Effects of Black Raspberry Extract on Myeloid Cell Differentiation and T lymphocyte Function

A Senior Honors Thesis

Presented in partial Fulfillment of the Requirements for graduation *with research distinction in Biology* in the undergraduate colleges of The Ohio State University

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ABSTRACT

Dietary agents are important factors in regulating the development of diseases such as cancer. Several in vitro and animal studies have provided evidence that dietary intervention can prevent cancer development. In addition, studies show that many cancer patients already use natural products or supplements so it is valuable to explore how these agents might benefit health. Black raspberries are a good example of a dietary super-food. Despite data showing that this whole food or metabolites within it can inhibit cancer cell growth in vitro and in animal models, we do not know the mechanisms by which they act. We studied the effect of lyophilized black raspberry extract (BRB-E) and black raspberry metabolites, cyanadin-3-rutinoside (C3R) and quercetin-3-rutinoside (Q3R), on T lymphocyte function and Myeloid Derived Suppressor Cell (MDSC) expansion in vitro. T lymphocytes are important for the ability to detect and eliminate cancer. In contrast, MDSCs can suppress the immune system and prevent cytotoxic cells from recognizing and eliminating tumor cells. Pro-inflammatory cytokines and proteins such as phosphorylated STAT3 drive MDSC expansion. We hypothesized that lyophilized black raspberries or bioactive compounds derived from them would reduce the inflammatory changes that promote tumor development or metastasis. We observed that BRB-E and its metabolites C3R and Q3R suppressed T cell proliferation. We also determined that BRB-E enhanced T cell migration via CXCR3 receptor upregulation. Furthermore, BRB-E and its metabolites inhibited in vitro MDSC differentiation and STAT3 phosphorylation. These data suggest mechanisms by which black raspberry can be immunomodulatory and can be applied to regulating diseases such as cancer.

ACKNOWLEDGMENTS

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INTRODUCTION

Dietary intervention is an expanding field, and may play a role in cancer prevention and therapy. It offers a natural means to physiologically counteract diseases such as cancer. Fruit and vegetable consumption has long been suggested as a means for disease prevention.[1] Research has demonstrated that black raspberries can act at the level of the tumor cell to inhibit growth.[2] However, few studies have addressed their effects on immunity. Black raspberries are good candidates for investigation due to their large content of anti-cancer compounds such as vitamin C and E, anthocyanins, flavonoids, selenium, dietary fiber, and plant sterols.[3] Several preclinical in vivo and in vitro models have demonstrated the effectiveness of whole lyophilized black raspberry.[4] We also investigated two specific metabolites produced by the body following black raspberry consumption in humans. Cyanidin-3-rutinoside (C3R), the main anthocyanin component of black raspberry, has been found to inhibit migration and invasion of

cancer cells, including lung carcinoma cells.[5] Quercetin, another metabolite, is a major flavonoid and has been shown to act as a strong antioxidant. [6] We hypothesized that an ethanol based black raspberry extract would modulate T cell function and myeloid cell differentiation.

T cells are important for immune function and cancer prevention as they may eliminate virally infected or cancerous cells in an antigen-specific manner. However, their actions are quite complicated as certain subsets of T cells may also serve to limit immune responses. Three major types of T lymphocytes in the immune system are T helper cells (CD4+), cytotoxic T cells (CD8+) and T regulatory cells (CD4+CD25+FoxP3+). T cell activation requires 2 signals, one at the CD3 receptor and another at the CD28 receptor. The CD3 receptor binds to an antigen molecule presented by an antigen presenting cell (APC). Costimulation at the CD28 receptor by an APC protein is the second signal needed to activate T cells. Activated T cells can proliferate and migrate to the periphery. In a normal, healthy immune system, CD4+ T cells are activated by APC via presenting peptides on major histocompatibility class I (MHC I) molecules to help other immune cells in their responses. CD8+ T cells are also activated by APC via peptides presented on MHC II molecules. Once they see antigen, these CD8+ cytotoxic T cells can lyse target cells via mechanisms such as perforin and granzyme B secretion. In contrast, T regulatory cells are immunosuppressive. They function in the body to regulate the immune response by preventing too strong of an inflammatory response, as well as preventing autoimmunity. In the setting of cancer, T regulatory cells can be immunosuppressive and act to inhibit the immune system from fighting tumor cells.[7] The ability of T cells to migrate is also important for normal immune function. This process is regulated by chemokines, which allow immune cells to move to sites of tissue damage, infection, or cancer.[8] Monokine induced by gamma (MIG) is a chemokine that binds to the CXCR3 T cell receptor. The CXCR3/MIG system is an example of an inflammatory chemokine system allowing for activated T cell to migrate.[9]

