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ORIGINAL ARTICLE

Immunological effects of a novel RNA-based adjuvant in liver cancer patients

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Abstract Evaluation of biological effects of adjuvants on immune cells has been assessed in a limited number of studies. Moreover, no data are available on samples derived from cancer patients who may have a severe immune impairment. The effects of a novel RNA-based adjuvant (RNAdjuvant® developed by CureVac) were assessed in an ex vivo setting on PBMCs obtained from 8 healthy volunteers and 17 HCC patients, using a multiparametric approach to analyze network dynamics of early immune responses. Evaluation of CD80, CD86 and HLA-DR expression, cytokine production as well as gene expression was performed. Moreover, the downstream effect on $CD4^+$ T cell phenotyping was evaluated. Treatment with RNAdjuvant® showed comparable effects on PBMCs of both HCC and healthy subjects. In particular, CD80, CD86 and HLA-DR expression was found up-regulated in circulating dendritic cells, which promoted a $CD4^+$ T cell differentiation toward an effector phenotype. A mixed Th1/Th2 cytokine pattern was induced, although a more predominant production of TNFα and IFNγ was observed in HCC patients versus healthy controls. The cytokine profile was further confirmed by gene transcriptional analysis, which showed up-regulation of several genes involved in innate and adaptive immune-related pathways. The present study is the first demonstration that HCC patients and healthy subjects are equally responsive to an adjuvant. This may suggest that the same vaccine formulation including the RNAdjuvant® might have similar potency in healthy subjects and cancer patients.

Keywords RNAdjuvant · Hepatocellular carcinoma · Immune response · Cancer vaccine

Abbreviations

APC	Antigen-presenting cell
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HPV	Human papilloma virus
IFN-γ	Interferon gamma
IL-6	Interleukin 6
IL-10	Interleukin 10
ISGs	IFN-stimulated genes
LPS	Lipopolysaccharide
mDC	Myeloid dendritic cell
MPL	Monophosphoryl lipid A
NLRs	Nod-like Receptors
PAMPs	Pathogen-associated molecular patterns
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
pDC	Plasmcytoid dendritic cell
PRRs	Pattern recognition receptors
RIG-I	Retinoic acid-inducible gene 1-like receptor
STING	Stimulator of interferon genes
TAA	Tumor-associated antigen
TE	Effector T cell
TCM	Central memory T cell
TEM	Effector memory T cell

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Th1/2	T helper 1/2
TLRs	Toll-like receptors
TNFα	Tumor necrosis factor alpha

Introduction

Since the discovery of human tumor antigens, the cancer immunotherapy field has rapidly progressed and several active (e.g., therapeutic cancer vaccine) as well as passive strategies (e.g., adoptive T cell transfer) are being investigated [1]. The anti-tumor efficacy of such strategies, however, is extremely variable and several lines of research are pursued in order to improve clinical outcome in cancer patients [2–4]. Several reasons may be taken into account for such low efficacy. Among others, the low immunogenicity of antigens delivered as peptides. Indeed, as for preventive anti-microbial vaccines, employment of the minimal antigenic determinant (e.g., epitope), devoid of all potentially risky adjuvanting "contaminants," results in a dramatically lower immunogenicity [4, 5]. This has required and continuously requires the need for developing adjuvants to improve the immune response elicited by vaccines. However, to date only a few number of adjuvants have been approved for use in humans [6, 7].

The increasing detailed knowledge about the central role of the innate immunity in igniting and directing the adaptive immune response has been crucial to the adjuvant field. In particular, the discovery of pattern recognition receptors (PRRs) in cells of the innate immune system, including Toll-like receptors (TLRs), Nod-like Receptors (NLRs), Retinoic acid-Inducible Gene 1-Like Receptor (RIG-I) or Stimulator of interferon genes (STING), has promoted an explosion in the discovery of novel adjuvants (reviewed in [8]). Indeed, they act as sensors for bacterial and viral components (Pathogen-Associated Molecular Patterns; PAMPs) and induce the innate immune response by activation of a signaling cascade leading to up-regulation of various immune genes, including inflammatory cytokines, chemokines, and type I IFNs. This will eventually end up in triggering a robust adaptive immune response [9]. Therefore, PRRs represent the ideal target for promoting an effective immune response.

In particular, several TLR ligands have been extensively evaluated as vaccine adjuvants in preclinical as well as human clinical setting (reviewed in [10]). Nevertheless, only the TLR4-ligand monophosphoryl lipid A (MPL) is licenced for human use as adjuvant of the preventive vaccines for hepatitis B virus (HBV) and human papilloma virus (HPV) [11, 12].

Moreover, most if not all of the licenced adjuvants induce only weak T cell immune response and their application in cancer immunotherapy would be very limited. Therefore, there is a continuous quest for new adjuvants and in particular for molecules able to elicit a balanced humoral and cellular immune response with potential application in both preventive and therapeutic vaccines.

For such a reason, a novel RNA-based adjuvant (RNA-djuvant®) has been recently developed by CureVac AG (Tübingen, Germany). The RNA component consists of a single-stranded, noncoding, non-capped RNA sequence containing several polyU-repeats. The RNA molecule is complexed with a polymeric carrier formed by a disulfide-crosslinked cationic peptide in order to increase its stability against RNase degradation. It was recently shown that RNAdjuvant® exhibited a favorable preclinical safety profile and induced Th1/Th2 balanced, long-lasting immune responses to HPV-16 E7 protein-derived long peptides, resulting in a strong activity against syngeneic TC-1 tumor model [13]. In this study, an extensive multiparametric analysis was conducted to clarify the immunological and molecular mechanisms underlying the biological effect observed in the animal model. Antigen-specific $CD8^+$ effector and memory T cells were induced only in the presence of the adjuvant. A gene expression analysis at the site of injection showed induction of Type I IFN and Type I IFN-stimulated genes (ISGs) as well as the up-regulation of inflammasome-associated genes. Moreover, distinct chemokines and immune activation markers were found up-regulated [13]. Overall, such data suggest that RNAdjuvant® is a safe and potent immunostimulator for cancer vaccines.

