ONCOIMMUNOLOGY
2023, VOL. 12, NO. 1, 2197370
https://doi.org/10.1080/2162402X.2023.2197370

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BRIEF REPORT

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Intratumoral neoadjuvant immunotherapy based on the BO-112 viral RNA mimetic

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ABSTRACT

BO-112 is a poly I:C-based viral mimetic that exerts anti-tumor efficacy when intratumorally delivered in mouse models. Intratumoral BO-112 synergizes in mice with systemic anti-PD-1 mAbs and this combination has attained efficacy in PD1-refractory melanoma patients. We sought to evaluate the anti-tumor efficacy of BO-112 pre-surgically applied in neoadjuvant settings to mouse models. We have observed that repeated intratumoral injections of BO-112 prior to surgical excision of the primary tumor significantly reduced tumor metastasis from orthotopically implanted 4T1-derived tumors and subcutaneous MC38-derived tumors in mice. Such effects were enhanced when combined with systemic anti-PD-1 mAb. The anti-tumor efficacy of this neoadjuvant immunotherapy approach depended on the presence of antigen-specific effector CD8 T cells and cDC1 antigen-presenting cells. Since BO-112 has been successful in phase-two clinical trials for metastatic melanoma, these results provide a strong rationale for translating this pre-surgical strategy into clinical settings, especially in combination with standard-of-care checkpoint inhibitors.

ARTICLE HISTORY
Received 6 March 2023
Revised 27 March 2023
Accepted 27 March 2023

KEYWORDS
BO-112; intratumoral immunotherapy; metastases; neoadjuvant; PD-1

Introduction

Preclinical and clinical studies are demonstrating the beneficial effects of the intratumoral delivery of immunotherapeutic agents with the idea of turning one of the metastatic tumor lesions into an in-situ vaccine capable of unleashing systemically efficacious immune responses against cancer[1,2]. However, the field of intratumoral immunotherapy is challenged by the results of two advanced melanoma phase-3 clinical trials failing to meet the survival endpoints when intratumorally using the recombinant herpes simplex virus-1 (HSV-1) viral vector Talimogene Laherparepvec (T-VEC) or the Toll-like receptor-9 (TLR9) agonist tilsotolimod in combinations with anti-PD-1 (pembrolizumab) or anti-CTLA-4 (ipilimumab) agents, respectively[3,4]. Hence, the field of intratumoral immunotherapeutics is in great need for similarly safe but more active substances.

Several lines of evidence have shown the advantages of neoadjuvant therapies for cancer, including the use of chemotherapy, radiotherapy, or hormone therapy[5]. Neoadjuvant immunotherapy is an approach pioneered in mouse studies by Michele Teng *et al.*[6], who prevented metastatic relapse from the 4T1 breast cancer model by combining neoadjuvant anti-PD-1 and anti-CD137 mAbs prior to surgery[6]. Sufficient evidence has accumulated to now make pre-surgical immunotherapy with checkpoint inhibitors the standard-of-care in resectable cases of melanoma[7,8], non-small cell lung cancer (NSCLC)[9,10] and MSI^{high} colon cancer[11,12]. However, local injection of immunotherapy agents has not yet been explored in the neoadjuvant setting for cancer patients, with the only exception of the herpes virus vector T-VEC[2,13].

BO-112 is a nanoplexed form of polyinosinic:polycytidylic acid (poly I:C) that aims to mimic viral particles loaded with double-stranded RNA of viral features[14]. This compound reportedly acts on TLR3, MDA5, and PKR[14–16]. BO-112 is active in the treatment of a variety of transplanted mouse tumors when given intratumorally in a manner dependent on anti-tumor immune responses[16]. In preclinical mouse models, its therapeutic efficacy can be synergistically enhanced by co-injection of a STING agonist[17], systemic delivery of checkpoint inhibitors[16,17], or radiotherapy[18].

This approach has been followed in the clinic for metastatic cancer cases bearing injection-amenable lesions in combination with anti-PD-1 agents[19]. The approach is safe and showed objective activity in cases refractory to checkpoint inhibitors[19]. Furthermore, evidence of a 28% overall response rate in PD-1 refractory melanoma cases has been recently reported upon combined treatment with intratumoral BO-112 + intravenous pembrolizumab[20].

