

OncoImmunology

ISSN: (Print) 2162-402X (Online) Journal homepage: https://www.tandfonline.com/loi/koni20

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To cite this article: Katia Lemdani, Nathalie Mignet, Vincent Boudy, Johanne Seguin, Edward Oujagir, Olivia Bawa, Frédérique Peschaud, Jean-François Emile, Claude Capron & Robert Malafosse (2019) Local immunomodulation combined to radiofrequency ablation results in a complete cure of local and distant colorectal carcinoma, OncoImmunology, 8:3, 1550342, DOI: 10.1080/2162402X.2018.1550342

To link to this article: https://doi.org/10.1080/2162402X.2018.1550342

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ONCOIMMUNOLOGY
2019, VOL. 8, NO. 3, e1550342 (14 pages)
https://doi.org/10.1080/2162402X.2018.1550342

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Local immunomodulation combined to radiofrequency ablation results in a complete cure of local and distant colorectal carcinoma

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ABSTRACT

Radiofrequency ablation (RFA) of colorectal liver metastases activates a specific T-cell response that is ineffective in avoiding recurrence. Recently, local immunomodulation garnered interests as a way to improve the immune response. We were interested in improving the RFA immune response priming to propose a curative treatment of colorectal cancer (CRC) based on antitumor immunity. First, we demonstrated that the RFA did not increase the tumor infiltrating lymphocytes in secondary distant tumors of patients and in mice model and could not avoid relapse. Remarkably, RFA and in situ immunomodulation with GM-CSF-BCG hydrogel induced complete cure of microscopic secondary lesions in mice, related to a strong specific immune response. Then, we demonstrated that the immune escape of large secondary lesions was reversed by addition of the systemic PD-1 blockade to the in situ immunomodulation. The lack of an effective distant immune response in patients treated with RFA confirmed the relevance of this new combination strategy. Increasing the in situ priming response of radiofrequency ablation provides effective adjuvants to induce an abscopal effect. In the case of large lesions, synergy between PD1 blockade inhibitor, ineffective alone or after single RFA, with in situ immunomodulation, could lead to reconsideration of the use of checkpoint inhibition in metastatic MSS CRC.

ARTICLE HISTORY
Received 20 July 2018
Revised 5 November 2018
Accepted 9 November 2018

KEYWORDS
Colorectal carcinoma; radiofrequency ablation; local immunomodulation; tumor infiltrating lymphocytes; immune response; distant tumor; anti-PD1 blockade

Introduction

Surgical resection has proved to be the most effective and potentially curative for liver metastases. Because of the location and the extension of the lesion, a curative surgical treatment can however be performed in less than 30% of the patients.[1,2] Moreover, patients with large numbers of colorectal liver metastases (CRLMs) are potential candidates for resection, but the benefit from surgery is unclear.[3] Furthermore, half of the patients, with liver resection considered complete, develop intrahepatic recurrence suggesting the existence of non-detectable metastases. Radiofrequency ablation (RFA), daily used in clinical practice, represents an alternative intervention modality[4] to treat primary and metastatic hepatic tumors, when surgery is not feasible and leads to an improvement of overall survival and disease-free survival.[5] RFA induces hyperthermia in tumor tissue which increases the release, exposure or denaturation of tumor antigens. The pro-inflammatory effects of necrotic cells are well documented and appear related to the release of endogenous adjuvants essential for the activation of a cellular response.[6,7] However, RFA results are compromised by high rates of local and systemic relapse.[8,9] Several studies showed a T-cell specific response after RFA in patients with malignant liver tumors. Nevertheless, this response was not correlated with a clinical efficacy.[10,11]

Clinical trials using autologous cancer cells, expressing all tumor antigens, combined with Bacillus Calmette-Guerin (BCG) showed a potential benefit for patients with colorectal tumor.[12,13] To improve immunogenicity, autologous cells were genetically modified to secrete Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF), required on the tumor site to induce specific immunity.[14,15] Efficiency of adjuvants, GM-CSF or BCG, was related to the local recruitment and maturation of DCs which represents the critical step in cell-mediated antitumor response.[16,17] However, high concentrations of systemic GM-CSF can also result in immune suppression.[18] Considering these

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adverse effects, the in situ controlled release of GM-CSF has been proposed. Biomaterials, such as gels, were used to enhance vaccine efficacy, avoiding the side effects of systemic delivery, and preventing protein degradation.[19] Some gels, such as thermosensitive hydrogels are FDA approved. Upon temperature and concentration increase, they undergo a sol-gel transition phase.[20] Because of their low toxicity and biocompatibility, poloxamer-based gels have been selected in this study for *in situ* immunomodulation approach.

Immune checkpoint inhibitors, particularly inhibitor of programmed cell death protein 1 (anti-PD-1), provide favorable results in colorectal carcinoma patients with mismatch repair deficiency (MMR), which represents only 5 to 15% of cases. Shi *et al.* demonstrated that anti-PD1 improved RFA efficacy in mice colorectal tumors.[21] The lymphocytes $CD8^+$ T infiltrating tumors are the only the ones expressing PD-1 phenotype.[22] In addition, vaccination strategies have been shown to stimulate antigen-specific $CD8^+$ T cells in patients with tumors. However, these $CD8^+$ T cells remain hypo-responsive at the tumor site and cannot eradicate the tumor.[23] Therefore, it appears necessary to strongly prime and amplify this immune response in order to gain significant clinical benefits.

In this work, we propose to prime a strong anti-tumor immune response during RFA of murine colorectal tumors. For this purpose, we combined RFA with local stimulation using GM-CSF and BCG. The local delivery of the drugs was performed thanks to a thermo-sensitive hydrogel in order to optimize the bioavailability of the immunomodulatory combination. This combination strategy was carefully chosen to be directly applied in standard protocols of colorectal cancer treatment.

Results

RFA treatment alone did not increase TILs in patients' colorectal hepatic metastases

It has been demonstrated that RFA induces a systemic immune response in patients with colorectal carcinoma.[10] However, only few studies were interested in the immune environment outside the tumor zone ablation. In order to investigate modification of the immune infiltrate after RFA in patients with liver metastases from colorectal carcinoma, we performed a retrospective study. Thirteen patients who received initial hepatic RFA followed by distant liver tumor resection were included in the 'RFA (+)' group, whereas 40 paired patients who received liver tumor resection only were identified as 'RFA (-)' group. Patients were matched according to age, sex, number and size of metastases and tumor status (Table. S1).

We evaluated lymphoid cells density in distant liver metastases by automatic count, as described by Allard et al.[24] Our data showed low lymphocyte densities of CD3, CD4 and CD8 on the patient tumors (Figure 1(a)). Furthermore, lymphocyte infiltration analysis showed similar patterns for all markers in both groups; characterized by a small increase of TILs on the tumor front (Figure 1(b)).

There was no difference in the density of $CD3^+$ TILs liver metastases between RFA (+) group and RFA (-) group in each of tumor tissue, tumor front, and the surrounding non tumor tissue (Figure 1(c), Table S2). In addition, we observed no difference on $CD8^+$ TILs and $CD4^+$ TILs expression between both groups of patients (Figure 1(d), 1(e), Table S2). Finally, the expression of FoxP3 TILs was similar (data not shown).