Vaccines have been shown to elicit similar antibody titers in healthy subjects as well as in cancer patients not undergoing chemotherapy although it is still uncertain whether such immune response is fully protective (reviewed in [14]).

Adjuvants, which are an essential component of vaccine formulations, are assumed to have comparable adjuvanting efficacy in healthy subjects and cancer patients. However, in order to prove that the same effective vaccine formulation in healthy subjects may be implemented in cancer patients as well, an experimental validation is needed. For such a reason, we took advantage of a multiparametric platform, developed and fully validated by our group, to assess ex vivo the innate and early adaptive response in human PBMCs [15–21].

The immune stimulating effects of the RNAdjuvant® were evaluated in PBMCs from healthy subjects and patients affected by hepatocellular carcinoma (HCC). Indeed, RNAdjuvant® is going to be employed in the therapeutic cancer vaccine formulation developed by the EU-funded HEPAVAC Consortium (www.hepavac.eu) specifically targeting liver cancer [22]. The overall results showed that HCC patients and healthy subjects are equally responsive to RNAdjuvant®.

Materials and methods

Enrolled subjects

Peripheral blood was obtained by venipuncture from 8 healthy volunteers and 17 HCV chronically infected HCC patients. All human specimens were obtained and processed at the National Cancer Institute in Naples under informed consent, as approved by the Institutional Review Board.

PBMC isolation

Fresh human PBMCs were isolated by Ficoll-Hypaque density gradient centrifugation and plated in six-well plates at a concentration of approximately 1×10^7 cells/well in a maximum volume of 3 ml/well. Isolated PBMCs were incubated for 24 h (short-term culture) or 7 days (medium-term culture) in RPMI 1640 medium.

Cell culture medium

PBMCs culture medium consisted of RPMI 1640 medium (Life Technologies, Carlsbad, CA) supplemented with 2 mM L-glutamine (Sigma), 10% fetal calf serum (Life Technologies) and 2% penicillin/streptomycin (5000 I.U./5 mg per ml, MP Biomedicals). Recombinant interleukin-2 (rIL-2; R&D Systems, Minneapolis, Minn.) was added at a concentration of 75 U/ml for medium-term culture (every 3 days).

Cell treatment

PBMCs were incubated for 24 h (short-term culture) with 20 μg/ml of RNAdjuvant® provided by CureVac AG (Tübingen, Germany). In parallel, cells were pulsed with 4 μg/ml of lipopolysaccharide (LPS), as positive control (from *Salmonella enterica serotype Minnesota*, purity >99.0%). Alternatively, PBMCs were incubated for 7 days (medium-term culture) with the same concentration of RNAdjuvant® or LPS, plus IL-2 added at day 0, 3 and 6. PBS was used as negative control. At the end of the incubation, PBMCs were harvested, washed with PBS 1X (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO4, 2 mM KH_2PO_4, pH 7.2) without calcium and magnesium and analyzed by flow cytometry. All cell supernatants were collected for quantification of cytokine production by enzyme-linked immunosorbent assay (ELISA).

Flow cytometry

Short-term culture PBMCs were incubated for 30 min at 4 °C with human monoclonal antibodies specific for CD80, CD83, CD86, HLA-DR, CD123, CD11c and CD14 (BD Pharmingen, San Diego, CA), washed and then analyzed with a FACScalibur flow cytometer (BD Pharmingen). Data analysis was carried out with WinMDI2.8 Software. A Paired t test was performed, all p values were considered significant if less than 0.05.

T-maturation analysis

Medium-term culture PBMCs were analyzed for markers of $CD4^+$ T cell phenotyping by flow cytometry. A T cell phenotyping was obtained after gating on the $CD3^+/CD4^+$ cells. Naïve $CD4^+$ T cells were identified as $RA^+/62L^+/CD27^+$; Effector cells as $RA^+/62L^-/CD27^-$; Central Memory cells as $RO^+/62L^+/CD27^+$; Effector Memory cells as $RO^+/62L^-/CD27^-$.

Cytokine analysis

At the time the cells were harvested, the supernatants were also collected and stored frozen until analyzed. IL-6, TNF, IFNg and IL-10 production was assessed using "Human ELISA MAX™ Deluxe (Biolegend)" for quantitative detection of each human cytokine, according to the manufacturer's instructions. Data acquisition was performed using the Sirio-S ELISA reader. A Paired t test was performed, and all p-values were considered significant if less than 0.05.

RNA extraction and PCR array

Total RNA was extracted using Qiagen RNEasy Mini Kit (Qiagen, Germany) according to the manufacturer's protocol and checked for quantity and quality using a NanoDrop 2000c. 250 ng of RNA of each sample was converted into cDNA using the RT2 First Strand Kit (SABiosciences, Frederick, USA). cDNA was added to the RT2 SYBR Green qPCR Master Mix (SABiosciences, Frederick, USA) and then aliquoted on the "Human Innate e Adaptive immune responses RT^2 Profiler PCR Array" (Quiagen) plat. All steps were done according to the manufacturer's protocol for the CFX96 Real time System (Bio-rad laboratories, Inc.)