The concept of intratumoral immunotherapy in the neoadjuvant settings is enticing and feasible[2], since the surgeon or

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Supplemental data for this article can be accessed online at https://doi.org/10.1080/2162402X.2023.2197370.

the interventional radiologist may inject immunotherapy agents into the primary tumor to be resected. The objective is to unleash an immune response able to deal with micrometastatic disease that might be lurking in the cases declared to be surgically resectable.

In this study, we provide evidence in mouse models for the efficacy of neoadjuvant intratumoral BO-112 to prevent metastatic relapse and for the involvement of CD8 T cell-responses in the beneficial effects.

Material and methods

Mice

Female or male C57BL/6 or female BALB/c mice were purchased from Harlan Laboratories (Barcelona, Spain). C57BL/6 Batf3tm1Kmm/J (BATF3 KO)[21] or wild-type counterparts were kindly provided by Dr. Kenneth M. Murphy (Washington University, St. Louis, MO) and bred at the CIMA animal facility. Mice were used at 8–12 weeks of age and housed under specific pathogen-free conditions. All animal protocols (E059–21 and its amendments) were approved by the Ethics Committee of Animal Experimentation at CIMA/University of Navarra.

Cell lines

C57BL/6-derived MC38 mouse colon carcinoma cell line was kindly gifted by Dr. Karl E. Hellström (University of Washington, Seattle, WA). BALB/c-derived 4T1 breast carcinoma cell line was originally provided by Dr. Claude Leclerc, (Institute Pasteur, Paris, France) and verified in the master cell bank at Institute Pasteur (Paris, France). These cells were authenticated by Idexx Radil (Case 6592–2012). These cells were grown in RPMI 1640 media supplemented with GlutaMAX™ (Gibco^{TM}), 10% heat-inactivated FBS, 50 µM 2-mercaptoethanol, 100 U/mL penicillin, and 100 µg/mL streptomycin at 37°C with 5% CO_2 (complete media). All cell lines were tested monthly for mycoplasma contamination (MycoAlert Mycoplasma Detection Kit, Lonza).

mCherry transfectant cell lines

When MC38 and 4T1 cells reached 80% confluence, cells were transfected with pCA665-mCherry expression plasmid, kindly given by Dr. Monsterrat Arrasate (CIMA, Pamplona), using lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. mCherry-positive cells were single cell-sorted in a MoFlo Astrios EQ cell sorter (Beckmann Coulter). Single-cell clones were expanded in culture using complete RPMI media.

MC38 neoadjuvant mouse tumor model

The MC38 neoadjuvant model was based on methods described by Aiken *et al.* for mouse melanoma[22]. 4×10^5 MC38 cells were subcutaneously injected in the ventral left size of C57BL/6 mice. On day 11, when the tumor reached 50–80 mm^3, and on day 14 post-tumor inoculation, 50 µg of BO-112 were intratumorally injected into the mice. Anti-PD-1 mAb (RPM1–14, BioXcell) was intraperitoneally given on days 12 and 14. Control mice received saline buffer supplemented with 5% glucose and/or rat IgG2a (BioXcell). On day +17, mice received intravenous injections of 5×10^5 mCherry + MC38 cells to sow liver metastasis. 24 h later, primary tumors were surgically excised. For the surgery, mice were anesthetized with 2.5% isoflurane and kept under anesthesia with 1% isoflurane. Buprenorphine was given 30 min previous surgery and every 12 h after that during the first two-three days post-surgery to control pain according to ECAE guidelines. In addition, mouse well-being was monitored every 2–3 days and animals were sacrificed when prespecified symptoms of illness appeared. On day+42, livers were surgically collected.

For depletion studies, mice received intraperitoneal injections of 100 µg anti-CD8β (clone Lyt 3,2, BioXcell) mAb on days nine and ten post-tumor inoculation, followed by weekly intraperitoneal injections until the end of the experiment to deplete endogenous CD8+ T cells without altering the CD8αα+ DC splenic compartment. Control mice received rat IgG (BioXcell) injections. To evaluate the role of cDC1, $BATF3^{-/-}$ mice or their corresponding counterparts were used.