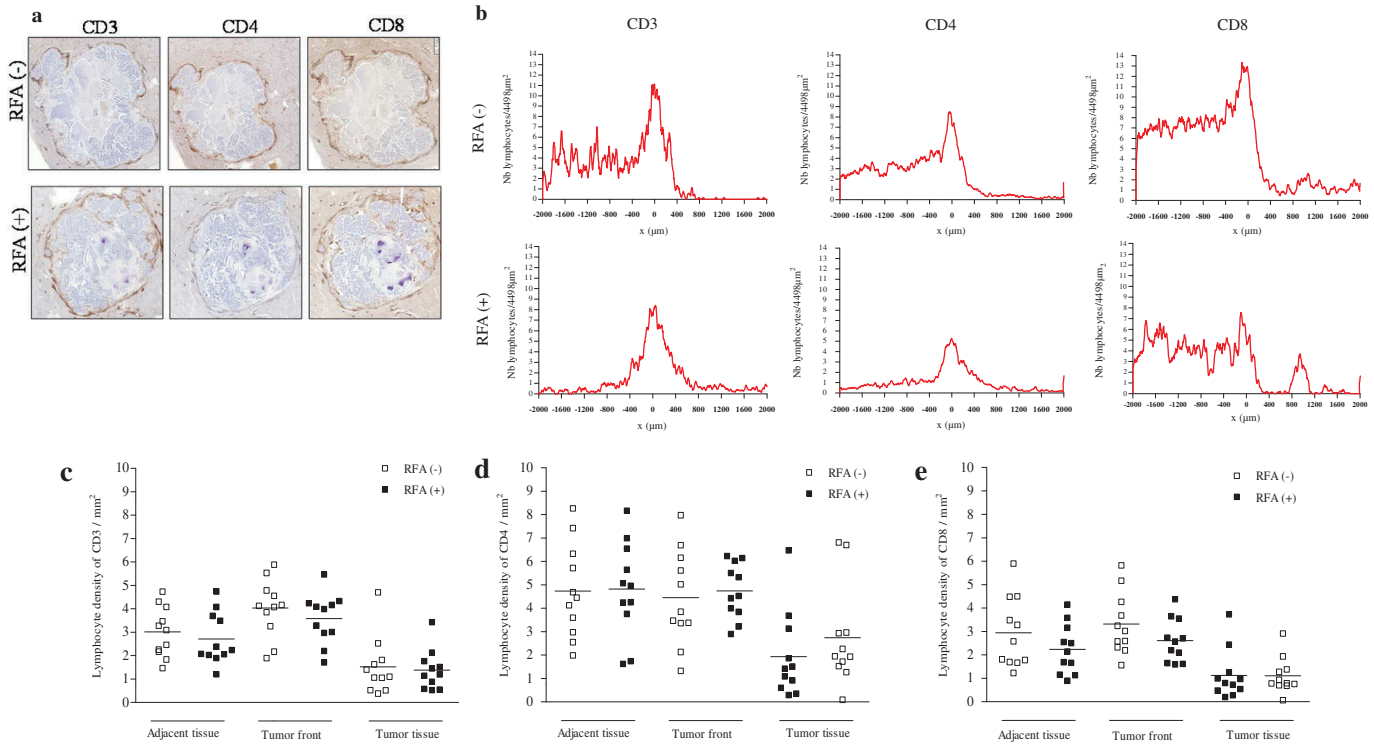


Figure 1. Tumor infiltrating lymphocytes (TILs) quantification in human liver metastases.

(a). Representative images showing CD3, CD4 and CD8 staining of patients from RFA (-) group and RFA (+) group. (b). Patterns of TILs CD3, CD4 and CD8 densities according to the distance from tumor invasive front. (c, d, e). Lymphocytes density of CD3, CD4, and CD8 T cells on tumor biopsies in adjacent tissue (negative distance), tumor front (0) and tumor tissue (positive distance).

These data suggest that RFA of liver metastases did not induce an increase of TILs count in distant lesions. The combination of RFA and local immunotherapy (BCG-GM-CSF) could improve anti-tumor immune response in liver metastases from colorectal cancer.

Combination of RFA and local immunostimulation resulted in complete cure of local and distant mice tumors in model of micro-metastases

The panel of the experiment is described on Figure 2(a). As no significant difference was observed between RFA and RFA + empty gel treatments, RFA + empty gel was chosen as a control group for all experiments. We found that GM-CSF-BCG loaded gel alone had a short effect on the local tumor whereas RFA induced a significant complete regression of the treated tumor (Figure 2(b)). The changes on distant tumor growth were visualized by bioluminescence imaging (Figure 2 (c)). RFA-empty gel treatment alone showed only a short inhibitory effect on distant tumor progression. RFA combined with GM-CSF and BCG liquid solution had a modest effect. By contrast, complete treatment (RFA+Gel-GM-CSF-BCG) resulted in a complete tumor regression at 15 days after treatment. Moreover, $CD8^+$ T cells depletion of mice treated with the complete combination completely abolished the control of tumor growth (Figure 2(d)), confirmed that the treatment effect was mediated by $CD8^+$ T cells. Mice survival was significantly improved with RFA with local complete combination as compared to all other groups (Figure 2(e)) with a survival of 50% of mice, 9 months after treatment.

Antitumor immune memory of cured mice

To evaluate the immunological memory of T lymphocytes, mice which showed a complete regression of distant tumors were re-challenged with CT26-Luc cells and a CT26-Luc fragment. As shown in Figure 2(f), no tumor growth was observed after tumor cells injection and rapid regression was observed after tumor fragment implantation. Our results show that RFA combined with local immunostimulatory hydrogel induced a strong antitumor immunity able to eliminate residual tumors and control tumor recurrences.

Combination treatment provides an increase in infiltrating T cells in the distant tumors.

Given the capacity of RFA combined to GM-CSF-BCG loaded gel to inhibit tumor growth, we sought to determine the intratumoral infiltration of $CD8^+$ cytotoxic T lymphocytes, $CD4^+$ T helper lymphocytes, $FoxP3^+$ regulator T lymphocytes and CD45R B lymphocytes The distant tumors were harvested at day 17 after treatment and TILs were evaluated by immunohistochemistry as described in material and methods. We observed a significant increase of $CD3^+$, $CD4^+$ and $CD8^+$ T cells infiltration in distant tumors with RFA and local immunostimulation. The level of FoxP3 expression was also enhanced (Figure 3(a, b)). However, B lymphocytes infiltration on distant tumors was similar in all groups (Fig. S1). These results indicate that treatment of the local tumor with RFA combined with Gel-GM-CSF-BCG induce a sustained immune response in the distant tumor, mediated by effector T cells.

Intratumoral Gel-GM-CSF-BCG administration increased systemic specific T cell immune responses induced by RFA

To determine the impact of local tumor immunomodulation on the systemic response, we analyzed $CD4^+$ and $CD8^+$ T cells populations from the spleen by flow cytometry. No differences were found between the percentage of cells in untreated, RFA and complete treatment groups (data not shown).

In order to study the function of peripheral T lymphocytes, we analyzed the ability of T cells from the spleen to produce IFN-γ and TNF-α. PMA/ionomycine stimulation, used as positive control, showed an increase of TNF-α and IFN-γ expression in all groups, confirming the features of T lymphocytes. We did not observe differences of both cytokines expression by $CD4^+$ and $CD8^+$ T cells between RFA group and the control. By contrast, the expression of IFN-γ and TNF-α by T cells was increased by a factor of 4 with the complete combination therapy (Figure 4(a)).

Cytokines expression within $CD4^+$ and $CD8^+$ T cells were significantly higher in the complete treatment group after stimulation with heated CT26-Luc (Figure 4(b)), which demonstrates the specificity of the antitumor immune response.

Association of RFA and local immunotherapy with anti-PD1 resulted in a synergistic antitumor effect in a model of distant macro-metastasis.

The efficacy of PD1 blockade therapy is related to a pre-existing T cell immune response.[25,26] As we shown in the previous experiments an increased T cells mediated immune response with the combined treatment, we investigated the addition of an anti-PD1 treatment to RFA-local immunomodulation to treat macro-metastases. For this purpose, we developed a model of large lesions and evaluated this association.

We observed a greater effect of RFA and Gel-GM-CSF-BCG combination compared to RFA therapy alone. The systemic administration of anti-PD1 enhanced RFA + Gel-GM-CSF-BCG effect on distant tumor and led to a complete regression of distant tumor on the majority of mice (Figure 5 (b)). The complete treatment had also a great effect on the survival of mice with 33% of mice survival after 4 months (Figure 5(c))

Effect of complete combination and anti-PD1 on lymphoid and myeloid cells infiltrating the tumor

To assess the involvement of immune cells in the different group of mice, we analyzed intratumoral TILs (defined as $CD3^+$ $CD4^+$ $CD8^+$) and MDSCs (defined as $Ly6\text{-}Gr1^+CD11b^+$ cells) on $CD45^+$ cells in the distant tumors of mice, 13 days post treatment. We observed a 2 fold to a 20-fold increase of the $CD45^+$ immune cells in the distant tumor of the complete treated mice as compared to untreated, RFA +anti-PD1, RFA and RFA-Gel-GM-CSF-BCG groups (data not shown). MDSCs analysis demonstrated a significant decrease of MDSCs $Ly6\text{-}Gr1^+$ $CD11b^+$ cells infiltrating tumors from complete treatment group mice as compared to the other groups (Figure 6(a)). There was no difference in macrophages F4/80 cells and dendritic cells $CD11c^+$ $CD80^+$ $CD86^+$ (Fig. S2). Inversely, T cell infiltration was characterized by a significant increase of $CD8^+$ T cells proportion, while the proportion of $CD4^+$ T cells was similar (Figure 6(b)).

