Abstract—Checkpoint inhibitor (CI) immunotherapy is playing an increasingly prominent role in the treatment of cancer but is effective and durable in only a subset of patients. There are concerted efforts to improve CI therapy through the use of multiple CIs or use of CIs in combination with other anti-cancer agents. Here we investigate the use of “anti-vascular” ultrasound-stimulated microbubble (USMB) treatments in combination with anti-PD-1 CI therapy. The colorectal cancer cell line CT26 was used to conduct longitudinal growth studies along with acute experiments to assess ultrasound-induced anti-tumor immune responses using flow cytometry and enzyme-linked immunospot (ELISPOT) analysis. Longitudinal experiments indicated that USMB + anti-PD-1 treatments significantly enhanced tumor growth inhibition and animal survival relative to monotherapies. Flow cytometry and ELISPOT data did not clearly support a T cell-dependent mechanism for the enhancement. Therefore, the results indicate the ability of anti-vascular USMBs to increase the anti-tumor effects of CI therapy; the specific mechanisms of enhancement remain to be established. (E-mail: goertz@sri.utoronto) © 2018 Published by Elsevier Inc. on behalf of World Federation for Ultrasound in Medicine & Biology.

Key Words: Anti-PD-1, Immunotherapy, CT26, Ultrasound, Microbubbles.
with immunotherapies has been reported (Vanpouille-Box et al. 2015; Victor et al. 2015). Yet the immune response to ablative therapies such as radiotherapy has been found to be both tumor stimulating and tumor suppressive, and the manner in which these therapies can be rationally combined with immunotherapies is the subject of active investigation (Formenti and Demaria 2013; Shaked 2016).

Therapeutic ultrasound can stimulate a spectrum of effects that are potentially relevant to immunotherapy (Unga and Hashida 2014). One path is to promote the local release or uptake of immunotherapies agents. A second is to exploit ultrasound to induce immunologically relevant bio-effects through thermal or mechanical mechanisms. Ablative therapy employs high-intensity focused ultrasound (HIFU) to thermally coagulate tissue and is well known to provoke both innate and adaptive immune responses (Deng et al. 2010; Unga and Hashida 2014; Wu et al. 2004; Xia et al. 2012). Pre-clinical and clinical studies have reported that ablative HIFU treatments can enhance the immune system through increases in tumor-infiltrating immune cells, T-cell activity (Xia et al. 2012), dendritic cell (DC) activation (Deng et al. 2010) and the upregulation of heat shock proteins (Hu et al. 2005; Hundt et al. 2007). In addition, there is evidence that systemic effects can be produced (Hu et al. 2007), which has implications for the treatment of metastatic disease. The mechanical liquefaction of tissue through the use of cavitation initiated by very high pressures, a process known as histotripsy, offers another potential avenue to promote immune effects. In this case, Hu et al. (2007) hypothesized that this may facilitate the exposure of DCs to tumor antigens. Initial pre-clinical studies have indicated that this can result in tumor growth inhibition and induce a systemic T cell-mediated immune response (Hu et al. 2007). There is also pre-clinical evidence that relatively low ultrasound intensities coupled with systemically circulating encapsulated microbubbles can elicit an immune response. In Hunt et al. (2015) and Liu et al. (2012), elevated levels of tumor-infiltrating leukocytes were observed after exposure of subcutaneously implanted murine tumors. In addition, Liu et al. (2012) indicated an increase in the ratio of T-cyt cells to (inhibitory) regulatory T (T-reg) cells after treatments.

The use of ultrasound-induced bio-effects in conjunction with conventional immunotherapy approaches remains largely unexplored. An important first report on ultrasound and a checkpoint inhibitor recently published by Silvestrini et al. (2017) indicates that ablative focused ultrasound could be used in combination with aPD-1 to induce an abscopal anti-tumor effect. In the study described here, we investigated the combination of a checkpoint inhibitor (aPD-1 antibody) with low-intensity ultrasound and microbubble (USMB) treatment. In particular, we employ an exposure regime that we have previously established to elicit pronounced vascular damage which can profoundly enhance the anti-tumor effects of chemotherapy (Goertz et al. 2012; Todorova et al. 2013). Longitudinal experiments were carried out to assess the impact of combination therapy on tumor growth inhibition. These data are supplemented by acute experiments that are conducted to investigate the impact of treatments on key T-cell populations with a view toward gaining relevant mechanistic insight into the interactions of USMBs with aPD-1 therapy.