4T1 neoadjuvant tumor model

The model to spontaneously produce lung metastasis and evaluate the anti-metastatic effects of intratumoral BO-112 in neoadjuvant settings was based on previous publications[6,23]. Briefly, 5×10^4 of mCherry-4T1 cells were orthotopically injected into the fourth left mammary fat pad of 8-week-old female BALB/c mice. 50 µg of BO-112 were intratumorally or subcutaneosly injected on days seven and ten post-tumor inoculation when tumors had reached approximately 50–80 mm^3. In some groups, mice received intraperitoneal injections of anti-PD-1 mAb (RPM1–14, BioXcell) on days eight and ten post-tumor inoculation. As a control, mice received saline buffer supplemented with 5% glucose and/or rat IgG2a (BioXcell). On day+14, primary tumors were surgically excised. For the surgery, mice were kept under anesthesia as explained above. The pain was controlled by Buprenorphine as stated above and mice were monitored for the onset of illness according to ECAE guidelines. Mice were sacrificed on day+39 to collect lungs for examination. In some experiments, daily intraperitoneal injections of 50 µg of the sphingosine 1-phosphate (S1P) receptor inhibitor FTY720 (Sigma-Aldrich) or PBS were given during the duration of the experiment to prevent T-cell recirculation from lymph nodes (LNs).

Quantitative analysis of liver and lung metastasis

mCherry-MC38 liver or mCherry-4T1 lung metastases were evaluated by epi-fluorescence microscopy (Zeiss LSM 880 NLO). At least three representative fluorescence images were acquired at 10× magnification from each individual mCherry+ tumor-metastasized organ. The percentage of surface area from the organs occupied by the metastasized tumor was assessed with Fiji image software[24]. The mean of metastasis area was estimated for each individual organ.

