OncoTargets and Therapy

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ORIGINAL RESEARCH

Antioxidant oils and *Salmonella enterica* Typhimurium reduce tumor in an experimental model of hepatic metastasis

This article was published in the following Dove Press journal:
OncoTargets and Therapy
28 May 2011
Number of times this article has been viewed

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Abstract: Fruit seeds high in antioxidants have been shown to have anticancer properties and enhance host protection against microbial infection. Recently we showed that a single oral dose of *Salmonella enterica* serovar Typhimurium expressing a truncated human interleukin-2 gene (*SalpIL2*) is avirulent, immunogenic, and reduces hepatic metastases through increased natural killer cell populations in mice. To determine whether antioxidant compounds enhance the antitumor effect seen in *SalpIL2*-treated animals, we assayed black cumin (BC), black raspberry (BR), and milk thistle (MT) seed oils for the ability to reduce experimental hepatic metastases in mice. In animals without tumor, BC and BR oil diets altered the kinetics of the splenic lymphocyte response to *SalpIL2*. Consistent with previous reports, BR and BC seed oils demonstrated independent antitumor properties and moderate adjuvant potential with *SalpIL2*. MT oil, however, inhibited the efficacy of *SalpIL2* in our model. Based on these data, we conclude that a diet high in antioxidant oils promoted a more robust immune response to *SalpIL2*, thus enhancing its antitumor efficacy.

Keywords: antioxidants, colorectal cancer, tumor models, metastasis

Introduction

Attenuated strains of *Salmonella enterica* (*S. enterica*) serovar Typhimurium were first explored as delivery vectors for anticancer therapeutics in the mid 1990s.[1,2] Since then, *S. enterica* tumor vaccines have shown efficacy in experimental animal models against multiple tumor types including colorectal, melanoma, breast, bone, spine, liver, pancreatic, prostate, and lung.[3–11] In our experimental models, we have focused on the efficacy of an avirulent and highly immunogenic strain, *S. enterica* Typhimurium χ4550, that was engineered to express a human interleukin-2 (IL-2) gene in primary and experimental metastasis models.[12–14] In these studies, *S. enterica* χ4550 expressing a human IL-2 gene was effective at reducing the number and volume of hepatic metastases both when administered before and after tumor introduction. Decreased tumor burden in these models was inversely correlated with increased hepatic natural killer (NK) cell populations and when depleted, *S. enterica* χ4550's antitumor properties were abolished.[15] Most recently, we have demonstrated that *S. enterica* Typhimurium expressing a C-terminal truncated human IL-2 gene (*SalpIL2*) reduced osteosarcoma lung metastases and significantly increased the number of detectable NK cells in the lung as compared to *S. enterica*-vector control or saline gavaged animals.[16,17] In addition, in the serum of animals with pulmonary osteosarcoma metastases, a single oral dose of *SalpIL2* altered the detection of several proinflammatory cytokines associated with tumor progression.[18] In *Rag-1* knockout mice, which do

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DOI: 10.2147/OTT.S17081

not have functional T-cells, *SalpIL2* continued to decrease tumor burden. Thus, *SalpIL2*'s antitumor properties appear to be independent of T cell contribution.

Adjuvants, such as cycloxygenase-2 (Cox2) inhibitors appeared to enhance the antitumor efficacy of our attenuated *S. enterica* strain. When administered with Cox2 inhibitors, *S. enterica* significantly reduced the number and volume of hepatic metastases as compared to all other treatment types.[19] However, due to insolubility of Cox2 inhibitors in water, controlled dose delivery of these drugs in the drinking water of mice required multiple suspension doses daily. Similar to pharmaceutical Cox2 inhibitors, fruit seed extracts from black cumin (BC; *Nigella sativa*), black raspberry (BR; *Rubus occidentalis*), and milk thistle (MT; *Silybum marianum*) have been shown to contain high levels of antioxidants and have inhibitory effects on Cox2.[20–23] In addition, BC and BR seed extracts suppress activity of NFκB, a proinflammatory transcription factor shown to be hyperactive in many cancers.[24,25] Anticancer properties of extracts from BC, BR, and MT oils have been demonstrated by observation of decreased tumor burden in oil treated animals.[26–28] The application of antioxidant compounds also appears to be clinically beneficial. Freeze-dried BR gels, when applied directly to premalignant oral lesions, induced regression of cancerous tissues.[21,29] However, to the best of our knowledge, BR oil has not been reported to affect the growth of syngeneic tumors in organs, nor have BC, BR, and MT oils been shown to be efficacious as adjuvants with immunomodulatory anticancer therapies.

In these studies, we sought to test the hypothesis that BC, BR, and MT antioxidant oils would enhance the efficacy of *SalpIL2* in an experimental murine model of hepatic metastases. In this report, we show that several antioxidant compounds augment the cell-mediated immune response to *SalpIL2* in nontumor bearing mice. In addition, antioxidant oils provide adjuvant antitumor properties to *SalpIL2* and appear to directly decrease the growth of experimental hepatic metastases. In contrast to BC and BR oils, MT oil abrogated the ability of *SalpIL2* to reduce the number of hepatic tumors in our model.

Materials and methods

Quantification of lipid-soluble antioxidants in selected oils

Fruit seed oils provided by Botanic Oil Innovation (Spooner, WI) were examined for lipid-soluble antioxidant capacities using an ACL kit to measure the capacity of lipophilic substances following the directions as described by the manufacturer (Analytik Jena, Spring, TX). Briefly, fruit seed oils were dissolved in hexane (20%) and methanol (79%) and run in triplicate to determine Trolox equivalents, a derivative of vitamin E, by chemiluminescence using the Photochem instrument (Analytik Jena).

Bacteria

The avirulent, but highly immunogenic *S. enterica* serovar Typhimurium strain χ4550, a well-characterized adenylatecyclase, cyclic adenosine monophosphate receptor protein, and aspartate semialdehyde dehydrogenase (*asd*) deletion mutant, was a kind gift from Dr Roy Curtiss III (Washington University, St Louis, MO). Plasmid pIL2, constructed by insertion of a C-terminal truncated human IL-2 cDNA immediately downstream from the trc promoter in pYA292[30] which contains an *S. enterica* asd cDNA for stable maintenance, was electroporated into *S. enterica* Typhimurium χ4550 and the resultant strain was renamed *SalpIL2*. *SalpIL2* was cultured overnight at 37°C in Luria-Bertani broth (Difco Laboratories, Detroit, MI), washed and reconstituted to approximately 10^9 mL^{-1} colony forming units (CFU) in Hank's Balanced Salt Solution (HBSS). Bacteria aliquots were stored as a 15% glycerol stock in liquid nitrogen until used in experiments. On the day of oral inoculation, bacterial stocks were allowed to thaw to room temperature and CFU concentration was confirmed by serial dilution on MacConkey agar plates (Becton, Dickinson, and Company, Bedford, MA). The University of Minnesota Institutional Biosafety Committee approved the use of *SalpIL2* in all experiments as described.

