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Bioactive compounds or metabolites from black raspberries modulate T lymphocyte proliferation, myeloid cell differentiation and Jak/STAT signaling

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Abstract

Bioactive phytochemicals from natural products, such as black raspberries (BRB; *Rubus occidentalis*) have direct anti-cancer properties on malignant cells in culture and in xenograft models. BRB components inhibit cancer progression in more complex rodent carcinogenesis models. Although mechanistic targets for BRB phytochemicals in cancer cells are beginning to emerge, the potential role in modulating host immune processes impacting cancer have not been systematically examined. We hypothesized that BRB contain compounds capable of eliciting potent immunomodulatory properties that impact cellular mediators relevant to chronic inflammation and tumor progression. We studied both an ethanol extract from black raspberries (BRB-E) containing a diverse mixture of phytochemicals and two abundant phytochemical metabolites of BRB produced upon ingestion (Cyanidin-3-Rutinoside, C3R; Quercetin-3-Rutinoside, Q3R). BRB-E inhibited proliferation and viability of CD3/CD28 activated human CD4⁺ and CD8⁺ T lymphocytes. BRB-E also limited *in vitro* expansion of myeloid-derived suppressor cells (MDSC) and their suppressive capacity. Pre-treatment of immune cells with BRB-E attenuated IL-6-mediated phosphorylation of signal transducer and activator of transcription-3 (STAT3) and IL-2 induced STAT5 phosphorylation. In contrast, pre-treatment of

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immune cells with the C3R and Q3R metabolites inhibited MDSC expansion, IL-6-mediated STAT3 signaling, but not IL-2 induced STAT5 phosphorylation and were less potent inhibitors of T cell viability. Together these data indicate that BRB extracts and their physiologically-relevant metabolites contain phytochemicals that affect immune processes relevant to carcinogenesis and immunotherapy. Furthermore, specific BRB components and their metabolites may be a source of lead compounds for drug development that exhibit targeted immunological outcomes or inhibition of specific STAT-regulated signaling pathways.

Keywords

Black raspberries; quercetin; myeloid derived suppressor cells; T lymphocytes; STAT3; STAT5

Introduction

Natural products have potential as a source of diverse bioactive compounds with immunomodulatory properties. Phytochemicals contained within black raspberries (BRB; *Rubus occidentalis*) or their active metabolites are of particular interest for modulating the response to inflammatory stimuli or dysregulated immunity in patients with cancer. The rationale for their utility in this setting originates from epidemiological studies in which fruit and vegetable consumption was inversely related to oral cancer incidence [1]. Among the fruits, BRB are of interest as they contain numerous compounds which some have demonstrated to having cancer fighting activity [2–6]. These dietary natural substances have been utilized with the intention of benefit either as a whole food, providing complex mixtures of phytochemicals or metabolites, as a topical intervention applied directly to cutaneous and mucosal surfaces, or as a source of lead compounds for drug development. In fact, black raspberry extracts have been shown to inhibit proliferation and promote apoptosis of both premalignant and malignant cells [2–4]. Other reports have demonstrated that administration of a diet enriched with black raspberries inhibits carcinogenesis in pre-clinical tumor models [2,7]. Despite these findings, little is known regarding the mechanisms by which bioactive phytochemicals present within black raspberries or their metabolites modulate immunologic factors relevant to tumor development and inflammatory processes.

T lymphocytes are essential cellular regulators of inflammatory processes and a key component in cancer immunosurveillance. Several phenotypic subsets of CD4⁺ and CD8⁺ T cells can be activated to proliferate and secrete cytokines via exposure to foreign antigens and inflammatory signals. Manipulation of T cells via either adoptive transfer [8], or checkpoint blockade [9] has also emerged as promising immunotherapeutic approaches against cancer. Conversely, regulatory T cells (T regs) are a distinct subset of lymphocytes that inhibit cytotoxic CD8⁺ T cell responses against tumors and their frequency is increased in patients with ovarian, breast, and pancreatic cancer [10–12]. Therefore, identifying how dietary phytochemicals and metabolites act upon specific T cell subsets could be relevant to understanding the impact upon inflammatory processes, carcinogenesis, and even immunotherapy. Indeed, the premise that T lymphocyte function can be positively or negatively modulated via compounds derived from natural products has been demonstrated.