Statistical analysis

Statistical analysis was performed using Graphpad Prism 6 software (Graphpad Software, La Jolla, CA, USA), and the results were considered statistically significant at a level of $p < 0.05$. The t Test, nonparametric Wilcoxon matched-pairs signed-rank test, was used to analyze intergroup comparisons. PCR array data were analyzed by the RT^2 profiler PCR array Data Analysis software which calculated two normalized average Ct values, a paired t test P value and

a fold change. Genes were identified to be differentially expressed on a significance level of $p < 0.05$.

Results

Clinical parameters of subjects included in the analysis

Twenty-five subjects were enrolled in the present study. Seventeen subjects were HCV chronically infected HCC patients, of whom eight were males and nine were females. Eight healthy donors were enrolled as controls matched for age and life style. Clinical parameters of enrolled subjects are described in Table 1.

Maturation markers induced in circulating APCs by the RNAdjuvant®

In order to evaluate the maturation markers induced by RNAdjuvant®, the expression of CD80, CD86 and HLA-DR molecules was examined in circulating antigen-presenting cells (APCs) by flow cytometry. In particular, HLA-DR$^+$CD14$^+$ monocytes, HLA-DR$^+$CD14$^-$CD11c$^+$ myeloid dendritic cells (mDCs) and HLA-DR$^+$CD14$^-$CD123$^+$ plasmacytoid dendritic cells (pDCs) were evaluated in parallel.

Table 1 Clinical parameters of enrolled subjects

	Sex	HCV	HCC
Healthy 1	M	Neg	No
Healthy 2	M	Neg	No
Healthy 3	F	Neg	No
Healthy 4	F	Neg	No
Healthy 5	F	Neg	No
Healthy 6	F	Neg	No
Healthy 7	F	Neg	No
Healthy 8	F	Neg	No
HCC 1	M	Pos	Yes
HCC 2	M	Pos	Yes
HCC 3	M	Pos	Yes
HCC 4	M	Pos	Yes
HCC 5	M	Pos	Yes
HCC 6	M	Pos	Yes
HCC 7	M	Pos	Yes
HCC 8	M	Pos	Yes
HCC 9	F	Pos	Yes
HCC 10	F	Pos	Yes
HCC 11	F	Pos	Yes
HCC 12	F	Pos	Yes
HCC 13	F	Pos	Yes
HCC 14	F	Pos	Yes
HCC 15	F	Pos	Yes
HCC 16	F	Pos	Yes
HCC 17	F	Pos	Yes

The basal expression of the evaluated markers was largely comparable between healthy and HCC subjects in all the three cell types (Suppl. Fig. 1). On the contrary, treatment with RNAadjuvant® in samples from the HCC subjects induced a statistically significant up-regulation of CD80 and CD86 in CD14$^+$ cells and all three markers in CD11c$^+$ cells. The up-regulation in the CD123$^+$ cell subset showed a trend which did not reach the statistical significance. On the contrary, the RNAdjuvant® treatment induced an up-regulation of the three markers in all cell subset from healthy subjects. Such effect was comparable to the one induced by control LPS (Fig. 1).

T cell maturation induced by the RNAdjuvant

The effect on CD4$^+$ T cell phenotyping induced by APCs treated with RNAdjuvant® was evaluated in PBMCs, after 6 days post-treatment. APCs treated with RNAdjuvant® induced a reduction of naïve CD4$^+$ T cells and a parallel increase in both effector (TE) and effector memory (TEM) cells. Such effects were comparable in samples from both healthy subjects and HCC patients. In both groups, the effect reached the statistical significance only for the naïve and TEM cells. On the contrary, the central effector memory (TCM) cells showed a trend to increase only in HCC patients (Fig. 2a, b).

The inter-group comparative analysis, both at baseline and after RNAdjuvant® treatment, showed a comparable percentage of all the CD4$^+$ T cell sub-populations in HCC patients versus healthy subjects. The lower levels observed in HCC patients, indeed, did not reach the statistical significance (Fig. 2c, d). The only exception was the TCM subpopulation, which was perfectly comparable after RNAdjuvant® treatment in both groups of samples.

Cytokine pattern induced in circulating APCs by the RNAdjuvant

The level of Th1 (IFNγ and TNFα), Th2 (IL-10) and pro-inflammatory (IL-6) cytokines was assessed by ELISA in supernatants of PBMCs after treatment with RNAdjuvant® (Fig. 3). The evaluation was performed after incubation for 24 h and 6 days to assess the persistence of the cytokine release.

The first observation was the comparable baseline production of all four cytokines by PBMCs from healthy subjects and HCC patients (Suppl. Fig. 2).

Treatment with RNAdjuvant® induced a balanced production of both Th1 and Th2 cytokines in both HCC and healthy subjects (p value <0.01). In particular, the highest