METHODS

Mice and tumors

The murine colon cell carcinoma cell line CT26.wt (CT26) was used (American Type Culture Collection, Manassas, VA, USA). This is a widely employed model for immunotherapy studies, and CT26 tumors have been reported to express PD-L1 (Dovedi et al. 2014; Kleinvink et al. 2017; Tian et al. 2016). For in vitro cell culturing, CT26 cells were grown in RPMI-1640 supplemented with 10% fetal bovine serum (Wisent, St-Jean, QC, Canada) at 37°C and 5% CO2. CT26 tumors were established in vivo by injecting cells subcutaneously (sc) into the right hindlimb (50 × 10⁵ of 1 × 10⁵ cells in phosphate-buffered saline [PBS]) of female Balb/c mice (Jackson Laboratory, Bar Harbor, ME, USA) 8–12 wks in age. All animal-related procedures were approved by the Institutional Animal Care Committee of Sunnybrook Research Institute.

Ultrasound treatments

The exposure apparatus and approach have been described previously (Goertz et al. 2012; Todorova et al. 2013). Briefly, mice were placed under anesthesia by subcutaneous injections of 0.1 mL/10 g of a 4:1 ratio of a ketamine–HCl (100 mg/mL) and (20 mg/mL) xylazine–HCl. The mice were then tail vein catheterized (26G indwelling), mounted on a stage and placed vertically into a 35°C water tank reaching to the shoulder region. The upper surface of the tumor was exposed to therapeutic US with a spherically focused 1-MHz transducer (3.75-cm diameter, 15-cm focal length, 1.05 cm–6 dB beam width at focal) (Valpy Fisher, Hopkinton, MA, USA). The sonication scheme employed was as follows: 50 0.1-ms-long pulses spaced 1 ms apart, which were repeated at 20-s intervals for a duration of 2 mins. Peak negative pressures at the focus were 1.65 MPa, as measured with a calibrated 0.2-mm needle hydrophone (Onda, Sunnyvale, CA, USA). For experimental groups receiving ultrasound exposures, the US pulsing sequenced commenced 10 s after the intravenous
injection of a 50-μL mixture of diluted MBs followed by
the 100-μL saline flush. This procedure was repeated
once after a 10-mins interval. Cavitation levels were
assessed in a subset of mice (n = 2) using a focused 0.75-
MHz transducer (2.5-cm diameter, 7.5-cm focal length)
(Valpy Fisher) with its focal zone situated on the tumors,
overlapping the therapy transducer focus (Goertz et al.
2012; Todorova et al. 2013). Received signals were
digitized (50-MHz sampling rate; Alazartech, Montreal,
QC, Canada) and the average spectra were calculated for
both baseline (pre-injection) and post-injection cases. In
particular, the spectral content was calculated for the first
exposure sequence, when the circulating agent concen-
tration was highest. In particular, the displayed power
spectrum was averaged over the first 10 bursts within the
sequence.

Ultrasound monitoring
Ultrasound contrast imaging was performed during
the course of exposures as a means of monitoring the
impact of exposures on tumor perfusion. An L12-5 trans-
ducer was employed with an EPIQ 7 system (Philips,
Amsterdam, Netherlands) operating in contrast mode at
a low mechanical index (0.07) and a frame rate of
11 Hz. The resulting data were then analyzed using Phi-
lips proprietary software (QLab Version 2.0). A region
of interest was drawn to encompass the tumor, and the
contrast intensity was plotted against time for each bolus
injection of MBs. Acquisitions were conducted for a
subset of acute group mice in which a third injection was
also administered to assess the cumulative effect of the
preceding two injections associated with treatment expo-
sures. The metric for the impact on perfusion was taken
as the ratio of the peak enhancement for the third/first
injections.

Tumor size measurements were made using volu-
metric ultrasound imaging and/or calipers. Three-dimen-
sional ultrasound (3-DUS) images of the tumors were
acquired using a high-frequency (25 MHz) Vevo770
ultrasound system (Visual Sonics, Toronto, ON, Can-
da). Tumor volumes were then calculated using the
Vevo770 (Version 3.0) software. All tumors (acute or
longitudinal) underwent 3-DUS measurements to estab-
lish sizing for entry into the study. For the longitudinal
study, tumors underwent 3-DUS measurements every 3
d until a size-dependent endpoint was reached.

Overview of experiments
Experiments were initiated when tumor volumes were
measured to be in the range 50–100 mm$^3$ (day 0). At this
point mice were randomly entered into one of four treat-
ment groups: MBs (a sham/control group), aPD-1, USMBs
and USMBs + aPD-1. All mice underwent identical proce-
dures with respect to anesthetization, catheterization, time
spent in the water (~20 mins) and the injection of MBs.
Each group of mice received their respective treatments as
outlined below and either were followed longitudinally or
were killed for acute experiments at appropriate time
points. A schematic overview of the acute and longitudi-
ual experiments is provided in Figure 1a and b, respectively.

