

Peer Review File

An in situ depot for the sustained release of a TLR7/8 agonist
in combination with a TGF β inhibitor promotes anti-tumor
immune responses



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REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

In this manuscript, the authors reported a CarboCell technology to form a sustained drug release depot in the tumor tissue and release immune stimulatory drugs for tumor immunotherapy. The authors provided profound evidences to prove this CarboCell TLR:TGFb technology is quite effective in inhibiting tumor growth, elicit system immune responses and prevent tumor recurrence. The authors also did a preclinical evaluation on a canine model. Overall, I think this is a meaningful study, which confirmed the necessity of prolonged release in eliciting efficient immune responses, and provided a potent new solution for cancer immunotherapy.

I have the following concerns for this manuscript.

1. Local drug release depot could also be formed by injectable hydrogel. Please comment and compare on the differences between these two technologies.
2. More details and information of the gel materials should be supplemented in Fig.1, including:
 - a, the mechanism of self-assembly and gelation;
 - b, the mechanism and period of degradation in vivo;
 - c, according to the Table S1, there are totally six formulations of CC. While different formulations were used in different experiments, the screening and optimization of formulations remained unclear. The functions and applications of different formulations need more introductions, and the imaging agents or components with special functions in the formulations should be introduced more clearly.
3. Fig.1D-E, the authors showed an ultralow concentration of TLR7/8a in the blood while much higher in the tumor for CarboCell TLR compared to other formulations. The question is how was the TLR7/8a metabolized? It is expected that a local depot would reduce the diffusion rate of small molecules to the blood, but a constant lower concentration was observed across one week compared to i.t. and i.v. injection.
4. Cytokine analysis (e.g. IL6, IL-1 β) in blood would help to confirm local depot leads to lower systemic inflammation compared to i.t. or i.v. injection.
5. A more comprehensive evaluation on the intratumor immune cells (e.g. CD4+ T Cells, CD8+ T cells, TAMs, MDSCs) would help to elucidate the exact changes in the tumor immune

microenvironment. In Fig.2H, only CD8+ T cells/Tregs were provided; in Fig.3E, only CD8+ T cell immune staining was provided; in Fig.3K, only TAMs were provided.

6. An analysis on the intrasplenic memory T cells would help in explaining the tumor rechallenge results.

7. Please provide the gating strategies and representative graphs for all the flow cytometry analysis in the supporting information.

8. In Fig. S3E-G, CarboCell TLR:TGFb with an extended release profile (CC-ER) was used for a single intratumoral injection to demonstrate the potential to decrease the total number and to increase the intervals between doses. But the results seem to lack a control group using original CarboCell TLR:TGFb (CC) for the same single injection, which is important to explain the advantage and necessity of CC-ER material.

9. In Fig. 4H-I, "CPX" may be used as the abbreviation of cyclophosphamide, but actually "CTX" is the more common abbreviation.

Reviewer #2 (Remarks to the Author):

Manuscript by Jensel et al describes a novel CarboCell technology for sustained intratumoral release of a TLR agonist + TGFb inhibitor. Studies were performed in multiple mouse tumor models and also in a canine model. These studies provide strong evidence for the efficacy this approach. Extensive characterization of local immune response and for generation systemic antitumor immunity has been performed. Some concerns include the lack of detailed mechanistic analysis utilizing knockout models to establish the role of specific immune subsets in generation of anticancer therapeutic effect and the relationship between the combination therapy and the observed therapeutic benefits. Further, the authors have used different controls in different experiments but there is no one experiment in which all the requisite controls are used.

Reviewer #3 (Remarks to the Author):

This is an interesting manuscript that describes the effect of using an intratumoral depot, CarboCell, to deliver a TLR agonist and TGFbeta inhibitor to the tumor microenvironment. The studies are well controlled and described, and indicate a clear positive effect in murine

models. A very large amount of data is presented, with significant components relegated to the supplemental materials due to the volume. Most of the figures are clear, easy to understand, and illustrate the appropriate experiments and results. Likewise, with the exception of few typographical errors, the text is well organized and generally easy to read and understand.

The introduction and Discussion would generally benefit from being more specific to this particular manuscript instead of broadly touting the perceived benefits of CarboCell, many of which are not tested in this manuscript.

The inclusion of canine studies seems disconnected from the rest of the manuscript. The lack of any figures related to these experiments in the manuscript (some are provided as a supplemental table and figure) leads to the question of why these studies were included. In addition, the section in which these experiments were described is titled “CarboCell improves the therapeutic performance of checkpoint inhibition and is tolerated in translational canine models”, yet two thirds or more of this section deals with murine models. Finally, no graphical, tabular, or image data is presented regarding the responses of the cancer-bearing dogs that were injected with CarboCell.

The manuscript states that CarboCell allows “individually tailored release profiles” – It is unclear how the release profiles are modified (or tailored) for individual components that are co-injected. It appears that all of the compounds in one formulation are subject to the same release profile.

Fig 2, CC-TGFb was not used alone as a control, thus it is not clear if the effect is synergistic when combined with CC-TLR or simply the effect of TGFbi. This does appear to be addressed in later studies, but would be reassuring to see addressed here as well.

Discussion: “Notwithstanding these efforts, no technology has yet successfully progressed into a clinical product.” – I would argue that anti-PD-1 antibody therapy is a clinical product.

Discussion: typo, “makescontinuous” needs a space added.

Discussion: "...CarboCell TLR:TGFb both in the tdLNs,.." – The use of the word both implies two subordinate clauses, but the sentence has three.

No description of methods or animal use approvals were provided for the canine work in the main manuscript, either with normal dogs for toxicity or in tumor-bearing dogs. The description provided in the supplement did not provide the number of dogs in each toxicity assessment group.