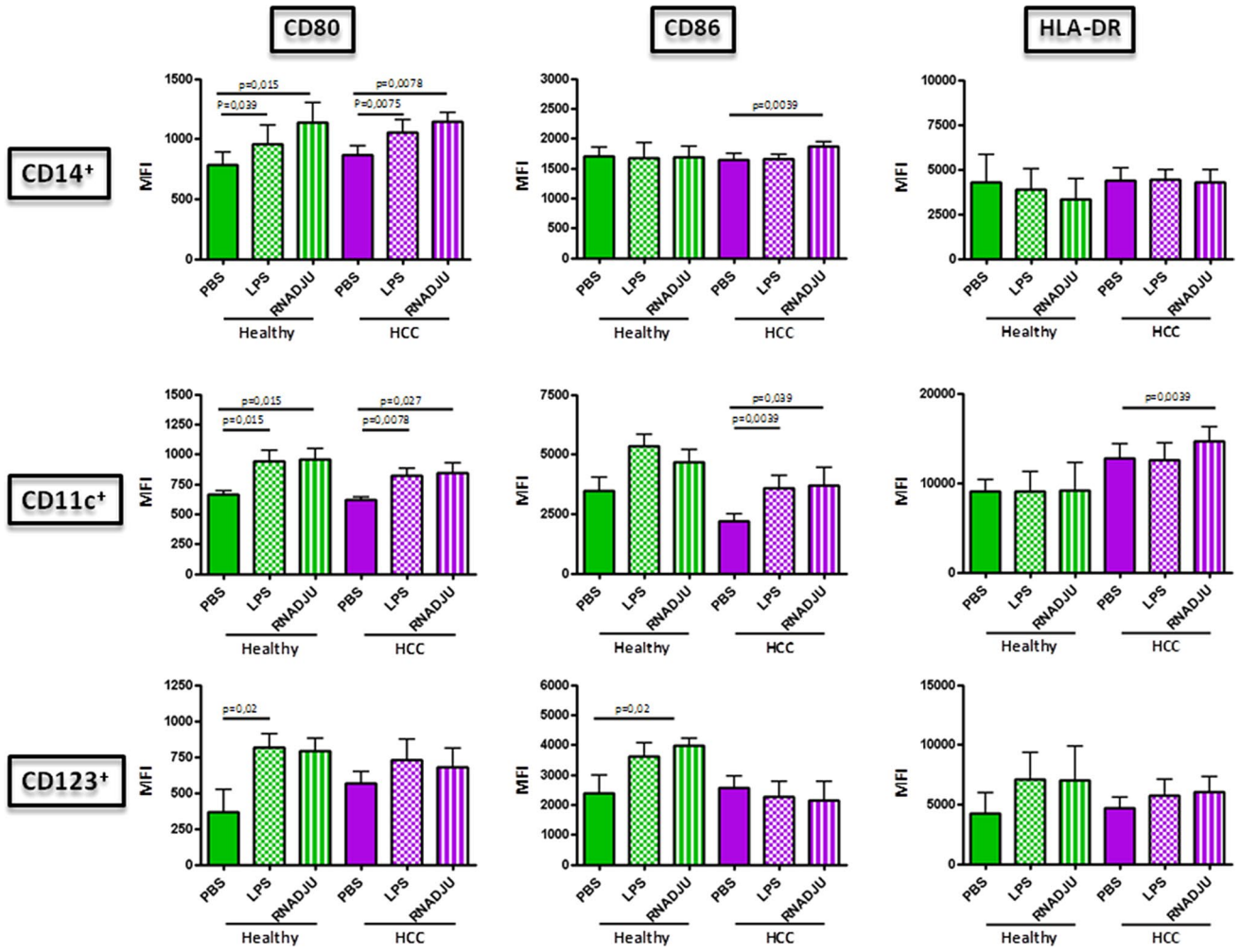

Fig. 1 Expression of surface circulating APC markers in PBMCs. Mean Fluorescence Index (MFI) for each of the indicated markers was evaluated on cells after 24 h treatment with RNAdjuvant and LPS by flow cytometry. Healthy group *green bars*; HCC group *violet bars*

fold change over the basal production was observed for the Th1 cytokine IFNγ (average of 117.8 in healthy and 142.6 in HCC) (Fig. 3). The increased production of the cytokines showed persistent fold changes for 6 days, especially in HCC samples. The only exception was the Th1 TNFα cytokine whose fold change dramatically dropped at 6 days in both groups of samples (Fig. 3). Interestingly, the levels of Th2 IL-10 and pro-inflammatory IL-6 cytokines were comparable to those induced by the LPS, used as positive control. On the contrary, RNAdjuvant® induced a statistically significant higher level of the Th1 cytokines TNFα (at 24 h) and IFNγ (at both 24 h and 6 days) compared to LPS.

Genes transcriptionally activated by RNadjuvant in PBMCs

To further characterize the immunological effects induced by the RNAdjuvant® in stimulated PBMCs, the gene expression profile was analyzed. Normalized array expression data were evaluated to assess the effect of the RNAdjuvant® treatment over the control (e.g., PBS). Samples from HCC subjects and healthy controls were collected after 24 h of treatment.

The analysis identified 30 differentially expressed genes in the healthy subjects (24 up-regulated and 6 down-regulated) and 45 differentially expressed genes in the HCC patients (31 up-regulated and 14 down-regulated) (Fig. 4a, b; Suppl. Table 1). The same pathways/signaling were modulated in the two groups (Fig. 4a, b). The comparative analysis showed that 27 differentially expressed genes were common to both groups, 22 up-regulated and 5 down-regulated (Fig. 5). In particular, the heatmap and the clustering generated with the expression values of shared genes showed that HCC subjects and healthy controls do not segregate into two different groups (Fig. 5). This suggests that they respond similarly to the adjuvant.