The second subset of cells we focused on were myeloid derived suppressor cells (MDSCs), which are immunosuppressive and originate from myeloid cells in the bone marrow. MDSCs are characterized by their cell surface makers and in humans have the phenotype $CD33^{+}CD11b^{+}HLADR^{-}CD15^{+}$. During inflammatory events such as infection or cancer, MDSCs migrate from bone marrow to peripheral tissues. In a healthy immune system, MDSCs function to protect against too extreme of an immune response. However, elevated MDSC populations are found at sites of inflammation. In peripheral blood samples of cancer patients, up to a 10-fold increased in MDSC numbers can be found.[10] MDSCs play an important role in promoting carcinogenesis by inhibiting natural killer cells (NK cell) and cytotoxic T cells (CTL) via iNOS and arginase-I (ArgI) enzymes. MDSCs can also promote another immunosuppressive subset of cells called regulatory T cells (T reg) through the proteins such as transforming growth factor beta (TGF-β).[11,12] In a host with cancer, cells from the tumor, stroma, and immune system can secrete cytokines such as interleukin-6 (IL-6) and granulocyte macrophage-colony stimulating factor (GM-CSF) that promote differentiation MDSCs **(Figure 1)**. IL-6 is a pro-inflammatory cytokine that activates Janus kinases (JAKs), which in turn phosphorylate and activate STAT3 (signal transducer and activator of transcription factor 3).

Phosphorylated STAT3 (pSTAT3) can move to the nucleus and bind to specific portions of DNA to regulate the transcription of genes involved in cell growth, movement, and apoptosis.[13] STAT3 is noted to be overactive in various forms of cancer, which is believed to contribute to the growth and division of cancer cells.[14] The IL-6/STAT3 pathway is also thought to promote immune suppression as it is a key pathway in MDSC generation.

Our data suggests that an ethanol based black raspberry extract and its metabolites, C3R and Q3R act to regulate lymphocyte function and myeloid cell differentiation. Understanding the mechanisms by which black raspberries can affect the immune system is important as it has the potential to be used as a novel therapy for inflammatory diseases such as cancer.

