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ORIGINAL RESEARCH

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Local therapy with combination TLR agonists stimulates systemic anti-tumor immunity and sensitizes tumors to immune checkpoint blockade

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ABSTRACT

Toll-like receptor (TLR) agonists are being developed as anti-cancer therapeutics due to their potent immunostimulatory properties. However, clinical trials testing TLR agonists as monotherapy have often failed to demonstrate significant improvement over standard of care. We hypothesized that the anti-cancer efficacy of TLR agonist immunotherapy could be improved by combinatorial approaches. To prevent increased toxicity, often seen with systemic combination therapies, we developed a hydrogel to deliver TLR agonist combinations at low doses, locally, during cancer debulking surgery. Using tumor models of WEHI 164 and bilateral M3–9-M sarcoma and CT26 colon carcinoma, we assessed the efficacy of pairwise combinations of poly(I:C), R848, and CpG in controlling local and distant tumor growth. We show that combination of the TLR3 agonist poly(I:C) and TLR7/8 agonist R848 drives anti-tumor immunity against local and distant tumors. In addition, combination of local poly(I:C) and R848 sensitized tumors to systemic immune checkpoint blockade, improving tumor control. Mechanistically, we demonstrate that local therapy with poly(I:C) and R848 recruits inflammatory monocytes to the tumor draining lymph nodes early in the anti-tumor response. Finally, we provide proof of concept for intraoperative delivery of poly(I:C) and R848 together via a surgically applicable biodegradable hydrogel.

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Introduction

The first successful immunotherapy was reported in the early 19th century following intratumoral injection of bacterial extracts, Coley's toxins, to a patient with sarcoma.[1] Today, interest in intratumoral immunotherapy remains high, with promising results reported using immunotherapeutics including cytokines, immune checkpoint blockade (ICB) antibodies, and innate immune agonists.[2] Toll-like receptors (TLRs) are pattern recognition receptors (PRRs) expressed on a range of immune cells. TLRs sense microbial products and initiate and co-ordinate innate immune responses via mediators including inflammatory or immunomodulatory cytokines.[3] There is ongoing interest in TLR agonists as anti-cancer therapeutics.[4] Numerous pre-clinical[5] and clinical studies[6] have demonstrated the anti-cancer potential of such agents. However, despite promising results, they have not been readily translated into the clinic, often failing in phase II/III clinical trials.[7,8]

Most pre-clinical and clinical studies assessing TLR agonists have used them as single agents. However, pathogens carry multiple pathogen-associated molecular patterns, which may simultaneously activate different PRRs, resulting in enhanced immune cell activation.[9] Signaling through multiple PRRs can work in cooperation, with subsequent cross talk between downstream signaling pathways leading to enhanced cytokine expression, which is important for generating a robust immune response.[9–12] To date, the few studies assessing TLR agonists in combination have demonstrated promising anti-cancer efficacy in preclinical studies.[11,13,14] However, several questions remain unanswered, including, which TLR agonists direct optimal systemic anti-tumor immunity, what is the nature and phenotype of the induced response,[15] and how best to deliver such agents. Additionally, there is increasing evidence that priming strong local anti-tumor immunity may enhance response to systemic ICB.[16] Here, we set out to identify an effective combination of TLR agonists, delivered intraoperatively using a controlled release hydrogel, which provides both local and systemic cancer control with the goal of improving the clinical translatability of this approach.

Materials and methods

Cell lines

CT26 was obtained from the National Institutes of Health Tumor Repository. WEHI 164 was obtained from CellBank Australia. M3–9-M was kindly gifted by C. Mackall (Stanford University, Stanford, CA). Cell lines were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum

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(FBS) (Fisher Biotech), 20 mM N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid (HEPES), 0.05 mM 2-mercaptoethanol, and 100 U/ml penicillin and streptomycin (all Invitrogen). Cell lines were validated yearly for strain specific major histocompatibility complex (MHC) class I alleles and tested for mycoplasma.

Tumor models

All animal experiments were carried out in accordance with institutional guidelines from the Animal Ethics Committee at the Harry Perkins Institute of Medical Research. BALB/cJAusb and C57BL/6J mice (all female, 8–12 weeks) were purchased from the Animal Resource Centre (Murdoch, WA) or bred onsite at the Harry Perkins Institute for Medical Research and maintained under specific pathogen-free conditions, with 12-hour light/dark cycle.

Mice were inoculated subcutaneously (s.c.) with 5×10^5 cells in 100 µl sterile phosphate buffered saline (PBS) on the right flank for single-tumor experiments or on both right and left flanks for bilateral tumor experiments. Once established, right flank tumors were treated intratumorally (i.t.) with single or pairwise combination of vaccigrade TLR agonists: poly(I:C), high molecular weight (HMW), cat# VAC-PIC; Resiquimod (R848), cat# VAC-R848; CpG ODN 2395, cat# VAC-2395-1; monophosphoryl lipid A (MPLA), cat# VAC-MPLS, (all from InvivoGen), daily for 6 days. For combination of TLR agonists and ICB treatment, mice received three intraperitoneal (i.p.) injections of 200 µg anti-programmed cell death protein-1(PD-1) (BioXcell, Clone RMPI-14), every 2 days, starting on day 8 after tumor inoculation in addition to i.t. treatment with combination of TLR agonists.

To assess the anti-tumor efficacy of poly(I:C)-R848 hydrogel after incomplete tumor resection, once right flank tumors were established, 90% of the right flank tumor was removed under anesthesia using isoflurane (4% isoflurane in oxygen, flow rate of 1 L/min for induction and 0.5 L/min for maintenance) while the left flank tumor was left intact. 200 µl of hydrogel loaded with either poly(I:C) or R848 or poly(I:C) and R848 was applied in the wound bed before closure with tissue glue (3M, Vetbond®).