Complete combination therapy and PD-1 checkpoint inhibitor enhanced systemic specific immune response

We next assessed the composition and the activation status of lymphocytes in the spleen to explore whether systemic

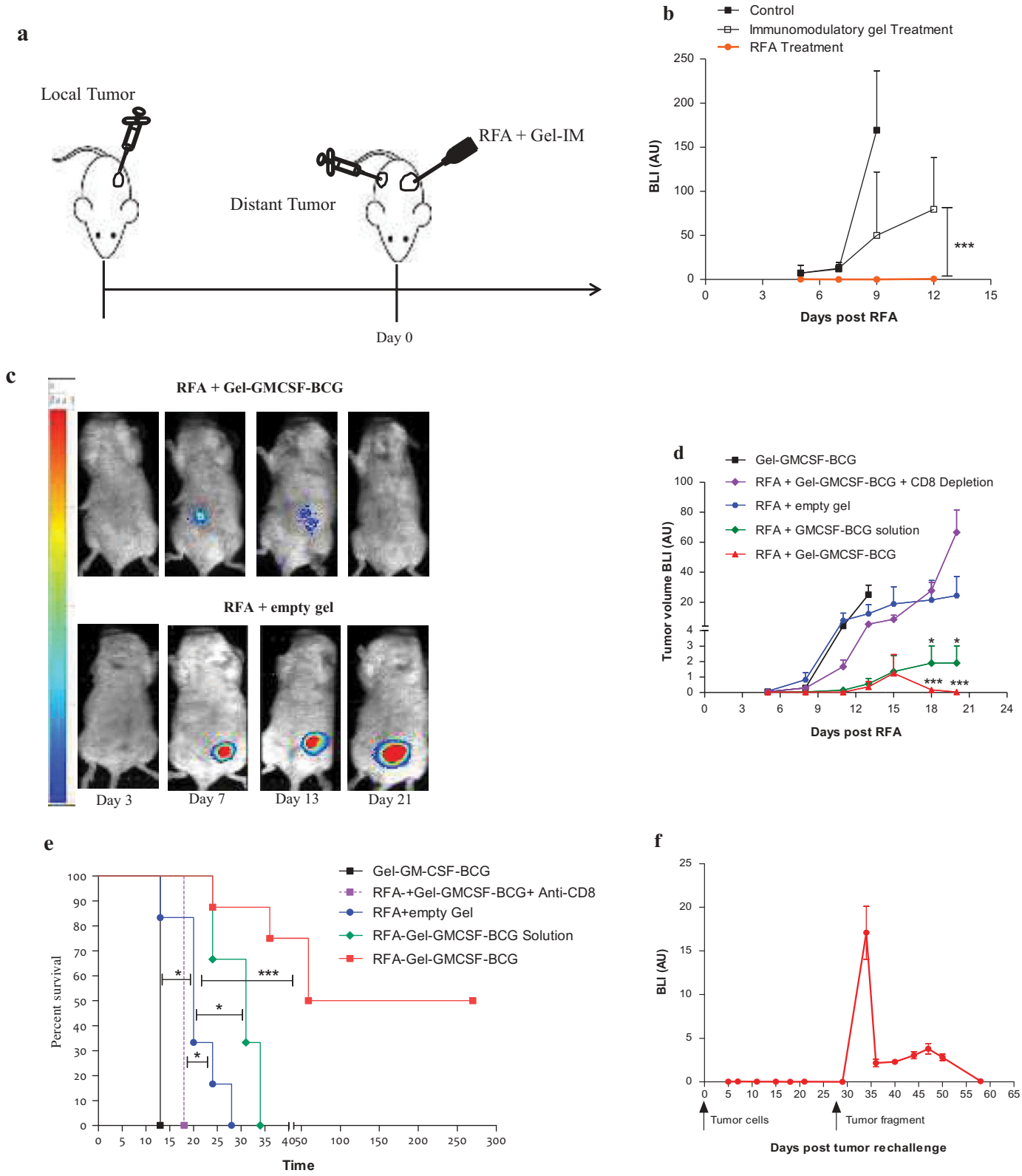


Figure 2. RFA and Gel-GM-CSF-BCG combination therapy improve antitumor effect of RFA in adjuvant model and maintain immunity against tumor re-challenge. (a). Panel treatment: Balb/c mice were implanted subcutaneously with CT26-Luc tumor fragment on the right flank, when the tumor reached about 300 mm^3, RFA was applied with respected parameters (Temperature above 60°C, 2 cycles time) and the hydrogel containing GM-CSF and BCG (Gel-IM) was injected in the treated tumor by the same route. The distant tumor was implanted (25000 CT26-Luc cells) on the opposite flank of the mice. (b). Effect of RFA on the local treated tumor. The tumor volume was measured with bioluminescence imaging every three days after RFA. Mice were injected by i.p. route with 2 mg of Luciferin and signal acquisition was performed with an iCDD camera during 10 minutes, (n = 10 per group). (c). Representation of the distant tumor growth with Bioluminescence imaging on day 3, 7, 13 and 21 after treatment. (d). Monitoring of distant tumor growth with bioluminescence (n = 10). Mice were injected by i.p. route with 2 mg of Luciferin and signal acquisition was performed with iCDD camera during 10 minutes (n = 10 per group). A two-way repeated measure ANOVA with Bonferroni posttest was performed. The results were represented with arbitrary unit. (e). Kaplan–Meier survival curves are shown. Long-rank test was performed, *** $P < 0{,}001$. All groups were compared to control (RFA+empty gel). (f). Memory antitumor response. Mice of RFA +Gel-GM-CSF-BCG group which presented a complete regression of distant tumor were re-challenged successively with tumor cells at day 0 and tumor fragments at day 29. Tumor volume was measured by bioluminescence every 3 days (n = 4). Error bars = SD, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