RESPONSE TO REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

In this manuscript, the authors reported a CarboCell technology to form a sustained drug release depot in the tumor tissue and release immune stimulatory drugs for tumor immunotherapy. The authors provided profound evidences to prove this CarboCell TLR: TGFb technology is quite effective in inhibiting tumor growth, elicit system immune responses and prevent tumor recurrence. The authors also did a preclinical evaluation on a canine model. Overall, I think this is a meaningful study, which confirmed the necessity of prolonged release in eliciting efficient immune responses, and provided a potent new solution for cancer immunotherapy.

I have the following concerns for this manuscript.

1. Local drug release depot could also be formed by injectable hydrogel. Please comment and compare on the differences between these two technologies.

Answer 1: We agree with the reviewer that hydrogel is an important comparator to CarboCell. Hydrogels are being used and translated into the clinic e.g. by Ascendis (<https://ascendispharma.us>) and SurgeTx (<https://surgetx.com>) for delivery of TLR agonists. However, CarboCell and hydrogels differ enormously in chemical composition and in the molecular interactions that govern drug release and depot integrity. Hydrogels comprise water and are held together by covalent bonds and crosslinks of the polymeric network, whereas CarboCell is non-aqueous and is held together by van der Waals interactions. As a consequence, i) CarboCell protects the embedded drug from hydrolysis which hydrogels do not, ii) CarboCell can release TLR agonists and TGFb inhibitors (and drugs in general) in their native form, whereas polymer prodrug conjugates of the drug is required to obtain sustained release (>days) from hydrogels, iii) CarboCell release is governed by controlled diffusion of the drug inside the depot whereas hydrogels incorporating prodrugs rely on linker hydrolysis, iv) CarboCell release rate can be modified by simple compositional changes whereas linker chemistry and chemical synthesis of prodrug libraries is required for hydrogels. Hydrogels may also be used for release unmodified small molecule drugs, but if not chemically linked, rapid and burst release is a likely outcome with risk of systemic spill-over causing toxicities.

To accommodate the reviewers request we have elaborated on this in the revised introduction:

“To date, successful administration of TLR7/8a is limited [27], but sustained intratumoral delivery technologies based on e.g. hydrogels are being explored [Park et al.. Sci Transl Med. 2018 Mar 21;10(433), Zúñiga et al. Cancer Cell International 2022 22:286]. Hydrogels, however, may suffer from rapid release of small molecules unless chemically linked to the hydrogel polymer, and sustained co-delivery of multiple agents with optimal dosing is inherently challenging.”

We have further elaborated on pros and cons of CarboCell and hydrogels in the revised discussion:

“TLR7/8 loaded hydrogels are being advanced both clinically and preclinically for intratumoral injection [Park et al.. Sci Transl Med. 2018 Mar 21;10(433), Zúñiga et al. Cancer Cell International 2022 22:286]. Hydrogels, however, display rapid release of small molecules or necessitate polymer-drug conjugates with increased chemical complexity, resulting in higher production cost. Whereas the integrity of hydrogels is governed by polymer-polymer and polymer-water interactions, CarboCell is non-polymeric and self-coalescent due to its hydrophobic nature. Hydrogels in addition contain water, posing the risk of premature drug

hydrolysis and imposing constraints on shelf-life. CarboCell is a non-aqueous liquid and has a completely scalable production setup which underlines the value of the current system. “

2. More details and information of the gel materials should be supplemented in Fig.1, including:

- a, the mechanism of self-assembly and gelation;
- b, the mechanism and period of degradation in vivo.

c, according to the Table S1, there are totally six formulations of CC. While different formulations were used in different experiments, the screening and optimization of formulations remained unclear. The functions and applications of different formulations need more introductions, and the imaging agents or components with special functions in the formulations should be introduced more clearly.

Answer 2a: The mechanism upon which the semi-solid CarboCell depot is formed is governed by Non-solvent Induced Phase Separation (NIPS). In this process, the solvent (ethanol), which is soluble in both CarboCell and water, leaches from the injected depot into the surrounding tissue and interstitial fluids forming the viscous CarboCell at the site of injection.

To clarify this, we have rephrased the section:

“CarboCell is a liquid composition comprising mixtures of a carbohydrate ester, a triglyceride, a polymer additive, and ethanol as solvent (Fig. 1B). Prior to administration, the CarboCell liquid is 700-800-fold more viscous than water and is injectable using standard procedures (Table S1, Fig. 1A). After administration, the semi-solid CarboCell depot is formed at the site of injection caused by efflux of the solvent. In this transition, the viscosity of CarboCell increases and it is immobilized in the tissue, from where high doses of a single or multiple drug(s) may be released. Several compositions were included in the optimization of the CarboCell TLR7/8a:TGFβ delivery system, which are abbreviated CC and sequentially described in Table S1. ~~In this transition, the semi-solid CarboCell is immobilized in the tissue, where high doses of a single or multiple drug(s) may be released.~~”

Answer 2b: The excipients of CarboCell are chemically linked by ester bonds and can be degraded by hydrolysis to disaccharides (sucrose or lactose), small fatty acids (benzoate, isobutyrate, acetate), glycerol and medium chain (C8 or C6) aliphatic fatty acids, which are all biocompatible. The physical elimination period for CarboCell in vivo depends on the injected volume. In current efficacy studies, 4x50uL depots were injected, with the outcome that most CarboCells were reduced in size, and some could not be recovered after 100 days.

We have elaborated on this point in the introduction:

“The carbohydrate esters and triglyceride excipients of CarboCell are biodegradable by hydrolysis and degradants are well-tolerated disaccharides and low molecular organic acids.”

Answer 2c: We appreciate that the reviewer’s interest in the formulation details and have updated table S1 so that excipients are listed in a consistent order, Carbohydrate esters (LAP, LOIB, SuBen) : triglycerides (GTH,GTO) : solvents (EtOH,PC) : Polymers (PLA,PLGA). The two final compositions are highlighted in grey whereas the development candidates are in white. A “rationale of design” column has been added to the table S1 highlighting the most important specification that led to the given composition. We believe this will increase the ability of the reader to follow differences between individual compositions.