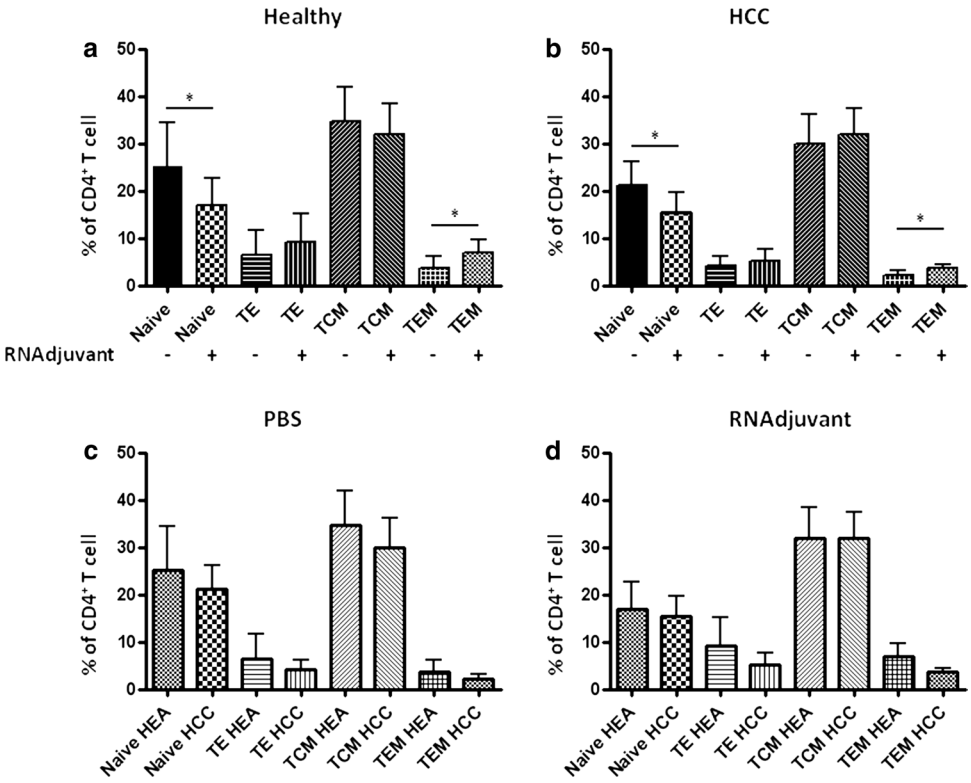

Fig. 2 Effect of RNAdj on ex vivo differentiation of naïve $CD4^+$ T cells from healthy subjects and HCC patients. Isolated PBMCs from healthy subjects (**a**) and HCC patients (**b**) were incubated with 20 μg/ml of RNAdjuvant. IL-2 was added every 3 days. After 6 days phenotype of $CD4^+$ T cells was assessed by flow cytometry. Naïve $CD4^+$ T cells were identified as $RA^+/62L^+/CD27^+$; Effector as $RA^+/62L^-/CD27^-$; Central Memory as $RO^+/62L^+/CD27^+$; Effector Memory as $RO^+/62L$-/CD27-. Side-by-side comparison between PBS and RNAdj in healthy subjects and HCC patients is shown in panels (**c**, **d**)

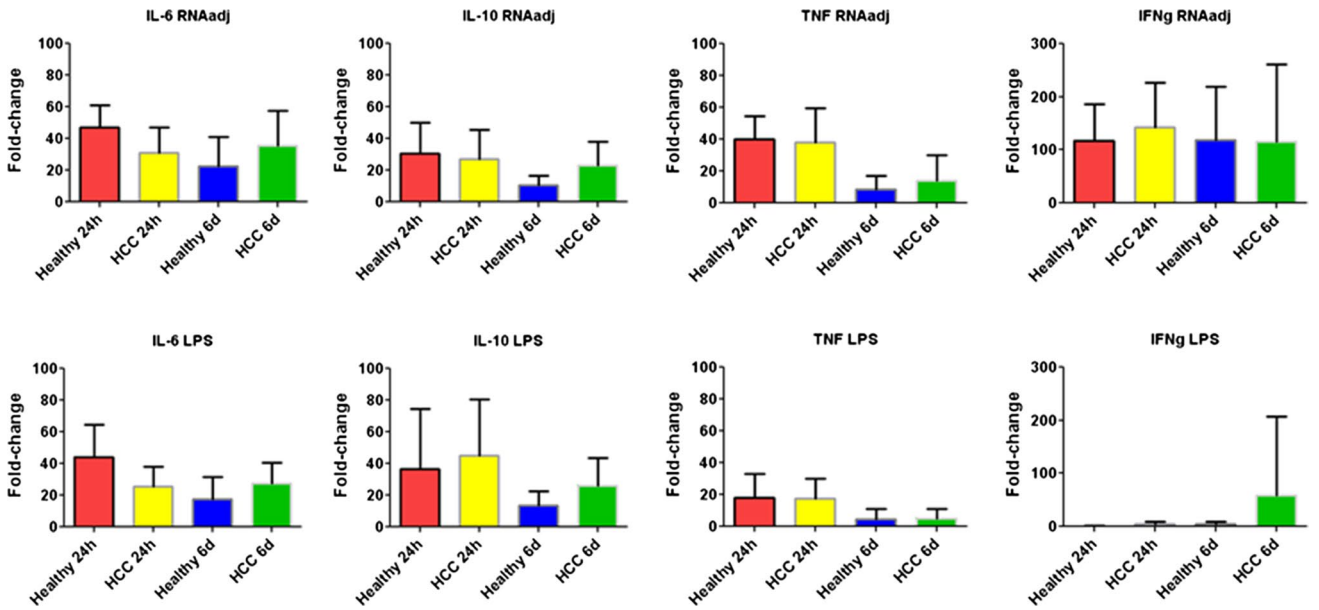

Fig. 3 Analysis of cytokine production in supernatants of PBMCs from HCC and healthy subjects. Isolated PBMCs were incubated for 24 h or 6 days with 20 μg/ml of RNAdjuvant or with 4 μg/ml of LPS), as positive control. IL-6, TNF, IFNg and IL-10 production was assessed in the cell culture supernatant using "Human ELISA MAX™ Deluxe (Biolegend)"

Overall, shared genes involved in several immune pathways were found up-regulated (Suppl. Table 1). In particular, the IL-6 and IFNγ genes were the most strongly up-regulated ones. The intra-cellular TLRs 3, 7 and 8 and their downstream mediators (e.g., MyD88, TICAM1, NFkB, Type I IFNs), the RIG-I (e.g., DDX58 and IRF7) as well as the inflammasome (e.g., Caspase 1) were found up-regulated. These findings suggest that the RNAdjuvant®