Mice

Female 6 to 8 week old C57BL/6 mice were purchased from Harlan Sprague Dawley (Indianapolis, IN) and allowed to acclimate for a minimum of 7 days in specific pathogen free conditions. After inoculation with *SalpIL2*, all experimental animals were transferred to biosafety level two for the remainder of the study. Antioxidant oils were mixed with ground standard mouse chow (Harlan) to make a homogenous 10% w/w meal. Experimental mice were given ground meal or meal mixed with antioxidant oil daily and drinking water was supplied ad libitum. All animals were housed in microisolator cages and cared for by trained personnel from Research Animal Resources. The University of Minnesota Institutional Animal Care and Use Committee approved all protocols.

Murine adenocarcinoma tumor model

MCA-38 syngeneic tumors were propagated in naïve C57BL/6 mice as previously described.[12] Briefly, 4×10^6 tumor cells were injected subcutaneously into the hind flank and allowed to grow until a palpable mass was present. Anesthetized animals were euthanized and the tumor mass was aseptically removed, minced and digested in 0.1% DNase, 0.1% hyaluronidase, and 0.1% collagenase (Sigma Co, St Louis, MO) in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (Sigma) for 2 hours with gentle agitation. The tumor solution was filtered through 150 μm Nitex mesh (Sefar America, Kansas City, MO), centrifuged at $500 \times g$ for 10 minutes and resuspended in HBSS twice before enumeration by trypan blue exclusion. 5×10^4 cells were injected into the spleen of anesthetized animals via transabdominal incision and allowed to circulate for 3 minutes. The spleens were removed and the animals were allowed to recover on warming pads until ambulatory. Animals were sacrificed and hepatic metastases were visually enumerated 14 days posttumor injection.

Splenic and hepatic lymphocyte enrichment

Spleens from nontumor bearing animals were collected for splenic lymphocyte population analysis on day 3, 7, 14, and 21-post oral gavage and start of antioxidant oil. After 14 days of tumor growth, tumor-burdened livers were ground in DMEM with 10% fetal goat serum using sterile glass stoppers. The tissue slurry was filtered through 150 μm Nitex mesh, layered on top of lymphocyte separation medium (Mediatech, Inc, Herndon, VA) and centrifuged at $300 \times g$ for 1 hour. Splenic, hepatic, and tumor lymphocytes were collected from the Ficoll-hypaque layer and serially washed with phosphate buffered saline with 1% bovine serum albumin and 0.1% sodium azide (Sigma).

Flow cytometry

Splenic and hepatic lymphocytes were examined for cells reactive with anti-NK1.1, anti-CD4, and anti-CD8 fluorochrome-conjugated anti-mouse monoclonal antibodies (BD Biosciences Pharmingen, San Diego, CA). Lymphocytes were incubated with the antibodies at 4°C for 30 minutes. After washing, analysis was performed with FACS cancyto-fluorometer (Becton-Dickinson, Grenoble, France) using Cell Quest Pro software (Becton-Dickinson). Lymphocytes were gated by side and forward scatter profiles. Data presented is based on 10,000 gated events.

Statistical analyses

Tumor number, volume, and splenic and hepatic lymphocyte populations were recorded for each mouse and used to calculate the mean values for each experimental cohort. All differences between groups were determined by Fisher's exact test and ANOVA using StatView software (v 5.0.1; SAS Institute, Cary, NC). In the following sections of the manuscript we describe the results as significant ($P < 0.05$) or "tended/marginally" significant as ($P < 0.10$).

Results

The lipid-soluble antioxidant capacities of BC, BR, BC+BR, and MT oils were examined. The combination BC+BR seed oil displayed higher antioxidant capacities (25.9 ± 0.2) in equal combination as compared to individual oils (BC, 21.0 ± 0.1 and BR, 19.8 ± 0.6). In contrast, MT seed oil displayed the lowest levels of lipid-soluble antioxidants (1.3 ± 0.2) of the oils tested.

BC and BR enhance cellular immune response to *SalpIL2*

On day 14, NK cell populations were increased ($P < 0.10$) in animals administered *SalpIL2* with or without BC+BR oil diet (10% w/w) compared to saline and oil-only groups (Figure 1A). On day 21, *SalpIL2* with BC+BR significantly increased NK cell populations over control and combination oil-only groups. *SalpIL2* with or without BC+BR increased $CD8^+$ T cell populations significantly by day 7 over saline control and oil-only groups (Figure 1B). By day 21, however, all experimental groups had significantly lower splenic $CD8^+$ and $CD4^+$ T cell populations than control animals (Figure 1B and 1C).

BC and BR enhance the NK cell response to *SalpIL2* in tumor burdened animals

We next used a hepatic metastasis model to screen whether NK cell populations, increased from BC+BR and *SalpIL2* administration (Figure 1A) would reduce tumor burden in vivo. Consistent with our previous studies, *SalpIL2* tended to reduce the volume of hepatic metastases by 78% in our model ($P < 0.10$). All experimental treatments, for example, BC+BR oil with and without *SalpIL2*, did not have significantly reduced tumor number and volume as compared to control animals (Figure 2A and 2B). Hepatic NK cell populations were significantly increased by *SalpIL2* alone (272%) or *SalpIL2* with BC+BR combination oil (412%) as compared to the controls (all $P < 0.05$). Mice given the combination of