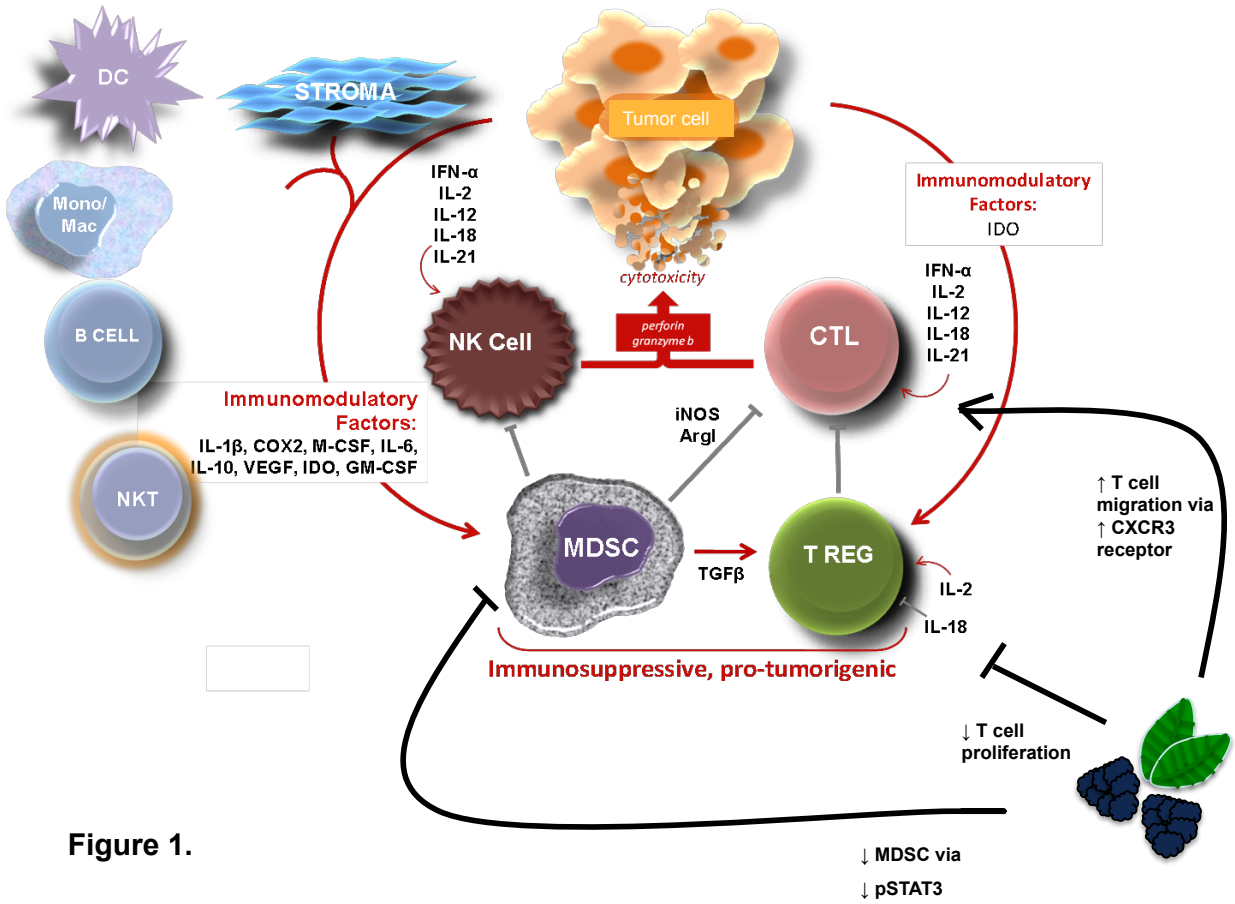


Figure 1.

MATERIALS AND METHODS

Reagents

Lyophilized black raspberry extract (BRB-E) was obtained from the laboratory of Dr. Christopher Weghorst.[4] Black raspberries were freeze-dried and the lyophilized black raspberry powder was extracted with ethanol to yield a residue. Next, this residue was resuspended in dimethyl sulfoxide (DMSO). Aliquots were stored at – 80°C. Cyanidin-3-rutinoside (C3R) and quercetin-3-rutinoside (Q3R) were purchased from Indofine and Sigma Aldrich, respectively. Recombinant human interleukin-6 (IL-6) and granulocyte macrophage colony stimulating factor (GM-CSF) were purchased from Peprotech (Rocky Hill, NJ).

Peripheral Blood Mononuclear Cell (PBMC) and T cell Isolation

Peripheral Blood Mononuclear Cells (PBMCs) were separated from healthy donor source leukocytes (American Red Cross, Columbus, OH) using density gradient centrifugation with Ficoll-Pacque (Amersham Pharmacia Biotech, Uppsala, Sweden) as previously described.[15,16] T cells were negatively selected using Rosette Sep reagents, as described by the manufacturer (Stem Cell Technologies, Vancouver, BC). Cells were cultured in RPMI 1640 (Gibco) supplemented with 10% fetal bovine serum and antibiotics.

CFSE T Cell Proliferation Assay

T cells were isolated from source leukocytes from donor blood using negative selection using Rosette Sep (Stem Cell Technologies, Inc., Vancouver, British Columbia, Canada). The isolated T cells were labeled with 1 μM CFSE, an intracellular fluorescent label (Invitrogen, Grand Island, NY). The labeled T cells were cultured for 3 days with CD3/CD28 beads (Invitrogen, Grand Island, NY) and DMSO (control), BRB-E (200 μg/mL), C3R (200 μM), or Q3R (200 μM). The cells were collected, stained, and analyzed for CD4+ or CD8+ T cells using flow cytometry on a FACS Calibur (BD Biosciences). The percent of T cell proliferation was determined by the dilution of CFSE. **(Figure 2.)**

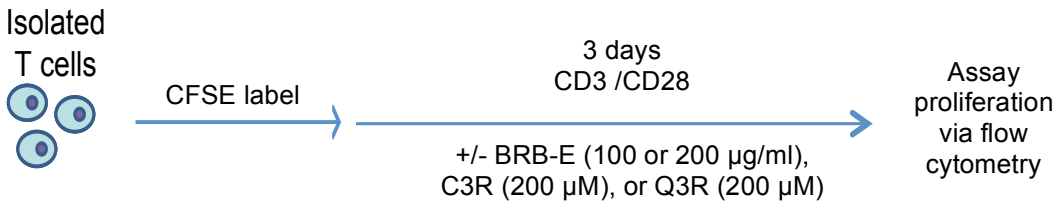


Figure 2.