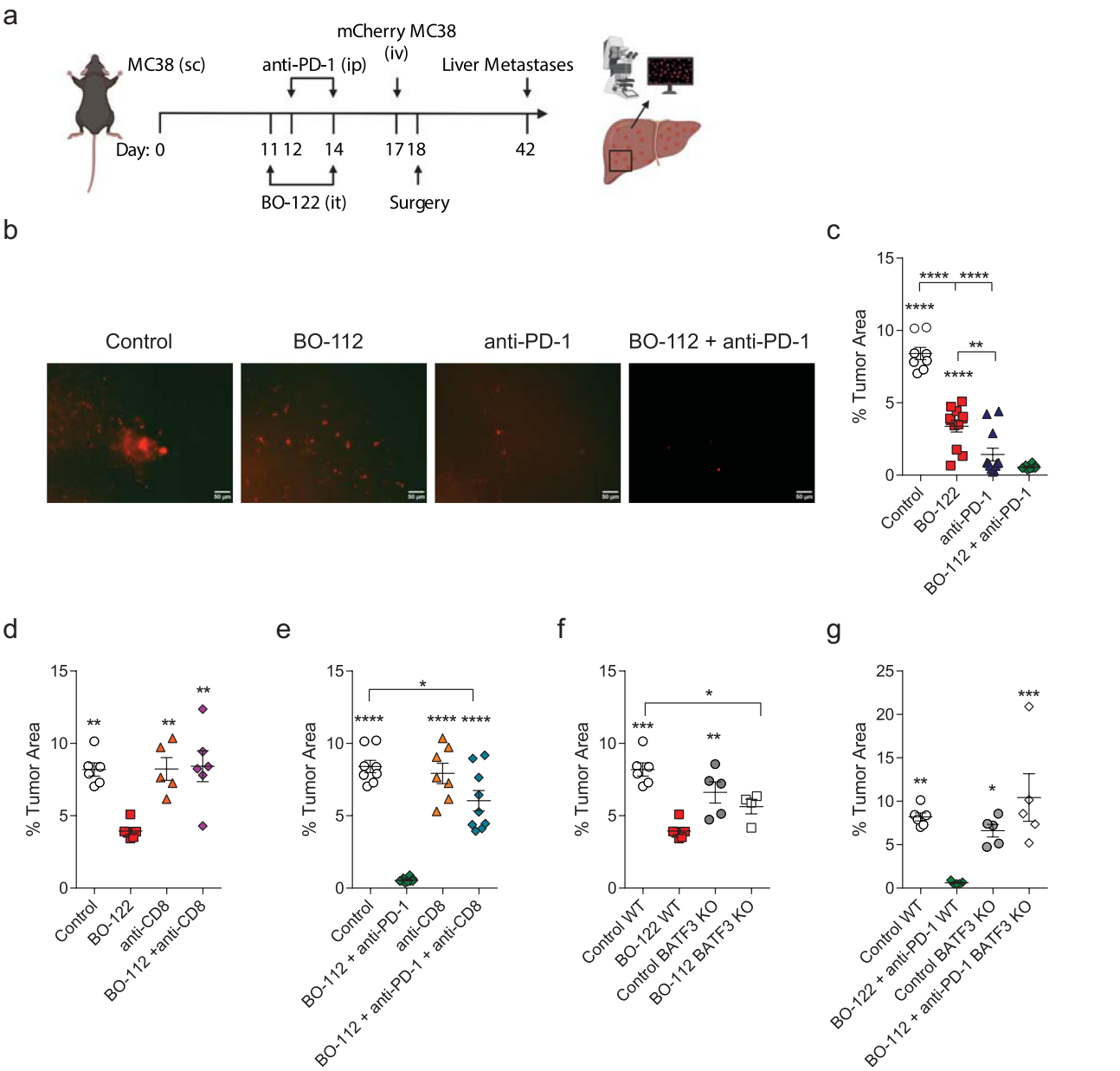

Figure 1. Anti-metastatic effects of intratumoral BO-112 + systemic anti-PD-1 mAbs in the MC38 colon cancer model. (A) Representation of the time-course of experiments in which MC38 was inoculated subcutaneously into syngeneic C57BL/6 mice treated intratumorally with BO-112 or saline as indicated and/or anti-PD-1 mAb given intraperitoneally. On day+17 mCherry MC38 stably transfected cells were given intravenously to induce spread of the disease to the liver. (B) Representative images of the surface of the liver under UV light showing fluorescent metastases. (C) Quantification of the percentage of the surface organ area covered with metastases in the indicated groups. (D-E) Similar experiments depleting CD8β T cells as indicated. (F-G) Experiments in wild-type mice as compared to BATF3$^{-/-}$ mice in the indicated treatment groups. The results are pooled together two similarly performed independent experiments with six mice per group in each condition (mean ± SEM). Statistical comparisons were performed with One-Way ANOVA tests. Significant differences are displayed for comparisons of each group with the BO-112 or BO-112 + anti-PD-1 groups (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).

Flow cytometry analysis of the tumor microenvironment

For analysis of the immune cell component within the excised primary tumors, MC38 and 4T1 tumors were collected on days +18 and+14 post-tumor inoculation, respectively. Tumors were mechanically disrupted, and single-cell suspensions were generated as previously described[16]. The cell suspensions were stained with mAbs to identify the CD8 T-cell and the cDC1-cell compartments following the gating strategy previously described[17]. In addition, CD8 T cells were also analyzed for tumor specificity using MHC-I pentamers loaded with dominant epitopes for H-2K^b or H-2 L^d corresponding to the endogenous retroviral antigen gp70 that is expressed both in MC38 (MHC pentamer H-2K^b KSPWFTTL, Proimmune) and 4T1 (MHC pentamer H-2 L^d SPSYVYHQF, Proimmune) tumor cells[16]. For a detailed description of the mAbs used, see supplementary Table 1.

Statistical Analysis

Each experiment was performed at least twice using 6 to 12 mice per group. One-way ANOVA tests with Tukey posttest analysis were used to determine statistical significance

(GraphPad Prism 6, La Jolla, CA). Findings were considered statistically significant when $p < 0.05$.

Results

To study the potential application of intratumoral BO-112 in neoadjuvant immunotherapy settings, we first studied a subcutaneous MC38-derived colon carcinoma model and intravenous injection of tumor cells to form liver metastasis. As described in Figure 1a, intratumoral treatment consisted of two injections of BO-112 given to established subcutaneous tumor nodules. In some experimental groups, anti-PD-1 mAb was systemically administered. To model hematogenous dissemination, intravenous MC38 cells expressing mCherry as a reporter gene were infused via the tail vein. Surgery completely resecting the subcutaneous tumors was performed on day +18 and liver metastases on the surface of excised livers were assessed on day+42 (Figure 1a). Representative images of the surface of the livers corresponding to each experimental treatment are shown in Figure 1b and quantitative compiled data are shown in Figure 1c. As can be seen, both intratumoral BO-112 and systemic anti-PD-1 mAb reduced metastatic liver burden, while the combination of the two treatments almost completely cleared the observable metastases (Figure 1b-c). The effect of BO-112 or the BO-112 plus anti-PD-1 mAb combination was lost upon depletion of CD8β+ T lymphocytes (Figure 1d-e). Moreover, when the experiments were performed in cDC1-deficient BATF3$^{-/-}$ mice, the benefit of BO-112 or BO-112 plus anti-PD-1 mAb treatments was also diminished (Figure 1f-g).