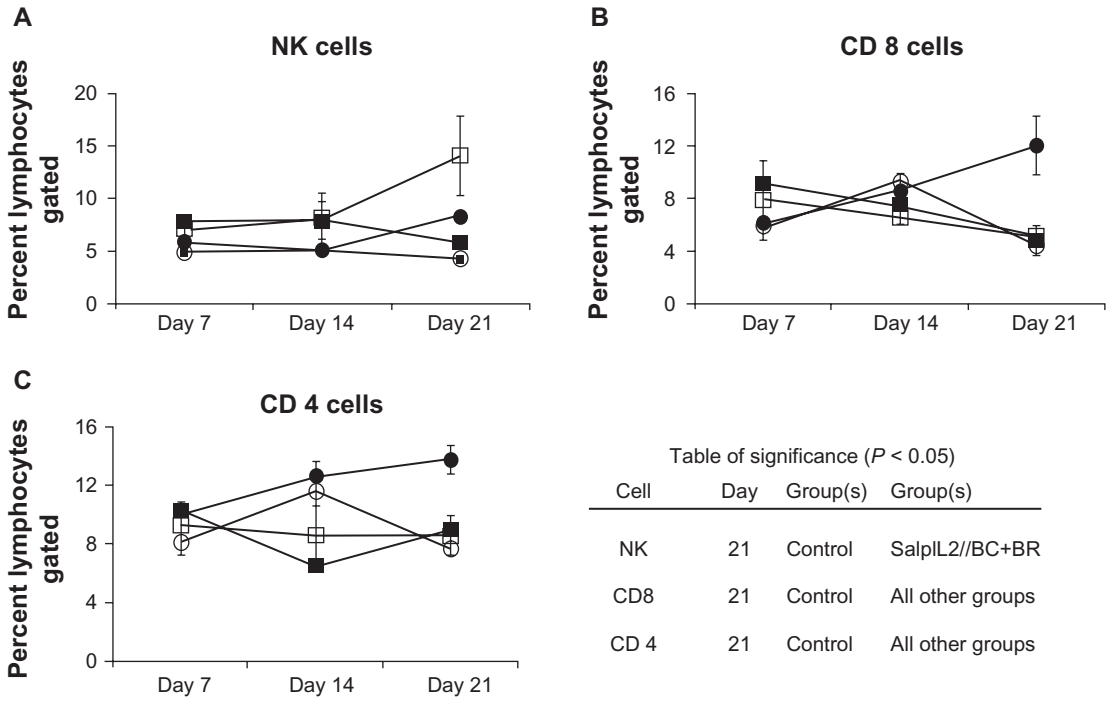

Table of significance ($P < 0.05$)

Cell	Day	Group(s)	Group(s)
NK	21	Control	SalpIL2//BC+BR
CD8	21	Control	All other groups
CD 4	21	Control	All other groups

Figure 1 Effect of combination antioxidant seed oil on the splenic lymphocyte response to *SalpIL2* in mice. Splenic natural killer (NK) (**A**), CD 8$^+$ T (**B**), and CD 4$^+$ T cell populations (**C**) as determined by flow cytometry in response in animals fed a diet consisting of equal amounts of black raspberry (BR; *Rubus occidentalis*), black cumin (BC; *Nigella sativa*) seed oils (○), administered a single oral dose of *SalpIL2* (□) or *SalpIL2*+BC+BR oil (■) starting on day 0 as compared to control animals (●).
Notes: The table insert indicates statistical significance between groups. Data are mean ± standard deviation from one experiment, N = 5 mice per group.

SalpIL2 with BC+BR oil tended to have increased NK cell populations compared to animals only given *SalpIL2* (Figure 2C; $P < 0.10$). CD8$^+$ and CD4$^+$ T cells populations were not significantly different across the experimental groups (Figure 2D and 2E).

BC oil diet reduces hepatic metastases

Although the combination BC+BR seed oils did not significantly decrease tumor number and volume in our hepatic metastasis model, we examined whether either BC or BR oil alone may enhance the antitumor efficacy of *SalpIL2*. Similar to the BC+BR combination oil diet, a BC seed oil diet did not significantly decrease the number of hepatic metastases. A diet with BC oil alone, however, reduced tumor volume by 74% and when administered with oral *SalpIL2*, significantly decreased tumor volume 75% as compared to control animals (Figure 3B; all $P < 0.05$). The reduction of tumor volume was similar to *SalpIL2*-only treated animals (91%). The antitumor effect observed by BC oil appeared to be independent of increased NK and CD8$^+$ T cell populations. Only *SalpIL2*-treated animals, with or without BC oil, displayed significantly higher NK cell populations as compared to controls (Figures 3C and 3D; $P < 0.05$). Lastly, *SalpIL2* with BC oil treated animals displayed a significant 29% reduction in CD4$^+$ T cell population at the experimental endpoint (Figure 3D) ($P < 0.05$).

BR oil diet has treatment and preventative potential on hepatic metastases

In our model, a diet high in BR seed oil significantly decreased tumor volume, not tumor number, by 47% as compared to controls (Figure 4A and 4B; $P < 0.05$). No significant additional benefit in reduction of tumor volume was observed in animals administered *SalpIL2* with (61%) or without BR seed oil (64%). In hepatic lymphocyte populations, however, only animals treated with *SalpIL2* and BR oil had significantly increased NK cells (219%) in the tumor-burdened animals when compared to control (Figure 4C; $P < 0.05$). There were no significant changes in T cell populations between treatment groups. Previously, we showed that inoculation of mice with *S. enterica* 7 days prior to tumor injection provided protection against the establishment of hepatic metastases. Similar to administering a diet of BR oil 3 days after tumor injection, BR oil alone significantly reduced the volume not number of hepatic metastases by 45% as compared to controls (Figures 5A and 5B; $P < 0.05$). Animals given *SalpIL2* with BR oil had similar reduction in