Mice were monitored for tumor growth and tumor size was measured using calipers as length x width. Tumor sizes at randomization for treatment are given for each model in Supplementary Table 3. Mice were euthanized once tumors reached a size of 100 mm^2.

Flow cytometry staining and FACS analysis

After 4 days of i.t. treatment with TLR agonists, mice were euthanized and bilateral CT26 tumors and tumor-draining lymph nodes (tdLNs) were harvested into fluorescence-activated cell sorting (FACS) buffer (PBS with 2% v/v FBS). Tumors were manually disrupted using a scalpel and dissociated using the gentleMACS and Mouse Tumor Dissociation Kit (Miltenyi). tdLNs were passed through a 70 µM nylon filter to obtain single cell suspensions. Murine Fc block (anti-CD16/CD32, BD) and Fixable Viability Stain 780 (BD) were incubated for 20 min. Next, cells were incubated with antibodies for surface markers (Supplementary Tables S1 and 2) for 30 min. The FoxP3 transcription factor staining buffer set (eBioscience) was used, according to manufacturer guidelines, prior to intracellular staining for 30 min. All incubations were carried out on ice, in the dark. Samples were resuspended in FACS buffer and data acquired on LSR Fortessa X-20 (BD) and analyzed using FlowJo (V10.8.1). For gating strategy, see Supplementary Figure S6.

Preparation of hydrogels

Hyaluronic acid (HA) hydrogels were prepared as previously reported[17] with slight modification to form R848-HA conjugated hydrogels. Briefly, 20 mg HA (Cat# FS-HA-ME0.5, Freshine Chem) was dissolved in 2 mL ultrapure water, then 4 mg (0.0128 mmol) of R848 (Cayman Chemical) was added into the solution followed by 2.4 mg (0.0172 mmol) of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride while adjusting pH to 4.75. Next, 0.9 mg (0.00104 mmol) of 3,3'-Dithiobis (propanoic hydrazide) (DTPH) was dissolved into the solution while stirring and maintaining pH at 4.75, for 1 hour, then left overnight. After overnight reaction, the pH was adjusted to 7.0 before addition of 1.8mg of dithiothreitol (0.057 mmol). The pH was adjusted to 8.5 and left overnight to reduce disulfides to free thiols. The product was then dialyzed by using a dialysis tube (3.5 kDa) in 0.1 M NaCl adjusted to pH 3.75 with 1 M HCl and then deionized water, before being lyophilized. HA-R848-DTPH polymer was stored in a desiccator at room temperature for further use.

To form 2.5% w/v empty HA hydrogels or HA-R848 conjugated hydrogels, in a typical representative reaction, 850 µl water and 100 µl 10× PBS and 50 µl DMSO were added to 25 mg of either HA-DTPH or HA-R848-DTPH lyophilized polymer and mixed by gentle inversion for 1–2 hours until completely dissolved, yielding a colorless, viscous solution. Poly(I:C)-loaded hydrogels were prepared by encapsulating 2500 µg poly(I:C) per ml of hydrogel solution using HA-DTPH polymer. Hydrogels containing both poly(I:C) and R848 were prepared by encapsulating 2500 µg poly(I:C) per ml in HA-R848-DTPH polymer. Hydrogels were aliquoted in 200 µl aliquots and allowed to set for 48 hours at room temperature, before being stored at 4°C.

Statistics and data analysis

GraphPad Prism v9.5.0 was used for all statistical analyses. FACS data were analyzed using FlowJo (v10.8.1).

Results

Local, low-dose intratumoral therapy with TLR agonists exhibits differential anti-tumor efficacy

We assessed TLR agonists poly(I:C), MPLA, R848, and CpG for their efficacy in controlling tumor growth as monotherapies. Local delivery of immunotherapy can induce immune activation within the tumor microenvironment (TME) at lower dosages compared to systemic delivery.[2] Therefore, we