T cell migration assay

T cells were isolated from healthy donor leukocytes as described above and activated with CD3 and CD28 beads for 7 days with or without BRB-E at 200 ug/mL. After 7 days, one million activated T cells were loaded into the top chamber of transwell inserts (5.0 µM pore size, Costar, Inc.). Media containing monokine-induced by gamma (MIG) was placed in the bottom well as a chemoattractant and positive control (R&D Systems, Inc.). RPMI media alone was used as a negative control. After the plates were incubated for 3 hours at 37°C, the percentage of T cells in the bottom chamber was calculated using trypan blue exclusion and/or flow cytometry to verify T cell phenotype. Flow cytometry was used to determine the percent of T cells expressing CXCR3 receptors and the mean fluorescent intensity of CXCR3. (**Figure 3.**)

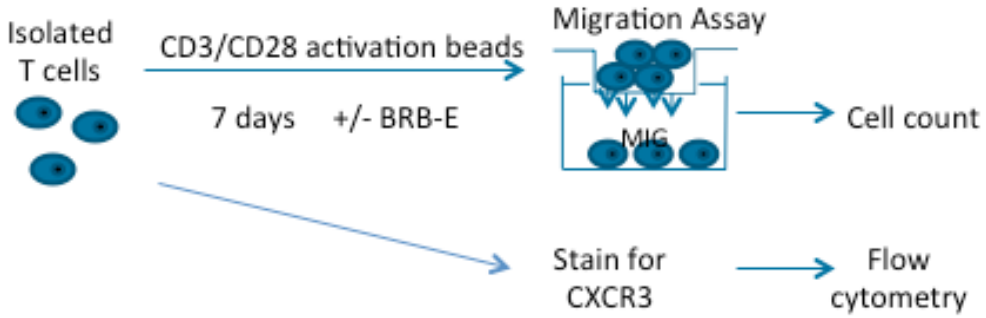


Figure 3.

MDSC generation in vitro

MDSCs were generated in vitro by adding IL-6 and GM-CSF to isolated PBMCs in media at 10ng/mL concentration each, as previously described.[15,16] Cells were treated BRB-E, C3R or Q3R at several concentrations. Cells were cultured at 37°C for 7 days with media and cytokine changed every 2-3 days. After a period of 7 days, cells were harvested, stained, and analyzed via flow cytometry. Cells were stained using the following mouse anti-human antibodies for MDSC surface makers: CD11b-PE, CD33-APC, CD15-FITC, HLA-DR- PE-Cy7. The isotype control was stained with the respective mouse antibodies. Following staining, cells were incubated for 30 minutes on ice. Cells were washed with FACS buffer and fixed in PBS with 1% formalin. Cells were run on a FACS calibur or LSRII flow cytometer (BD Biosciences, San Jose, CA). The data was analyzed using FlowJo software. (**Figure 4)**

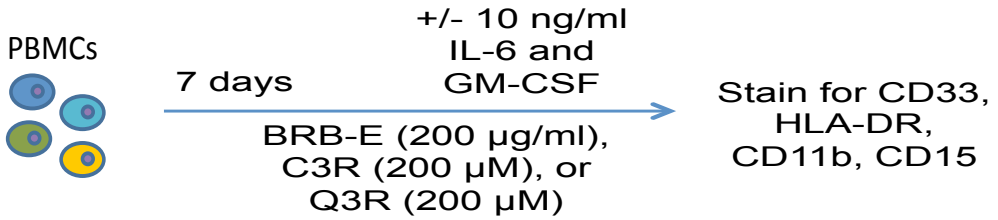


Figure 4.