The classical model of spontaneous metastasis is the orthotopic inoculation of 4T1 triple-negative breast cancer cells in the mammary glands of female BALB/c mice from which successful tumor metastases reach the lungs[6,23]. In these experimental settings, the original observations on neoadjuvant immunotherapy were made[6]. As depicted in Figure 2a, experiments involved BO-112 intratumoral injections of the orthotopic tumor on day+7 after tumor-cell inoculation and systemic intraperitoneal anti-PD-1 to some of the groups. In this case, tumor cells also expressed mCherry and the ensuing metastasis on the surface of the lungs could be quantified. Figure 2b shows representative lung images used to quantify results in Figure 2c. Again, both intratumoral BO-112 and anti-PD-1 mAb given prior to surgery reduced metastases, but it was the combination treatment that achieved a dramatic reduction in the number of observable metastases (Figure 2bc).

Trafficking of T cells is presumably required to attain systemic control of the nascent metastases. In this regard, inhibition of recirculation from LNs with the sphingosine

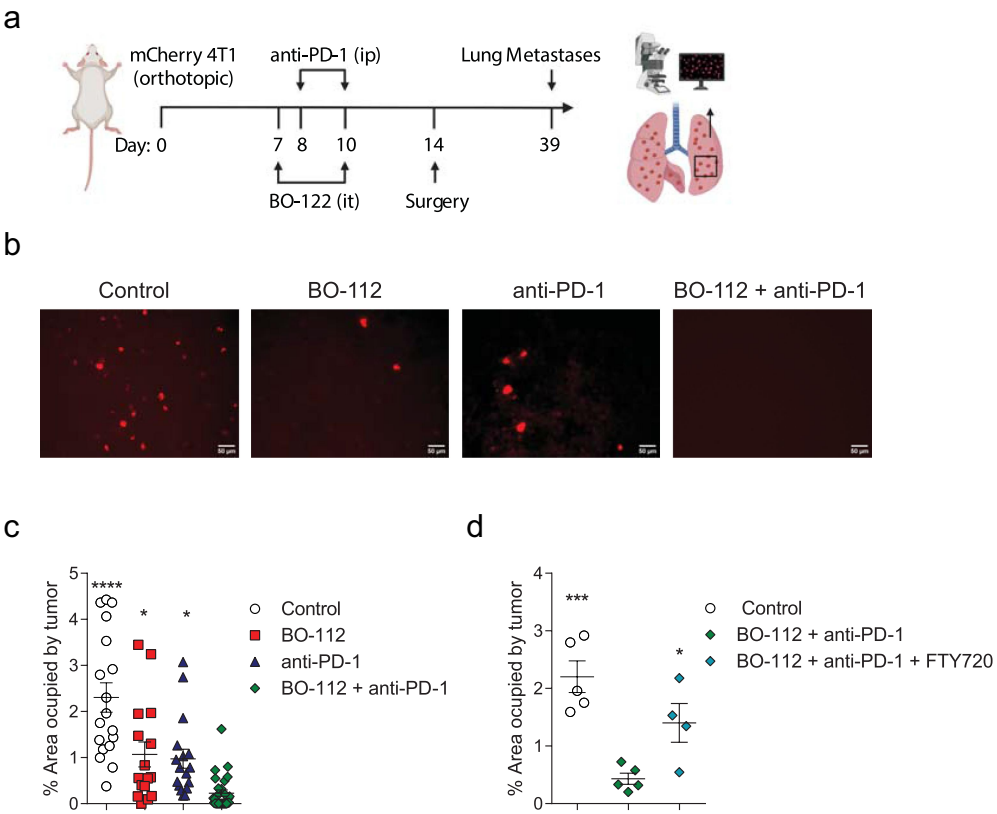

Figure 2. Neoadjuvant treatment using intratumoral BO-112 and systemic anti-PD-1 blockade against orthotopic 4T1-derived breast carcinomas. (A) Scheme of the experiments implanting in one of the mammary glands of female BALB/c mice the stably transfected 4T1 cell line to express mCherry fluorescent protein. Treatments were given as indicated to the corresponding groups and spontaneous lung metastases were evaluated at the end of the experiment. (B) Representative images of the surface of the excised lungs from the indicated treatment groups. (C) Quantitative data with 16 to 29 mice per group pooled from three similarly performed experiments. (D) Shows an experiment in which mice treated intratumorally with BO-112 and systemically with anti-PD-1 mAb received daily injections of FTY720 as indicated. Data in B and C represent pooled findings from three independent experiments with six to 12 mice per group (mean ± SEM). Data in C represent an independent experiment with six mice per group (mean ± SEM). One-Way ANOVA tests were used to assess statistical significance. Significant differences are displayed for comparisons of each group with the BO-112 + anti-PD-1 group (*p < 0.05, ***p < 0.001, ****p < 0.0001).