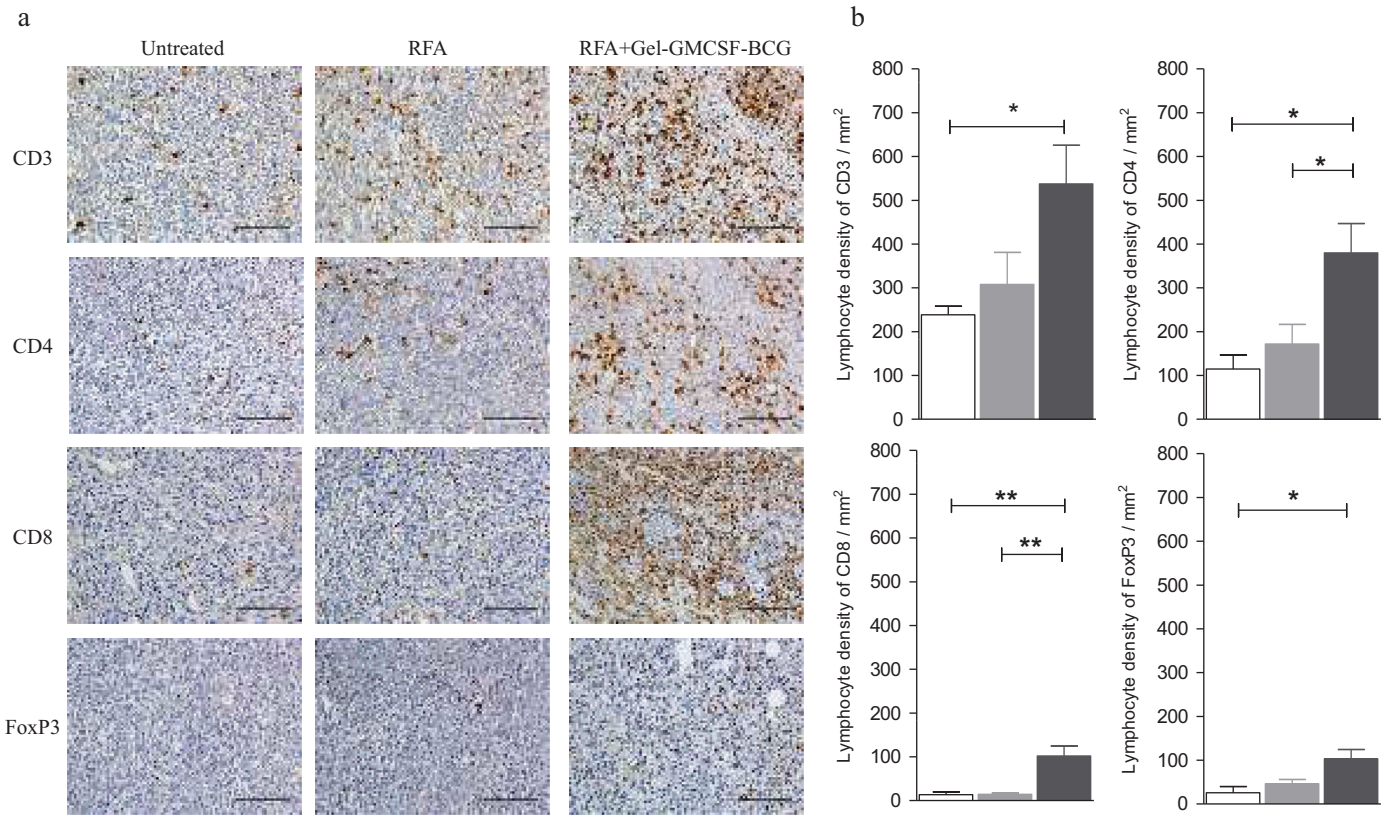


Figure 3. IHC analysis demonstrated an increase of TILs with complete combination therapy.

Tumors from Untreated, RFA and RFA + Gel-GM-CSF-BCG groups were removed 17 days and fixed for further analysis (n = 5). IHC's staining CD3, CD4, CD8, FoxP3 were performed. (a). Representative images of Tumor-infiltrating lymphocytes in the different groups. Scale bar 50 µm. (b). Quantification of lymphocytes density on the tumor using macro image J as described on supplementary materials (S3, S4, S5, and S6). White bar represents the untreated group, grey bar, the RFA treated group and the black column the group which received the complete treatment. One way Anova with Bonferroni Comparison Test was performed, *P < 0.05, **P < 0.01.

tumor antigen specific T cells had been induced. We detected a significant increase of TNF-α producing $CD4^+$ and $CD8^+$ T cells and a significant increase of IFN-γ producing $CD8^+$ T cells in the complete treatment group compared with the untreated group and the RFA +anti-PD1 group (Figure 7).

These data suggest that the *in situ* immunomodulation with the thermosensitive hydrogel containing GM-CSF-BCG boosted RFA- anti-PD1 antitumor response and induced adaptive specific $CD8^+$ T cell immune response.

Discussion

In this study, we demonstrated that RFA alone does not increase Tumor infiltrating lymphocytes on distant tumor in both mice model and patients with liver metastases from colorectal carcinoma. It has been established that RFA enhanced specific T cells response in patients with malignant liver tumors. Indeed, hyperthermia increases the release of tumor antigens[27] and the induction of pro-inflammatory effect triggering the recruitment and maturation of DCs. These pro-inflammatory effects appear related to the release of essential endogenous adjuvants, such as B1 nucleoproteins, hsp70 and gp96 heat shock proteins required for the activation of an effective antitumor immunity.[28]

Some analyses have shown that RFA is specifically associated with a systemic immune response and lymphocyte infiltration in the treated tumor.[29] However, systemic effects induced by RFA are controverted[30] and the abscopal effects might depend upon activation of antitumor immune response.

In our retrospective study of patients, we were interested on the analysis of TILs within distant hepatic metastasis. Patients who received RFA followed by resection were matched with patients who received resection alone. We used an automatic lymphocyte counting method to study lymphoid infiltration on the surrounding non tumor tissue, the tumor front and the tumor tissue of complete patient's biopsies. We showed that RFA did not increase T CD4 and T CD8 infiltration in the tumor microenvironment of liver distant lesions, explaining the hepatic and extrahepatic relapse observed (lung metastases, peritoneal carcinomatosis). These results confirmed the necessity of enhancing the priming of T-cell immune response for an efficient immunotherapy in metastatic colorectal carcinoma.

To enhance immune response after RFA, we have chosen two adjuvants, BCG and GM-CSF. BCG vaccine is widely used in the treatment of bladder carcinoma.[31] Live BCG vaccine has also been associated with irradiated tumor cells in immunotherapy trials for colon cancer.[32] BCG induces a non-specific immune response after binding to TLR2/TLR4 receptors. This link induces activation of complex signaling pathways which leads to the

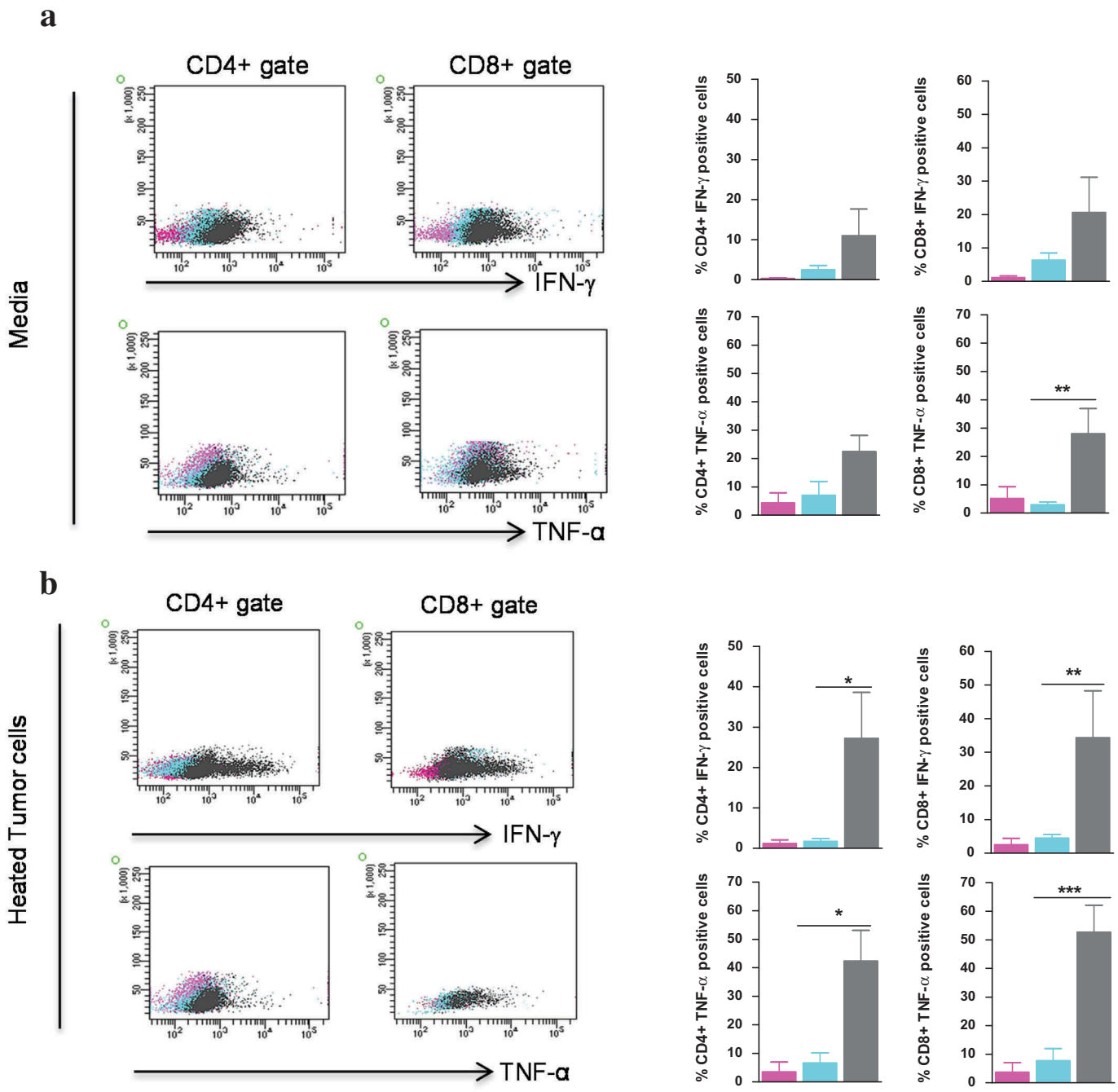


Figure 4. Induction of specific T cells response after RFA+ Gel-GM-CSF-BCG treatment.