T cell suppression assay by in vitro generated MDSCs

In vitro generated MDSCs were tested for functionality using a T cell suppression assay. MDSCs were isolated using CD33+ magnetic bead selection and co-cultured with CFSE labeled isolated T cells for 3 days in the presence of CD3/CD28. T cell proliferation was assayed using flow cytometry. (**Figure 5**)

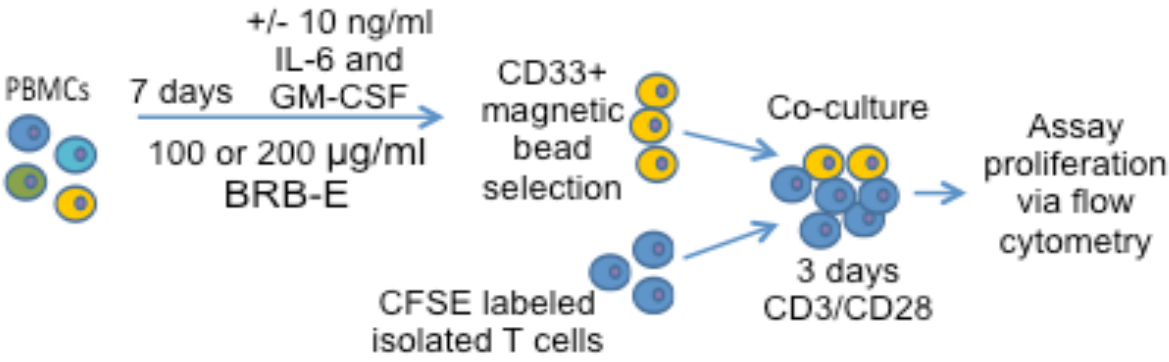


Figure 5.

Immunoblot analysis

PBMCs were cultured with black raspberry extract or its metabolites. Lysates were prepared in Laemelli buffer and assayed for protein expression as previously described via immunoblot analysis with antibodies (Ab) to STAT3, pSTAT3, and beta actin (Sigma).[17] After incubation with horseradish-peroxidase-conjugated secondary Ab, protein expression was detected using the SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Rockford IL). (**Figure 6**)

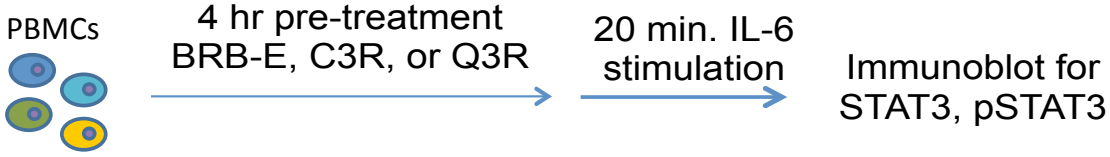


Figure 6.

RESULTS

Immune cell viability is not affected by ethanol extract from black raspberries

PBMCs from several healthy donors were cultured for 3 days with the ethanol based black raspberry extract (BRB-E). Cell survival was analyzed by annexin V/PI staining and flow cytometric analysis. Concentrations of BRB-E ranging from 0-400 µg/ml showed no significant effects on cell viability. (**Figure 7**)

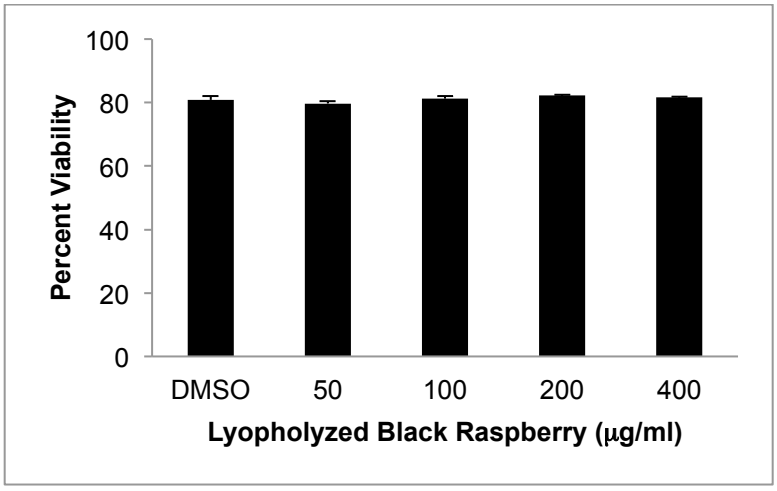


Figure 7.

T cell proliferation is inhibited by black raspberry extract and its metabolites

CD3+ T cells were isolated from PBMCs from donor blood from the Red Cross via negative selection as described above. (**8A)** The T cells were fluorescently labeled with CFSE to assess proliferation. The T cells were also stimulated with beads that activated CD3 and CD28 surface markers or unstimulated (negative control). When BRB-E was added to the T cell cultures (**8B**) CD4+ T lymphocyte and (**8C**) CD8+ T lymphocyte proliferation was inhibited. The effects of the BRB-E were noted to behave in a dose dependent manner.