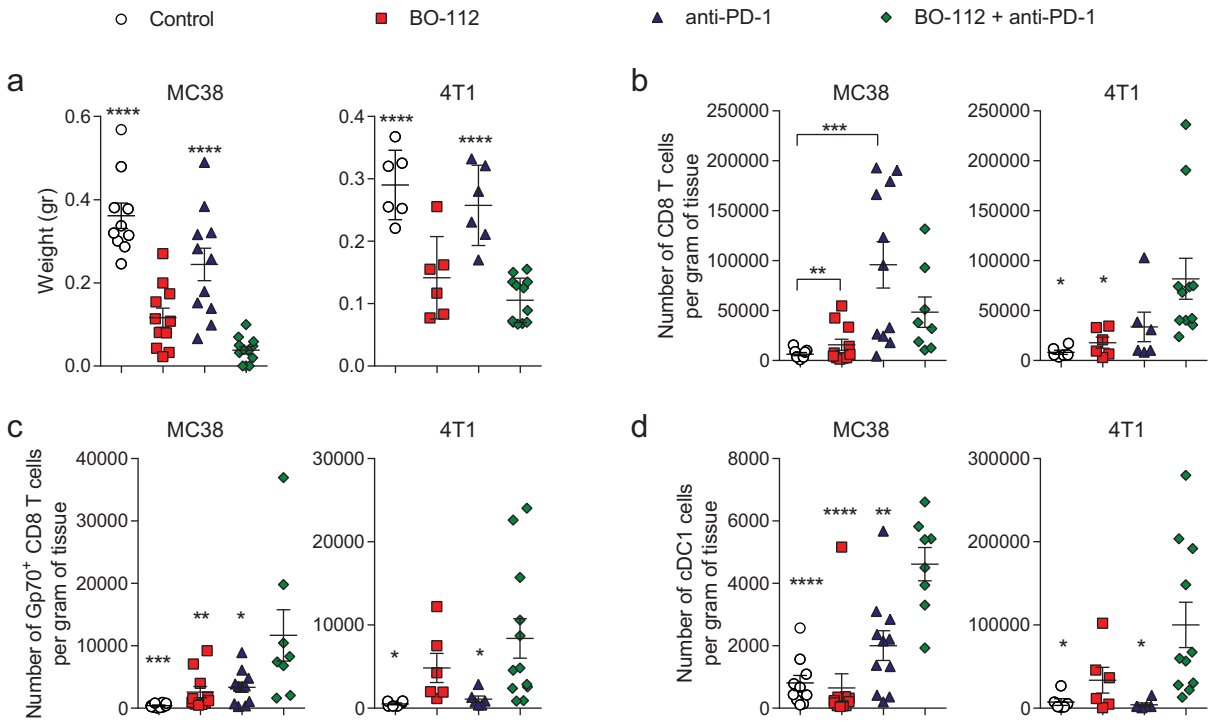

Figure 3. Changes in the cellular composition of the tumor microenvironment of the primary tumors upon neoadjuvant treatment. Note: (A) Weight of excised primary tumors treated as in Figure 1a and Figure 2a from mice bearing either MC38 (left panel) or 4T1 (right panel) as indicated. (B) Density of CD8+ T cells (CD45+CD19$^-$F4/80$^-$TCRβ+CD4$^-$CD8+) inside such tumors, as assessed by flow cytometry on single-cell suspensions derived from the tumors following the indicated treatments. (C) Density of gp70-specific CD8+ T cells by multimer MHC-I staining in each tumor model following the indicated treatments. Of note, different multimers and gp70 epitopes for H-2K^b and H-2 L^d were used to recognize specific CD8 T cells in C57BL/6 and BALB/c backgrounds respectively. (D) Density of cDC1 cells (CD45+CD19$^-$F4/80$^-$TCRβ$^-$MHCII+CD11c+CD11b$^-$) in the indicated excised tumors. For the MC38 model, data represents pooled findings from two independent experiments with 5 to 6 mice per group (mean ± SEM). For the 4T1 model, data are representative of four independent experiments with 5 to 12 mice per group (mean ± SEM). Statistical comparisons were performed with One-Way ANOVA Tests. Significant differences are displayed for comparisons of each group with the BO-112 + anti-PD-1 group (*p < 0.05, ***p < 0.001, ****p < 0.0001).

1-phosphate (S1P) receptor inhibitor FTY720 reduced the therapeutic effects against 4T1 lung metastasis (Figure 2d).

Next, experiments were carried out in the MC38 and 4T1 models to study immune effects in the microenvironment of the primary tumor that may account for the beneficial effects against metastases since this was sensitive to CD8 depletion and cDC1 absence. In terms of weight of the tumors, these experiments recapitulated a partial effect of intralesional BO-112 injections and of anti-PD-1 systemic blockade, while the combination drastically reduced tumor weight in both MC38 and 4T1 primary tumors (Figure 3a). Our previous studies had shown increases in the number of CD8 T cells infiltrating the tumors[16,17]. As shown in Figure 3b, such increases took place in both tumor models and the combined treatment gave rise to synergistic increases. Indeed, only combined treatment meaningfully enhanced the CD8 T-cell content in the 4T1 neoadjuvant model (Figure 3b). Part of those CD8 T lymphocytes were tumor-specific since their TCRs were recognized by MHC-I pentamers with dominant epitopes for the endogenous retroviral antigen gp70 that is shared by MC38 and 4T1 (Figure 3c).