Acute experiments were carried out to assess the
immune cell status of tumors either 3 or 7 d after the ini-
tial treatment day (n = 52 animals). At 3 d, the animals
received a single USMB or MB treatment with or with-
out aPD-1 at day 0. At 7 d, the animals received a single
USMB or MB treatment with or without aPD-1 at days
0, 3 and 6. At the endpoint, the tumors were harvested
and bisected, with half used for histology and the other
half used for flow cytometry analysis. Tumor-draining
lymph nodes (TDLNs), identified as the inguinal lymph
nodes (Harrell et al. 2008; Kwon and Sevick-Muraca
2010), were excised to undergo enzyme-linked immuno-
spot (ELISPOP) analysis. For flow cytometry, the fol-
lowing immune cells were assessed: tumor-infiltrating
leukocytes (TILs); cytotoxic T (T-cyt) cells, a primary
cell type engage in tumor cell killing; helper T (T-help)
cells, which facilitate tumor cell killing; and regulatory
T (T-reg) cells, which act to inhibit the activity of T-cyt
cells.

Longitudinal experiments were carried out to assess
the impact of treatments on tumor growth and mouse
survival (n = 22 animals). Tumor growth data were nor-
malized by expressing the data as a percentage increase
relative to the entry volume at day 0. Mice were catego-
rized as reaching their survival endpoint when the tumor
exceeded 1000 mm$^3$ in size, consistent with institutional
ethical requirements.

Drug and microbubbles
The immunotherapy agent used in this study was
anti-mouse aPD-1 (clone: RMP1-14, Bioxcell), which
was administered intraperitoneally at a dosage of 200 μg
0.5 h before USMB or MB treatment and then subse-
quently every 3 d for a total of five doses. It was found
that the isotype control (clone: 2A3, Bioxcell) for anti-
mouse aPD-1 did not significantly alter CT26 tumor
growth compared with control mice (i.e., devoid of iso-
type control) (n = 5) (data not shown). The MBs used in
this study were an experimental agent obtained from
Artenga Inc. (Ottawa, ON, Canada). The MBs consist of
octafluoropropane gas cores encapsulated by pegylated
phospholipid shells. These MBs have number- and vol-
ume-weighted mean diameters of 1.1 and 3.7 μm,
respectively, based on Coulter counter measurements in
the size range 0.7–20 μm. The MBs were administered
in bolus form in a 50-μL injection corresponding to a
dose of 9.6 × 10$^8$ MBs/kg.
Flow cytometry analysis of tumors

To generate a single-cell suspension from tumors for the acute experiments (day 3 or 7), tumors were diced and then incubated with 7 mL of digestion buffer at 37˚C for 1 h. The digestion buffer consisted of 1 mg/mL collagenase type IV (C5138; Sigma-Aldrich, Oakville, ON, Canada), 0.2 mg/mL DNase 1 (DN25; Sigma-Aldrich) and 10% fetal bovine serum (Wisent) in RPMI-1640 medium. After digestion, the cells were passed through a 70-μm nylon mesh filter and centrifuged at 1000 rpm for 10 mins. Cell pellets were re-suspended in RPMI-1640 medium and filtered for a second time through a 70-μm nylon mesh filter. The resulting suspensions then underwent flow cytometry analysis.

For cell surface staining, the following antibodies and reagents were used: Live/Dead Fixable Aqua stain (ThermoFisher Scientific, Eugene, OR, USA), anti-CD16/32 (2.4 G2; BD Biosciences, Mississauga, ON, Canada), anti-CD45-FITC (30-F11, BD Biosciences), anti-CD4-PE (RM4-5; BD Biosciences), anti-CD8-(PE-CF594) (53-6.7, BD Biosciences) and Stain Buffer (BD Biosciences). For intracellular staining, the following antibodies and reagents were used: Foxp3/Transcription Factor Staining Buffer Set (ThermoFisher Scientific), anti-Foxp3-APC (FJK-16s, Thermofisher Scientific) and rat IgG2a K-isotype control APC (eBR2a; Thermofisher Scientific). To discern distinct T-cell populations within the tumor, 1 × 10⁶ cells from the tumor digestion isolate were used per sample for antibody labeling. Cells were stained with the Live/Dead Fixable Aqua stain as per the manufacturer’s instructions for 30 mins. Cells were then washed and incubated in anti-CD16/32 antibody for 5 mins, followed by a PBS wash and then incubated with the appropriate cell surface antibodies at 4˚C for 20 mins in the dark. Unbound antibodies were removed by washing with stain buffer and then fixed and permeabilized according to the manufacturer’s instructions for the Foxp3/Transcription Factor Staining Buffer set. Cells were then incubated with Foxp3 or Foxp3 isotype for 45 mins at room temperature in the dark. After the incubation period, the unbound antibodies were removed by washing them twice with permeabilization buffer, and the cells were re-suspended in stain buffer.