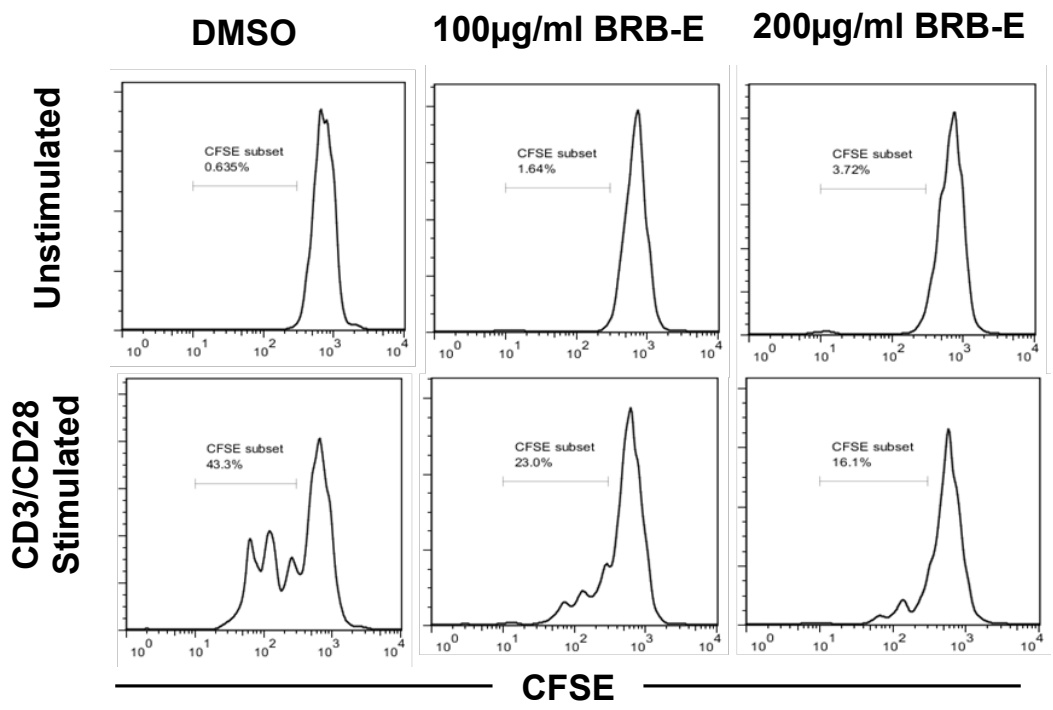


Figure 8A.

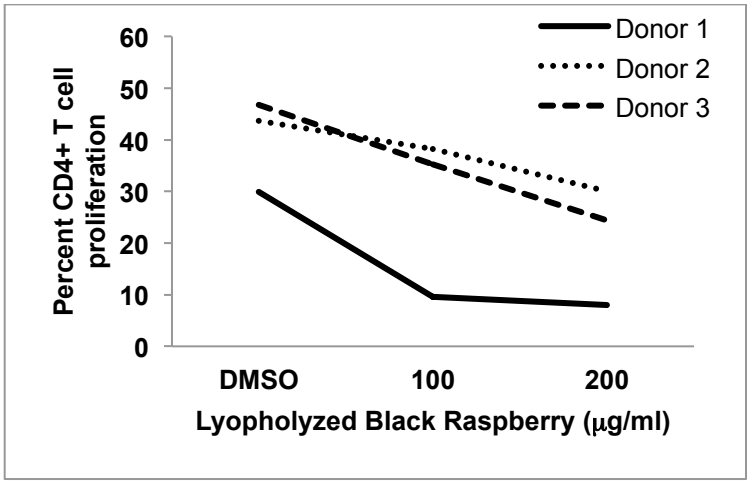


Figure 8B.