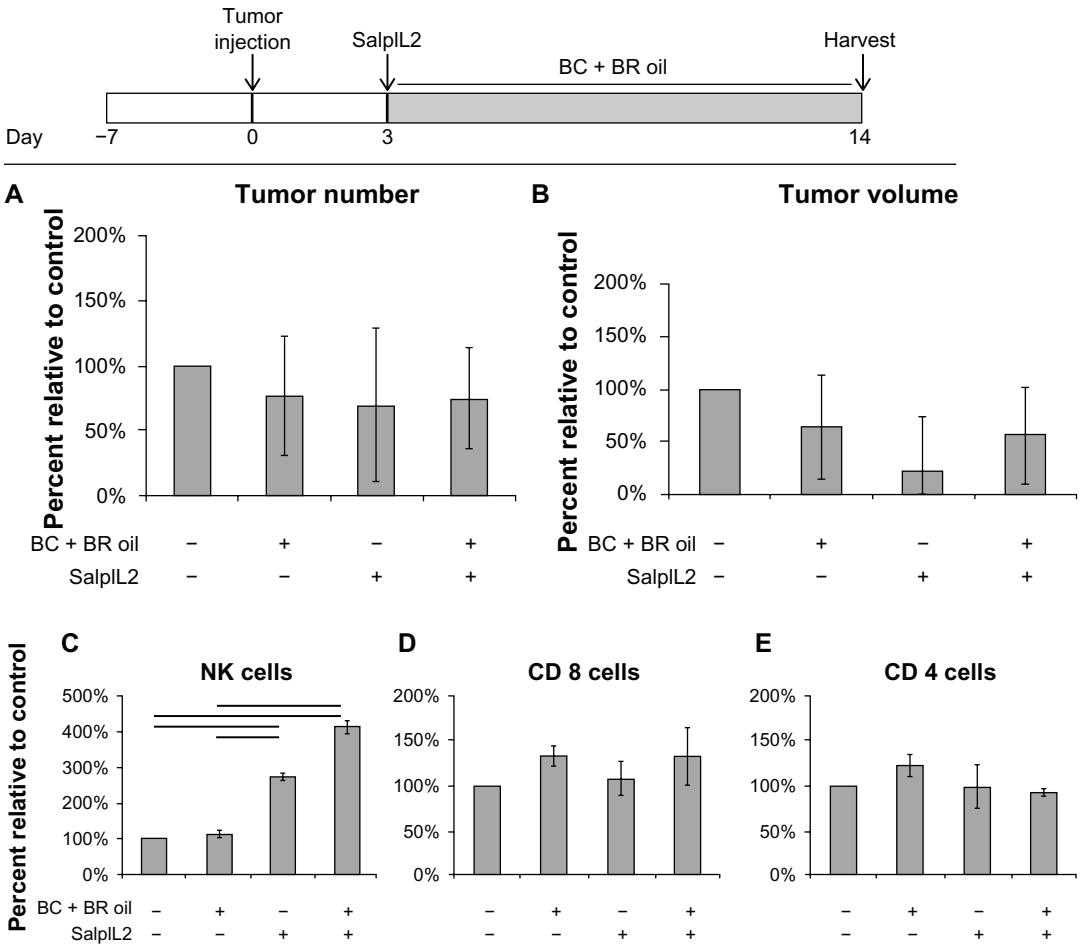

Figure 2 Effect of combination antioxidant seed oil on the *SalpIL2* anti-tumor response in mice. On Day 0, 5×10^4 MCA-38 cells were injected intrasplenically to naïve animals. A single oral administration of *SalpIL2* and initiation of a diet consisting of equal amounts of black raspberry (BR; *Rubus occidentalis*), black cumin (BC; *Nigella sativa*) seed oils 10% w/w was given to animals on Day 3. Animals were maintained for a total of 14 days prior to collection of tumor and hepatic lymphocyte data (see diagram above). Tumor number (**A**) and tumor volume (**B**) in animals fed a BC+BR with or without *SalpIL2*. Hepatic natural killer (NK) (**C**), $CD8^+$ T (**D**), and $CD4^+$ T cell response (**E**) to *SalpIL2* and antioxidant oils in tumor burden mice at the experimental endpoint.

Notes: Bars indicate significance between groups. Data was normalized to percent of control and presented as ± standard deviation from one experiment with N = 4 mice per group.

tumor number and volume to *SalpIL2* alone, 81%, 98% and 70%, 96% respectively as compared to controls ($P < 0.10$). Only animals administered *SalpIL2* with BR oil showed a significant 298% increase in NK cell populations as compared to control animals (Figure 5C; $P < 0.05$). *SalpIL2*-only animals had a marginally significant increase of 260% in NK cell populations compared with controls ($P < 0.10$). Only $CD8^+$ T cell populations were significantly altered between BR oil *SalpIL2* treatment groups (Figure 5D; $P < 0.05$).

BR oil diet augmented cellular immunity to *SalpIL2*

To further elucidate the antitumor efficacy of BR seed oil, we examined the effect of BR oil diet on the splenic lymphocyte populations in *SalpIL2*-treated mice. BR oil with *SalpIL2* increased NK cells by day 3 over control animals (5.7 to 4.7 respectively; Figure 6A; $P < 0.05$), which has not previously been observed in *SalpIL2*-treated animals.[18] By days 7 and 14 however, increased NK-cell populations by *SalpIL2* were independent of BR oil treatment. With respect to $CD8^+$ T cell populations, *SalpIL2* with BR oil significantly increased $CD8^+$ T cell populations on days 3 and 14 compared to *SalpIL2*-treated and control animals (Figure 6B; $P < 0.05$). *SalpIL2* and BR oil treatment also affected $CD4^+$ T cell populations. By day 14, *SalpIL2* significantly decreased $CD4^+$ T cell populations ($P < 0.05$), while *SalpIL2* with BR oil appeared to negate the *SalpIL2*-dependent drop in $CD4^+$ T cells (Figure 6C).

MT seed oil abrogates the antitumor efficacy of *SalpIL2*

As demonstrated in Figure 5, the antitumor effect of *SalpIL2* is most evident in animals when delivered prior to