At day 17 after treatment, spleen from Untreated (pink color), RFA (blue color) and RFA + Gel-GM-CSF-BCG (grey color) groups were removed (n = 5). The organs were dissociated and splenocytes were collected in fresh media. Flow cytometry analysis of IFN-γ and TNF-α secreted by T $CD4^+$ and T $CD8^+$ was performed. (a). IFN-γ and TNF-α positive cells analysis on fresh non stimulated splenocytes. (b). 2.10^4 CT26-Luc cells were incubated at 46°C for 1 hour and co-culture with 2.10^5 fresh splenocytes. IFN-γ and TNF-α expressed by stimulated splenocytes was analyzed by flow cytometry. One way Anova with Bonferroni's Comparison Test. *P < 0.05, **P < 0.01, ***P < 0.001.

expression of inflammatory cytokines, TNF-α, IL-12, and up-regulates MHC class I molecules, playing a crucial role in the maturation of DCs.[33] GM-CSF is an important chemokine for anti-tumor immune response regulation involved in the activation of both innate and adaptive immunity. The cytoplasmic domains of GM-CSF receptor are associated with the phosphorylation of Janus kinase 2 (JAK2) and regulates differentiation and maturation of macrophages.[34] Nevertheless, through in situ interactions in the tumor micro-environment, GM-CSF was described as a potent tumor-promoting factor, increasing tumor cell growth in multiple cancer types.[35,36] RFA provided all tumour antigens like thermally inactivated autologous whole cells and reduces the risks of tumor escape with GM-CSF.[37]

The advantage of a continuous release of GM-CSF has previously been shown, particularly in increasing to increase protein half-life and reducing the immunosuppressive effects

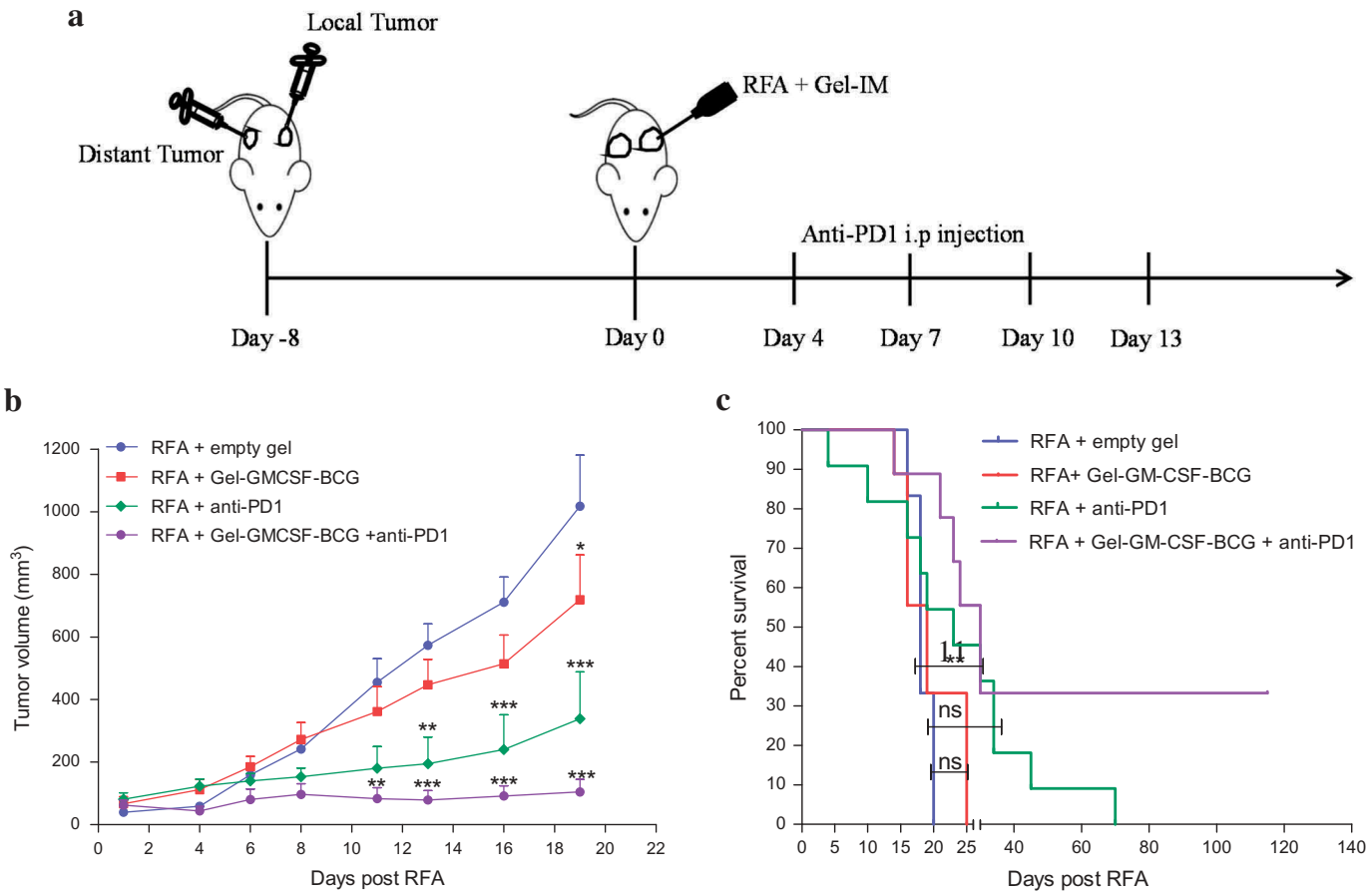


Figure 5. RFA and Gel-GM-CSF-BCG combination therapy improve PD1 response in macro metastases model.

(a) Panel treatment. Balb/c mice were implanted subcutaneously with CT26-Luc tumor fragment on both flanks. The local tumor was treated with RFA and the hydrogel containing GM-CSF and BCG was injected by the same route. Anti-PD1 was injected 4 times after treatment at days 4, 7, 10 and 12. (b) Tumor volume of distant tumor was measured every 3 days with calliper and the volume was calculated according to the formula (Width*Width*Length/2). n = 10 per group. A two-way repeated measure ANOVA with Bonferroni posttest was performed. (c) Kaplan–Meier survival curves are shown in this graph; long-rank test was performed. All groups were compared to control (RFA+empty gel) ns: non-significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

observed at high doses. Here, we propose to control the intra-tumoral release of GM-CSF with the use of bio-adhesive thermosensitive hydrogel based on a previous physico-chemical study. The prepared solution is compatible with biomolecules and gelify at physiological temperature, allowing a larger surface of interaction and a prolonged effect. The formulation has been optimized for a prolonged release of GM-CSF over several days to allow the induction of an effective immune response (unpublished data).

Immunocompetent mice were grafted subcutaneously with local and distant tumors. The local lesion was treated with RFA associated with in situ immunotherapy to induce an immune response according two different protocols, distant microscopic and distant macroscopic tumors. This design simulated two very common clinical situations explaining the evolution of distant occult or unresected lesions at the time of RFA treatment. In the adjuvant protocol targeting distant micrometastases, RFA combined with Gel-GM-CSF-BCG resulted in a complete response in mice, while the local administration of the immunomodulatory gel alone or RFA alone was ineffective. The abscopal effect resulted in a regression of distant lesions mediated by cytotoxic T cells response and increase animal survival. In human cancer, involvement of tumor infiltration of T cells to obtain an antitumor immune response is considered to be predictive factors, associated with long-term survival of patients.[24,38] In our strategy, RFA and local combination therapy showed a strong increase of TILs in the distant tumors.

In some cancers, it has been shown an increase of Th1/Th2 ratio induced by B cells infiltrating tumors.[39] Indeed, CD20 + B cells within the tumor microenvironment represent important and clinically relevant markers.[40,41] However, the role of CD20 + B cells in CRC remains unclear.[42] It was suggested that a lower density of CD20 + B cell infiltration correlated with poor clinical outcomes in the primary CRC[39]A high density of CD20 + B cells was therefore significantly correlated with an improved of the overall survival of patients.[43] In our strategy, RFA and local combination therapy did not induce an increase of B cells infiltrating the distant tumors.