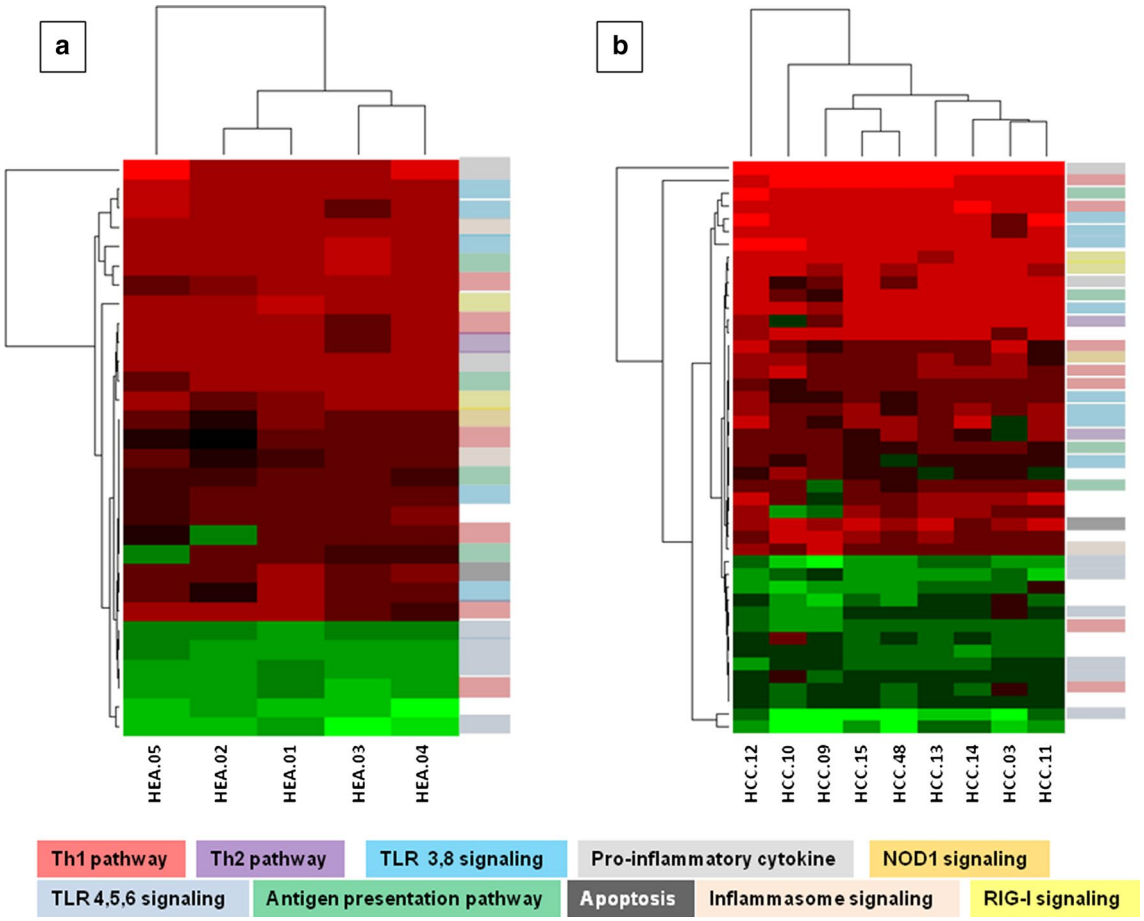

Fig. 4 Gene expression profile after RNAdjuvant treatment. **a** Heatmap and the clustering generated with the expression values of 30 differentially expressed genes in Healthy group; **b** heatmap and the clustering generated with the expression values of 45 differentially expressed genes in HCC group

treatment is able to activate the innate immunity via more than one Pathogen-Related Receptor (PRR) signaling. Moreover, in support of the cytokine ELISA data, both Th1 (e.g., IFNγ) and Th2 (e.g., IL-10) cytokines were activated along with pro-inflammatory cytokines (e.g., IL-6).

Expression of co-stimulatory CD40 and CD80 genes was up-regulated, along with HLA-A and E molecules, indicating a potentiated antigen-presentation activity to effector T cells.

Concerning the down-regulated genes, it was very interesting to find a set of genes correlated with the TLR4 and 2 signaling (e.g., CD14, LY96 and MAPK1). Of note, the down-regulation of TLR 5 and 6 was specifically identified only in samples from HCC patients (Fig. 4b; Suppl. Table 1).

The expression levels of the described genes in the two groups of samples are further described in a Circos image (Fig. 6). The IL-6 and IFNγ are confirmed to be the most expressed genes upon RNAdjuvant® treatment of PBMCs. However, their differential expression pattern is striking. Indeed, IL-6 is equally expressed in PBMCs from healthy and HCC subjects, while IFNγ is mostly expressed in PBMCs from HCC subjects. Considering the other highly expressed genes, type I IFNs and MX1 are equally expressed in both groups; CXCL10 and CD80 are mostly expressed in HCC samples; IRF7 is mostly expressed in healthy samples.

Discussion

Several tumor-associated antigens (TAAs) have been characterized to date for development of anti-cancer vaccines. However, due to their low intrinsic immunogenicity peptides are not efficient in eliciting immune responses and require formulations with adjuvants to both potentiate immunogenicity and induce a Th1/Th2 balanced immunity.