Flow cytometry was performed using an LSR II (BD Biosciences) and analyzed offline on Flowjo Software version 10.00 (Tree Star Inc., Ashland, OR, USA) (Supplementary Fig. S1, online only). The total leukocyte population was identified by the expression of CD45+ and quantified as a percentage of the total live cell population. Helper T cells were identified by the expression of CD45+CD4+ and quantified as a percentage of the total live cell population. Cytotoxic T cells were identified by the expression of CD45+CD8+ and...
quantified as a percentage of the total live cell population. Regulatory T cells were defined as CD4+CD4+Foxp3+ and quantified as a percentage of the total live cell population.

Analysis of PD-L1 expression on CT26 cell line

Analysis of PD-L1 expression on the CT26 cell line was determined by flow cytometry. Briefly, CT26 cells grown in culture were stimulated with IFN-γ (2 μg/mL) where indicated, harvested and incubated with APC anti-mouse PD-L1 antibody (10 F.9 G2, BioLegend) or isotype control (RTK4530, BioLegend) in PBS. Stained cells were washed with PBS, run through the LSR II (BD Biosciences) and analyzed offline on Flowjo Software Version 10.00 (Tree Star Inc, Ashland, OR, USA)

Identification of TDLNs

A mouse with a subcutaneous CT26 tumor on the hindlimb in the size range 50–100 mm³ was used to identify the corresponding TDLN (i.e., inguinal lymph node). By use of a 0.5-ml syringe with a 29G needle (ELIMEDICAL, Markham, ON, Canada), three intratumoral injections of 5% Evan’s Blue (dissolved in Hank’s buffered salt solution) were given, totaling a volume of 50 μL, while the mouse was under anesthesia. The animal was kept warm during the anesthesia period by being placed atop a warm saline bag. The animal was killed 45 mins after injection of Evan’s Blue, and the inguinal lymph node was exposed to confirm labeling with Evan’s Blue.

Interferon γ ELISPOT analysis

Spleens and TDLNs were aseptically harvested from euthanized mice at days 3 and 7 post-treatment. Single-cell suspensions were prepared by passing organs through a 40-μm cell strainer (Corning, Corning, NY, USA). Total cell number and viability were determined using the trypan blue dye exclusion assay, with viability typically exceeding 90%. Cells were suspended and cultured in RPMI-1640 medium supplemented with penicillin (100 U/mL), streptomycin (100 μg/mL), 0.05 mM 2-mercaptoethanol and 10% fetal bovine serum. Tumor-specific IFNγ-secreting cells were assayed by ELISPOT (R&D Systems; Minneapolis, MN, USA) according to the manufacturer’s protocol. Briefly, TDLN cells (3 × 10⁵) or splenocytes (5 × 10⁵) were co-cultured with 2 × 10⁹ gamma-irradiated CT26 or EMT6 tumor cells in 96-well ELISPOT plates containing immobilized IFNγ capture antibody. After 48 h, cells were removed, and IFNγ spot-forming units (SFU) developed using the ELISPOT blue development kit (R&D Systems). Each well was imaged using an automated ELISPOT plate counter (Cellular Technologies Inc; Shaker Heights, OH, USA), and spots were enumerated manually by a blinded investigator.

Histologic analysis

Tumor tissue was flash-frozen with Tissue-Tek Optimal Cutting Temperature (O.C.T) (VWR, Missis- sauga, ON, Canada) and stored at −80°C. Sections were subsequently sliced at 8-μm thickness and stained with hematoxylin and eosin (H&E). The samples were scanned with the Axio Imager.M2 (Carl Zeiss Microscop- copy, Jena, Thuringia, Germany) using a 20 x objective and subsequently analyzed for the percentage area of necrosis (Goertz et al. 2012).

Statistical analysis

A one-way analysis of variance (post hoc: Fisher’s least significant different) test was used to compare all treatment groups. Differences between groups were deemed significant when p < 0.05. Kaplan–Meier survival curves were compared with a log rank (Mantel–Cox) test. All statistical calculations were done using GraphPad Prism Version 7.00 (GraphPad Software, La Jolla, CA, USA). Results are expressed as the mean ± standard error of the mean (SEM).

RESULTS

Validation of PD-L1 expression on CT26 cells

Consistent with previous studies using the CT26 tumor model, we found that CT26 cells express PD-L1, and its expression is upregulated when stimulated by IFN-γ (produced by activated T cells) (Supplementary Fig. S2, online only). This is in line with the understanding of the mechanism underlying immune evasion used by tumor cells (Garcia-Diaz et al. 2017).