Given that there is synergy in inducing CD8 T cell-responses in the tumors, we studied the numbers of cDC1 dendritic cells in the tumor microenvironment (Figure 3d) because such dendritic-cell subpopulation, that is deficient in BATF3$^{-/-}$ mice, is almost exclusive in its ability to cross-present tumor antigens to CD8 T lymphocytes[25].

In some instances with visceral metastases, intratumoral delivery can be cumbersome. Hence, we assessed whether BO-112 could be given subcutaneously instead of intratumorally. Of note, subcutaneous injections were performed in the center of the back of the animals while the tumors were located in the mammary gland. When comparing such treatment conditions, as in the experiments in Figure 2, we found that the number of assessed metastases were quite similar in subcutaneously and intratumorally treated mice (Supplementary Fig. S1A-B). However, the therapeutic effect on the growth of the primary tumors until their surgical removal was clearly better in the intratumorally treated group (Supplementary Fig. S1C). This is in spite of the fact that the mice treated intratumorally and subcutaneously showed similar counts of CD8+ T cells in their excised primary lesions (Supplementary Fig. S1D). Nonetheless, in the cases of intratumoral delivery of BO-112, more intratumoral CD8+ T lymphocytes were specific for the gp70 antigen (Supplementary Fig. S1E). Noticeably, cDC1 numbers were similarly increased by both routes of BO-122 administration (Supplementary Fig. S1F).

Discussion

Our experiments provide data supporting the use of an RNA viral mimetic given intratumorally for the neoadjuvant treatment of resectable cancers. The experimental evidence in the 4T1 spontaneously metastatic model is especially relevant and reminiscent of the original observations on systemic neoadjuvant immunotherapy[6]. The concept of intratumoral delivery might be especially appropriate for early-stage resectable tumors, since the goal in such cases is to temporally use the primary tumor as an in-situ vaccine expressing all the relevant tumor antigens[1,2]. Additionally, often between diagnoses and surgeries there is a waiting time that can be exploited to test neoadjuvant approaches once the patient is staged as resectable.

The involved train of effects starts with the priming of CD8 T cells by cDC1 cells. BO-112 could be enhancing cDC1-cell numbers and function, and setting the adequate cytokine milieu for a convenient reshaping of the tumor immune microenvironment. The contribution of the tumor-draining LNs as previously observed remains to be elucidated[23]. In any case, PD-1 blockade is probably facilitating both priming and eradication of nascent micrometastases in the target organs. These phenomena most likely underlie the observed synergy between intratumoral BO-112 and systemic PD-1 blockade.