For example, dietary grape proanthocyanidins were shown to enhance CD8⁺ T cell responses and the production of IL-12 and IFN- γ in a murine model of UVB radiation-induced skin carcinogenesis [13,14]. Other studies have shown that topical administration of an extract from lyophilized BRB could limit skin carcinogenesis and decrease the number of intratumoral T regs [15]. Finally, curcumin has been shown to negatively regulate T cell proliferation [16]. These data highlight the potential for pleiotropic effects of natural products on T cell function.

Activation of the signal transducer and activator of transcription-3 (STAT3) signal transduction pathway in both transformed and inflammatory cells contributes to inflammation and carcinogenesis [17]. This pathway is also important for the expansion of myeloid-derived suppressor cells (MDSC). This heterogeneous cell population mobilizes from bone marrow to peripheral tissues during inflammatory events such as infection, sepsis, and cancer [18]. Recent work by our group and others indicates that MDSC prime metastatic sites (the so-called pre-metastatic niche) prior to arrival of tumor cells, and elevated MDSC are a poor prognostic marker in patients with malignancy [19–22]. MDSC maintain a feed-forward loop of pro-inflammatory cytokines and inhibit cytotoxic lymphocytes from eliminating tumors [18]. Therefore, studying factors that regulate MDSC development and targeting these cells represent areas of priority for understanding how chronic inflammation leads to cancer. Despite their role in carcinogenesis, only limited studies using 25-hydroxyvitamin D3, curcumin, Withaferin A, or Polyphenol E (green tea catechins) have examined how natural products regulate MDSC biology [23–26].

We hypothesized that compounds within black raspberries or their physiologically relevant metabolites are capable of eliciting potent immunomodulatory properties that impact cellular mediators of chronic inflammation and tumor progression. In the present report, we assessed the immunomodulatory actions of an ethanol extract from black raspberries (BRB-E), and two abundant phytochemical metabolites of BRB produced upon ingestion (Cyanidin-3-Rutinoside, C3R; Quercetin-3-rutinoside, Q3R). Although not present within the complex BRB-E, C3R is a major anthocyanin metabolite of black raspberry while Q3R is an abundant quercetin metabolite observed following ingestion of berries [27]. These compounds were capable of limiting human T cell proliferation, survival and MDSC differentiation *in vitro*. Further mechanistic studies revealed that BRB-E, C3R, and Q3R could inhibit cytokine-induced phosphorylation of specific STAT proteins. Together our studies provide novel data identifying how mixtures of black raspberry bioactive components and specific metabolites may impact T cell and MDSC biology that is relevant to carcinogenesis and cancer immunotherapy.

Materials and Methods

Reagents

An ethanol extract from lyophilized black raspberry (BRB) power, intended to contain a complex mixture of BRB phytochemicals, was prepared by the Nutrient and Phytochemical Analytical Shared Resource at The Ohio State University. Black raspberries were first freeze-dried. The seeds were removed, ground and then the seed powder was added back into the BRB mixture. This finely ground freeze dried BRB powder (mortar and pestle) was

extracted twice with 50mL of hexanes to remove non-polar compounds which were not assessed in these studies. Residual BRB material was extracted thrice with 50mL of 80% (v/v) ethanol/water, centrifuging after each extraction and those fractions pooled. The ethanol in the extract was removed by rotovap resulting in an aqueous suspension of hydrophilic BRB material which was frozen in liquid nitrogen and freeze dried overnight while protecting from light. This hydrophilic fraction accounted for one third to one half the starting mass of lyophilized black raspberry extract. Analysis of the extract was conducted by high performance liquid chromatography (HPLC) as previously described to determine the most abundant classes of bioactive compounds present within the ethanol extract [5,28]. The anthocyanin metabolite, Cyanidin-3-Rutinoside was purchased from Indofine Chemical Company (Hillsborough, NJ). The second, Quercetin-3-rutinoside was purchased from Sigma (St. Louis, MO). Recombinant human interleukin-6 (IL-6) and granulocyte macrophage colony stimulating factor (GM-CSF) were purchased from Peprotech (Rocky Hill, NJ).