General principles for how drug release rates can be tailored is described in the manuscript

“A reduced release rate was obtained by lowering the triglyceride and polymer content (Table S1).”. It is further mentioned that the drug release and synchronization of release rate is governed by the hydrophobicity (logP) of the API in *“Here, the TLR7/8a Resiquimod (R848, logP = 1.7, Fig. 1B) and the TGFβi RepSox (logP = 2.5, Fig. 1B) were co-formulated in CarboCell, as both compounds are lipophilic with comparable logP, rendering these drugs optimal for synchronized dual release.”* We believe these statements in combination with the newly introduced “rationale of design” column in table S1 effectively capture the core of how CarboCell API release can be tuned.

The intrinsic imaging properties and X-ray contrast of CarboCell (MR, US and CT) have been described in a previous publication by the authors (Hansen et al, Science Adv 2020) and has thus not been extensively discussed in the current manuscript.

However, in order to clarify this point and guide the reader to the previous publication regarding CarboCells image properties we have rephrased the sentence

“In a previous publication, CarboCell was demonstrated to be intrinsically visible by ultrasound, magnetic resonance imaging, as well as by computed tomography (CT) upon co-formulation of iodine rich carbohydrate esters (Fig. S1D-E)³¹”

Revised Table S1 and legend

Reference name	CarboCell composition	Rationale of design	Viscosity [cP] 25°C/37°C
CC	SuBen:GTO:EtOH:PLA (60:25:15:0.5 %w/w)	Final composition based on SuBen with optimized R848 stability improving shelf-life and storage conditions compared to CC-4.	790 / 270
CC-ER	SuBen:GTO:EtOH (63:17:20 %w/w)	Final composition with extended release (ER) compared to CC having reduced GTO and PLA content.	700 / 230
CC-1	LAB:xSAIB:GTO:EtOH:PC:PLGA (63:5:15:5:10:2 %w/w)	Development candidate containing xSAIB providing CT contrast.	
CC-2	LAP:GTO:EtOH:PC (70:15:5:10 % w/w)	Development candidate with equivalent carbohydrate ester:triglyceride ratio as CC-1.	
CC-3	LAP:GTH:PC (55:40:5 %w/w)	Development candidate with increased triglyceride content (GTH) for release of the more hydrophobic TGFβ inhibitor SD-208.	
CC-4	LOIB:GTO:EtOH (82.5:7.5:10)	Lead candidate based on LOIB with optimized compositional stability.	

Table S1. CarboCell reference name, composition, rationale of design and viscosity.
CC: CarboCell, ER: extended release.

3. Fig.1D-E, the authors showed an ultralow concentration of TLR7/8a in the blood while much higher in the tumor for CarboCell TLR compared to other formulations. The question is how was the TLR7/8a metabolized? It is expected that a local depot would reduce the

diffusion rate of small molecules to the blood, but a constant lower concentration was observed across one week compared to i.t. and i.v. injection.

Answer 3: Thank you for the question, we believe that the observation in the tumor and blood is consistent the CarboCell serving as a sustained release depot. Here the systemic spill-over is lowered for CarboCell and is constantly declining ~10-fold throughout the experiment.

Regarding the tumor, one important detail is that the tumor measurement reports the total R848 content in the tumor tissue as well as the injected depot as these cannot be separated experimentally. According to our interpretation, we see a gradual transfer of R848 from the CarboCell compartment to the tumor tissue compartment during the first 72h and hence little change in the sum of these compartment is seen. After 1 week, the CarboCell is depleted and the total R848 signal (CarboCell + tumor tissue compartment) is reduced as R848 is transferred to other compartments. Transfer of R848 to other compartment may be through systemic spill-over, uptake in cell populations and migration of these or biological degradation of R848. The current data however do not allow for exact interpretation/prediction of how R848 was lost from the tumor compartment and is considered outside the scope of the current experiment, which aimed to investigate initial systemic spill and tumor+gel retention. Still, the overall conclusion remains that CarboCell lowers systemic spill-over and extends the duration and concentration of R848 in the tumor.

To accommodate the reviewer's comments and questions we have clarified the manuscript to emphasize that the R848 signal from the tumor reports the combined CarboCell + tumor tissue compartment activity.

We have changed the result section (inserted text in bold):

~~*"The low systemic spill-over of ^3H -R848 from intratumoral CarboCell TLR was clearly demonstrated by the high intratumoral activity throughout the seven day evaluation period (Fig. 1e). The total activity within the depot and tumor tissue is represented by the intratumoral CarboCell ^3H -R848, as depicted in Figure 1e. This, combined with the sustained release from CarboCell, supports the assertion that ^3H -R848 offers continuous activity within the tumor tissue, accompanied by a low and decreasing systemic spill-over."*~~

*In comparison, less than 19% of the intratumorally injected free ^3H -R848 could be detected in the tumor after 30 minutes, and less than 1% were found at all time points in intravenously injected mice (**Fig. 1e**)."*

And in Fig 1. text legend subfigure e (inserted text in bold):

*"e. ~~tumor retention~~ **intratumoral activity** of R848 administered intratumorally (i.t.), intravenously (i.v.), or intratumorally via CarboCell"*

4. Cytokine analysis (e.g. IL6, IL-1 β) in blood would help to confirm local depot leads to lower systemic inflammation compared to i.t. or i.v. injection.

Answer 4: Thank you for your suggestion to evaluate cytokine profiles such as IL-6 and IL-1 β , which would indeed offer additional insight into the systemic inflammatory response following different R848 administration routes. We agree that cytokine analysis is a valuable tool for distinguishing the systemic impact of local versus systemic drug delivery.