The complete response after tumor rechallenge confirmed the activation of a strong immune response. In this model, the cells effectors were not altered by the immunosuppressive microenvironment. The tumor infiltration was associated to

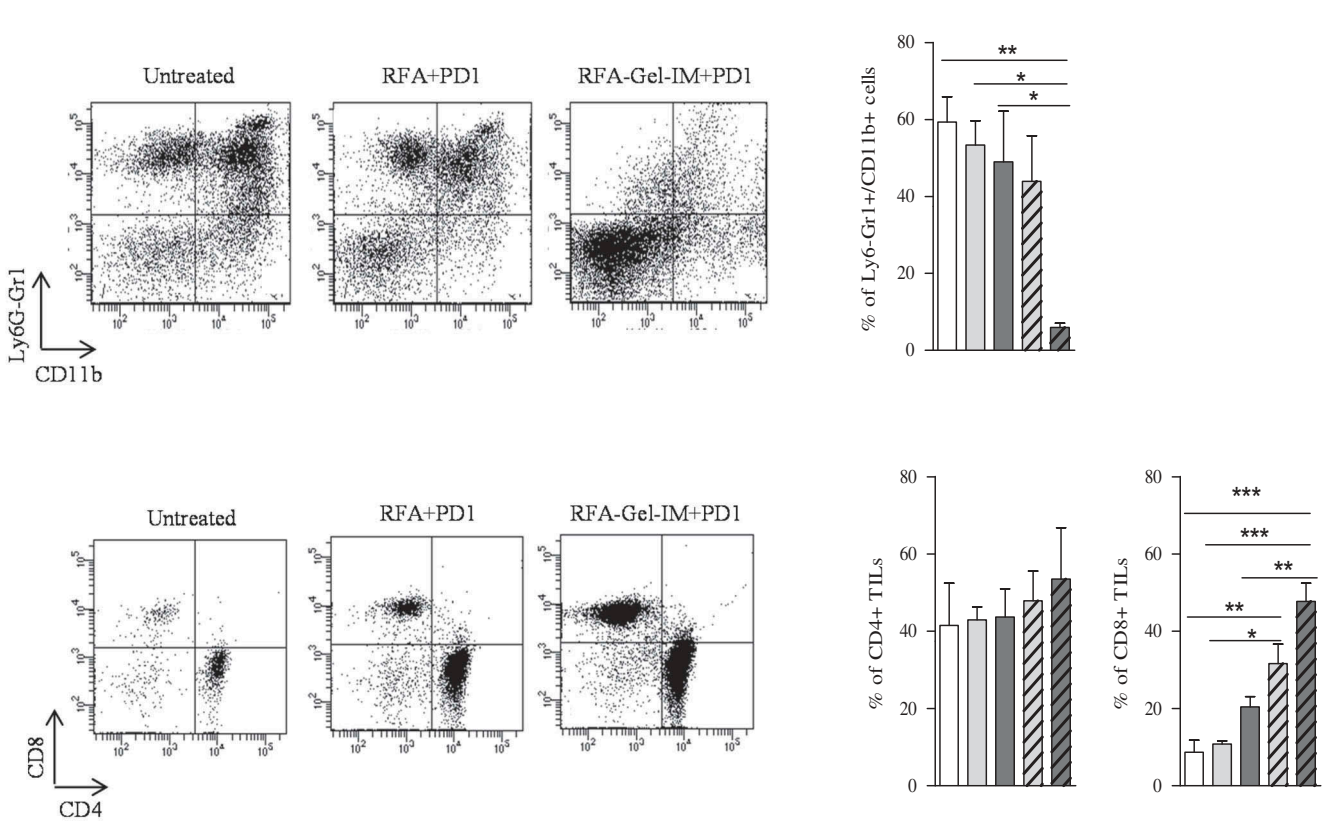


Figure 6. Complete treatment of primary tumors increased cytotoxic T lymphocytes and decreased MDSC in distant tumors.

Mice were treated as described in Figure 5. At day 13 after treatment, secondary tumors (n = 5/group) from untreated (white bars), RFA (light grey bars), RFA – Gel-GM-CSF-BCG (dark grey), RFA + anti-PD1 (slashed light grey bars), and RFA-Gel-GM-CSF-BCG + anti-PD1 (slashed dark grey bars) groups were removed and hematopoietic cells were extracted as described in Materials and Method. MDSC and T lymphocytes were analyzed with anti CD11b and Ly6-G, anti CD3, CD4 and CD8 antibodies, antibodies respectively. Gel-GMCSF-BCG was replaced by 'Gel-IM' in these figures. One way Anova with Bonferroni's Comparison Test. *P < 0.05, **P < 0.01, ***P < 0.001.

the activation of peripheral lymphocytes with increase of IFN-γ and TNF-α synthesis inducing a Th1 immune response. This process might be an important mechanism underlying the synergistic effect of these combination therapies.

However, RFA and local immunomodulation increased TILs and controlled the distant tumor in adjuvant situation only. The combination strategy was not sufficient to treat macro metastases, possibly due to the exhaustion of TILs and the overexpression of PD-1 phenotype. Monoclonal antibodies targeting the immune checkpoints, PD1 particularly provided a clinical efficacy in gastrointestinal cancers with MMR. These tumors had more mutations producing neo-antigens, recognized and targeted by anti-tumor immune response. However, in microsatellite stable (MMS) colorectal cancers, checkpoint blockade did not demonstrate any effect suggesting that the efficacy of anti-PD1 is associated with pre-existing antitumor immunity. Radiotherapy and checkpoint inhibitors combined have been shown to synergistically enhance antitumor immunity in preclinical studies.[44] Moreover, Shi *et al.* have shown that the association of RFA and anti-PD1 is efficient in the treatment of metastatic colorectal cancer in mice.[21] Therefore, for the treatment of residual macro-disease, we proposed the association of local therapy (RFA +Gel-GM-CSF-BCG) with systemic anti-PD1 injection. We demonstrated a synergy between the local combination treatment and PD1 checkpoint inhibitor. Indeed, this association inhibited growth of distant tumor, enhanced T cells infiltration and decreased MDSCs infiltration on distant tumors.

MDSCs have been functionally defined by their ability to suppress T cells in tumor-bearing mice, as well as cancer patients and then play an important role in tumor evasion of immunosurveillance.[45] Recent reports have suggested that the survival, differentiation, and suppressive activity of MDSCs are influenced by TLR signaling.[46] As MDSCs express TLRs and accumulate in cancer, they, (as well as DCs), appear to be primary targets of TLR ligands when administered into tumor-bearing hosts. Activation of TLRs by administration of an appropriate ligand leads to loss of the suppressive activity of MDSCs, resulting in the inhibition of tumor growth by restoring anti-tumor T cell responses.[47] Granulocyte-macrophage colony promotes the proliferation and differentiation of bone marrow stromal cells into MDSCs by activating nuclear factor kappa B (NF-κB) and Janus kinase/signal transducers and activators of transcription (JAK/STAT) signal pathway.[48]