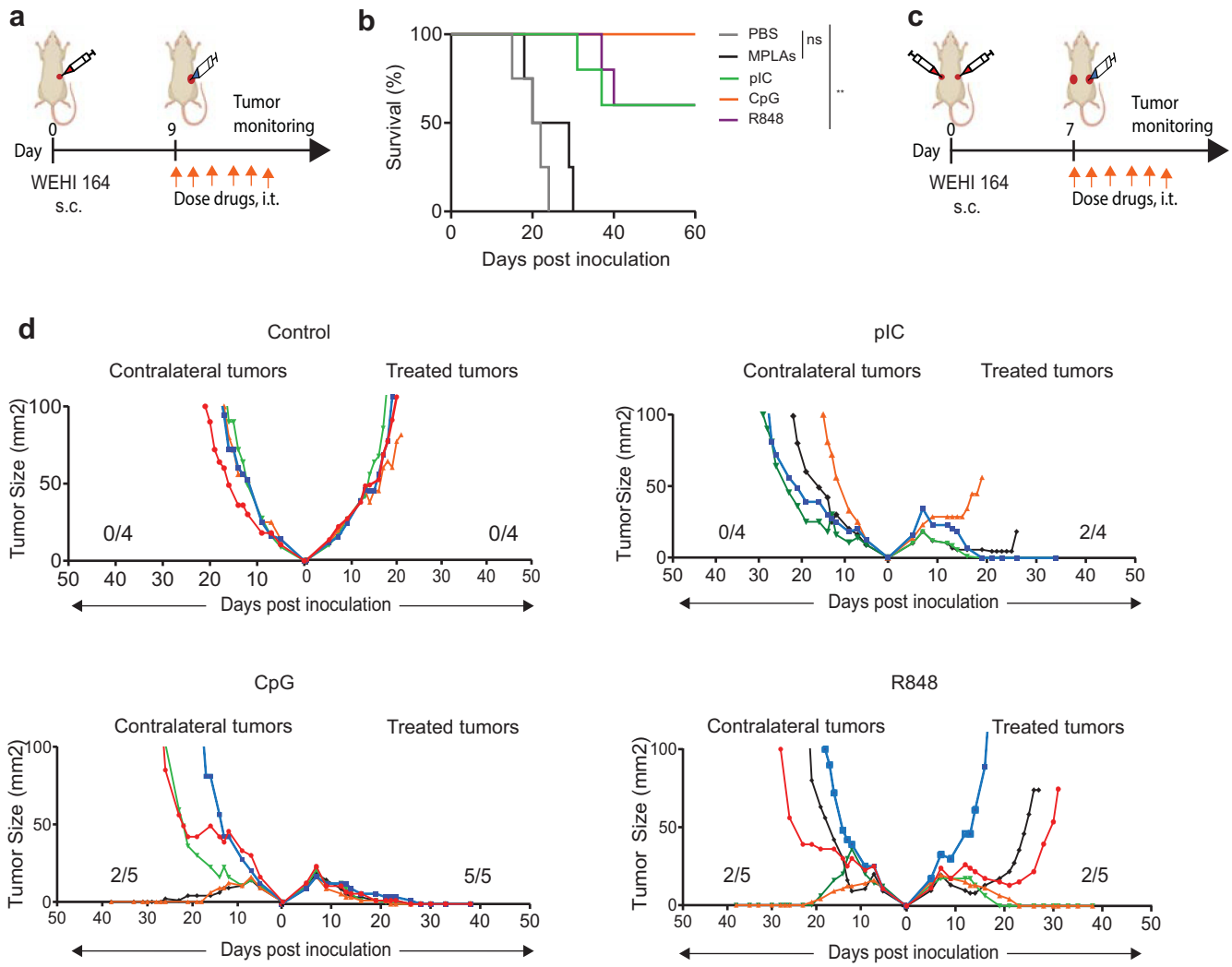

Figure 1. Repetitive, local, low dose of TLR agonists shows different efficacy in controlling local and distant tumors. (a) Established tumors were treated by i.t. injection with either poly(I:C), 10 µg/d; CpG, 10 µg/d; R848, 8 µg/d; MPLA, 4 µg/d; or vehicle daily for 6 days. (b) Survival curves of WEHI 164 tumor-bearing mice; $n = 4–5$ mice per group. (c) For bilateral tumors, mice were inoculated with 5×10^5 WEHI 164 cells on the right and left flanks. The right flank tumor was treated by i.t. injection with either poly(I:C), R848, CpG ODN, or vehicle, daily for 6 days using same doses as in (a). (d) Tumor growth curves for the right and left flank tumors; $n = 4–5$ mice per group. In (b), statistical analysis was performed using Log-rank (Mantel–Cox) test. $^{**}P < 0.005$. ns: not significant.

delivered agonists via intratumoral injection (Figure 1a) at 20% of the commonly reported systemic dosage.[18–20] The three TLR agonists, poly(I:C), R848, and CpG, that target endosomal TLRs demonstrated strong anti-tumor effect against WEHI 164 tumors, whereas MPLA, the agonist for TLR4, provided no survival benefit (Figure 1b, Supplementary Figure S1).

Because metastatic disease is the major cause of cancer-related death,[21] we next assessed the ability of poly(I:C), R848, and CpG as monotherapies to control distant tumor growth, using a bilateral tumor model[22] (Figure 1c). Both poly(I:C) and CpG provided strong local tumor control. However, treatment with poly(I:C) did not control distant tumors, whereas CpG or R848 treatment could induce regression of distant tumors (Figure 1d). Interestingly, the strong CpG-induced local response did not universally translate into a strong response against the distant tumor. While R848 showed less robust local tumor control, there was a consistent symmetry of response for the treated and distant tumor (Figure 1d). These data demonstrate that strong local tumor control is not always an indicator of distal efficacy, suggesting that combination of different TLR agonists may be needed to achieve the full potential of TLR-targeted immunotherapy.

Combination of intratumoral poly(I:C) and R848 demonstrates additive effect in controlling distant tumors

The efficacy of combination TLR agonists for local tumor control has been demonstrated elsewhere.[14,23] We focused on their ability to control distant CT26 adenocarcinoma and M3–9-M rhabdomyosarcoma tumors. Established bilateral tumors (Supplementary Table S3) were treated by injecting the right flank tumor with single or pairwise combination of poly(I:C), R848, or CpG ODN, daily for 6 d (Figure 2a). Tumor size was