Identification of TDLN of the CT26 tumor

Injection of 5% Evan’s Blue into the subcutaneous CT26 tumor situated on the mouse’s hindlimb labeled the ipsilateral inguinal lymph node; the contralateral lymph node was not labeled blue (Supplementary Fig. S3, online only), thereby confirming the inguinal lymph node to be a TDLN of the CT26 tumor. This, in turn, confirms our IFNγ ELISPOT analysis of a genuine TDLN.

Flow shutdown

The USMB treatment resulted in a shutdown of blood flow within the tumors, with the peak enhancement intensity being reduced by 88 ± 3.6% relative to the MBs (p < 0.05) treatment. An example is illustrated in Figure 2, where a profound perfusion reduction is evident in the post-treatment image. As observed in our previous reports (Goertz et al. 2012; Todorova et al. 2013), the exposures resulted in the presence of broadband emissions, a hallmark of inertial cavitation indicating the presence of violent microbubble oscillations (Fig. 3).
Individual and average tumor growth curves are provided in Figure 4a and b, respectively, normalized by expressing each tumor volume as a percentage increase relative to its entry volume at day 0. The intergroup statistical differences between means as a function of time are summarized in Table 1. Tumors in the USMB + aPD-1 treatment group were significantly smaller than tumors in all other treatment groups at days 3, 6 and 9. One (1/6) of the mice treated with USMBs + aPD-1 exhibited complete regression of its tumor and was subsequently subjected to a re-challenge experiment (see below). The Kaplan–Meier survival curves in Figure 4c indicate that USMB + aPD-1 treatment resulted in significantly longer times to reach survival endpoints compared with the other treatments. Statistical differences between treatment groups are summarized in Table 2. Finally, growth delays were quantified as the number of days to reach 500% of initial tumor size (Fig. 4d). The groups treated with USMBs and USMBs + aPD-1 had longer growth delays compared with the group treated with MBs ($p < 0.05$ and $p < 0.001$).

The mouse in the USMBs + aPD-1 treatment group that exhibited complete regression of its tumor underwent a re-challenge experiment. This entailed another subcutaneous injection of tumor cells (100,000 CT26 cells/50 $\mu$L) into the contralateral flank. After 90 d, no tumor was evident on ultrasound imaging, consistent with the development of an adaptive immune response to the CT26 cells.

No significant differences in TIL counts were observed between groups at either day 3 or 7 (Fig. 5a, b). For T-cyt cells, there was a considerable spread in the data for the MB and aPD-1 treatment groups at day 3 or 7, but no differences between any groups (Fig. 5c, d). Data on T-help cells indicated an increased spread in non-control groups at day 3 but no significant inter-group differences (Fig 5e). At day 7, the aPD-1 treatment group had a significantly elevated number of cells compared with the groups treated with MBs ($p < 0.05$), USMBs ($p < 0.01$) and USMBs + aPD-1 ($p < 0.05$) (Fig. 5f). For T-regs, the day 3 results for the aPD-1 and USMB + aPD-1 treatment groups revealed an increased spread relative to the other treatment groups, but with no significant differences (Fig. 5g). At 7 d, however, cell numbers in both the aPD-1 and USMB + aPD-1 treatment groups were significantly elevated relative to the non-aPD-1-treated groups (Fig. 5h). Finally, the T-cyt/T-reg ratio results are illustrated in Figure 5i and j for days 3 and 7, respectively. Significant differences were observed at day 7, where the ratio for the group treated with MBs was higher than that for the group treated with aPD-1 ($p < 0.05$).

Cell counts and IFN$\gamma$ ELISPOT analysis. The TDLN cell count data are illustrated in Figure 6, with the USMB group having a significantly elevated number of cells relative to the groups treated with MBs ($p < 0.05$) and aPD-1 ($p < 0.05$) (Fig. 6a). At day 7, there was a significant elevation in the number of cells in the group treated with USMBs + aPD-1 compared with the groups treated with MBs ($p < 0.05$) and USMBs ($p < 0.05$), but not compared with the aPD-1-treated group ($p > 0.05$) (Fig. 6b).
Increased expression of IFNγ was observed in the USMB + aPD-1-treated group compared with the MB-treated group \((p < 0.05)\) at day 3 (Fig. 6c). By day 7, an increase in IFNγ was seen only in the aPD-1-treated group compared with the group treated with MBs \((p < 0.05)\) (Fig. 6d). Cells and IFNγ expression were quantified for

**Table 1.** \(p\) Values for differences between treatment groups in the longitudinal growth experiments at days 3, 6 and 9