With regard to the clinical application of the strategy, we already know that the combination of intratumoral BO-112 and intravenous anti-PD-1 is well tolerated in patients[19,20]. In those studies, heavily metastatic cases were treated, and bulky disease and multiple immune evasion mechanisms probably hampered the efficacy of the treatment. In the case of primary tumors at high risk of post-surgical relapse, the strategy might be especially efficacious.

At present, intratumoral injections of BO-112 are being tested to improve radiotherapy results for oligometastatic diseases in NSCLC patients treated concurrently with nivolumab cycles (NCT05265650) in a scheme based on experimental results in mice[18]. For neoadjuvant development, some solid malignant diseases might be more adequate due to accessibility for injection, such as breast cancer, melanoma, squamous skin cancer and resectable hepatocellular carcinoma cases[1]. In these types of neoadjuvant trials, availability of the surgical specimen will be most suitable to observe differences in the tumor microenvironment composition as those reported here in mouse tumors and such changes may perhaps predict outcome. However, in the mouse experiments, we do not observe such correlations in a clear way. Considering that cross-priming is involved, other co-treatments eliciting immunogenic cell death[26] or further enhancing cDC1 functions[17] will be tested in triple combinations.

For clinical development, more aspects need to be considered. Repeated injections could be risky and inconvenient. Hence, some of the BO-112 administrations can be performed subcutaneously since this route of administration also causes beneficial effects. There is also a theoretical risk of disease dissemination in the tract of the needle that needs to be mitigated. Improvements in delayed pharmaceutical formulations of BO-112 could be envisaged for these purposes. The involvement of LNs also needs to be carefully considered[23], since the surgical procedures should either include or not regional lymphadenectomies[5]. Indeed, there is already clinical data supporting that tumor-draining LNs are important for the efficacy at depleting Tregs of local injections of the anti-CTLA-4 mAb ipilimumab in cases of resectable melanoma[27].

The multiple elements needed for neoadjuvant clinical trial development of intratumoral BO-112 are already in place. Defining the optimal schedules and the combinations with standard-of-care neoadjuvant regimes, including checkpoint inhibitors, require study in the clinical arena.

Acknowledgments

We would like to acknowledge both CIMA/University of Navarra shared flow cytometry (Diego Alignani), image platform and animal facilities (Eneko Elizalde and Elena Ciordia) for their technical support. Professional English editing by Dr. Paul Miller is also acknowledged. Finally, we wish to thank all the members of Dr. Melero's and Dr. Berraondo's groups for the continuous support and helpful discussions.

Disclosure statement

CM, SG, MER, GG, AC, IO, JG, JG, CL AA, EB, AT, PB declare no competing interests. MA declares receiving a commercial research grant from Highlight Therapeutics. MCO reports receiving a commercial research grant from AstraZeneca. MQ is full-time employee of Highlight Therapeutics. IM reports receiving commercial research grants from AstraZeneca, BMS, Highlight Therapeutics, Alligator, Pfizer Genmab and Roche; has received speakers bureau honoraria from MSD; and is a consultant or advisory board member for BMS, Roche, AstraZeneca, Genmab, Pharmamar, F-Star, Bridget Peak, BioNtech, Bioncotech, Bayer, Numab, Pieris, Gossamer, Alligator and Merck Serono. IM has received consultancy fees from Highlight therapeutics.

Funding

This work was supported by the PID2020-112892RB-100 funded by MCIN/AEI/10.13039/501100011033, PDC2021-121769-C21 grant funded by MCIN/AEI/10.13039/501100011033 and The European Union Next GenerationEU/PRTR, Instituto de Salud Carlos III (PI22/00147) co-financed by Fondos Feder, the Fundación La Caixa (HR21-0083), the AACR-AstraZeneca career development award for physician-scientist in honor of Jose Baselga and the Fundació La Marató TV3 (488/C/2019). MA was supported by a Spanish Association Against Cancer's Investigator grant (INVES19041ALVA) and currently receives "Ayudas Ramon y Cajal" (RYC2021-033381) from the MCIN/AEI/10.13039/501100011033.

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