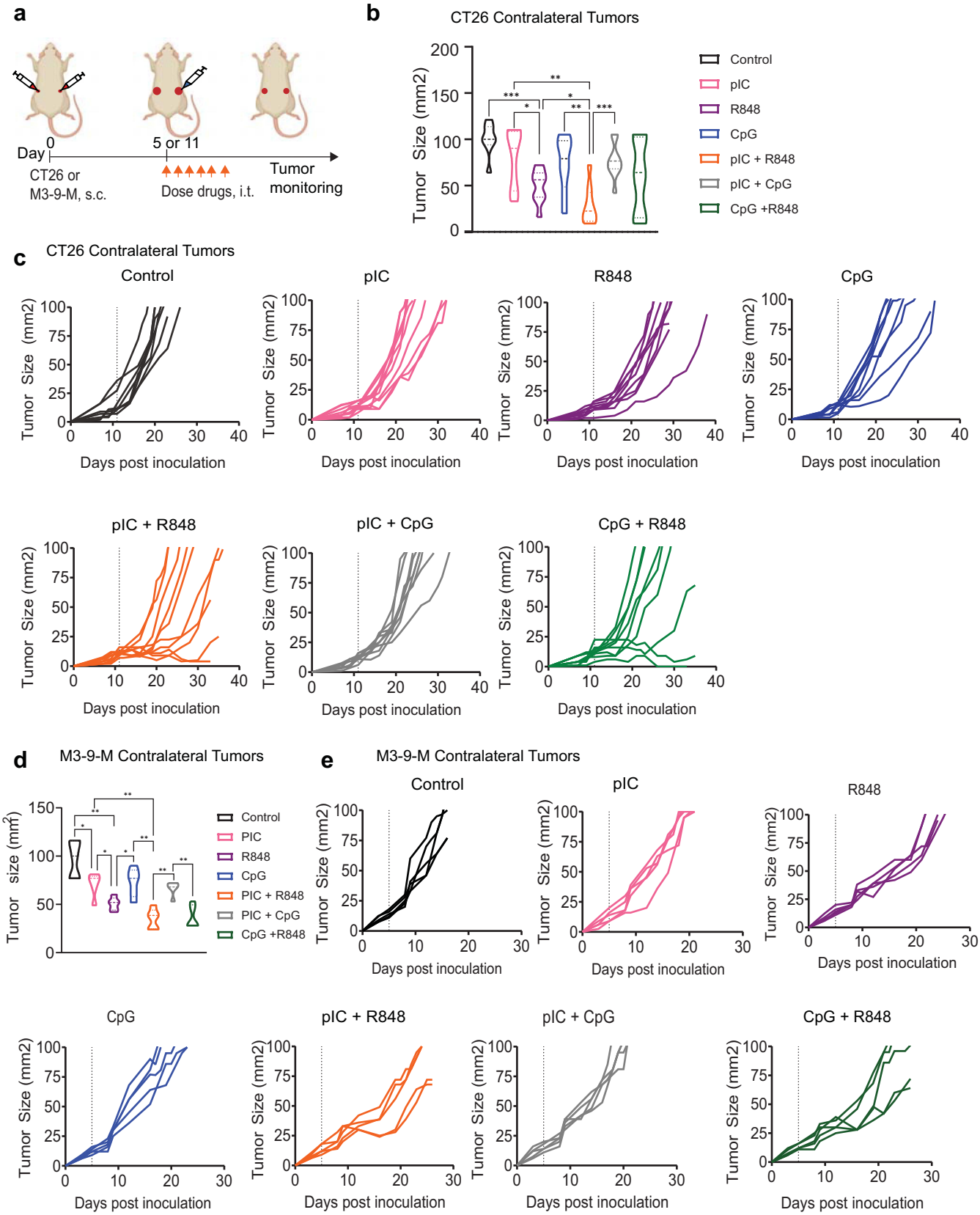

Figure 2. Intratumoral injection with pairwise combinations of TLR agonists delays distant tumor growth in immunotherapy-resistant models. (a) On day 5 (M3-9-M) or day 11 (CT26), the right flank tumor was treated with single TLR agonist: poly(I:C), 10 μg/d; R848, 8 μg/d; or CpG ODN, 10 μg/d; or vehicle, or pairwise combination of TLR agonists, daily for 6 days. (b) Size of contralateral CT26 tumors on day 23 when all control tumors had reached endpoint. (c) Tumor growth curves for contralateral CT26 tumors. $n = 8–10$ mice per group. (d) Size of contralateral M3-9-M tumors on day 17 when all control tumors had reached endpoint. (e) Tumor growth curves for contralateral M3-9-M tumors. $n = 5$ mice per group. In (b) and (d), statistical analyses were performed by Brown–Forsythe and Welch ANOVA tests. $^{*}p \le 0.05$, $^{**}p \le 0.005$, $^{***}p \le 0.0005$.

compared at day 22 (CT26) or day 17 (M3–9-M), the timepoint when all distant tumors in control groups had reached experimental endpoint. All monotherapies were ineffective in controlling distant tumor growth. The combination of poly(I:C) with R848 and CpG with R848 delayed outgrowth of local and distant CT26 tumors (Figure 2b–c). All pairwise combinations demonstrated a strong local control (Supplementary Figure S2). Although the anti-tumor effect was less pronounced against M3–9-M tumors, the R848-poly(I:C) and R848-CpG combinations again delayed distant tumor outgrowth (Figure 2d–e). Together, these data demonstrate that local delivery of poly(I:C) and R848 can improve local and distant tumor control in the two models tested.

TLR7/8 agonist R848 recruits inflammatory monocytes to local and distant lymph nodes

To determine the impact of local poly(I:C) and R848 therapy on the immune compartment, we assessed infiltrating immune cells in local and distant tumors, as well as their tdLNs, after 4 d of i.t. treatment (Figure 3a). We chose an early time point because, later, the strong response against the local tumor made analysis of the

