CI	*p*-value
Age	0.99	0.96–1.02	0.72			
Gender (female)	0.58	0.29–1.14	0.10			
Alcohol intake	1.03	0.58–1.82	0.91			
Hepatitis C	1.33	0.74–2.37	0.33			
HCC size ≥ 3 cm	1.16	0.63–2.11	0.62			
Serum AFP level	1.001	1.00–1.002	0.008	0.99	0.99–1.02	0.40
NKp30^{+} dynamic	0.47	0.25–0.88	0.016	0.61	0.29–1.29	0.20
Total NK dynamic	**2.21**	**1.13–4.29**	**0.018**	**2.35**	**1.15–4.81**	**0.018**
CD56^{bright} NK dynamic	**2.36**	**1.19–4.65**	**0.012**	**2.41**	**1.15–5.07**	**0.019**

Discussion

Our study reports a prospective immuno-monitoring cohort of cirrhotic patients with early HCC (n = 80) treated with percutaneous RFA, collected at an expert liver center, and analyzed on a certified immune-monitoring platform. Based on analysis of sequentially collected samples, we found that RFA led to a variation of systemic immune innate cells (NK, Dendritic Cells) and adaptive immune cells (CD8+, CD28+ CD8). We also showed that an early increase of activation marker NKp30 between D1/M1 was associated with a favorable prognosis, whereas a delayed increase of total NK cells, CD56^{bright} NK cells, and mDC PDL1^{+} between D1/M1 was associated with more tumor recurrence after RFA.

Our data reinforce current evidence that a local treatment can trigger systemic immunological effects. Actually, most of the knowledge in this field is coming from radiotherapy studies, suggesting that focal radiation leads to numerous systemic immune variations: increase of inflammation (macrophages, cytokines release), improvement of dendritic cells priming (through calreticulin, HMGB1, and TLR4),[15] broadening of TCR repertoire and enhancement of T cells killing properties (via ICAM, Fas, and MHC-1).[16] These findings explain how the irradiated tumor is considered an "immunological hub" and why radiotherapy is combined to ICB in numerous immunotherapy trials.[17] While RFA is a standard treatment since the beginning of the 2000s, there is still few data about immune dynamics induced by this ablation technique. In animal models, RFA increased circulating levels of cytokines (Heat Shock Protein, TNFα, IFN type 1),[18,19] enhanced neoantigens presentation, and induced anti-tumor reactivity transferrable by splenocytes.[20] In humans, Zerbini et al. performed studies investigating the immune dynamics generated by RFA. In 2006, in 20 patients, they found that 1month after RFA, patients developed T cell responses against tumoral tissue measured by IFNγ ELISPOT and intracellular staining of IFNγ.[21] No association was found between T cell responses and protection from hepatocellular carcinoma, which is consistent with the results of our larger cohort of patients. They also showed that RFA cell lysates induced *in vitro* maturation of monocytes and monocytes derived dendritic cells[22] and increased the number and cytotoxicity of systemic NK cells in 37 patients.[23]

Our data contribute several new findings to the preliminary data available in existing literature. First, using large panels of antibodies, we studied altogether the main populations of myeloid (monocytes, DCs, MDSC) and lymphoid immune cells (CD4 and CD8 T cells subpopulations, NK cells, γδ T cells). Thanks to this broad analysis, we pointed out that the main actors of RFA immune dynamics were innate immune cells (NK and DC) and that they were closely linked to HCC recurrence. Second, we included in our timepoints an early evaluation of RFA induced immune dynamics at D1, which is rarely reported in existing