Our initial submission focused on pharmacokinetics and biodistribution (PK/BD) utilizing tritium-labeled R848 (3H-R848). The data demonstrated low systemic spill-over from the intratumorally administered CarboCell TLR, suggesting its potential to form stable intratumoral depots and maintain localized drug activity within the tumor microenvironment (TME). This was further verified using radiocontrast material to confirm depot formation via μ CT imaging, as detailed in our manuscript and illustrated in Supplementary Figures S1A-C and S2A-E. While we recognize the value of cytokine profiling, our current research scope and resource allocation have been directed toward the PK/BD analysis, which we believe provides a strong indication of the therapeutic's localized effect, and we strongly believe that the findings presented here, is representative of increased safety and lower systemic spill-over.

5. A more comprehensive evaluation on the intratumor immune cells (e.g. CD4+ T Cells, CD8+ T cells, TAMs, MDSCs) would help to elucidate the exact changes in the tumor immune microenvironment. In Fig.2H, only CD8+ T cells/Tregs were provided; in Fig.3E, only CD8+ T cell immune staining was provided; in Fig.3K, only TAMs were provided.

Answer 5: Thank you for your insightful comments regarding the evaluation of intratumoral immune cells following treatment with our CarboCell technology. Your feedback has provided us an opportunity to elaborate on the comprehensive immune profiling conducted in our study, particularly concerning the role of T cells in mediating the therapeutic efficacy of CarboCell TLR:TGF β . In addition to the previously mentioned immune analyses, we conducted further studies in both immunocompetent and immunodeficient BALB/c mice to specifically understand the contribution of T cells in our treatment approach. In these studies, CT26 tumors were established in both BALB/c (immunocompetent) and BALB/c-nu (immunodeficient, lacking T cells) mice. The mice were then treated with intratumoral CarboCell TLR:TGF β , with untreated mice serving as controls. Our findings show the difference in tumor control between immunocompetent and immunodeficient mice. While the CarboCell-treated BALB/c-nu mice exhibited some delay in tumor growth compared to untreated controls, it was only in the immunocompetent BALB/c mice that we observed substantial tumor control over the evaluated period. This result emphasizes the necessity of T cells for full therapeutic efficacy and adaptive activation following CarboCell TLR:TGF β . It underscores the synergy between innate and adaptive immune responses, particularly highlighting the role of CD8+ T cells in mediating anticancer immune responses following TLR7/8 agonist treatment. This additional study not only reinforces the importance of the T cell component in the tumor immune microenvironment but also aligns with the aim of our manuscript to demonstrate the benefits of a localized, slow-release platform for immunotherapy. The ability of CarboCell TLR:TGF β to effectively modulate T cell responses

in the TME is central to its therapeutic advantage, as evidenced by our comprehensive analysis across different immune landscapes. Considering your feedback, we have included these findings in our revised manuscript, providing a more detailed depiction of the critical role of T cells in the therapeutic success of our CarboCell platform.

The following has been added to the manuscript and figure 2 has been revised:

Methods:

Female mice were obtained at 5 – 7 weeks of age (BALB/c, **BALB/c-nu (BALB/cAnN-Foxn1nu/nu/Rj)** or C57BL/6 from Janvier and Charles River).

Results:

“To investigate the therapeutic significance of CD8+ T cells in the context of CarboCell TLR:TGFβ treatment, we conducted a comparative efficacy study between immunocompetent BALB/c mice and immunodeficient BALB/c-nu mice, which are devoid of T cells. CT26 tumors were established in both mouse strains, followed by two biweekly intratumoral administrations of CarboCell TLR:TGFβ (Fig. 2I). In immunodeficient BALB/c-nu mice treated with CarboCell, a delay in tumor progression was noted when compared to their untreated controls. Conversely, the immunocompetent BALB/c mice exhibited sustained tumor suppression over study period (Fig. 2J). These findings underscore the critical role of T cells in mediating the antitumor efficacy of CarboCell TLR:TGFβ intratumoral therapy.”

6. An analysis on the intrasplenic memory T cells would help in explaining the tumor rechallenge results.

Answer 6: We appreciate your suggestion to analyze intrasplenic memory T cells to further elucidate the tumor rechallenge results presented in our study. In response to your comment, we would like to provide additional insights into our investigations of immunological memory formation.

In our efforts to characterize the antigen-specific T cell response following treatment with CarboCell TLR:TGFβ, we conducted studies to assess the presence of AH-1 antigen-specific T cells in the spleen post-tumor rejection. While we observed an increase in these antigen-specific T cells, the change did not reach statistical significance. We attribute this finding to the contraction phase of the immune response, which typically ensues following the clearance of antigenic stimuli, such as tumor eradication. During this phase, there is a reduction in the number of effector T cells as the immune system reverts to a state of homeostasis, and consequently, the remaining population of antigen-specific memory T cells constitutes only a small fraction of the total T cell pool. This biological process presents inherent challenges for detection and analysis by flow cytometry due to the reduced frequency of these cells long after the tumor has been cleared.

Despite the absence of statistical significance in the increase of splenic AH-1 specific T cells, the functional capacity of these cells to mediate tumor rejection was convincingly demonstrated through our adoptive transfer experiments (Fig. 4I-J). The complete responder rate of 100% in recipient mice receiving these splenocytes starkly contrasts with

the rates observed in mice receiving splenocytes from naïve or CT26 tumor-bearing donors, providing compelling evidence of the durable anticancer immunological memory induced by our treatment.

This functional demonstration of immunological memory underscores the efficacy of CarboCell TLR:TGFb in generating a sustained immune response capable of eliminating established tumors, even though the quantifiable presence of antigen-specific T cells in the spleen may be limited due to the natural dynamics of the immune response.