Isolation and differentiation of human blood cells

Peripheral blood mononuclear cells (PBMCs) were isolated from source leukocytes of healthy adult donors (American Red Cross, Columbus, OH) with Ficoll-Paque (Amersham, Uppsala, Sweden) as previously described [29,21]. For negative selection of CD4⁺ and CD8⁺ T lymphocytes, Rosette Sep reagents were employed per manufacturer's instructions (Stem Cell Technologies, Vancouver, BC).

T cell proliferation and flow cytometric analysis of T cell activation checkpoint receptors

T cells were labeled with 1 M CFSE (Invitrogen, Grand Island, NY), cultured with CD3/CD28 beads (Invitrogen, Grand Island, NY) for 3 days, stained with antibodies against CD4 or CD8 (Beckman Coulter, Indianapolis, IN) and fixed for flow cytometric analysis on a FACS Calibur (BD Biosciences). Percent proliferation was determined based on dilution of CFSE in CD4⁺ or CD8⁺ cells. For analysis of T cell activation checkpoint receptor expression, T cells were stained with CD4-APC, CD8-FITC (Beckman Coulter) and PD-1 or CTLA-4 antibodies (BD Biosciences, San Jose, CA) after 3 days of culture with CD3/CD28 beads. For analysis of apoptosis, cells were stained with APC-Annexin V (BD Biosciences, San Jose, CA) and propidium iodide (BD Biosciences, San Jose, CA) as recommended by the manufacturer.

In vitro generation of human MDSC

The protocol for *in vitro* generation of MDSC was adapted from Lechner *et al* as described [29]. After 7 days, suspension and adherent PMBC were harvested and myeloid cells were isolated from culture using the Easy Sep Myeloid Isolation Kit (Stem Cell Technologies, Inc). Cells were labeled with anti-CD33/66b magnetic microbeads and positively selected using an Easy Sep magnet. PBMC isolated from the same donor but not treated with cytokines were used as controls. Cells were analyzed for MDSC phenotypic markers by flow cytometry. To test immunosuppressive function, MDSC were co-cultured with autologous, CFSE-labeled T cells stimulated with CD3/CD28 beads. T cells and MDSC

were co-cultured at different ratios (5:1, 3:1, and 1:1; T cells to MDSC) to determine suppressive activity.

Flow cytometric analysis of myeloid derived suppressor cells

Cells from MDSC cultures of each donor were incubated with fluorochrome-labeled antibodies at 4°C. Specific antibodies included CD33 PE, HLA-DR PE-Cy7, CD11b APC (Beckman Coulter). Appropriate isotype control antibodies for each fluorochrome were used as negative controls. All samples were run on a BD LSR II flow cytometer, and analyzed with FlowJo (Tree Star, Inc.). MDSC were defined as cells positive for CD11b and CD33, and low expression of HLA-DR [21].

Analysis of STAT protein signal transduction

Lysates were prepared from cells using Laemmli buffer and assayed by immunoblot analysis with antibodies (Ab) to STAT3, pSTAT3 (Y705), or β -actin (Sigma). Following incubation with the appropriate horseradish-peroxidase-conjugated secondary Ab, immune complexes were detected using the SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific/Pierce, Rockford IL). To determine pSTAT5 levels by flow cytometric analysis, PBMC were fixed using Invitrogen Fix & Perm Cell Permeabilization Reagent and ice cold methanol. Cells were then stained with specific antibodies for pSTAT5 (BD Biosciences), run on a BD FACS Calibur flow cytometer, and were analyzed with FlowJo (Tree Star, Inc.).

Statistical Analysis

Paired t-tests were used to evaluate change in the outcome within donor between DMSO and the highest administered dose of the agent for the majority of the experiments. For multi-dose T cell proliferation experiments, a mixed effects regression model was used including a random effect for donor to adjust for inter-donor differences and a fixed effect for the log-transformed dose. Including dose as a continuous predictor enabled the testing of a linear trend. All analyses were performed in SAS v9.3 (SAS Institute, Cary, NC) and no adjustments were made for multiple comparisons.