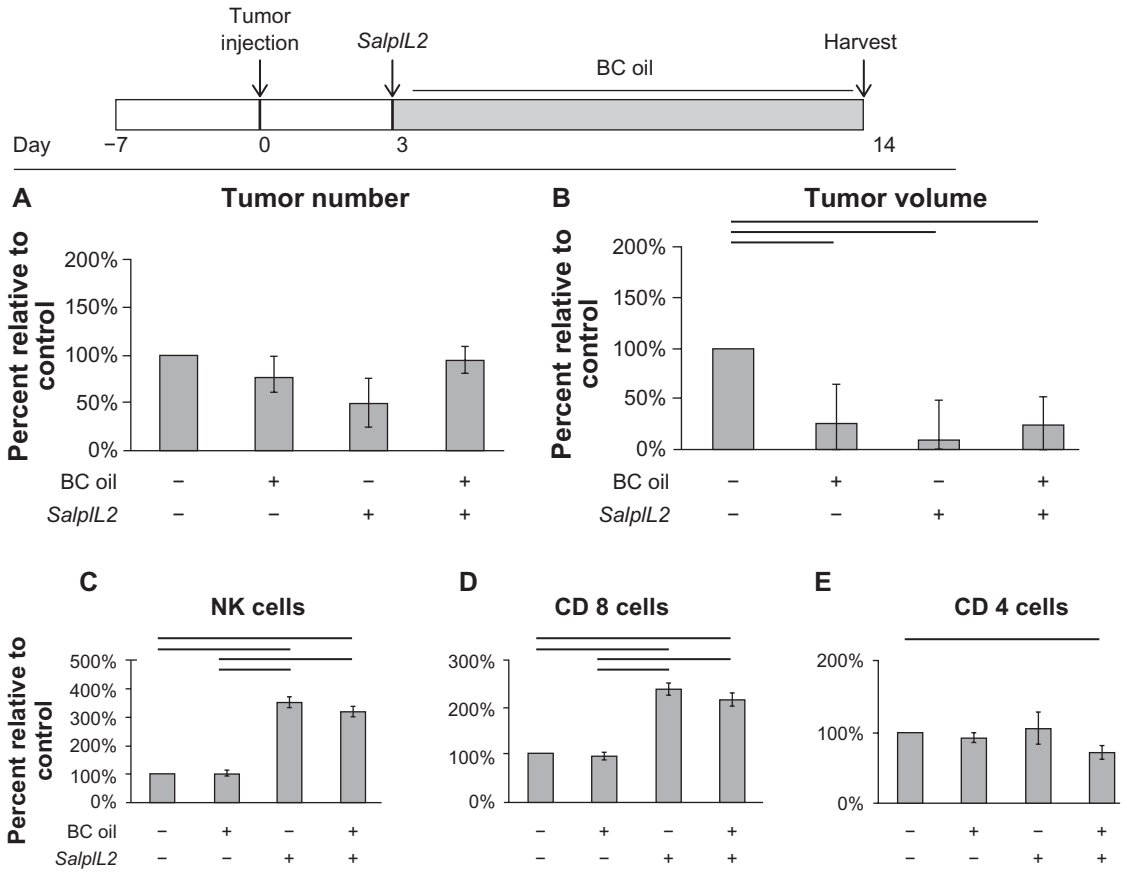

Figure 3 Effect of black cumin (BC; *Nigella sativa*) seed oil on the *SalpIL2* anti-tumor response in mice. On Day 0, 5×10^4 MCA-38 cells were injected intrasplenically to naïve animals. A single oral administration of *SalpIL2* and initiation of BC (shaded area) seed oil diet 10% w/w was given to animals on Day 3. Animals were maintained for a total of 14 days prior to collection of tumor and hepatic lymphocyte data (see diagram above). Tumor number (**A**) and tumor volume (**B**) in animals fed a diet BC seed oil with and without *SalpIL2*. Hepatic natural killer (NK) (**C**), $CD8^+$ T (**D**), and $CD4^+$ T cell response (**E**) to *SalpIL2* with and without BC oil on Day 14.

Notes: Bars represent statistical significance between groups. Data is from one experiment, normalized to percent of control, N = 5, control and *SalpIL2*; N = 10, BC oil and *SalpIL2*+BC oil; and presented as ± standard deviation.

tumor administration. MT oil, however, appeared to inhibit *SalpIL2*'s ability to reduce tumor number (98% to 37%; $P < 0.10$; Figure 7A) and volume (99% to 7%; $P < 0.01$; Figure 7B). MT oil appeared to reduce the NK cell response to *SalpIL2* as NK cell populations were significantly increased in animals given *SalpIL2* with MT oil (255%) and *SalpIL2* alone (335%; Figure 7C). Interestingly both MT oil (315%) and *SalpIL2* alone (387%) increased $CD8^+$ T cell populations as compared to controls (Figure 7D), whereas, *SalpIL2* with MT oil (176%) showed a significant reduction in $CD8^+$ T cell populations as compared to *SalpIL2* alone. *SalpIL2* increased the number of hepatic $CD4^+$ T cells (376%) as compared to controls, which was significantly increased with respect to all other experimental groups.

Discussion

In this report, we investigated whether several known antioxidant seed oils could reduce hepatic metastasis or enhance the antitumor efficacy of attenuated *S. enterica* Typhimurium. Consistent with previous reports on synergistic antioxidant capacities upon mixing oils,[31] we show, compared to individual oils, the combination of BC+BR had increased antioxidant capacity. When given to mice without tumors, the combination of BC+BR oil with *SalpIL2* resulted in an increase in splenic NK cells at day 21. The BC+BR combination oil was synergistic with *SalpIL2* and increased hepatic NK cells populations, however, the number and volume of hepatic metastases were not significantly decreased. Perhaps due to the increased cell-mediated immune response to *SalpIL2*, the addition of BC+BR oil to the diet may have provided an increased clearance of *SalpIL2*. Thus, additional doses may be required to maintain the same level of *SalpIL2* infection and demonstrate a significant difference between *SalpIL2* with and without BC+BR oil diet.

Since, BC and BR oils individually have displayed significant antitumor properties in other models,[26,27,32] we next asked whether individual BC or BR oils have the ability to reduce tumor burden in our hepatic metastasis model. BC