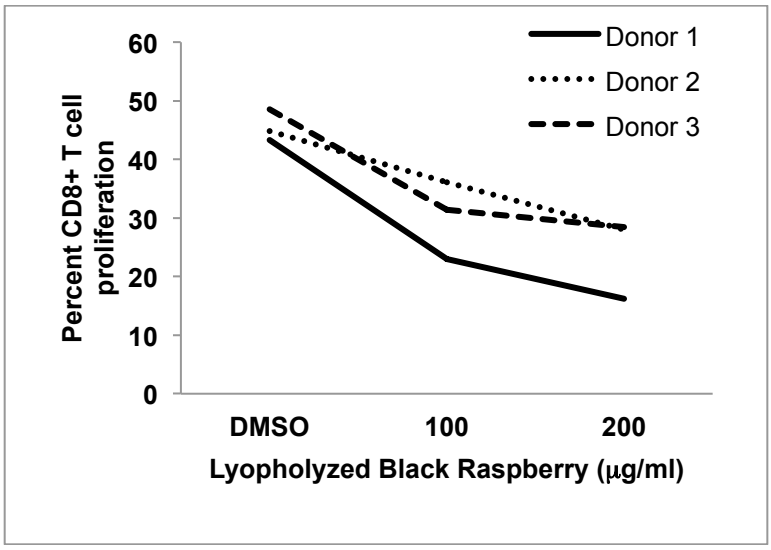
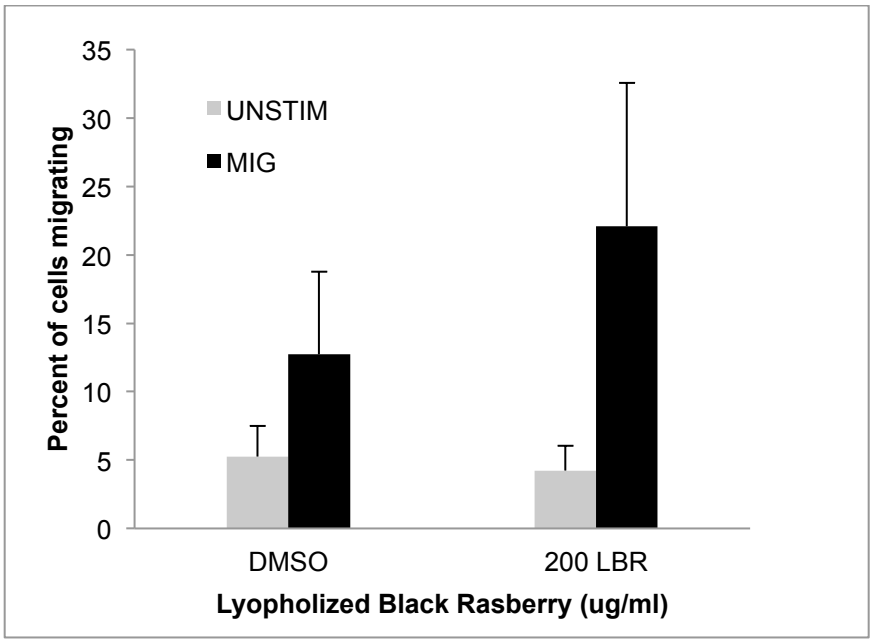


Figure 8C

T cell migration is enhanced by black raspberry extract via chemokine receptor upregulation

T cells activated by CD3/CD28 beads were placed in a transwell plate containing MIG (monokine induced by gamma). (**9A**) A significant increase in T cell chemotaxis (p=0.01) was observed in T cells incubated with BRB-E. Using flow cytometry, CXCR3 (the receptor for MIG) was found to be upregulated in both CD4+ and CD8+ T cells. An increase in the (**9B**) percentage of cells expressing CXCR3 and the (**9C**) mean fluorescent intensity of CXCR3 was observed.



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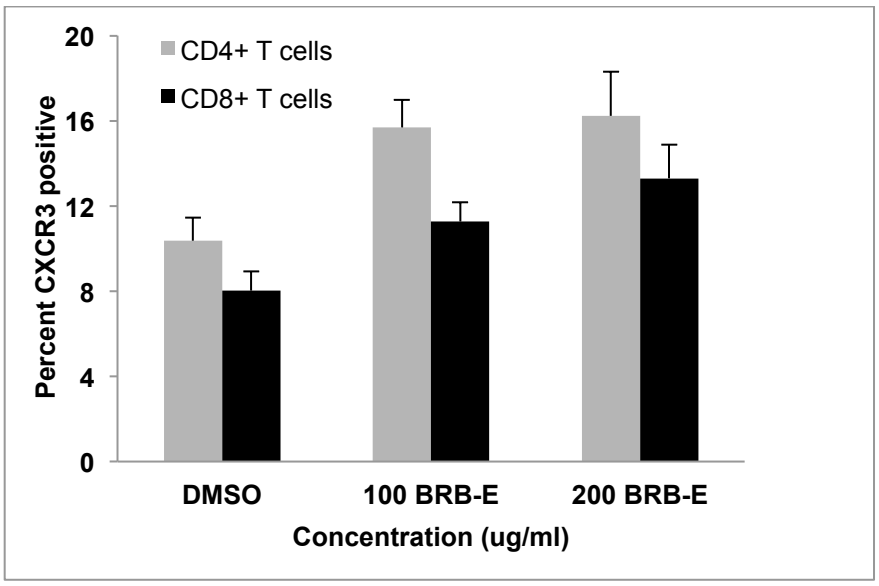