Results

An ethanol extract from black raspberries (BRB-E) inhibits T cell proliferation

The phytochemical composition of an ethanol extract derived from lyophilized black raspberry powder (BRB-E) was determined by HPLC (Fig 1A). As intended, this extract contained a mixture of bioactive compounds present within BRB including abundant levels of phenolics such as anthocyanins, quercetin glycosides, and ellagitannins (Fig 1B). We next investigated the effects of BRB-E at a variety of concentrations on T cell proliferation in response to CD3/CD28 activation beads (Fig 2A). The presence of BRB-E (200 g/ml) during activation significantly inhibited proliferation of both CD4⁺ (1.93 fold reduction; Fig 2B) and CD8⁺ (1.88 fold reduction; Fig 2C) T lymphocytes (p-values 0.021 and 0.020, respectively). These concentrations were consistent with prior studies showing a growth inhibitory effect in malignant cells, and represent what might be physiologically achievable following dietary consumption of black raspberries [2,3]. Furthermore, these concentrations

would most certainly be achieved in carcinogenesis studies in which BRB phytochemicals come into direct contact with the target tissue such as cutaneous or mucosal surfaces [6]. Consistent with our cell proliferation data, BRB-E (200 g/ml) also decreased the percentage of both CD4⁺ (Supplementary Fig S1B) and CD8⁺ (Supplementary Fig S1C) T cells expressing the CTLA4 activation checkpoint receptor. A similar decrease in the percentage of CD4⁺ (Supplementary Fig S1D) and CD8⁺ (Supplementary Fig S1E) T cells expressing the PD-1 marker was also evident upon culture with BRB-E.

BRB-E inhibits T lymphocyte survival and IL-2 induced STAT5 phosphorylation

The dramatic inhibition of T cell proliferation prompted an investigation as to whether BRB-E altered T cell viability. The effect of BRB-E was evaluated by annexin V/PI staining of T cells activated in the presence of CD3/CD28 beads or under more physiologic conditions, in the presence of IL-2. These data indicated that BRB-E significantly reduced viability of both CD3/CD28 (18.15% decrease in viability at 200 g/ml; $p=0.0019$) and IL-2 (21.75% decrease in viability at 200 g/ml; $p=0.0157$) stimulated T cells (Fig 3A). In contrast, there were no alterations in the viability of bulk PBMCs cultured in the presence of BRB-E for 72 hours ($p=0.4829$; Fig 3A). Further analysis revealed that pre-treatment of negatively selected T cells with BRB-E led to a concentration-dependent reduction in IL-2 induced STAT5 phosphorylation cells (Fig 3B), a key pathway regulating T cell viability and proliferation (Fig 3C–D; 28.47% decrease in pSTAT5 expression at 200 g/ml BRB-E in CD4⁺ T cells, $p=0.0001$ and 25.04% decreased expression at 200 g/ml BRB-E in CD8⁺ T cells, $p=0.0195$).

BRB-E inhibits in vitro generation and suppressive activity of MDSC

We next examined whether bioactive compounds present within BRB could modulate cytokine-induced MDSC generation *in vitro*. MDSC were induced from PBMC of healthy donors [2,29,21] by culture for 7 days in the presence of GM-CSF and IL-6 as previously described [29,21,30]. Cellular surface markers consistent with an MDSC phenotype (CD33⁺HLADR^{low}CD11b⁺) were assessed by flow cytometry (Fig 4A). Addition of BRB-E to these long term cultures consistently inhibited the generation of MDSC as compared to cells cultured with DMSO vehicle control (Fig 4B). MDSC differentiated in the presence of BRB-E were also less suppressive as determined by co-culture with activated autologous CD4⁺ and CD8⁺ T cells from two separate donors (Fig 4C–D).