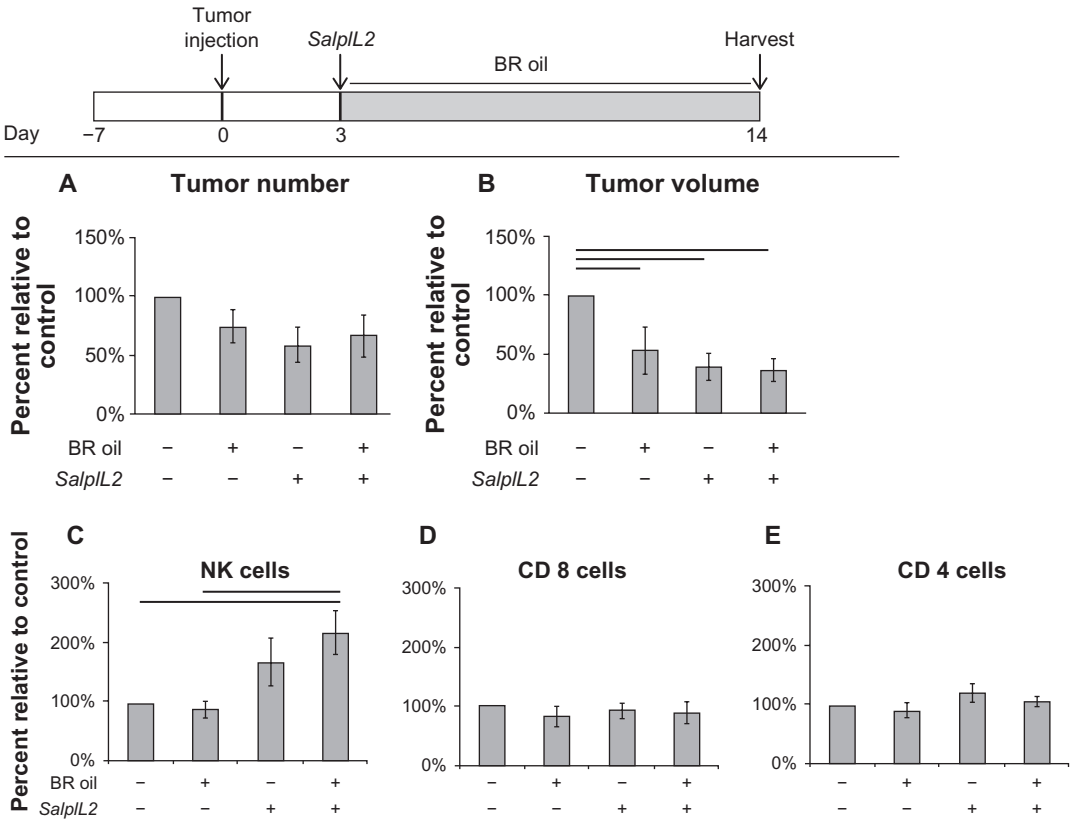

Figure 4 Effect of black raspberry (BR; *Rubus occidentalis*) seed oil on the *SalpIL2* anti-tumor response in mice. On Day 0, 5×10^4 MCA-38 cells were injected intrasplenically to naïve animals. A single oral administration of *SalpIL2* and initiation of BR (shaded area) seed oil diet 10% w/w was given to animals on Day 3. Animals were maintained for a total of 14 days prior to collection of tumor and hepatic lymphocyte data (see diagram above). Tumor number (**A**) and tumor volume (**B**) in animals fed a diet consisting of BR with and without *SalpIL2*. Hepatic natural killer (NK) (**C**), $CD8^+$ T (**D**), and $CD4^+$ T cell response (**E**) to *SalpIL2* with and without BR oil on Day 14.
Notes: Bars represent statistical significance between groups. Data was normalized to percent of control and presented as ± standard error of mean from three independent experiments with N = 5 mice per group.

oil significantly reduced the volume, but not the number of hepatic tumors, suggesting that the consumption of BC oil can reduce tumor growth in the liver. The effect of BC oil in our model appears to be dose-dependent, as BC oil mixed equally with BR oil did not significantly reduce tumor burden without *SalpIL2*. The protective properties of BC oil against tumor initiation may be the result of the combination of antioxidant compounds within the extract,[33] and appears to not be directly correlated to its lipid-soluble antioxidant content. Thymoquinone (TQ), a component of BC seed oil, has demonstrated inhibitory effects on Cox-1 expression and prostaglandin E-2 production; necessary components of the Cox-dependent inflammatory response.[34] Similar to our data, TQ and BC oil reduced tumorigenesis and xenograft growth in animal models of colon and prostate cancer.[32,35] The anti-inflammatory properties of BC oil may account for the decreased $CD4^+$ T cell populations, however, increased NK or $CD8^+$T cells in *SalpIL2*-treated animals may suggest a shift in the type of inflammatory response in tumor-bearing mice. Some $CD4^+$ T cell populations, specifically "regulatory" T cells, appear to contribute to a tumor's ability to resist killing by the immune system.[36] In contrast, regulatory T cells have also been correlated with less advanced colorectal disease.[37–39] Therefore, depending on the severity of disease, regulatory T cells may be an attractive target for reprogramming local tumor immune cell populations. In order to more fully interpret our results in this regard, further characterization of tumor $CD4^+$ T cells must be performed.

Similar to BC oil, BR oil alone significantly reduced tumor volume and appeared to enhance the NK cell response to *SalpIL2* in mice with established metastases. Since tumor number was not significantly reduced, we interpreted these data to suggest that BR oil reduced the growth rather than induced regression of tumor. Although *SalpIL2* alone marginally increased hepatic NK cells, the addition of BR oil significantly increased NK cells in tumor bearing mice, suggesting BR oil enhanced the NK cell response to *SalpIL2*. This observation was consistent in our experiments in nontumor burdened animals, as BR oil appeared to also increase the NK cell response to *SalpIL2* (Figure 6). Oral administration of *SalpIL2,* with and