<table>
<thead>
<tr>
<th>Day</th>
<th>USMBs + aPD-1 vs. MBs</th>
<th>USMBs + aPD-1 vs. USMBs</th>
<th>USMBs + aPD-1 vs. aPD-1</th>
<th>MBs vs. USMBs</th>
<th>MBs vs. aPD-1</th>
<th>USMBs vs. aPD-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>(p &lt; 0.0001)</td>
<td>(p &lt; 0.01)</td>
<td>(p &lt; 0.001)</td>
<td>(p &lt; 0.05)</td>
<td>(p &lt; 0.05)</td>
<td>n.s.</td>
</tr>
<tr>
<td>6</td>
<td>(p &lt; 0.0001)</td>
<td>(p &lt; 0.01)</td>
<td>(p &lt; 0.01)</td>
<td>(p &lt; 0.05)</td>
<td>(p &lt; 0.05)</td>
<td>n.s.</td>
</tr>
<tr>
<td>9</td>
<td>(p &lt; 0.0001)</td>
<td>(p &lt; 0.01)</td>
<td>(p &lt; 0.05)</td>
<td>(p &lt; 0.05)</td>
<td>(p &lt; 0.01)</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

aPD-1 = anti-PD-1; MBs = microbubbles; n.s. = not significant; USMBs = ultrasound-stimulated microbubbles.
the spleen as well, but no inter-group differences were found (data not shown).

**Histology**

An example of an H&E-stained section of the tumor at day 3 from the USMB treatment group is provided in Figure 7a. A quantitative analysis of the H&E sections for all groups at day 3 is illustrated in Figure 7b. Necrosis was significantly higher in the USMB treatment group relative to the MB (\(p < 0.05\)) and aPD-1 (\(p < 0.05\)) treatment groups. In addition, necrosis was significantly higher in the USMB + aPD-1 treatment group relative to the MB (\(p < 0.01\)) and aPD-1 (\(p < 0.05\)) treatment groups.

**DISCUSSION**

It has been described here that anti-vascular USMB treatments can significantly enhance the anti-tumor effects of aPD-1 therapy. The CT26 colon carcinoma tumor cell line employed exhibits only a partial response to aPD-1 therapy (Wang et al. 2016), as is the case with many clinical tumors (Pardoll 2012; Sharma and Allison 2015). The ability to locally enhance tumor treatments is of clinical relevance to a range of tumor types for which therapies are conducted with curative intent or for survival prolongation and palliative purposes. In the case of colorectal metastases to the liver, for example, the successful surgical resection of tumors results in sharply improved survival rates (Fong et al. 1999). The majority (~70%–80%) (Adam et al. 2004; Leonard et al. 2005) of patients with colorectal liver metastases are not initially eligible for resection, and such patients undergo chemotherapy to reduce tumor burden to enable subsequent resection. However, the rates of conversion from unresectable to resectable are limited (Adam et al. 2004), and improved methods for local tumor therapy are required in a peri-operative setting.

To the authors’ knowledge the only previously published study reporting the combination of ultrasound treatments with a checkpoint inhibitor examined the impact of ablation coupled with combined aPD-1 and toll-like receptor agonist therapy (CpG) (Silvestrini et al. 2017). The anti-tumor effects were assessed on the ablated tumor(s) as well as an additional distal tumor to assess abscopal effects.

It was observed that regardless of the relative timing of drugs and ablation, the primary tumor growth inhibition was not enhanced with ablation. Indeed, for the case of a single treated tumor, the distal tumor had a faster growth rate with the combination compared with aPD-1 + CpG only, unless the drugs were administered before ablation, in which case the USMB + aPD-1 group was comparable to the drug-only group. The latter timing strategy was shown to have the effect of depleting pro-tumoral macrophage- and myeloid-derived suppressor cell populations, while enhancing tumor-suppressing CD8+ cells (T-cyt cells). An important finding was that when two tumors were treated rather than one, abscopal effects were achieved such that the additional non-ablated tumor underwent regression, and survival was improved relative to drug-only treatments. In addition to suggesting the potential of local thermal ablation to affect the treatment of metastatic disease, this highlights the complexity of local treatment on immune effects in that they can be deleterious as well as complementary. The present work studied the feasibility of the enhancing effect of aPD-1 monotherapy on the treated tumor but did not assess abscopal effects.

Our previous work with anti-vascular USMB tumor treatments indicated that as a monotherapy, it can induce rapid vascular damage and shut down blood flow within tens of seconds (Goertz et al. 2012). This is followed by widespread apoptosis and necrosis, with the central regions of the tumor being preferentially affected by the exposures. We have found that coupling this treatment with chemotherapy, such as metronomic cyclophosphamide (Todorova et al. 2013) or low-dose docetaxel (Goertz et al. 2012), potently enhances anti-tumor effects. The former approach is achieved at least in part by blunting the anti-angiogenic rebound occurring after vascular collapse, analogous to that ensuing with treatments with small-molecule vascular disrupting agents (Daen et al. 2009; Shaked et al. 2006). When combining anti-vascular USMB treatments with aPD-1, it is of interest to consider the potential anti-tumor mechanisms involved, and whether they have their origin in engaging the immune system or are complementary to checkpoint immunotherapy by other means.