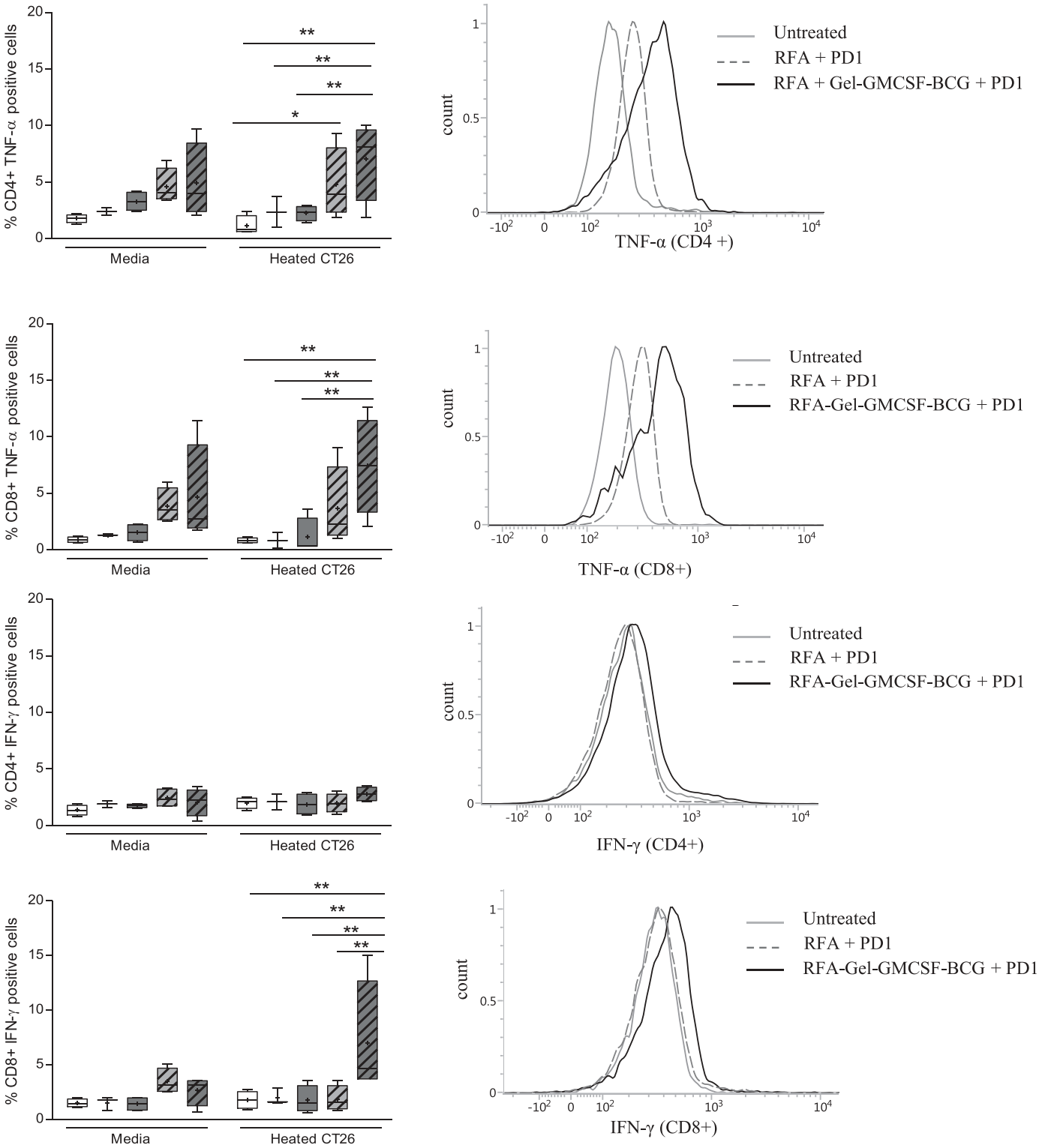


Figure 7. The combination of RFA gel GM-CSF BCG with anti-PD1 enhanced tumor specific T cell response.

Mice were treated as described in Figure 5. At day 13 after treatment, spleen from all groups (n = 5/group) were removed and treated as described in Figure 4. Flow cytometry analysis of IFN-γ and TNF-α expressed by $CD4^+$ and T $CD8^+$ T lymphocytes were performed. (a) Quantification of IFN-γ and TNF-α secreted by unstimulated and stimulated splenocytes with heated CT26 tumor cells from untreated (white box), RFA(light grey box), RFA- Gel-GM-CSF-BCG (dark grey box), RFA + anti-PD1 (light grey hatched box), RFA -Gel-GM-CSF-BCG + anti-PD1 (dark grey hatched box) groups. Anova test was performed. (b). Overlays representation of IFN-γ and TNF-α cytokines expressed on stimulated splenocytes by heated CT26 from untreated, RFA + anti PD1 and RFA-Gel-GM-CSF-BCG + anti-PD1 groups. Two way Anova with Bonfonni's post-test. Error bars: SD, Cross: Mean, Horizontal bar: median * $P < 0.5$, ** $P < 0.01$

However, this activation is related to GM-CSF administrated dose. In our study, we demonstrated that the control of GM-CSF release with the hydrogel avoids MDSCs stimulation and promotes T effectors activation.

In human CCR, several studies have shown that lymphocytic infiltration was a good prognostic factor,[38,49] even in the colorectal liver metastasis. We have shown an increase of T cells infiltration on distant tumor with complete treatment.

Moreover, specific T cell response was activated as demonstrated by the increase of IFN-γ and TNF-α synthesis in lymphocytes from spleen after co-culture with heated tumor cells. Interestingly, RFA and PD1 combination were less effective. In fact, the in-situ induction of a strong T cells mediated immune response leaded to the sensibility of the macroscopic distant tumor to PD1 antibody. Both $CD8^+$ T cells and IFN-γ expression are critical for anti-tumor immunity.[50] IFN-γ secreted by immune cells in the tumor microenvironment induces growth arrest, MHC class I expression, contributes to the recruitment of effector cells, causes T-reg fragility and coordinates the process of innate and adaptive antitumor response.[51] Th1 cells promotes durable tumor specific CTL responses, are particularly important for activated T Cells maturation[52] and induction of strong immunological memory against tumor rechallenge. In addition, IFN-γ signature is associated with better survival for pembrolizumab treated melanoma patients.[53] Otherwise, TNF-α cytokine is produced by immune cells and has a capacity to suppress tumor cell proliferation and induce tumor regression. TNF-α is also a potent anti-tumor cytokine which enhances the activity of macrophages, NK cells and cytotoxic T cells.[54] It has been shown that in situ vaccination using IL-12 and TNF-α in microsphere generates a systemic anti-tumor immune response capable of eradicating distant metastasis.[55]

Here, we demonstrated that RFA associated with local immunomodulation increased specific T cells immune response and induced a complete tumor regression on adjuvant model.

BCG is currently used to treat superficial bladder cancer.[56] The injection of BCG in the liver treated RFA zone could induce adverse effects. We should consider another TLR agonist to replace BCG in the immunomodulatory gel. The microenvironment is different according to the tumor implantation site.[57] It could be interesting to evaluate RFA associated with local immunomodulation on a liver metastases mice model.

Our data provided a strong rational for a clinical assay evaluating the combination of RFA with in situ immunotherapy and PDL1/PD1 blockade therapy in patients with metastatic colorectal carcinoma, regardless of micro-satellites stability. The predictive biomarker for response to the proposed immunotherapy should be analyzed on patients. After the liver treatment, this strategy could allow the control of a distant residual macroscopic disease on liver, lung, lymph nodes metastasis or untreated primitive lesion in reverse strategy.

Materials and methods

Study patient

In the digestive surgery department of Ambroise Paré hospital, between 2002 and 2012, 250 patients had a liver metastases resection for colorectal carcinomas. We retrospectively studied cancer tissue specimen from matched 53 patients. Among them 13 patients who received initial hepatic RFA followed by local tumor resection were included in the 'RFA (+)' group whereas other paired 40 patients who received initial local tumor resection were identified as 'RFA (-)' group. One patient from 'RFA (+)' group was coupled with 3 or 4 patients from 'RFA (-)' group according to the criteria described in Table. S1. Paraffin-embedded samples were obtained from the Pathology Department and the tissue bank of Ambroise Paré Hospital, which has been registered with the French Ministry of Research (# DC 2009–933). Immunohistochemistry was performed with a Bond autostainer (Leica, Biosystems Newcastle Ltd) as described by Allard MA *et al.*[28] The primary antibodies were mouse monoclonal anti-CD3 (1/50 dilution, rabbit polyclonal, Dakocytomation.), (anti-CD4 (1/30 dilution, clone 4 B12, Novocastra); anti-CD8 (1/25 dilution, clone C8/144B, Dakocytomation) and anti-FoxP3 (1:100 dilution; 623801, Biolegend, France) antibodies. Virtual tumor slides were obtained by scanning with Mirax Desk (Zeiss, Germany). Images were analyzed with Visilog 9.0 software (Noesis, Saclay, France); the area of quantification included both tumor tissue and the surrounding non tumor tissue.

Patients included in this retrospective study provided informed consent for translational research. This study was conducted in accordance with the Declaration of Helsinki and with local ethical rules.

Mice

Six to 8 week old female BALB/c mice were obtained from Janvier laboratories (Le Genest de l'ile, France). Studies were conducted following the recommendations of the European Convention for the protection of vertebrates Animals use for Experimentation and the local Ethic Committee on Animal care and Experimentation (APAFIS #11352).