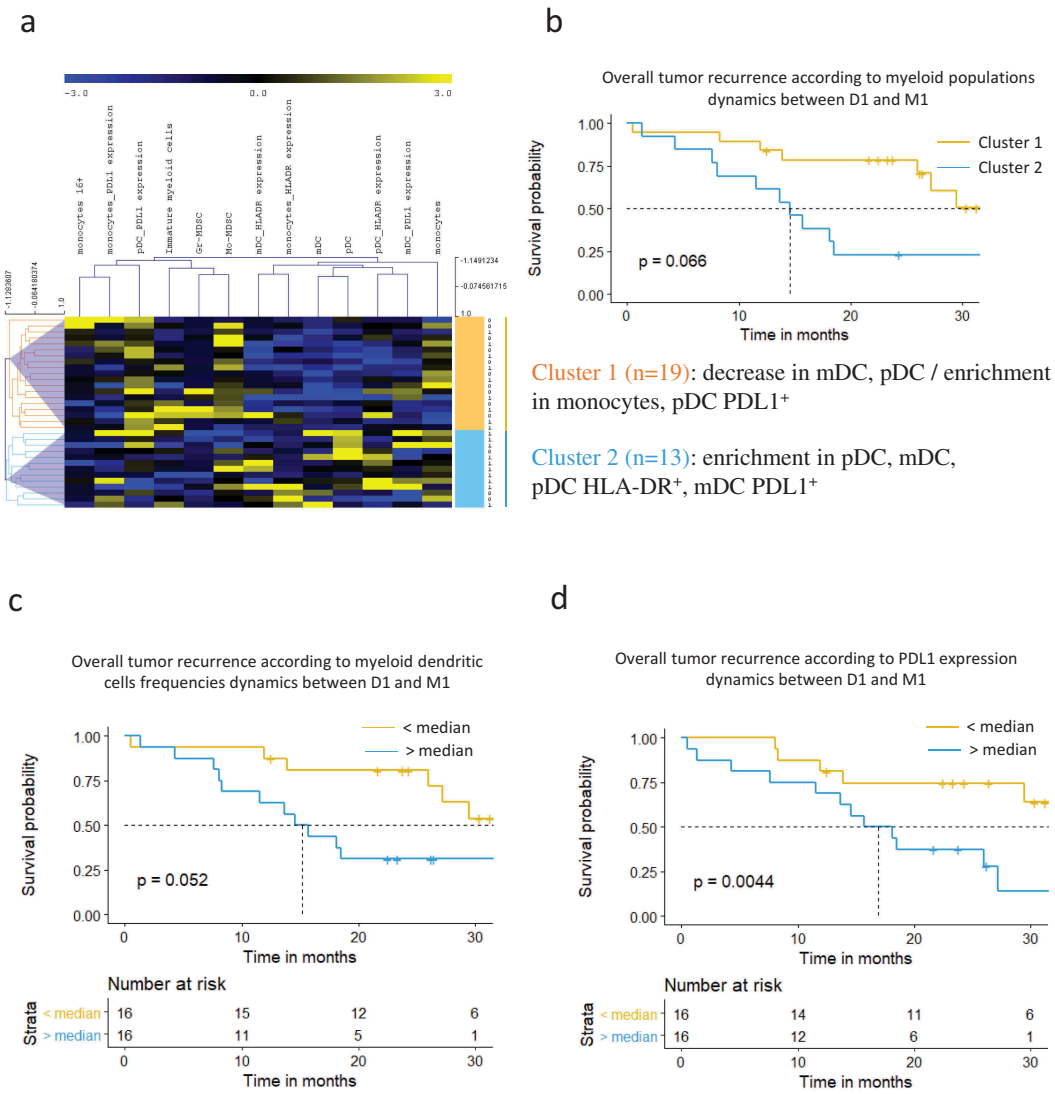

Figure 5. Overall tumor recurrence according to the variation of myeloid cells after radiofrequency ablation. (a) Exploratory unsupervised hierarchical clustering analysis of variation of frequencies of myeloid cells and expression of myeloid cells markers after RFA using the MeV software with Pearson correlation (one sample per row; at the right of the row: 0 = no overall tumor recurrence; 1 = tumor recurrence). Abbreviations: pDC = Plasmacytoid Dendritic Cells; mDC = Myeloid Dendritic Cells; Gr-MDSC = Granulocytic Myeloid-Derived Suppressor Cells; Mo-MDSC = Monocytic Myeloid-Derived Suppressor Cells. (b) Overall tumor recurrence according to the two different myeloid clusters, (c) according to myeloid dendritic cells dynamics (d) and according to PDL1 expression in myeloid dendritic cells between day 1 (D1) and month 1(M1) in the validation cohort (exploratory analysis). Results were represented using the Kaplan Meier Method and compared using the log-rank test with the numbers at risk under the *X*-axis.