Anti-PD-1 treatments promote the activity of T-cyt cells by blocking inhibitory signals (Buchbinder and Desai 2016). For T-cyt cells to be effective, they must be

### Table 2. \(p\) Values from log-rank (Mantel–Cox) test for statistical differences between treatment groups on the Kaplan–Meier survival curves

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>USMBs + aPD-1 vs. MBs</th>
<th>USMBs + aPD-1 vs. USMBs</th>
<th>USMBs + aPD-1 vs. aPD-1</th>
<th>MBs vs. USMBs</th>
<th>MBs vs. aPD-1</th>
<th>USMBs vs. aPD-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Value</td>
<td>(&lt;0.01)</td>
<td>(&lt;0.01)</td>
<td>(&lt;0.005)</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

aPD-1 = anti-PD-1; MBs = microbubbles; n.s. = not significant; USMBs = ultrasound-stimulated microbubbles.
activated through exposure to tumor-specific antigens in the lymph nodes and trafficked into the tumor, where they are then subject to microenvironmental factors. Although a host of cell types affect the activity T-cyt cells, the presence of T-help cells within the microenvironment will in general facilitate their action, whereas higher levels of T-reg cells will inhibit their activity. The acute experiments therefore focused on assessing the effect of treatments on these key T-cell subpopulations with flow cytometry. The results did not, however, support a clear T-cell-dependent mechanism of action in that there were no significant increases in T-cyt or T-help populations when comparing USMBs + aPD-1 with MBs. For T-reg cells, there was a significant increase with aPD-1 treatment compared with MB and USMB treatments which was also mirrored by the USMB + aPD-1 treatment. The increase in Tregs with the administration of aPD-1 treatment has been observed previously by Ngiow et al. (2015).

It is useful to compare the above findings with those of Lui et al. (2012) and Hunt et al. (2015). In Lui et al. (2012), CT26 tumors were also employed, and flow cytometry was carried out at 1, 3 and 18 d after treatment. No significant impact on T-reg cells was observed, and a modest increase in T-cyt cells was observed at 3 and 18 d (\( \sim 20\% \) and \( \sim 50\% \), respectively). This work, however, was carried out under conditions that did not appear to shut down the vasculature but rather produced a degree of erythrocyte extravasation and enhanced vascular permeability that was not accompanied by necrosis. In Hunt et al. (2015), anti-vascular effects were produced in the form of acute perfusion shutdown, which was accompanied by subsequent necrosis. At 24-h post-treatment, immunohistochemistry revealed that there was an increase in non-specific T-cell (CD45+/CD3+) infiltration into the treated tumors. In that study, experiments were carried out with continuous wave ultrasound under conditions that induced hyperthermia (Wood et al. 2008), which itself is known to elicit immune effects (Toraya-Brown and Fiering 2014). Thus, it is possible that the differences between these studies and the present work with respect to not observing T-cyt cell enhancement may at least in part be due to differences in the particular exposure conditions employed.

In addition, the flow cytometry T-cell data were supplemented by the TDLN analysis and the re-challenge experiment. The TDLN ELISPOT data quantified the expression of the IFN\( \gamma \) cytokine in the presence of CT26 cells, which provides an indication of activated tumor-specific T cells. The results indicated that a larger number of IFN\( \gamma \) SFU were formed with the USMB + aPD-1 treatment compared with MB treatment only at day 3, which indicates
that there was a partial reversal of T-cell anergy. However, this was not observed at day 7. This is an interesting observation as it could be the result of USMB therapy having a transient effect on increasing cell-mediated immunity in conjunction with aPD-1 therapy at day 3, but this effect disappears by day 7. In addition, at day 7, aPD-1 therapy had increased cell-mediated immunity compared with USMB treatment, but this effect was not observed in USMB + aPD-1 treatment group compared with any of the monotherapy groups. This finding could potentially be due to the fact that only some CT26.wt tumors respond to aPD-1 treatment (Wang et al. 2016), and the animals within the combination group were not as responsive to aPD-1 treatment. Lastly, it was also seen that an increase in cell counts in the lymph nodes of the USMBs + aPD-1 group at day 7 did not translate into an increase in IFNγ SFU. A potential reason for this is that the increase in cell count was not necessarily in cytotoxic and helper T cells, which are the primary producers of IFNγ in cell-mediated immunity. It could have been other immune cells, such as dendritic cells, macrophages and B cells that do not produce IFNγ, hence explaining the discrepancy. The absence of immunophenotyping of the cells within the lymph node was a shortcoming in the experiment. Thereby, no strong inferences can be made with these data, but nonetheless it is an interesting observation. Lastly, with respect to the re-challenge experiment conducted on the mouse that had complete regression of its tumor with USMB + aPD-1 treatment suggests the engagement of an adaptive memory response.