To further support that anticancer T-cells are generated in the CarboCell TLR:TGFb injected tumor and that these traffic to the contralateral (un-injected) tumor we performed T cell receptor repertoire analysis on tumor tissue using immune sequencing of TCR β CDR3. Encouragingly, we observed that CarboCell injected and un-injected tumors on displayed an average nucleotide sequence overlap of 731 sequences. In contrast, in the untreated tumors, an average overlap of 41 nucleotide sequences between the two tumors. Despite the high overlap between tumors on individual mice we observed no correlation in shared sequences between injected or un-injected tumors between different mice.

The following has been added to the manuscript and figure 4 has been revised:

Results

“To explore anti-cancer T-cell responses in CarboCell TLR:TGFb-treated and untreated tumors, TCR β CDR3 immune sequencing was conducted on whole tumor tissue from mice with bilateral CT26 tumors. Encouragingly, despite no increase in overall TCR clone count (suppl. Fig S8 (Venn diagrams), the treated tumor and contralateral tumors showed sequence overlap of 731, compared to only 41 sequences for controls (Fig 4e). Notably, while individual CarboCell TLR:TGFb-treated mice displayed high overlap, minimal overlap was observed between mice as illustrated by the Morisita index map (Fig 4f).”

METHODS

TCR β CDR3 immune sequencing

“Mice bearing bilateral CT26 tumors (established as above) received three unilateral CarboCell TLR:TGFb injections. Tumors were harvested one week after CarboCell treatment (31 days after inoculation). Untreated tumors were harvested 17 days after inoculation. Genomic DNA was extracted and purified from tumors using the DNeasy Blood & Tissue Kit (QIAGEN) according to the manufacturer's instructions. RNA-free genomic DNA was obtained by adding 4 μ l RNase A (100 mg/ml, QIAGEN). Immunosequencing of TCR β CDR3 regions was performed using the survey ImmunoSeq Assay (Adaptive Biotechnologies, Seattle, WA). ”

Statistics

“Sequencing data were analyzed using immunoSEQ ANALYZER 3.0 (Adaptive Biotechnologies).”

Discussion

“The TCR sequence overlap between the injected and uninjected tumors in the same mouse is an important observation. This suggests a systemic immune response induced by the local immunotherapy. The high degree of overlap implies that T cells in the treated tumor are also recognizing and responding to the untreated tumor following circulation of activated T cells.”

The following has been added to the legend of the revised figure 4

“E. TCR β CDR3 nucleotide sequence overlap between intratumoral TCR β CarboCell injected tumors and uninjected tumors from mouse bilateral tumor model. F. Morisita overlap index of intratumor TCR β in CarboCell injected (R) and contralateral uninjected tumor (L) from CarboCell CarboCell TLR:TGF β treated mice (n=4, M01-M04).”

7. Please provide the gating strategies and representative graphs for all the flow cytometry analysis in the supporting information.

Answer 7: Please find the gating strategy for flow cytometry data as described below:

- Figure 2- G: Gating strategy found in supplementary figure S14.
- Figure 2- H: Gating strategy found in supplementary figure S15.

Both references in provided in figure legend, have now also been added to main text, to make it more clear to the reader.

- Figure 3- J: Gating strategy found in supplementary figure S16
- Figure 3- k: Gating strategy found in supplementary figure S17

Both references in provided in figure legend, have now also been added to main text, to make it more clear to the reader.

- Figure 4- D: Gating strategy found in supplementary figure S18
- Figure 3- G: Gating strategy found in supplementary figure S20

Both references in provided in figure legend, have now also been added to main text, to make it more clear to the reader.

8. In Fig. S3E-G, CarboCell TLR:TGF β with an extended release profile (CC-ER) was used for a single intratumoral injection to demonstrate the potential to decrease the total number and to increase the intervals between doses. But the results seem to lack a control group using original CarboCell TLR:TGF β (CC) for the same single injection, which is important to explain the advantage and necessity of CC-ER material.

Answer 8: Thank you for your insightful comment. In response to your comment, we have updated Fig. S3E-G with results from a revised efficacy study that includes the requested control group. The updated data demonstrate that both the extended-release formulation (CC-ER) and the original CarboCell TLR:TGF β (CC) achieved significant therapeutic outcomes, with 50-75% complete response rates after a single injection in this tumor model. Despite

the similar efficacy observed between the two formulations, we maintain that the development of a slow-releasing variant (CC-ER) capable of delivering a higher dose of R848 over an extended period, without diminishing therapeutic effectiveness, is a crucial advancement for enhancing clinical applicability and patient compliance. Figure S3 in the revised supplementary material has been revised accordingly.

The following has been added to the manuscript:

“Therefore, the influence of release rate on therapeutic efficacy was investigated by comparing the CarboCell TLR:TGFb standard formulation with a CarboCell TLR:TGFb extended drug release composition with double drug concentration in CT26 tumor-bearing mice (Fig. S3e). For both formulations, a single intratumoral injection of CarboCell TLR:TGFb resulted in complete responses in the majority (50-75%) of the treated animals (Fig. S3f-g). Thus, increasing the dose and reducing the burst effect can achieve comparable treatment outcomes, potentially allowing for fewer and less frequent doses, enhancing clinical relevance.”

9. In Fig. 4H-I, “CPX” may be used as the abbreviation of cyclophosphamide, but actually “CTX” is the more common abbreviation.

We appreciate this comment and have accordingly changed this in fig 4 H-J and legend.

Reviewer #2 (Remarks to the Author):

Manuscript by Jensel et al describes a novel CarboCell technology for sustained intratumoral release of a TLR agonist + TGFb inhibitor. Studies were performed in multiple mouse tumor models and also in a canine model. These studies provide strong evidence for the efficacy this approach. Extensive characterization of local immune response and for generation systemic antitumor immunity has been performed. Some concerns include the lack of detailed mechanistic analysis utilizing knockout models to establish the role of specific immune subsets in generation of anticancer therapeutic effect and the relationship between the combination therapy and the observed therapeutic benefits. Further, the authors have used different controls in different experiments but there is no one experiment in which all the requisite controls are used.