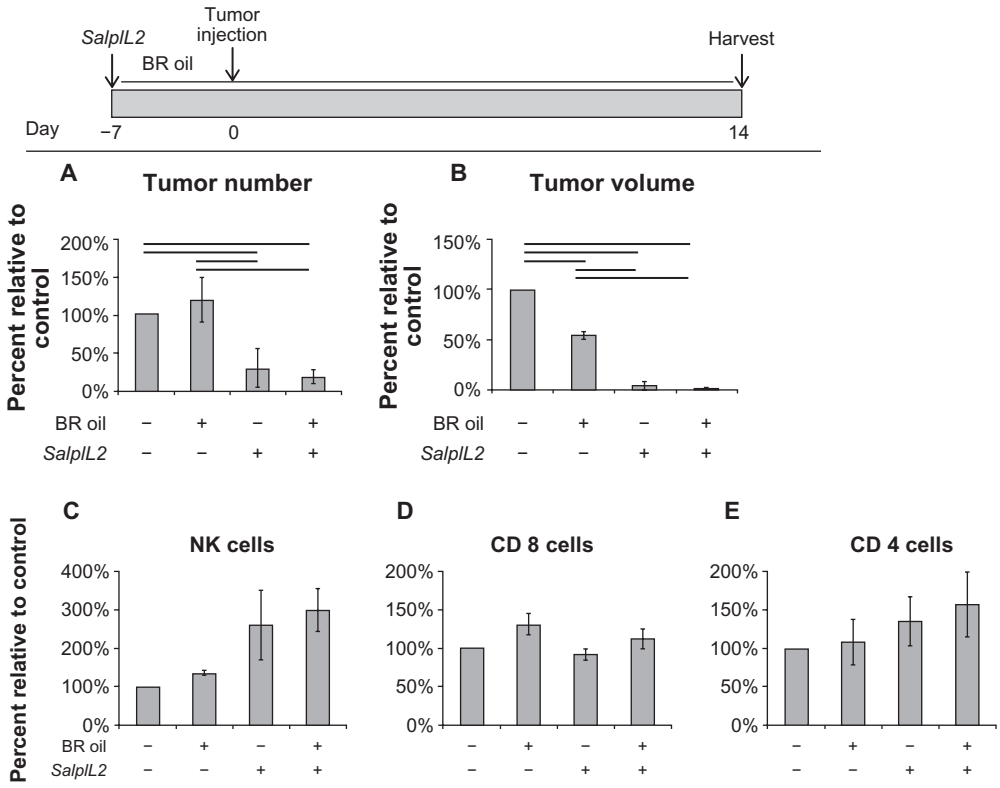

Figure 5 Effect of black raspberry (BR; *Rubus occidentalis*) seed oil and *SalpIL2* on the prevention of experimental hepatic metastases in mice. A single oral administration of *SalpIL2* and initiation of BR (shaded area) seed oil diet 10% w/w was given to animals on Day –7. On Day 0, $5x10^4$ MCA-38 cells were injected intrasplenically and animals were maintained for 14 additional days. Tumor and hepatic lymphocyte data collected on Day 14 (see diagram above). Tumor number (**A**) and tumor volume (**B**) in animals fed a diet consisting of BR with and without *SalpIL2*. Hepatic natural killer (NK) (**C**), $CD8^+$ T (**D**), and $CD4^+$ T cell response (**E**) to *SalpIL2* and BR oil in tumor burden mice on Day 21.
Notes: Bars represent statistical significance between groups. Data was normalized to percent of control and presented as ± standard error of mean from three independent experiments with N = 5 mice per group.

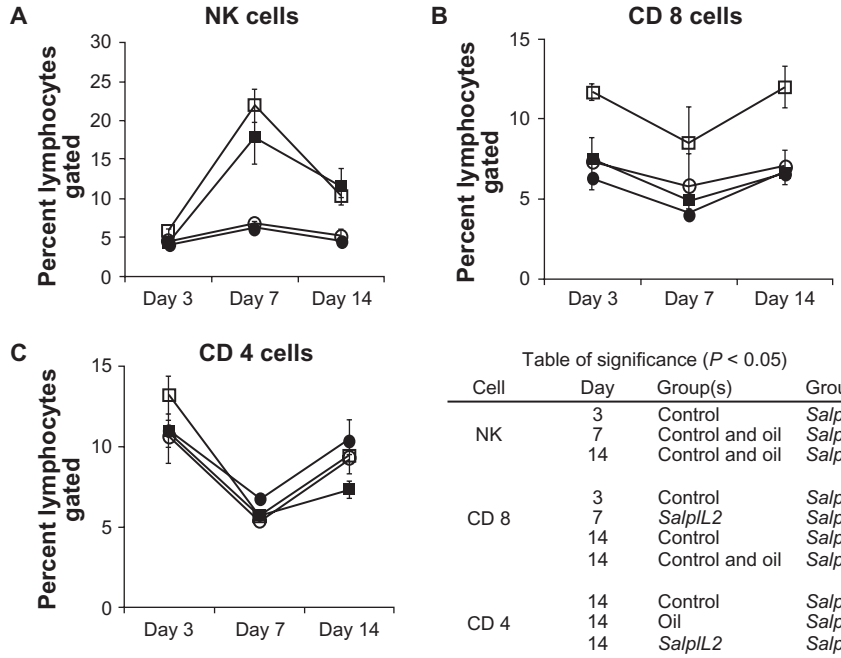

Table of significance ($P < 0.05$)

Cell	Day	Group(s)	Group(s)
NK	3	Control	*SalpIL2*/oil
	7	Control and oil	*SalpIL2* and *SalpIL2*/oil
	14	Control and oil	*SalpIL2* and *SalpIL2*/oil
CD 8	3	Control	*SalpIL2*/oil
	7	*SalpIL2*	*SalpIL2*/oil
	14	Control	*SalpIL2*/oil
	14	Control and oil	*SalpIL2*/oil
CD 4	14	Control	*SalpIL2*
	14	Oil	*SalpIL2*/oil
	14	*SalpIL2*	*SalpIL2*/oil

Figure 6 Effect of black raspberry (BR; *Rubus occidentalis*) seed oil on the splenic lymphocyte response to *SalpIL2* in mice. Splenic natural killer (NK) (**A**), $CD8^+$ T (**B**), and $CD4^+$ T cell populations (**C**) as determined by flow cytometry in response in animals fed a diet consisting of BR seed oil 10% w/w (○), administered as single oral dose of *SalpIL2* (□) or *SalpIL2* + BR oil (■) starting on day 0 as compared to control animals (●).
Notes: The table insert indicates statistical significance between groups. Data are mean ± standard deviation from one experiment, N = 5 mice group.