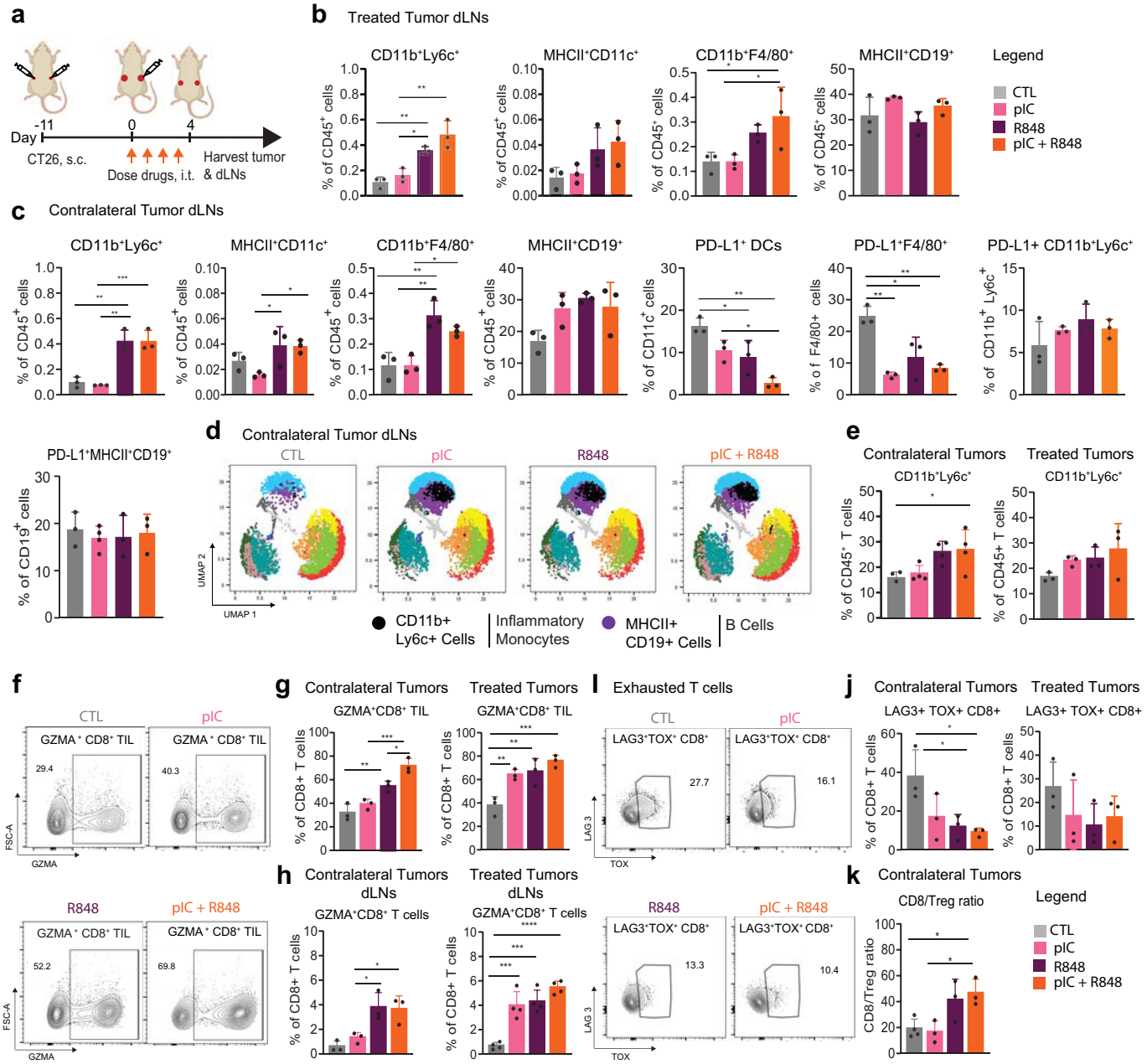

Figure 3. Combination of poly(I:C) + R848 modulates the local and distant immune microenvironment. (a) Tumors were treated with four daily injections of TLR agonists poly(I:C) and R848, as single agents or combination. Local and contralateral tumors and tdLNs were harvested 24 hours after the final treatment for flow cytometry analysis. (b–c) Graphs showing the proportion of immune cells in the treated tdLNs (b) or contralateral tdLNs (c). Data are mean ± s.d.; $n = 3$ mice per group. Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparison test. *$p \le 0.05$, **$p \le 0.005$, ***$p \le 0.0005$. (d) UMAP showing clustering of cell populations across different treatment groups in the contralateral tdLNs. (e) Graphs showing the proportion of $CD11b^+$ $Ly6C^+$ cells in contralateral and treated tumors. (f) Representative FACS plot showing GZMA expression on $CD8^+$ T cells in contralateral tumors. (g–h) Graphs showing GZMA expression in tumors (g) and tdLNs (h). (i) Representative FACS plot showing exhausted $CD8^+$ T cells ($Lag3^+$ TOX^+) in contralateral tumors. (j) Graphs showing exhausted T cells in tumors. (k) Graph showing CD8/Treg ratio in contralateral tumors. Data are mean ± s.d.; $n = 3$ mice per group. Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparison test. *$p \le 0.05$, **$p \le 0.005$, ***$p \le 0.0005$, ****$p \le 0.0001$.

tumor-infiltrating leukocytes impossible. Most strikingly, a significant increase in the number of immature ($CD86^{low}$) inflammatory monocytes ($CD11b^{+}Ly6c^{+}$) was observed in both the treated and contralateral tdLNs of mice treated with R848 alone or in combination with poly(I:C) (Figure 3b–d) and in contralateral but not treated tumors (Figure 3e). R848 treatment alone or combined with poly(I:C) also increased the percentage of dendritic cells ($MHCII^{+}CD11c^{+}$) and macrophages ($CD11b^{+}F4/80^{+}$) in contralateral, but not treated, tdLNs (Figure 3b,c) and decreased PD-L1 expression on these populations (Figure 3c). However, PD-L1 expression on tumor cells remained unchanged regardless of treatment (Supplementary Figure S3h) and expression of CD86, Arg-1, and CD206 on $F4/80^{+}$ macrophages did not vary across treatment groups (Supplementary Figure S3a, b, e–g).

$CD8^{+}$ T cells in tumors and tdLNs demonstrated significant upregulation of the cytotoxic molecule Granzyme A, which was driven primarily by R848 in contralateral tumors and both poly(I:C) and R848 in treated tumors (Figure 3f–h). Consistent with their increased function, $CD8^{+}$ cells had significantly reduced expression of exhaustion markers LAG3 and TOX after all TLR agonist treatments in contralateral tumors but not in treated tumors (Figure 3i,j). We also observed a significant increase in the CD8:Treg ratio in contralateral tumors but only after treatment with R848 alone or combined with poly(I:C) (Figure 3k). $CD8^{+}$ T cell expression of effector/memory markers CD44 and CD62L was unchanged across treatment groups, while conventional $CD4^{+}$ T cells had consistently reduced expression of the activation markers CD25 and ICOS (Supplementary Figure S3C/D).