We value the highly positive and constructive feedback received. In response, we have supplemented the manuscript with additional data demonstrating the dependence on generation of anticancer T cells. We have included the nude mouse study in Fig. 2I-J to show the crucial role of T cells in maintaining the therapeutic response. To support that anticancer T-cells are generated in the CarboCell TLR:TGFb injected tumor and that these traffic to the contralateral (un-injected) tumor we performed T cell receptor repertoire analysis on tumor tissue using immune sequencing of TCRβ CDR3.

We do completely accept the importance of including appropriate controls. We are obligated to strictly adhere to national legislation and studies are evaluated by the institutional board to assure that the number of animals included are reduced to what is scientifically and statistically justified. In the case of CarboCell TGFbi monotherapy this was

demonstrated to provide no effect in supplementary Fig S5. Based on this observation we believe that repeated evaluation was not justified.

To further bring attention to importance of Fig S5 for stating the synergistic activity we have therefore changed the text to now read:

“In connection to this observation no therapeutic activity of CarboCell TGFβi monotherapy in the identical model was observed (Fig S5) which support the synergistic effect of the therapeutic combination.”

Reviewer #3 (Remarks to the Author):

This is an interesting manuscript that describes the effect of using an intratumoral depot, CarboCell, to deliver a TLR agonist and TGFβ inhibitor to the tumor microenvironment. The studies are well controlled and described, and indicate a clear positive effect in murine models. A very large amount of data is presented, with significant components relegated to the supplemental materials due to the volume. Most of the figures are clear, easy to understand, and illustrate the appropriate experiments and results. Likewise, with the exception of few typographical errors, the text is well organized and generally easy to read and understand.

The introduction and Discussion would generally benefit from being more specific to this particular manuscript instead of broadly touting the perceived benefits of CarboCell, many of which are not tested in this manuscript.

We appreciate your thoughtful evaluation of our manuscript and the positive feedback on the overall presentation of the study. We have carefully considered your comments regarding the introduction and discussion sections, specifically regarding the need for more specificity about the CarboCell formulation and its role in delivering Resiquimod (TLR7/8 agonist) and RepSox (TGFβ inhibitor) to the tumor microenvironment.

In response to your suggestion, we have revised the introduction to focus more narrowly on the aspects of using the CarboCell technology for delivering Resiquimod and RepSox.

Additionally, we reduced generalized statements about CarboCell technology to more directly therapeutic performance and formulation related to the presented research data. Instead, the revised introduction now outlines the objectives of intratumoral delivery of Resiquimod and RepSox.

The introduction now reads (changes in bold):

*“To overcome these obstacles, we **formulated the TLR7/8 agonist Resiquimod (R848) and TGFβi RepSox in a lipophilic carbohydrate ester based injectable depot technology (CarboCell TLR7/8:TGFβi).** ~~designed the CarboCell platform to provide an immune cell hot spot directly in the cancerous tissue (Fig. 1A) and associated lymph nodes.~~ Upon injection into the tumor, CarboCell **TLR7/8:TGFβi** transforms from a liquid into a semi-solid scaffold from which **Resiquimod and TGFβi RepSox** ~~single or multiple small molecule drug(s) may be released~~ **single or multiple small molecule drug(s) may be released** ~~each with individually tailored release profiles.~~ **The CarboCell TLR7/8:TGFβi***

technology can thereby form an immune cell hot spot directly in the cancerous tissue (Fig. 1A) and associated lymph nodes. Previously, the practicality of intratumoral injections has been subject for debate; and, variation and operator dependent outcomes have been reported²⁸. Some of the central challenges are associated with the rapid wash out of free drug from the TME and lack of methods for validating the positioning of injected drugs²⁸. The CarboCell TLR7/8:TGFβ1 technology overcomes these limitations.”

The inclusion of canine studies seems disconnected from the rest of the manuscript. The lack of any figures related to these experiments in the manuscript (some are provided as a supplemental table and figure) leads to the question of why these studies were included. In addition, the section in which these experiments were described is titled “CarboCell improves the therapeutic performance of checkpoint inhibition and is tolerated in translational canine models”, yet two thirds or more of this section deals with murine models. Finally, no graphical, tabular, or image data is presented regarding the responses of the cancer-bearing dogs that were injected with CarboCell.

We agree that only very limited data is available on the therapeutic performance of CarboCell TLR:TGFβ in spontaneous cancer bearing dogs and have therefore excluded these from the manuscript. We have maintained the data presented for the laboratory beagle dogs as this support the tolerability in a large animal model and release kinetics for larger sized depots.

To make the division between the data in mice concerning improved performance of checkpoint inhibitors and data on tolerability in canines clearer, we have divided the section in to two separate sections including mice and canine data, respectively.

The section headline has been changed from

~~CarboCell improves the therapeutic performance of checkpoint inhibition and is tolerated in translational canine models~~

To be two separate sections intitled

CarboCell improves the therapeutic performance of checkpoint inhibition

and

CarboCell is tolerated in experimental canine model

All data and information concerning clinical canine cancer patients have been removed and the following text has therefore been removed from the primary manuscript and supplementary materials:

“Tolerability was evaluated in mouse models and laboratory beagle dogs ~~and clinical canine cancer patients.~~”

~~“Accordingly, we injected highly advanced and high-grade canine cancer patients with CarboCell TLR and CarboCell TLR:TGFb to investigate tolerability after injection in spontaneous cancers (Fig. S12 I-K and Table S2). Both CarboCell compositions were well tolerated, and the clinical examinations showed tumor size reductions in three out of five dogs and the fourth dog displayed long-term tumor control of a normally aggressive osteosarcoma with a short, expected survival period. Although these pilot data were collected in a small heterogeneous group of aggressive high-grade or recurrent stage cancers, they support that the CarboCell technology is promising and that further clinical evaluation as monotherapy or in combination therapy is highly interesting.”~~

~~The demonstrated therapeutic efficacy, in combination with the validated injectability in large translational animal models, make the CarboCell technology highly attractive for clinical advancement.~~

And supplementary figure S12 I-K and Table 2 have been removed.