Most of the adjuvants are developed for preventive vaccines, mainly aiming at eliciting a Th2-type response. On the contrary, the purpose of therapeutic cancer vaccines is

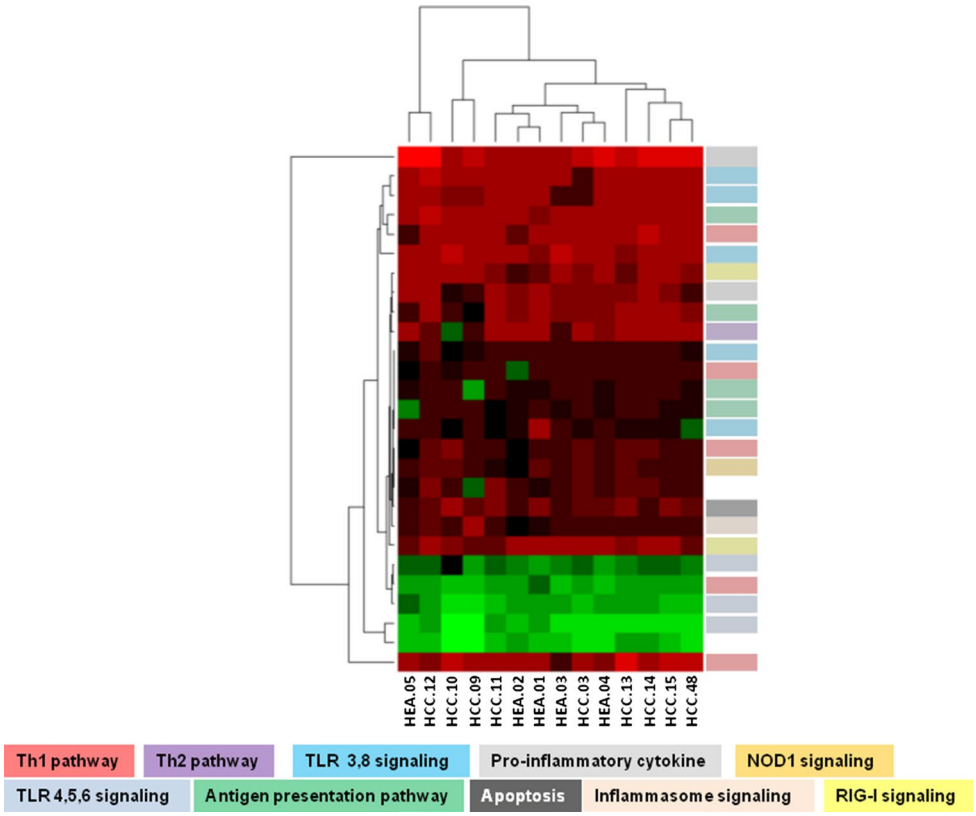

Fig. 5 Gene expression profile after RNAdjuvant treatment. Heatmap and the clustering generated with the expression values of 27 shared genes in healthy and HCC groups

to elicit a Th1-type response and very few adjuvants are available for this purpose. Given the availability of very limited information, in the present study we aimed at verifying the responsiveness to an adjuvant in cancer patients versus healthy subjects. This was performed employing a multiparametric analysis in an ex vivo setting, exposing PBMCs from liver cancer patients and healthy controls to a novel RNA-based adjuvant (RNAdjuvant®, CureVac AG).

Results in samples from the HCC subjects showed that the CD80 and CD86 were statistically up-regulated in $CD14^+$ monocyte as well as in $CD11c^+$ myeloid DC (mDC), together with HLA-DR. The up-regulation in the $CD123^+$ cell subset showed a trend which did not reach the statistical significance. These results are largely comparable between HCC and control groups.

Such observations confirm that RNAdjuvant® is a strong immune potentiator activating maturation of APCs and expression of co-stimulatory molecules to promote an efficient adaptive response. Moreover, they suggest that the HCC status does not impair the responsiveness of circulating APC populations to the immunogenic stimulus of RNAdjuvant®.

Cells from HCC patients and healthy subjects showed a comparable basal production of Th1/Th2 cytokines. The RNAdjuvant® treatment induced a balanced production of both Th1 and Th2 cytokines in both HCC and healthy subjects which persisted for 6 days. This would strongly suggest an in vivo long-lasting driving milieu for development of a balanced immune response [23]. In particular, when compared to LPS, RNAdjuvant® induced a much higher level of the Th1 cytokines, confirming that it has the required properties to be an eligible adjuvant for a therapeutic cancer vaccine formulation. The induction of the anti-inflammatory IL-10, as previously reported by our group, suggests the activation of a positive homeostatic mechanism to neutralize an excessive pro-inflammatory status which would be detrimental to an effective immune response [18].

The ability of APCs treated with RNAdjuvant® to induce activation of naïve $CD4^+$ T cells into effector cells was assessed ex vivo. The results showed that, in samples from both healthy subjects and HCC patients, reduction of naïve $CD4^+$ T cells was associated with a parallel increase in both effector (TE) and effector memory (TEM) cells. The central effector memory (TCM) cells showed a trend to increase only in HCC patients. All the $CD4^+$ T cell sub-populations showed a comparable percentage in HCC patients versus healthy subjects, both at baseline and after RNAdjuvant® treatment. Such observation strongly suggests that RNAdjuvant® is able to induce a shift in the phenotype of $CD4^+$ T cells from naïve to effector, which are the main actors in helping and boosting the T and B cell immune response. In particular, the observed increase in both effector memory (TEM) and central memory (TCM) is of high relevance,