Combination of local poly(I:C) and R848 sensitizes tumors to systemic ICB

Combination therapy with poly(I:C) and R848 is reported to enhance STAT1-IFN signaling with increased pro-inflammatory cytokine production[9] and local IFN signaling sensitizes tumors to ICB.[19] Therefore, we designed experiments to test whether combination of local poly(I:C) and R848 with systemic anti-PD-1 ICB (Figure 4a) could further increase anti-tumor efficacy against M3–9-M and CT26 tumors. Combination of local poly(I:C) and R848 with ICB significantly increased survival of mice bearing M3–

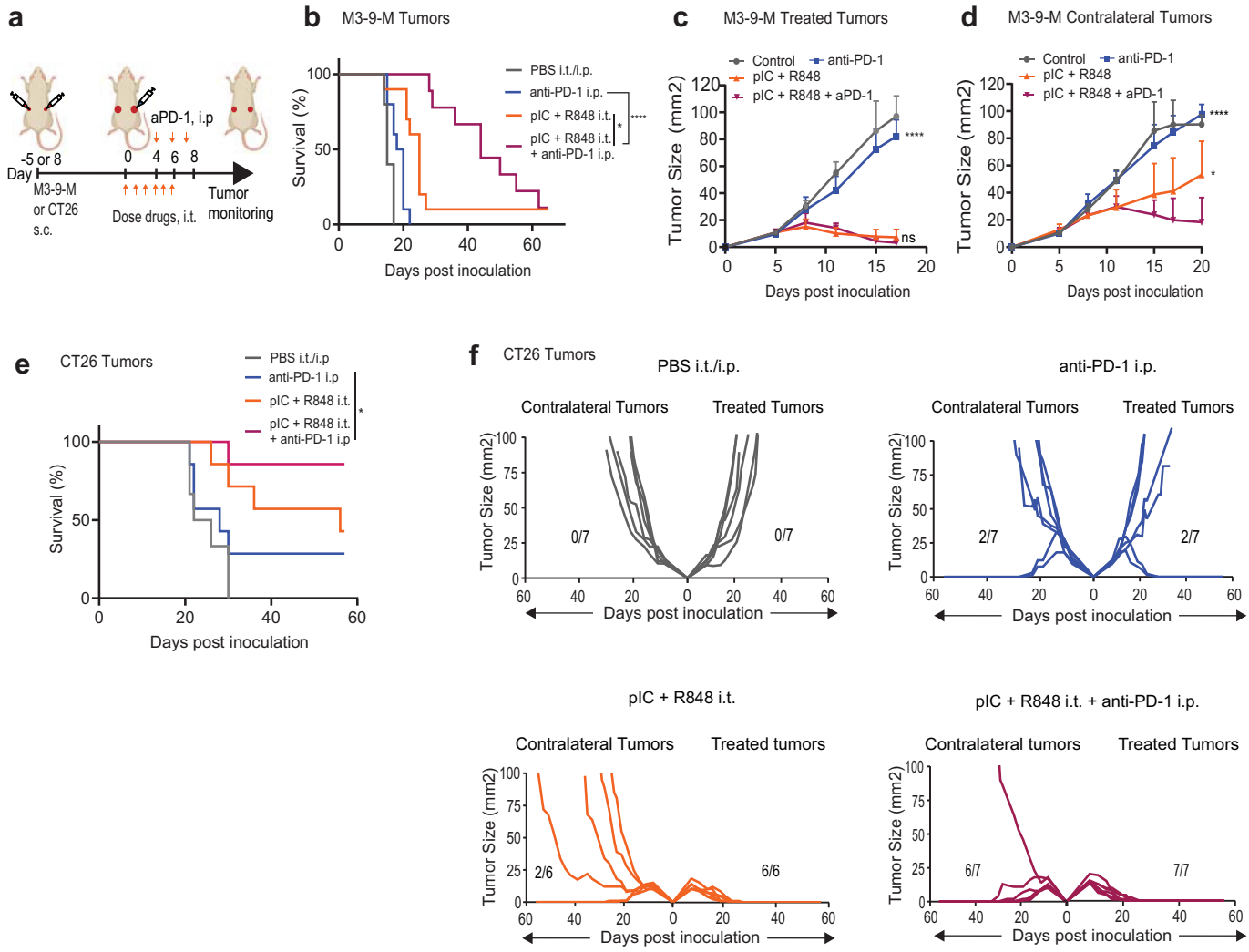

Figure 4. Combination of local poly(I:C) and R848 sensitizes tumors to systemic ICB. (a) Starting on day 5 mice received six daily injections of poly(I:C) (10 µg/d) and R848 (8 µg/d) plus anti-PD1 (BioXcell, Clone RMPI-14), 200 µg i.p., every 2 days for three doses, starting on day 8. (b) Survival of M3-9-M tumor-bearing mice. (c–d) Mean tumor size for treated (c) and contralateral (d) M3-9-M tumors. (e) Survival of CT26 tumor-bearing mice. (f) Tumor growth curves for treated and contralateral CT26 tumors; $n = 8$–10 mice per group for (b–d) and $n = 6$–7 mice per group for (e/f). In (b) and (e), statistical analysis was performed using Log-rank (Mantel–Cox) test. In (c) and (d), statistical analysis was performed using a two-way analysis of variance (ANOVA). $^{*}p \le 0.05$, $^{****}p \le 0.0001$.

9-M tumors compared to TLR agonists or ICB alone (Figure 4b), inducing strong local control and sensitizing distant tumors to respond to ICB therapy (Figure 4c–d). Notably, there were several partial responses in the combination therapy group, where distant tumors initially regressed but later grew out (Supplementary Figure S4). CT26 tumors demonstrated stronger responses after combination TLR and ICB therapy, with 28% and 33% of mice surviving in anti-PD-1 and TLR agonist single arm therapy groups, respectively, compared to 85% in the TLR agonist and ICB combination group (Figure 4e,f). These data suggest that local therapy with low-dose TLR agonists sensitizes tumors in a fashion that is supportive of combination with systemic immunotherapies.