Fig. S12. CarboCell TLR:TGFb is well tolerated in laboratory canines and display clinical activity in canine with spontaneous advanced high-grade cancers.

A. Beagle dogs (11.9±0.3 kg) injected s.c. in the neck region with 1 x 250 µl (blue and green) or 2 x 250 µl (purple) CarboCell TLR:TGFb (2.4 mg/mL:8 mg/mL, CC-4, Table S1). Injected CarboCell depots were surgically recovered after 7 days (blue) or 14 days (green and purple) (n = 4). **B.** Retained TLR7/8a and TGFβi of initial concentration in CarboCell TLR:TGFb. **C.** Body weight. **D.** Rectal temperature. **E.** Serum C-reactive protein. **F.** Serum cytokine levels of TNF-α, IFN-γ and MCP-1. **G.** Hematology. **H.** Serum biochemistry. (n = 3-4). Dotted lines indicate upper and lower standard reference limits. Results are presented as mean ± SEM. **I-K.** Diagnostic imaging of three of the included canine cancer patients. **I.** Left: cross-sectional CT image of the nasal cavity of dog no. 1 demonstrating the extent of a grade-3 apocrine adenocarcinoma (arrows), middle: Sagittal SPECT/CT image (^{99m}Tc-MIBI) of the head-and-neck region demonstrating high activity in the rostral part of the nasal cavity and muzzle region correlation with the extent of the cancer (arrows) and enlargement of, and activity in, the mandibular lymph (arrow heads), right: Enlarged mandibular lymph tracer activity (arrow heads) corresponding with lymph node metastasis (cytologically confirmed). **J.** Axial (left), coronal (middle) and sagittal (right) CT images of the recurrent transitional cell carcinoma (arrows) of dog no. 2 after retrograde infusion of radiographic contrast in the urinary bladder. **K.** Left and middle axial SPECT/CT (^{99m}Tc-MIBI) images of the recurrent high-grade soft tissue sarcoma (arrows) of dog no. 5 and right sagittal CT of soft tissue pulmonary mass compatible with a pulmonary metastasis (not histologically confirmed) (radiotracer: ^{99m}Tc-MIBI, Mediradiopharma, Hungary).

The supplementary materials and methods for procedures in canine cancer patients have furthermore been removed.

Pilot evaluation of intratumoral CarboCell in clinical canine cancer patients

~~Clinical canine cancer patients, for which no alternative therapeutic interventions were available or accessible, were included in the pilot evaluation of CarboCell tolerability and therapeutic performance. Owners of dogs provided informed consent and all procedures and evaluations were performed by experienced veterinary oncologist. All dogs had histologically confirmed diagnoses and extensive diagnostic work-up was performed to provide accurate~~

~~clinical grade, stage, and local extension of disease (Table S2 and Fig. S12 P-R). For CarboCell injections the areas to be injected were aseptically prepared and injection performed using a 1 mL syringe and 23 or 25 Ga needles. Dogs were anesthetized or sedated at the discretion of the veterinary surgeon undertaking the procedure. CarboCell composition and injection volume, number of injections and frequency are provided in Table S2. All injections and follow up clinical investigations were performed on an out patient basis and regular post injection visits were conducted during the first week after injection. Clinical tolerability and performance were evaluated by the team of experienced veterinary oncologist based on external tumor measurements and diagnostic imaging and all dogs were followed throughout the period from enrollment to euthanasia (Table S2).~~

The manuscript states that CarboCell allows “individually tailored release profiles” – It is unclear how the release profiles are modified (or tailored) for individual components that are co-injected. It appears that all of the compounds in one formulation are subject to the same release profile.

Answer: As also requested by reviewer 1, we have clarified the rationale for design of the individual compositions by updating of table S1 (See answer 2c reviewer 1 for further details). However, it is important to emphasize that the two drugs are not only co-injected, but also co-formulated meaning that both are released from the same CarboCell.

The underlying mechanisms for tuning of release rates are governed by 1) the viscosity of the depot which is set by the ratio of the carbohydrate ester and triglyceride and 2) the interaction of the drug and the CarboCell material which can be gauged by the logP of the drug. Synchronized release as observed for the current pair of drugs can therefore be obtained as their hydrophobicity (logP values) is of similar magnitude.

We believe these general principles for how drug release rates can be tailored are described in the manuscript:

“A reduced release rate was obtained by lowering the triglyceride and polymer content (Table S1).”. It is further mentioned that the drug release and synchronization of release rate is governed by the hydrophobicity (logP) of the API in “Here, the TLR7/8a Resiquimod (R848, logP = 1.7, Fig. 1B) and the TGFβi RepSox (logP = 2.5, Fig. 1B) were co-formulated in CarboCell, as both compounds are lipophilic with comparable logP, rendering these drugs optimal for synchronized dual release.”

And we think these statements in combination with the newly introduced “rationale of design” column in table S1 effectively captures the core of how CarboCell API release can be tuned.

Fig 2, CC-TGFβ was not used alone as a control, thus it is not clear if the effect is synergistic when combined with CC-TLR or simply the effect of TGFβi. This does appear to be addressed in later studies, but would be reassuring to see addressed here as well.

We do agree to the importance of including appropriate controls. However, as it is correctly referenced the exact same TGFβ concentration in the identical cancer was investigated in

the study of supplementary Fig S5. We are obligated to strictly adhere to national legislation and studies are evaluated by institutional board to assure that the number of animals included are reduced to what is scientifically and statistically justified.