literature. This early time point at D1 was chosen for practical reasons (patients were still hospitalized) and because it has been described that innate populations can have a very early variation after a local treatment.[24,25] Interestingly, if the magnitude of immune variation was limited between D0/M1, dynamics were more informative between D0/D1 and D1/M1. It is concomitant with other studies[24] reporting that after local treatment, pDC and NK cells can be recruited very early into lymph nodes and RFA site to process neoantigens, prime adaptive immunity and have an antitumoral effect.[25] Thirdly, with one of the largest cohort (n = 80) and the longest follow-up (median = 56 months), our clinical correlations shed important insight in this field, where the size of studied populations is usually very small. In the same way as Zerbini et al. (2006–2010), we also reported the dynamics of CD8, pDC and NK cells induced by RFA, but we had more statistical power to highlight the leading role of NK cells on tumor recurrence. Interestingly, two recent studies of immunomonitoring after stereotaxic body radiation therapy for liver and lung tumors also described systemic changes of NK cells in peripheral blood after treatment.[26,27]

NK cells are cytolytic innate lymphoid cells involved in the early response to viral infections and cancer.[28] In liver, NK cells are the main component of lymphocytic populations (30–50%), and their cytotoxicity is dependent of the equilibrium between activating receptors (NKp30, NKp44, NKp46, NKG2D, DNAM-1) and inhibitory receptors (KIR, LIR, CD94).[29] Our study showed that a high D0/D1 dynamics of the NK cells expressing NKp30 activating receptor were associated with less tumor recurrence. This is consistent with several recent studies describing NKp30 as a major favorable prognosis biomarkers for HCV induced liver fibrosis[30] and for different malignancies such as acute myeloid leukemia,[31] prostate cancer,[32] gastrointestinal stromal tumors,[33] lung cancer,[34]

and melanoma.[35] The early variation of NKp30 expression found in our analysis is supported by several studies reporting NKp30 dynamic in the earliest stage of infections or treatments,[36–38] notably after interferon therapy where NKp30 expression changed within hours of treatment initiation.[39] Conversely, our results reported that late persistence of NK cells (total NK and CD56^{bright} NK cells) 1month after RFA was associated with more HCC recurrence. However, this double-edged effect of the innate immune response has already been described: several studies in radiation oncology, cardiology, and infectious diseases have demonstrated the detrimental effect of chronic inflammation and continuous innate immune cell activation.[40,41] Notably, NK cells have the potential to restrict tumor-induced CD8 + T cell priming and generation of Effector Memory CD8+ cells.[42,43] In our study, the persistence of an innate immune response 1month after RFA may reflect the incapacity of patients immunity to switch from an immature pro-inflammatory phenotype towards an efficient adaptive immune response.

Furthermore, NK cells are closely linked to dendritic cells: they can recruit DCs to the tumor site and regulate their maturation through PD1/PDL1 checkpoint.[42,44] Like in NK cells, our results showed that the persistence of a higher level of dendritic cells 1month after RFA has a negative outcome. Indeed, in the context of cancer, dendritic cells are defective in their differentiation and are poor stimulators of immune response.[45] Moreover, previous data described that the expression of inhibitory molecules, like PDL1, contributes to alter functionality of DCs[45,46] and that PDL1^{+} DCs could confer T and NK cell immune suppression.[47] In our data, a delayed overexpression of PDL1^{+} in mDC was associated with more tumor recurrence following RFA treatment for HCC. These results could partially explain the positive effects of anti-PD1 and anti-PDL1 treatment in advanced HCC,[9,48] and raise the question of their use as an adjuvant therapy for early HCC treated with RFA.