Intraoperative delivery of poly(I:C) and R848 via a surgically optimized hydrogel

Current challenges associated with local intratumoral immunotherapy include invasiveness of injections, repetitive dosing, and lack of access to the tumor site for viscerally located cancers.[24] Therefore, we adapted a published hydrogel platform for intraoperative delivery of immunotherapy,[17] to deliver poly(I:C) and R848 intra-operatively during incomplete local tumor resection[25] with the presence of a smaller distant tumor, inoculated 4 d after the primary, to model undetected metastatic disease.

Poly(I:C), a high molecular weight dsRNA, can be easily encapsulated within porous hydrogels for sustained release. R848, a small-molecule agonist, easily diffuses out of such matrices. To overcome this, R848 was covalently conjugated to functional groups on the HA polymer, allowing for targeted delivery while tying R848 release kinetics to the degradation of the biomaterial (Figure 5a). This method allowed for a maximum of 9 µg of R848 to be conjugated per 200 µL of hydrogel. Conjugation efficiency was characterized by proton nuclear magnetic resonance (^{1}H NMR) spectroscopy, showing distinct peaks characteristic of R848 and HA (Figure 5b). The degree of conjugation and R848 recovery were characterized by UV spectrophotometer at 320 nm using an R848 standard curve (Supplementary Figure S5a).

We assessed the ability of the poly(I:C)-R848 loaded hydrogels to control both local and distant tumors after incomplete tumor debulking and intraoperative delivery. Using CT26 bilateral tumors and a 90% surgical debulk of the right flank tumor (Figure 5c), poly(I:C)-R848 hydrogel provided strong control of residual local disease (Supplementary Figure S5b), with modest improvement against distant tumor growth compared to either single poly(I:C) or R848-loaded hydrogels (Figure 5d). One complete and three partial responses were observed with the poly(I:C)-R848 hydrogel compared to a single complete response with the R848-hydrogel and a partial response with the poly(I:C)-hydrogel (Figure 5d). Importantly, the hydrogel itself does not show anti-tumor effect above the background effect of surgery alone (Supplementary Figure S5c). These data demonstrate the utility of biomaterials for local application of combination immunotherapies, in the surgical context.

Discussion

In this study, we assessed local delivery of pairwise combinations of clinically advanced TLR agonists, poly(I:C), R848, and CpG, for

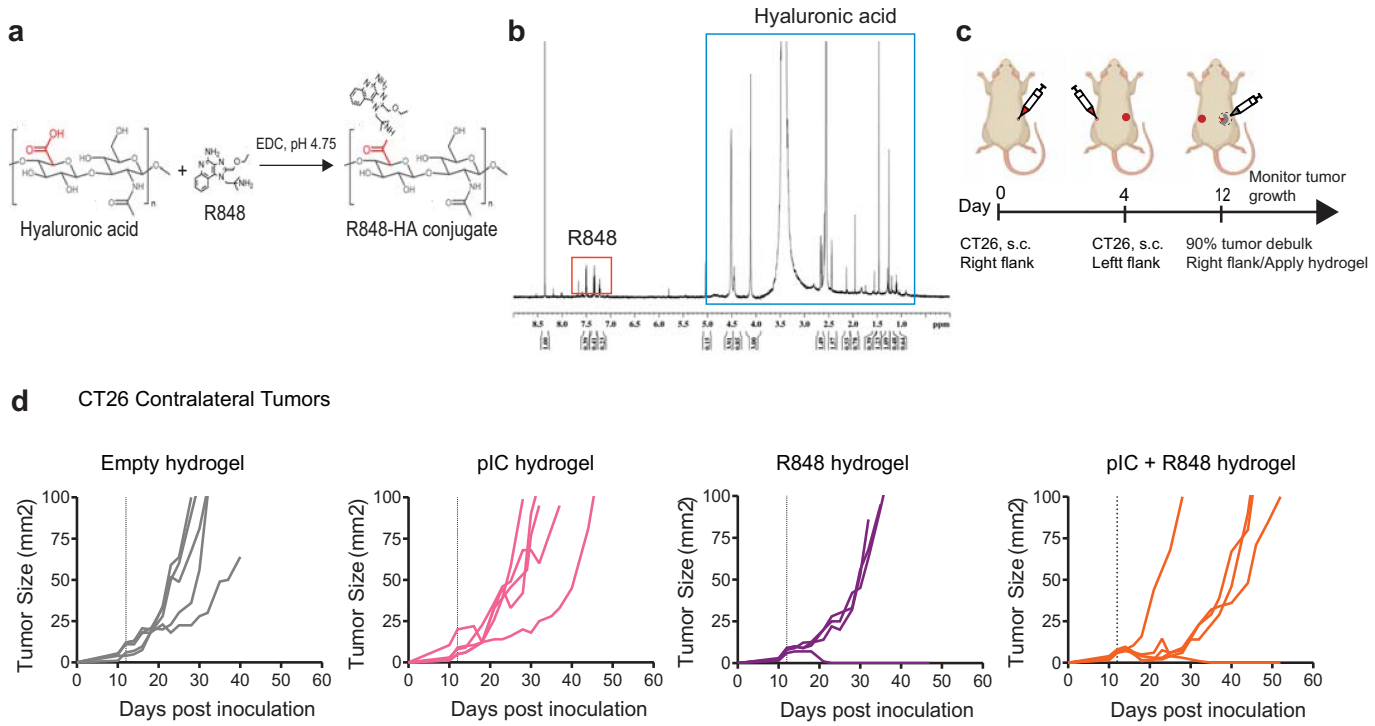

Figure 5. Intraoperative delivery of combination poly(I:C) and R848 using a biomaterial hydrogel delays distant tumor growth. (a) Strategy for conjugation of R848 to the HA polymer backbone. (b) ^{1}H NMR spectra of R848 conjugated HA. (c) A 90% tumor debulk was performed on the right flank tumor. 200 µl of either poly(I:C) and R848-HA hydrogel (250 µg poly(I:C), 9 µg R848), or poly(I:C) hydrogel (250 µg poly(I:C)), or R848-HA hydrogel (9 µg R848), or empty hydrogel was placed in the resection cavity. (d) Tumor growth curves for contralateral CT26 tumors. $n = 4$–5 mice per group.