Based on this we felt that observations in Fig S5 did not justify the need for a repeated evaluation in the same model. To further bring attention to importance of Fig S5 for stating the synergistic activity we have therefore changed the text to now read (changes in bold):

*“Conversely, only 22% complete responses were evinced in the CarboCell TLR-treated group (Fig. 2B-C), confirming the synergistic effect of TLR7/8 activation and TGFβ inhibition over single agent activity. **In connection to this observation no therapeutic activity of CarboCell TGFβi monotherapy in the identical model was observed (Fig.S5) which support the synergistic effect of the therapeutic combination.**”*

Discussion: “Notwithstanding these efforts, no technology has yet successfully progressed into a clinical product.” – I would argue that anti-PD-1 antibody therapy is a clinical product.

We completely agree on this being wrong and it was supposed to only refer to innate immune activating therapies (TLRs, RIG-1, STING agonists).

The text now reads:

“Notwithstanding these efforts, no innate activating immunotherapy technology has yet successfully progressed into a clinical product.”

Discussion: typo, “makescontinuous” needs a space added.

Thanks for observing this, space has been added

Discussion: “...CarboCell TLR:TGFβ both in the tdLNs,..” – The use of the word both implies two subordinate clauses, but the sentence has three.

Thanks for noticing this – text changed (changes in bold)

“...CarboCell TLR:TGFβ ~~both~~ in the tdLNs,..”

No description of methods or animal use approvals were provided for the canine work in the main manuscript, either with normal dogs for toxicity or in tumor-bearing dogs. The description provided in the supplement did not provide the number of dogs in each toxicity assessment group.

We appreciate this comment and regret that we have missed to include the number of dogs (n=4) included in each group. This has now been updated accordingly. The experimental procedures in canines were performed under Hungarian Animal Experiments License and this is included in experimental section in supplementary materials and methods.

Supplementary material (changes in bold)

*“On day 7 (group A, **n=4**) or day 14 (group B, **n=4** and C, **n=4**) dogs had the CarboCell depots surgically recovered under general anesthesia and analyzed for remaining concentration of TLR7/8a and TGFβi”*

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

In the revision, the authors responded well to the questions in the comments. There are no problems with the supplementary experimental data and the text description in the revision, and there are no further comments to this article.

Reviewer #3 (Remarks to the Author):

The authors were responsive to the 1st round of reviews. I have no further review comments.

Reviewer #4 (Remarks to the Author):

This is an interesting study (performed in several cell-based tumor models in mice including a canine model) which employs CarboCell technology to deliver TLR7/8 agonists together with TGF beta inhibitors directly to tumors.

The authors have responded to the original concern of "lack of detailed mechanistic analysis utilizing knockout models to establish the role of specific immune subsets in generation of anticancer therapeutic effect and the relationship between the combination therapy and the observed therapeutic benefits", raised by the original reviewer 2 by repeating the experiments in nude mice and showing that T-cells are crucial for maintaining the therapeutic response. These results are important and strengthen the study, which however is still falling short in explaining the immunological mechanism in more depth. Moreover, the approach seems to be very effective in tumors generated by subcutaneous injections of tumor cells. Validation in a more relevant tumor model would have been important, e.g. at least in cell-based orthotopic models to show that the approach has translational potential which goes beyond subcutaneous tumors.

minor comment:

The antibody employed for PD-L1 staining with A647 seems to be an anti-human antibody. If

the number in the manuscript is correct (Biolegend 329728), there is no cross reaction with mouse. Is this product number correct?

Reviewer #1 (Remarks to the Author)

In the revision, the authors responded well to the questions in the comments. There are no problems with the supplementary experimental data and the text description in the revision, and there are no further comments to this article.

Answer: We would like to thank the reviewer for the constructive comments and suggestions which has improved the manuscript

Reviewer #3 (Remarks to the Author)

The authors were responsive to the 1st round of reviews. I have no further review comments.

Answer: We would like to thank the reviewer for the constructive comments and suggestions which has improved the manuscript

Reviewer #4 (Remarks to the Author)

This is an interesting study (performed in several cell-based tumor models in mice including a canine model) which employs CarboCell technology to deliver TLR7/8 agonists together with TGF beta inhibitors directly to tumors.

The authors have responded to the original concern of "lack of detailed mechanistic analysis utilizing knockout models to establish the role of specific immune subsets in generation of anticancer therapeutic effect and the relationship between the combination therapy and the observed therapeutic benefits", raised by the original reviewer 2 by repeating the experiments in nude mice and showing that T-cells are crucial for maintaining the therapeutic response. These results are important and strengthen the study, which however is still falling short in explaining the immunological mechanism in more depth. Moreover, the approach seems to be very effective in tumors generated by subcutaneous injections of tumor cells. Validation in a more relevant tumor model would have been important, e.g. at least in cell-based orthotopic models to show that the approach has translational potential which goes beyond subcutaneous tumors.

Answer: We appreciate the positive comments on the CarboCell technology and performance. The aim of the study was to demonstrate the therapeutic value of a sustained intratumoral drug delivery system providing dual release of both a TLR7/8 and TGFb inhibitor. Considering the extensive characterisation of both drug classes available in the literature we feel that repeating these would not improve the manuscript and potentially confuse the primary aim. Yet we believe that the current manuscript fully supports that we provide sustained activation and inhibition intended.

minor comment:

The antibody employed for PD-L1 staining with A647 seems to be an anti-human antibody. If the number in the manuscript is correct (Biolegend 329728), there is no cross reaction with mouse. Is this product number correct?

Answer: We would like to sincerely thank for observing this. The antibody used was in all experiments involving PD-L1: anti-mouse PD-L1 (B7-H1), clone 10F.9G2, BioXcell. This has been changed in the methods section.

The changed text is in green:

Anti-PD-L1 antibody labelling

Anti-PD-L1 antibody (anti-mouse PD-L1 (B7-H1), clone 10F.9G2, BioXcell) was labelled with Alexa Fluor (AF)647 fluorophore using the Rapid Conjugation System (Lightning-Link®, Expedeon).