Overall, this study has several limitations. First, as two sequential cohorts were performed to validate the biomarkers, antibodies panels were larger in the validation cohort, and some populations were not studied in the pilot cohort. Second, as our biological material was blood only, it is not possible to know if the observed variations were due to tumor infiltration or solely blood dynamics. Third, it has been described that cryopreservation can have an impact on myeloid cells viability (particularly in granulocytes that we did not study), but for practical considerations, it was not possible to process fresh blood samples in this study. Finally, the impact of RFA on innate immunity should be further explored with functional tests to link these phenotypic variations with cytokine secretions or cytotoxic capabilities.

As HCC diagnosis is mainly based on radiological imaging and given that RFA is a non-invasive technique, oncologists often lack traditional HCC pathologic markers (satellites nodules, microvascular invasion) to predict prognosis and risk of tumor recurrence. In this context, blood biomarkers are appealing and some of the most studied non-immune biomarkers are pre-treatment serum levels of AFP and DCP (des-γ-carboxy prothrombin).[49,50] Interestingly, our immune findings in NK cells (total, CD56^{bright}, and NKp30^{+}) and DCs (PDL1^{+} mDC) present several advantages: they have been assessed prospectively, they are based on the biological dynamics induced by RFA, and they identify targetable immune populations. Indeed, several treatments are now available to promote NK cells activation, such as anti-KIR antibodies and TLR agonist.[28] Moreover, anti-PDL-1 durvalumab showed promising activity in a phase I/II clinical trial in unresectable HCC[48] and is currently tested in a randomized phase II (NCT02519348).

In conclusion, percutaneous RFA induced systemic changes in adaptive and innate immunity closely linked to the risk of tumor recurrence. These results suggest a potential role for the combination of percutaneous RFA with immunotherapy to decrease tumor recurrence in cirrhotic patients with HCC.

List of abbreviations

AFP	Alpha fetoprotein
CM	Central Memory
CT scan	computed tomography scan
DCs	Dendritic Cells
EM	Effector Memory
HR	Hazard Ratio
HCC	Hepatocellular Carcinoma
HBV	Hepatitis B Virus
HCV	Hepatitis C Virus
Gr-MDSC	Granulocytic Myeloid Derived Suppressor Cells
ICB	Immune Checkpoint Blockade
IMC	Immature Myeloid Cells
mDC	Myeloid Dendritic Cells
MDSC	Myeloid Derived Suppressor Cells
Mo-MDSC	Monocytic Myeloid Derived Suppressor Cells
MFI	Median Fluorescence Intensity
Mono	Monocytes
MRI	Magnetic resonance imaging
NASH	Nonalcoholic steatohepatitis
NK	Natural Killer
OS	Overall Survival
PBMC	Peripheral Blood Mononuclear Cells
PD1	Programmed cell death 1
pDC	Plasmacytoid Dendritic Cells
PDL1	Programmed death-ligand 1
PFS	Progression-free Survival
RFA	Radiofrequency Ablation
TEMRA	CD45RA+ Effector Memory T cells

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Authors' contributions

PR, JCN, NG, and DO designed the research and wrote the paper. PR and FM performed the flow cytometry experiments. PR, JCN, AJG, ASC, NG, and DO interpreted the data. All the authors collaborated on the paper's conception, reviewed the paper and approved the final version of the article to be published.

Availability of data and material

The datasets used during the current study are in the Institut Paoli Calmettes and are available from the corresponding author on reasonable request.

Competing Interests

O. Seror received personal fees and non-financial support from Angiodynamics, Olympus, and Bayer Schering Pharma and received personal fees from GE as a consultant. N. Ganne and P. Nahon received personal fees from Bayer Schering Pharma. D. Olive is founder of Imcheck Therapeutics. Other authors have no conflict of interest to declare.

Consent for publication

Written informed consent was obtained from every patient included in this study.

Ethics approval and consent to participate

This study was approved by the French Institutional Review Board (N° IRB00006477) from Hopitaux Universitaires Paris Nord Val De Seine on March 30th 2011.

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