their efficacy in controlling local and distant tumor growth. Therefore, we chose a setting where development of robust systemic anti-tumor immunity is required to control distant tumors, and models where contralateral tumors do not respond to single-agent TLR therapy. We demonstrate that local delivery of the TLR3 agonist poly(I:C) in combination with the TLR7/8 agonist R848 can slow the outgrowth of local and contralateral tumors and sensitize them to systemic ICB. A similar synergistic effect for combination of innate immune agonists was reported by Alvarez et al.[26] In this study, the authors demonstrate that a combination of a nanoplexed poly(I:C) and STING agonist, 5,6-dimethylxanthenone-4-acetic acid (DMXAA), resulted in local and distant anti-tumor effect against MC38 and B16.OVA tumors.

Our data demonstrate an increased number of inflammatory monocytes in tdLNs after treatment with R848, supporting the idea that specific immunotherapeutics can enhance the priming of systemic anti-tumor immunity, outside the local TME. R848 increased infiltration of myeloid cells including inflammatory monocytes, dendritic cells (DCs), and macrophages in tdLNs alone or in combination with poly(I:C). Additionally, we identified reduced PD-L1 expression on populations of antigen presenting cells in the contralateral tdLNs in both R848 and R848-poly(I:C) treated groups, which may improve T cell priming. However, expression of M1 and M2 macrophage markers CD86, Arg-1, and CD206 remained consistent across treatment groups at the timepoint analyzed and did not suggest a repolarization of macrophage subsets, at odds with a previous study demonstrating that a combination of poly(I:C) and R848 polarized macrophages toward an M1-like phenotype with improved anti-tumor efficacy.[14] Further in-depth phenotyping of the innate immune populations observed in this study may help to elucidate their role during local therapy and response to ICB.

We found that local low-dose R848-poly(I:C) combination could drive abscopal responses in untreated tumors on the contralateral flank as previously reported for this class of agents[27,28] and sensitize the TME to systemic anti-PD-1 therapy, in CT26 and M3–9-M tumor models. Similar results have been demonstrated with i.t. delivery of poly(I:C) + DMXAA in combination with systemic anti-PD-1 in the B16.OVA model.[26] However, there are challenges regarding the translation of STING agonists into the clinic, due to the high toxicity of these agents.[4] Preclinical studies do suggest that the addition of local therapy with innate agonists could open new patient populations to effective ICB therapy and enhance response rates in cancer types where ICB is already indicated.[29,30]

Local cancer immunotherapies currently require repeated i.t. injections, which can be invasive for patients and complex to administer. Therefore, we designed a biomaterial hydrogel to deliver poly(I:C) and R848 together during tumor debulking surgery. HA hydrogels are biomaterials that can be tuned for prolonged release of immunotherapies and specific degradation when applied intraoperatively.[17] However, delivering multiple agents in a single formulation presents challenges, as different therapeutics may require unique spatial and temporal dynamics. In this study, we chemically conjugated the small molecule R848 to the HA polymer using the carbodiimide chemistry,[31] which has been used to conjugate cytokines such as TGFβ and other small molecules.[32] With this conjugation strategy, we could only conjugate a relatively low dose of R848 (9 μg per 200 μL hydrogel) to the HA polymer. Nonetheless, our data show that this strategy can result in anti-tumor responses against unresected distant tumors, albeit with a moderate response. Further studies are required to optimize the delivery of nucleic acid and small-molecule drugs in the same biomaterial, including modification of biomaterials properties (e.g., pore size, viscosity, functional groups)[33] or the development of novel delivery strategies.[34]

This study demonstrates the potential of local immunotherapy with combinations of TLR agonists, to drive unique and differing effects on systemic anti-tumor immunity. Further, preclinical studies are required to fully elucidate the underlying mechanisms behind these responses. In addition, the utility of biomaterials for delivery of such agents into the TME remains to be explored. With clinical translation in mind, it will be critical to conduct comprehensive analyses of toxicology and pharmacokinetics using biomaterial delivery, similar to recent work by Zúñiga et al.[35] who assessed a slow-release depot formulation for prolonged delivery of a TLR agonist. Focusing on solid cancers, the advantage of biomaterials to deliver a prolonged release depot of innate receptor agonists raises the opportunity of using surgery as a window opportunity to target drugs more precisely to the TME.

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Disclosure statement

The authors declare a patent application related to the hydrogel biomaterial used in this work.

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Conceptualization, B.W, K.S.I., and W.J.L.; methodology, FX.R., T.W., H. Z., O.E., J.K., M.N., C.W.E., B.W., R.M.Z., K.S.I., W.J.L., and B.W; formal analysis, FX.R., B.W., O.E.; investigation, FX.R., T.W., H.Z., X.Y., M.L.O. M, J.S., and B.W.; writing – original draft, F.X.R., W.J.L., and B.W.; writing – review & editing, all authors; supervision, W.J.L. K.S.I., and B. W.; project administration, F.X.R., W.J.L., and B.W.; funding acquisition, W.J.L. and B.W.

Data availability statement

The data supporting this study are available within this manuscript and/or its supplementary materials.

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