Collectively the results therefore present a complex picture. The cytometry results did not support that the USMB treatments shifted T-cell subpopulations to a more favorable anti-tumor state. There was a trend toward increased IFNγ expression and a negative re-challenge experiment for the USMB + aPD-1 treatment. In this regard it is useful to note the recent work of Wang et al. (2016), who found that there is a large degree of variation in the response to aPD-1 therapy in the CT26 tumor model. It was found that a majority of

**Fig. 6.** Cell counts from the TDLN and associated cell-mediated anti-tumor immunity at days 3 and day 7 after immune checkpoint blockade. The TDLNs of all treatment groups were harvested and cells were quantified for (A) day 3 and (B) day 7. After quantification, the cells were co-cultured with either EMT6 cells (control) or CT26 cells at a 15:1 (effector:target) ratio. IFNγ SFUs were determined by enzyme-linked immunosorbent assay and then quantified for each treatment group to assess the degree of cell-mediated anti-tumor immunity conferred by each treatment for (C) day 3 and (D) day 7. Mean and standard errors of the mean are plotted. *p < 0.05. aPD-1 = anti-PD-1; IFNγ = interferon γ; MBs = microbubbles; SFU = spot-forming units; TDLN = tumor-draining lymph node; USMBs = ultrasound-stimulated microbubble.
CT26 tumors (~56%) were insensitive to aPD-1 treatment, and the remaining tumors fell between low responders and high responders. Therefore, it is possible that the insensitivity to aPD-1 treatment exhibited by some CT26 tumors was masking the cell-mediated immunity (via T-cyt cells) initiated by aPD-1 treatment in another subset of CT26 tumors when grouped together as a whole. As such, although evidence was not found to strongly support a T-cyt cell-dependent mechanism, it is plausible that one does exist.

From a mechanistic perspective, the investigation of the potential role for a wider range of immune cell types is warranted. These include macrophages and myeloid-derived suppressor cells, which correlates with a
poor prognosis in patients receiving checkpoint inhibitor therapy (Davis et al. 2017), as well as natural killer cells and B cells. Indeed, macrophages play a prominent role in the response to treatment with small-molecule vascular disrupting agents (Jassar et al. 2005; Welford et al. 2011). With combretastatin-A4 (a vascular disrupting agent), for example, the increased tumor infiltration of macrophages ensuing vascular collapse appears to limit treatment effectiveness, and addressing this with combinatorial therapy directed toward macrophages may be a means by which to improve this approach (Welford et al. 2011). It is also relevant to investigate if there is a degree of immunogenic cell death induced by USMBs, which may be responsible for the observed memory effect by increasing tumor-specific antigen presentation. In this light it will be appropriate to also investigate a wider range of exposure conditions along with the effects of relative timing of the CI administration and potential abscopal effects.

Separate from adaptive immune system engagement, it is also plausible that the enhanced therapeutic effect was achieved by other complementary effects. For example, the USMB treatments preferentially affect the vasculature in the central portions of the tumor, as is evident from Figure 2. In previous work, we reported that this results in high levels of necrosis (~50%–70%), primarily within the central regions of the tumor (Goertz et al. 2012; Todorova et al. 2013). In the present study, necrosis levels were measured to be lower (~15% in the case of USMB groups), though it should be noted that the measurements were made at the 3-d point when the lower necrosis levels may be attributable to a degree of regrowth of tumor tissue in the intervening period. It is possible that the enhanced tumor effects present within the combined treatment group may therefore be associated with the action of USMBs on a portion of the tumor, while the aPD-1 acts on the remaining tumor tissue. This is a longstanding hypothesized mechanism for the combined action of vascular disrupting agents and chemotherapeutic agents (Horsman and Siemann 2006). Regardless of the mechanisms in play, the results described here indicate the feasibility of achieving enhanced aPD-1 activity in a treated tumor.

CONCLUSIONS

Immunotherapy is arguably assuming a role as the fourth pillar in cancer treatment, next to surgery, radiation and chemotherapy. Checkpoint blockade inhibitors are the forefront of immunotherapy, yet despite their success in clinical trials for the treatment of a growing number of cancers, the responses have been heterogeneous. The use of multiple checkpoint inhibitors or their combination with conventional anti-cancer agents such as chemotherapeutic drugs is being investigated, but it is recognized that the increased toxicity is a significant issue. Physical methods such as ultrasound may offer a way of overcoming these limitations. In our study we found that coupling anti-vascular ultrasound with a key checkpoint aPD-1 was more efficacious at inhibiting tumor growth than aPD-1 monotherapy. The mechanism of enhancement remains a subject for future work.

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SUPPLEMENTARY MATERIAL

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REFERENCES