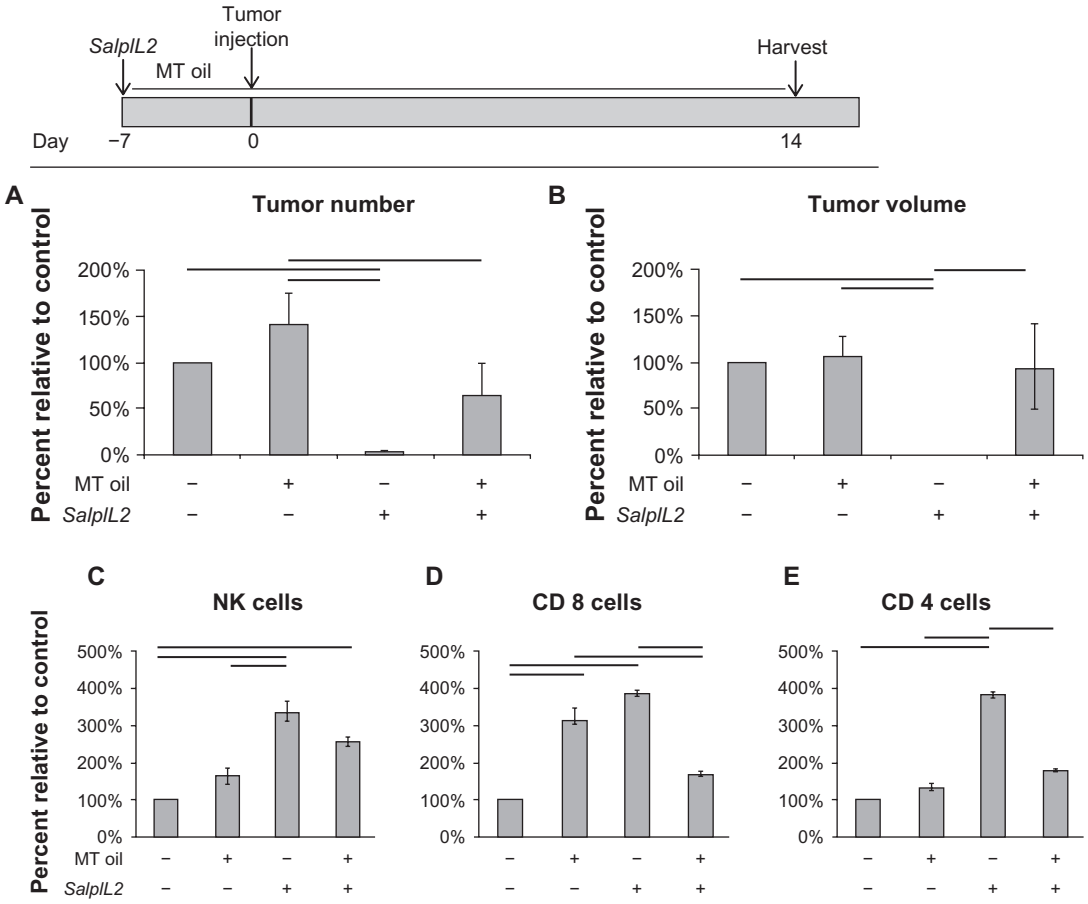

Figure 7 Milk thistle (MT; *Silybum marianum*) seed oil inhibits *SalpIL2*-dependent prevention of experimental hepatic metastases in mice. A single oral administration of *SalpIL2* and initiation of MT (shaded area) seed oil diet 10% w/w was given to animals on Day −7. On Day 0, 5×10^4 MCA-38 cells were injected intrasplenically and animals were maintained for 14 additional days. Tumor and hepatic lymphocyte data collected on Day 21 (see diagram above). Tumor number (**A**) and tumor volume (**B**) in animals fed a diet consisting of MT oil and or *SalpIL2*. Natural killer (NK) (**C**), $CD8^+$ T (**D**), and $CD4^+$ T cell response (**E**) to *SalpIL2* and MT oil on Day 21.

Notes: Bars represent statistical significance between groups. Data was normalized to percent of control and presented as ± standard deviation from one experiment with N = 5 mice per group.

without BR oil, induced a significant increase in splenic NK cells after as short a time period as 3 days and peaked 7 days after oral inoculation (Figures 1A and 6A). Tumor delivery at the peak of the NK response to *SalpIL2* and BR (Figure 5) resulted in greater than 90% reduction of tumor, though there was no statistical difference between the two *Salmonella* groups. BR oil alone, however, significantly reduced tumor volume suggesting BR oil can induce a preventive effect to the liver within 7 days to reduce tumor growth.

Not all oils tested in our models were shown to have measureable antitumor effects. MT oil, which had the least detectable amount of lipid soluble antioxidants, did not provide any protection against hepatic metastasis. Extracts from milk thistle seeds have shown antiproliferative effects with colon cancer cell lines and showed synergism with the chemotherapeutics doxorubicin and paclitaxel in vitro.[40] Most importantly milk thistle extracts have demonstrated promise as adjuvant treatment with minimal adverse effects in human clinical trials as reviewed by Tamayo and Diamond.[41] Silibinin, a flavinoid contained in MT seeds has been shown to inhibit tumorigenesis in rats[42] and promotes cell cycle arrest of human colon cancer cells.[43] Our results with MT oil seem to contradict previous reports, however, mice were only fed the diet of MT oil 1 week prior to tumor injection, whereas others dosed silibinin for 3 weeks prior to prostate tumor xenograft.[44] Even though our tumor cells must survive metastasis from the spleen to the liver, the exposure to MT oil appeared to have little effect on tumor cell death or survival. In contrast, MT oil treatment alone slightly increased tumor burden and reversed the antitumor efficacy of *SalpIL2* in our model. Silibinin has been described to be hepatoprotectant against infection and may have blocked the ability of *SalpIL2* to mount an antitumor response, perhaps by enhancing host resistance to infection in the liver.[45,46]

In these studies, we show antioxidant oils with *SalpIL2* induce a robust NK cell-mediated response and reduction of tumor burden. By administering *SalpIL2* and the antioxidant oil

diet 3 days after establishment of hepatic metastases, we model the clinical situation in which patients are diagnosed with distant metastases. In the clinical setting, soon after surgical resection, systemic chemotherapy is used to reduce undetectable micrometastases or unresectable metastatic lesions. Despite these efforts, patients diagnosed with metastatic colorectal cancer have an average 3-year survival rate of 11%.[47] Because there is a propensity for malignant colon cancers to form hepatic metastases, the use of *S. enterica,* whose primary site of infection is within the liver, is an ideal vector for colorectal hepatic metastases. Furthermore, based on the data in this report, the administration of antioxidant oils with *SalpIL2* induces a more robust NK cell response as compared to *SalpIL2* alone; even when tumors are not present. Hence, when administered prior to tumor development or under low metastatic tumor burden, *SalpIL2* with antioxidant oils may create an environment in the liver that is abundant with NK cells and thus inhospitable for tumor metastasis. By reducing the occurrence of hepatic metastasis, *SalpIL2* and antioxidant oils may provide a significant survival benefit, perhaps similar to the 90% 5-year survival of colon cancer patients without metastatic disease.[47] Therefore, additional studies on the mechanism by which antioxidant oils and *SalpIL2* suppress metastatic tumors is warranted.

Disclosure

BS, AL, and DS are stockholders in Botanic Oil Innovation. All other authors certify that they have no commercial associations that may pose a conflict of interest in connection with this manuscript.

Acknowledgments

This study was supported by Botanic Oil Innovations and The Arnold S Leonard Cancer Research Fund. The Flow Cytometry Core Facility of the Masonic Cancer Center, a comprehensive cancer center designated by the National Cancer Institute, is supported in part by P30CA77598.

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