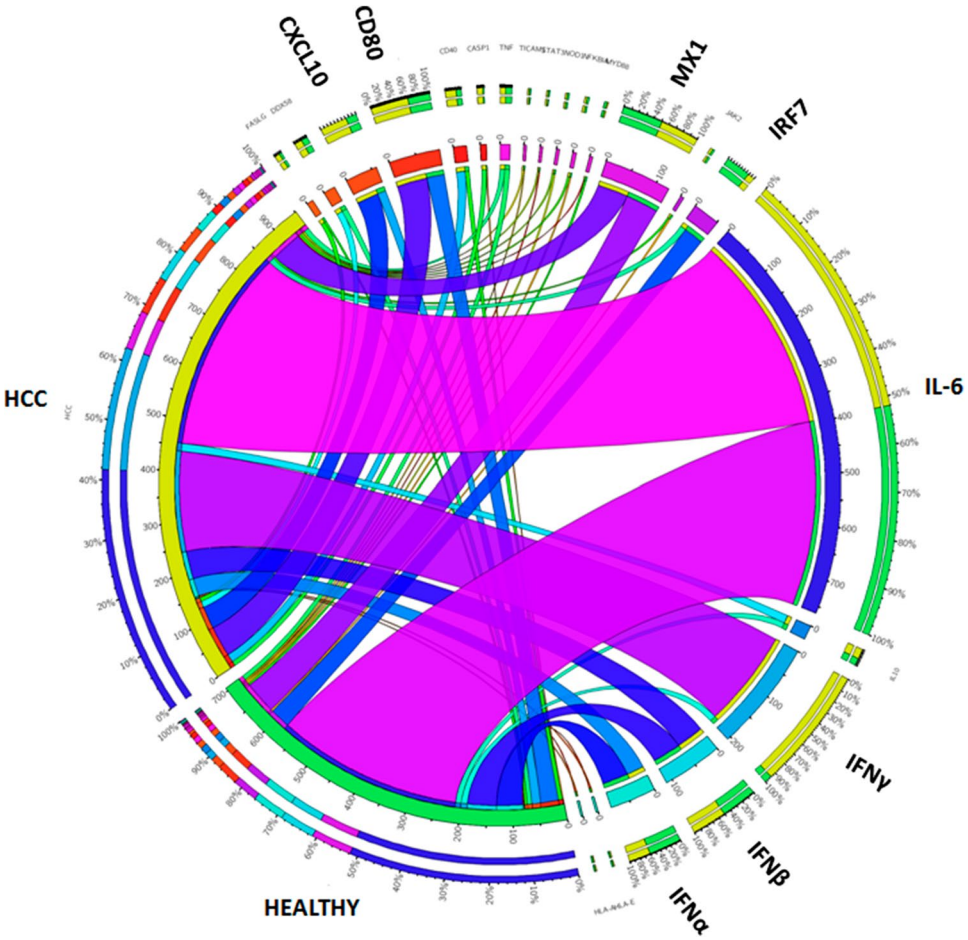

Fig. 6 Circos image. Circos plot showing log fold change of shared up-regulated genes in HCC and Healthy group. Log fold changes for the up-regulated genes are indicated by the width of ribbons and the presence of each gene is indicated by connection between genes and groups

suggesting that vaccines formulated in RNAdjuvant® is able to generate subsets capable of circulating in lymphoid (TCM) as well as non-limphoid compartments (TEM) [24, 25]. Upon contact with the appropriate antigen, effector memory cells can instantly execute effector functions, whereas central or lymphoid memory cells can rapidly proliferate, expanding and acquiring effector functions. This is of high relevance for vaccine development.

The gene expression analysis performed on PBMCs treated with RNAdjuvant® showed in both healthy subjects and HCC patients the modulation of genes involved in several innate and adaptive immune-related pathways. Overall, the results showed a similar gene expression profile in the two groups, with overlapping pathways. The RNAdjuvant® was able to induce the antigen-presentation activity of APCs to effector T cells and to activate the innate immunity probably via more than one Pathogen-Related Receptor (PRR) signaling (e.g., TLRs, RIG-I and inflammasome). In particular the signaling of intra-cellular TLRs (e.g., TLR 3, 7, 8) was activated, according to the RNA nature of the RNAdjuvant® [13, 26]. Interestingly, the signaling of the cell-surface TLRs (e.g., TLR 2, 4, 5, 6) was down-regulated. This observation suggests that the RNAdjuvant® might turn on a signaling and, at the same time, shut down a possible "interfering" signaling. Further experimental evidences on a larger sample size representing different cancers are required to validate such observation.

The balanced activation of Th1/Th2 pathways assessed by ELISA was confirmed also by the gene expression analysis although, on average, the Th1 pathway was much more activated in cells from HCC patients than healthy subjects. Moreover, activation of type I IFNs is of high relevance due to their key role in activating effector and memory Th1 cells and counteracting Th2 and Th17 differentiation [27].

Collectively, the data show that RNAdjuvant® is a very promising candidate as a potent immunostimulator in therapeutic cancer vaccination formulations. Nonetheless, given the induction of a balanced Th1/Th2 response, its inclusion in preventive vaccine formulations is foreseeable, as currently evaluated in a safety Phase I clinical trial conducted in healthy subjects in combination with rabies virus vaccine (ClinicalTrials.gov Identifier: NCT02238756).

Moreover, our results showed that the effects of RNAdjuvant® are comparable in HCC and healthy subjects, although specific minor differences were observed. In this respect, the present study is the first demonstration that

cancer patients and healthy subjects are equally responsive to an adjuvant, suggesting that the same vaccine formulation may have similar potency in healthy subjects and cancer patients.

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Authors' contributions LC, MT and AP carried out all experimental procedures. RH, MLT and FMB participated in the design of the study and analysis of data. LB conceived the study, participated in its design and coordination. LC and LB drafted the manuscript. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest Regina Heidenreich is employee of CureVac AG. All other authors declare that they have no competing interests.

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