Cyanidin-3-Rutinoside (C3R) and Quercetin-3-Rutinoside (Q3R) are immunomodulatory bioactive metabolites derived from black raspberries

Upon introduction into a physiological system, phytochemicals within black raspberries can be converted into many different bioactive compounds in the body including metabolites of anthocyanins and quercetin. Of particular interest to us were two physiologically-relevant BRB metabolites not present within the complex BRB-E, Cyanidin-3-Rutinoside (C3R) and Quercetin-3-Rutinoside (Q3R). C3R is one of the main phenolic antioxidant anthocyanin metabolites of black raspberry [27]. Similarly, Q3R is one of the important abundant quercetin metabolites observed following ingestion of berries. In contrast to experiments using BRB-E (Fig 3), culturing PBMC or activated T cells with either C3R (Fig 5A) or Q3R (Fig 5B) metabolites had no significant effect on the viability of healthy donor T cells

activated with CD3/CD8 beads in the presence of C3R and Q3R for 3 days. A significant decrease in both CD4⁺ (Fig 5C–D; p-value for trend in log dose = 0.001 and 0.008 respectively) and CD8⁺ (Fig 5E–F; p-value for trend in log dose = 0.001 and 0.004 respectively) T cell proliferation was also observed when cells were cultured with the anthocyanin metabolite C3R and quercetin metabolite Q3R. The effect of these two metabolites on MDSC differentiation was also evaluated. PBMCs from healthy donors were co-cultured with Q3R and C3R and stimulated for 7 days in the presence of GM-CSF and IL-6 to induce CD33⁺HLADR^{low}CD11b⁺ MDSC. Similar to BRB-E, C3R and Q3R consistently reduced IL-6 and GM-CSF-mediated differentiation of PBMCs into MDSC (Fig 6A). However, addition of C3R or Q3R to MDSC generation cultures did not alter the functional ability of the cells to suppress autologous T cell proliferation (Supplementary Fig S2A–D) as observed with BRB-E (Fig 4C–D).

BRB-E, C3R and Q3R promote differential modulation of cytokine-induced STAT3 and STAT5 phosphorylation

We postulated that the ability of BRB-E, C3R and Q3R to inhibit MDSC differentiation might be due to modulation of downstream signal transduction pathways. The STAT3 pathway was of particular interest due to its documented role in mediating MDSC differentiation, and the fact that both IL-6 and GM-CSF promote its phosphorylation [17]. Furthermore, prior studies have shown that anthocyanins and quercetin can inhibit STAT3 signaling [31,32]. To determine the effect of BRB-E or the physiologically-relevant C3R and Q3R metabolites on STAT3, PBMCs from normal donors were pre-treated for 4 hours with BRB-E and then stimulated with IL-6. Immunoblot analysis revealed that pre-treatment with higher concentrations of BRB-E reduced STAT3 phosphorylation on the Tyr⁷⁰⁵ residue in response to IL-6 (Fig 6B). However, BRB-E could also inhibit IL-2 induced STAT5 phosphorylation in a concentration dependent manner (Fig 6F) suggesting a broad degree of specificity for cytokine-induced activation of STAT proteins. A concentration dependent, reduction in pSTAT3 was also observed when PBMC were pre-treated with the Q3R metabolite prior to IL-6 exposure (Fig 6C). However, no appreciable effect on IL-6 induced pSTAT3 was observed following with pre-treatment with the C3R metabolite (Fig 6D). Pretreatment of PBMC for 4 hours also inhibited IL-2 induced STAT5 phosphorylation with increasing concentrations of BRB-E (Fig 6E–F; p=0.049 at 200 g/ml), with little or no effect when cells were cultured with C3R or Q3R, even at concentrations of up to 200 μM (Fig 6E and Supplementary Fig S3).