Local and distant tumors graft

The CT26 colon adenocarcinoma cell line was purchased from American Type Culture Collection (ATCC, CRL-2638, LGC Standards, Molsheim, France). The CT26-Luc cell line was generated by transfection of wt-CT26 cell line with luciferase gene as reporter and cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco Life Technologies) containing 10 % fetal bovine serum (FBS, Gibco Life Technologies), 100 µM of streptomycin, 100 U/mL of penicillin and 0,4 mg/ml of Geneticin (G418 sulfate, Gibco Life Technologies at 37°C in a 5 % CO2-humidified atmosphere. A subcutaneous CT26-Luc tumor was resected, placed into sterile Phosphate buffer (Dulbecco's phosphate buffer, Sigma), cut into fragments of 30 mm^3 and inserted subcutaneously using a 12-gauge trocar (38 mm) into the mouse flank, as local tumor. The mice were treated in 2 separate protocols. Protocol I was designed as a model of a clinical adjuvant situation with distant tumors established by $2.5.10^4$ CT26-Luc cells injected into the other flank of mice, forming a microscopic tumor, at the time of the RFA. In the protocol II, the local and distant tumors were simultaneously grafted in the opposite flanks, as synchronous macroscopic distant lesions at the time of RFA.

Treatments and RFA procedure

Three weeks earlier, the mice were vaccinated by subcutaneous injection of BCG (BCG SSI, Sanofi Pasteur, France). Treatments were initiated when the tumor volume reached

about 500 mm^3. Indeed, animals were anesthetized by i.p injection of Ketamin (100 mg/ml) and Xylazin (10 mg/ml). The ablation was performed using a radiofrequency probe (Cool-tip, Covidien, USA) inserted into the center of the tumor. The probe was removed when temperature reached 60°C within the tumor to ensure complete ablation of the target lesion.

Anti-PD-1 (200 µg, clone: J43, BioXCell) was administered through i.p. injection to mice every 3 days for a total of four times. $CD8^+$ T cell depletion was realized by *i.p*. injection of 250 µg of anti-CD8 (clone 2.43; Bio-XCell) four times every 3 days, starting from 1 day before RFA.

Hydrogel injection

A 21 % solution of poloxamer 407 (Kolliphor, BASF Germany) containing 0.1 % of Satiaxane UCX930 mucoadherent gum (Kolliphor, BASF Germany) was prepared with deionized water. The solution was kept at 4°C until use. Concentrations of the components are expressed as weight/volume percentage (% w/v). The physicochemical properties of the hydrogel have been defined (unpublished data). 60 µl of hydrogel containing 5 µg of recombinant GM-CSF (granulocyte macrophage colony stimulating factor, Miltenyi Biotec, France) and 5×10^5 CFU of BCG (Sanofi Pasteur, France) was injected in the treated tumor zone using a 23-gauge needle, five minutes after RFA. In all experiments, tumors were measured with a digital caliper every 3 days. Tumor volumes were calculated in cubic millimeter using the following formula: length x width x width/2. Data shown are mean ±SD.

Tumor growth monitoring by optical imaging

Luciferin potassium salt (D-luciferin, K+ salt Fluoprobes, Interchim) diluted in PBS was injected through *i.p* route at 2 mg per mouse which is in large excess relative to the luciferase amount. Optical imaging was performed with a cooled intensified charge-coupled device (CCDi) camera (Biospace, PhotonImager Paris, France). Luminescence acquisition was initiated 20 min after the injection of the substrate with duration of 10 min. The luminescence level was evaluated by an ROI applied to the tumor zone (software M3 Vision+ from Biospace Mesure, Paris, France). The results are expressed according to the equation:

BLI (AU) = ROI value of tumor (ph/s/sr/cm2)/ROI value of control (ph/s/sr/cm2). Where ROI is an international LED positive control.

Flow cytometry analysis

To study tumor infiltrating lymphocytes (TILs), tumors were cut into small pieces, incubated in tumor dissociation kit mouse (Miltenyi Biotec, France) and then dissociated using the Gentlemacs dissociator (Miltenyi Biotec, France). The cell suspension was filtered through a cell mesh of 70 µm and resuspended in Phosphate buffer with 0.5 % BSA (Bovin Serum Albumin, ID Bio, France) for further analysis. All antibodies were purchased from BD Bioscience. TILs were analyzed with rat anti-mouse CD45 V500 (clone 30-F11), rat anti-mouse PE-Cy7 CD3 (clone 17A2), rat anti-mouse APC CD4 (clone RM4-5), rat anti-mouse APC-Cy7 CD8 (clone 53–6.7).

Tumor infiltration by dendritic cells, macrophages and myeloid-derived suppressor cells (MDSC) was analyzed with rat anti-mouse PE CD11c (clone HL3), and rat anti-mouse APC CD86 (clone GL1), rat anti-mouse BV421 F4/80 (clone T45-2342), rat anti-mouse Alexa fluor488 CD11b (clone M1/70), rat anti-mouse APC-H7 Ly-6G (clone 1A8).

Analysis of spleen lymphocytes was also performed. The spleens were dissociated in a potter and filtered through a cell mesh 70 µm. The red blood cells were lysed with PharmLyse (559759, BD Bioscience) and cells were suspended in RPMI media 1640 (11875093, Invitrogen) containing 10 % of FBS. Freshly spleen cells were incubated at 2.10^5 cells per well plates in presence of heated CT26 cells (10 µg/mL), medium, or PMA (10 ng/mL), Ionomycin (1 µg/mL) and Brefeldin A (10 µg/mL) overnight at 37°C. The intracellular cytokines were analyzed as described by BD Bioscience protocol. Briefly, cells were stained with rat anti-mouse CD45 V500 (clone 30-F11), rat anti-mouse CD3 PE-Cy7 (clone 17A2), rat anti-mouse CD4 APC (clone RM4-5), rat anti-mouse CD8 APC-Cy7 (clone 53–6.7), fixed and permeabilized with Fixation/Permeabilization Solution Kit (554714, BD Bioscience) and stained with rat anti-mouse Alexa Fluor 488 IFN-γ (clone XMG1.2), rat anti-mouse Alexa Fluor 488 TNF-α (clone MP6-XT22) and rat anti-mouse Alexa Fluor 488 IL-2 (clone JES6-5H4).Flow cytometry analysis was performed using a flow cytometer (FACS CANTO II, Becton Dickinson, France).

Immunohistochemical staining

Freshly collected tumors were placed in Zinc fixative (0.5 g Calcium Acetate, 5.0 g Zinc Acetate, 5.0 g Zinc Chloride, 0.1 M Tris buffer, pH 7.4) 24 hours at room temperature. After fixation, dehydrate tissues were embedded in paraffin and sections (4 µm) were stained with hematoxylin eosin and safran. Paraffin sections were processed for heat-induced antigen retrieval, incubated with rabbit anti-mouse CD3 antibody (Dako) and rabbit anti-mouse Fox P3 antibody (Abcam, 1:1000). Staining was visualized by using the peroxidase/diaminobenzidine Rabbit PowerVision kit (ImmunoVision Technologies). For CD8 immunohistochemistry, paraffin sections were incubated with a rat monoclonal anti-CD8 antibody (1:100) (Neomarkers; LabVision). The membrane signal was revealed with the Polink 2 plus HRP detection kit (GBI Labs).

All slides were immunostained in cover plates the same day, guaranteeing a perfectly standardized intensity of staining. Each slide was examined using a microscope. A single representative whole tumor tissue section from each animal was digitized using a slide scanner. Five tumors/group were analyzed. For one tumor, the lymphocyte density was quantified on 10 images extracted from the virtual slice at x20 magnification, with the help of image J software.[58] The lymphocyte density was quantified with image J as described in SI1

Statistical analysis

Graph Pad Software was used to analyze data and determine statistical significance between groups. Data are shown as the means ±SD. Mann-Whitney test was used to compare the difference between two groups. Anova test with Bonferroni posttest was used for multiple comparisons P values < 0.05 were considered significant.

Acknowledgments

We would like to thank G. Carvalho from Milteny Biotec for her help on tumor dissociation protocol and Dr Mostafa El Hajjam for providing BCG vaccine.

We thank the Animal Platform, Faculty of Pharmacy, and Paris Descartes University, France.

Disclosure of Potential Conflicts of Interest

Authors declare that they have no conflict of interests.

Funding

This work was supported by grants from the 'Ligue contre le cancer', the association of research in digestive oncology (A.R.O.L.D) and the SATT IDF Innov

Author contributions

RM, JFE conceived the study. NM, VB, KL conceived the bioadhesive hydrogel. KL, JS, RM developed the mice model and performed in vivo experiments. KL, CC, JS did and analyzed the experiments. RM, JFE, FP, KL and CC participated to the patient's study. OB did immunohistochemistry experiments. KL, NM, RM, CC and JFE wrote the manuscript.

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