Discussion

The present report demonstrates that an ethanol extract containing bioactive compounds from black raspberries has immunomodulatory properties relevant to human T lymphocyte proliferation, survival, and myeloid cell differentiation. We also found that physiologically-relevant anthocyanin (C3R) and quercetin (Q3R) phytochemical metabolites (not present within BRB-E) likely to be encountered by the body following BRB consumption could modulate human T lymphocyte proliferation and MDSC differentiation, respectively. These findings are relevant as there is currently interest in black raspberries, anthocyanins, or quercetin as natural-product based complementary and alternative medicine (CAM)

interventions for both cancer prevention and inhibiting processes associated with chronic inflammation [33,34]. In fact, recent estimates indicate that 38% of adults and likely greater proportions of oncology patients in the United States use some form of CAM [35]. Thus, although the use of natural products as a means to impact disease processes is attractive in terms of safety and compliance and is founded on epidemiologic data [1], well controlled studies on the biologic impact of berry components or other CAM upon human immune cells are greatly lacking. Although various bioactive fractions of black raspberries are known to have anti-proliferative and pro-apoptotic effects against premalignant and malignant cells, this report represents the first demonstrating these fractions or physiologically relevant metabolites have modulatory activity upon human immune cells that may positively or negatively influence cancer therapy or prevention.

The notion that bioactive components or metabolites derived from foods impact immune function could have implications in a number of clinical settings in patients with established cancers. In theory these interactions could alter the response to immune based cancer therapies, or the ability of the body's endogenous immune system to recognize and eliminate developing tumors. To date, a limited number of other studies suggest BRB may have anti-inflammatory effects in a clinical setting [15,36,37]. For example, Mentor-Marcel *et al.*, conducted a clinical trial in colorectal cancer patients using oral administration of freeze-dried black raspberry powder in a slurry of water. Following three weeks of treatment, serum levels of increased GM-CSF and decreased IL-8 were observed [36]. In a second study, BRB was orally administered at 5% in the diet, based on average body weight to patients with Barrett's esophagus, a premalignant condition that confers increased risk for esophageal cancer. The authors observed a reduction in oxidative stress markers (8-epi-prostaglandin F2 α and 8-hydroxy-2'-deoxyguanosine) important for malignant transformation in this subset of patients [38]. These early stage clinical studies support the idea that BRB might decrease inflammatory mediators tied with induction of immunosuppressive populations (MDSC, T regs, macrophages) relevant to patients with cancer. However, the mechanism by which BRB or their metabolites modulate these or other populations of human immune cells has not been considered to date. Results from this report indicate that BRB and its prominent phytochemical metabolites can directly modulate the proliferation, survival and differentiation of immune cells.

This study represents the first report demonstrating that bioactive components in BRB or metabolites can modulate differentiation of human MDSC. Not only did these studies conclude that BRB-E can limit MDSC differentiation *in vitro* but also a portion of their suppressive activity. However, the metabolites C3R and Q3R did not alter the suppressive activity of MDSC. This is in contrast to the more complex mixture of BRB-E and this data suggest the mixture of individual bioactive compounds within BRB-E may have distinct immunomodulatory properties as compared to the metabolites. MDSC are upregulated in patients with cancer, and represent a significant barrier to immune-mediated recognition of tumors [39]. Thus, there is great interest in identifying novel compounds or strategies that could control MDSC expansion. Many studies have demonstrated that agents with direct activity against tumor cells also inhibit expansion or viability of MDSC. For example, several chemotherapeutic approaches including docetaxol, gemcitabine and all-trans retinoic

acid are directly toxic to MDSC [40,41]. A limited number of targeted, small molecule inhibitors, including sunitinib can also reduce MDSC expansion [42]. Of all the studies examining the use of natural products for cancer, only 25-hydroxyvitamin D3, curcumin, Withaferin A and Polyphenol E (green tea catechins) have been investigated as a means to modulate MDSC and resulting immune responses [23,25,24,26]. These data suggest that dietary products may serve as novel sources of compounds that can be used to modulate phenotypic and functional properties of the immune system.

In addition to altered myeloid cell differentiation, BRB-E inhibited survival and proliferation of human T lymphocytes *in vitro*. These data were quite interesting and could be interpreted a number of ways. It is important to appreciate the diverse role for multiple T lymphocyte subsets in regulating the antitumor immune response. For example, inhibiting proliferation of immune suppressive T regs could be beneficial to the host. In line with this, our data indicated that BRB-E limit IL-2 induced STAT5 phosphorylation, a pathway of importance to T regulatory cell expansion and survival. Further, prior *in vivo* studies showed that topical administratin of BRB inhibited skin carcinogenesis and decreased intratumoral T regs [15]. Conversely, there remains the possibility that the BRB effect might also abrogate expansion or survival of tumor-specific, cytotoxic T lymphocytes. This is particularly relevant to potent and promising immune therapies including T cell checkpoint blockade (i.e. antibodies targeting PD-1, CTLA-4) or adoptively transferred T cells.

One area of potential importance is delineating how BRB can interact with epithelial or mucosal cell layers to modulate the host response to various innate immune stimuli. These stimuli may be derived from biofilms, normal commensal bacteria, or cellular debris created in response to inflammatory insults. This is particularly relevant as very high concentrations of BRB can be achieved at these sites. The opportunity for direct and longer exposure at these areas may provide for a more potent local immune modulating effect. Indeed prior studies have provided evidence that anthocyanins and quercetins can reduce biofilm development, formation, and adhesion [43,44].

Implementation of BRB or their bioactive components as a therapeutic or preventative intervention in humans is attractive, but will require a number of important considerations. For example, a number of factors influence tissue distribution of bioactive compounds from berries [45]. First, with regard to dietary administration, the food matrix has a profound impact upon metabolism and absorption [46]. Second, genetic differences between individuals may influence metabolism of bioactive compounds within raspberries. Third, the oral, intestinal, or cutaneous microbiota can process raspberry-based interventions to generate additional metabolites that could vary between individuals. Thus, although this approach holds promise, implementing it for benefit of patients will require a concerted, multi-disciplinary effort and complex analytical capabilities to detect metabolites in human tissue.

Finally, data from this study also suggest that black raspberries may serve as a source of compounds that could be further manipulated to develop potent, pathway specific inhibitors. This 'reductionist' approach using natural products often serves as a foundation for drug development. In particular, our data point to BRB-E or its metabolites as potential inhibitors

of the STAT3 and STAT5 signal transduction pathways. These pro-inflammatory pathways are particularly attractive for cancer therapy due to their dual role as both oncogenes in malignant cells and dominant pathways promoting immune suppression in myeloid and lymphoid cells [17,47]. Interestingly, we observed that the quercetin metabolite Q3R, but not C3R, inhibited STAT3 phosphorylation with little effect on STAT5 phosphorylation. Thus, the specific compounds within BRB-E with inhibitory activity against STAT signaling or its upstream activating stimuli have yet to be identified, however this remains an area of future research.

In summary, we have demonstrated that black raspberries are a source of bioactive components and metabolites that have potential for modulating T lymphocyte and myeloid cell survival and differentiation. These natural products also serve as a novel source of inhibitors of STAT-mediated signal transduction. These studies suggest that black raspberries may have utility for both therapeutic intervention and as a potential source of lead compounds for drug development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

BRB	Black raspberry
BRB-E	black raspberry extract
C3R	Cyanidin-3-Rutinoside
MDSC	myeloid-derived suppressor cell
Q3R	Quercetin-3-rutinoside
STAT3	signal transducer and activator of transcription 3
STAT5	signal transducer and activator of transcription 5

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Figure 1. Phytochemical composition of an ethanol extract derived from lyophilized black raspberry (BRB-E)

(A) HPLC chromatogram of black raspberry powder extract showing anthocyanins (anth), ellagitannins (ET), quercetin glycosides (Q), ellagic acid (EA) and methyl ellagic acid derivatives (MeEA). (B) Proportions of each structural class of phenolic.

Figure 2. BRB-E inhibits T lymphocyte proliferation

T lymphocytes were labeled with CFSE then stimulated for 72 hours with anti-CD3 and anti-CD28 antibodies in the presence or absence of 100 µg/mL or 200 µg/mL BRB-E. (A) CFSE dilution was measured by flow cytometry. Histograms are from a representative donor from a total of 3 total donors. Data are representative of both CD4⁺ (B) and CD8⁺ (C) T cells from three normal donors (n=3). *, 200 /ml BRB-E statistically significant compared to DMSO at p-value <0.02.

Figure 3. BRB-E inhibits T lymphocyte survival and IL-2 induced STAT5 phosphorylation (A) PBMC and negatively selected T lymphocytes from healthy donors stimulated with CD3/CD28 activation beads or 4nM recombinant IL-2 were cultured with a range of BRB-E concentrations (50–200 g/ml) for 72 hours and assayed by annexin-V/PI staining. (B) PBMC were cultured for 4 hours with a range of BRB-E concentrations (50–200 g/ml) and stimulated with 4nM IL-2 for 20 min (unstimulated = dashed line and IL-2 stimulated = solid black line) and stained for phosphorylated STAT5. Data are representative of (C)

CD4⁺ and **(D)** CD8⁺ phosphorylated STAT5 cells from four normal donors (n=4). *, 200 /ml BRB-E statistically significant compared to 0 /ml BRB-E at p-value <0.02.

Figure 4. *In vitro* generation and suppressive activity of human myeloid derived suppressor cells (MDSC) is inhibited by BRB-E

Culture of PBMCs with IL-6 and GM-CSF (10 ng/mL) resulted in increased levels of MDSC. (A) Representative flow cytometric analysis of human MDSC generated *in vitro*. Quadrants represent dual CD11b and CD33 staining on cells gated in lymphocyte and monocyte regions based on forward scatter, side scatter, and HLA-DR^{low} expressing cells. (B) Addition of BRB-E (100 or 200 g/ml) to these cultures inhibited differentiation. MDSC (CD33⁺ cells) were also co-cultured with CFSE-labeled, autologous donor T cells to assess

proliferation in response to activation with CD3 and CD28 antibody-coated beads. *In vitro* generated MDSC inhibit proliferation of autologous (C) CD4⁺ and (D) CD8⁺ positive T cells that are stimulated with CD3 and CD28 antibody coated beads. Addition of BRB-E to MDSC generation cultures limited the ability of MDSC to suppress autologous T cell proliferation. Error bars are derived from standard deviation of independent experiments involving three separate donors (n=3).

Figure 5. Bioactive BRB-E metabolites are immunomodulatory

PBMC and negatively selected T lymphocytes from healthy donors stimulated with CD3/CD28 activation beads were cultured with a range of C3R (A) and Q3R (B) for 3 days and assayed by Annexin-V/PI staining. Negatively selected T lymphocytes were labeled with CFSE then stimulated for 72 hours with anti-CD3 and anti-CD28 antibodies in the presence or absence of C3R (C, E) or Q3R (D, F). Data are representative for both CD4⁺ (C–D) and CD8⁺ (E–F) T cells from three normal donors (n=3). *, C–D; p-value for trend in log dose =

0.001 and 0.008 respectively and E-F; p-value for trend in log dose = 0.001 and 0.004 respectively.

Figure 6. Effects of BRB-E and bioactive metabolites on MDSC differentiation, STAT3, and STAT5 phosphorylation

Normal donor PBMC were treated with IL-6 and GM-CSF (10ng/ml) for 7 days in the presence of BRB-E (200 g/ml), C3R or Q3R (200 M). (A) Cells were stained for the percentage of HLA-DR^{low}CD11b⁺CD33⁺ MDSC. PMBCs were pre-treated with (B) BRB-E, (C) Q3R, and (D) C3R for 4 hours and stimulated for 20 min with IL-6 and STAT3 phosphorylation was determined by immunoblot. Total STAT3 and β-actin served as controls for equal loading. (E) PBMC were pre-treated with BRB-E, C3R or Q3R for 4

hours and stimulated for 20 min with 4nM IL-2. STAT5 phosphorylation was determined by flow cytometry in a representative donor from 3 healthy normal subjects (unstimulated = dashed line and IL-2 stimulated = solid black line). (F) PBMC with varying doses of BRB-E for 4 hours and then stimulated for 20 min with 4nM IL-2 to assess STAT5 population. Error bars were derived from the standard deviation from independent experiments involving four separate donors (n=4). *, 200 /ml BRB-E statistically significant compared to DMSO at p-value = 0.049.