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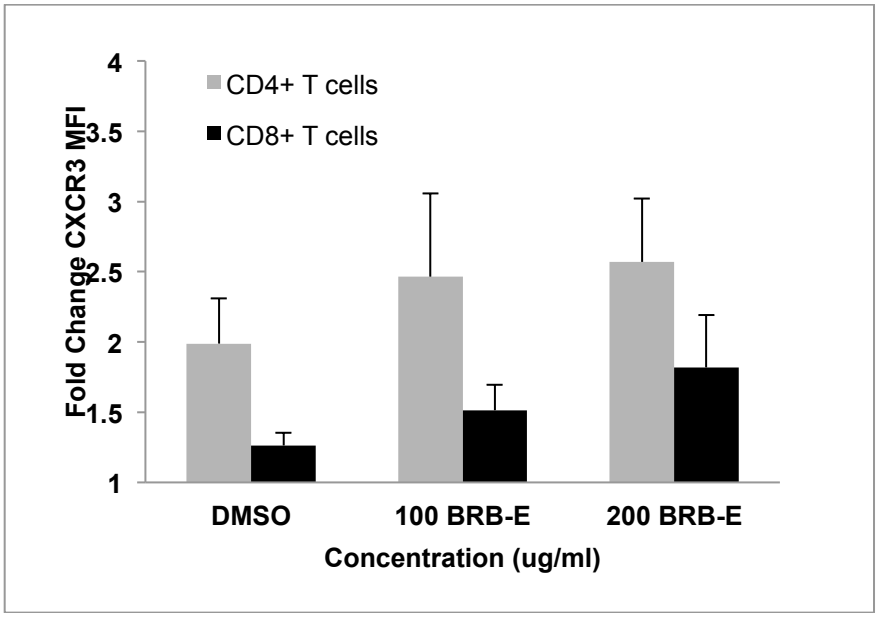

Figure 9C.

Black raspberry extract inhibits MDSC differentiation in vitro

Donor blood from the Red Cross was used to isolate PBMCs using Ficoll-Paque as described above. PBMCs were stimulated with IL-6 and GM-CSF (10ng/mL each) for 7 days with media and cytokine replacement every 2-3 days. Adherent and confluent cells

were collected, stained and analyzed via flow cytometry. Cells were stained for an MDSC phenotype. (**10A**) Representative flow cytometric analysis of MDSC generation in vitro. When comparing unstimulated (top panel) versus stimulated (bottom panel) conditions, we see an increase in CD11b+, CD33+ MDSC cells. (**10B**) Addition of black raspberry extract was noted to consistently inhibit MDSC generation in vitro as compared to DMSO, the vehicle that was used as a control. (**10C**) These in vitro generated MDSCs were tested for functionality in that they were able to suppress autologous T cell proliferation.

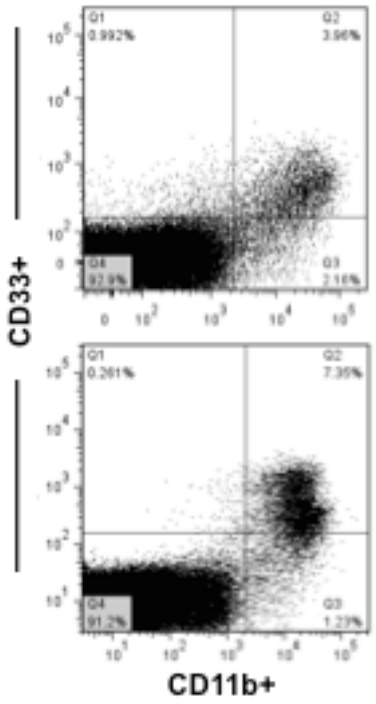

Figure 10A.

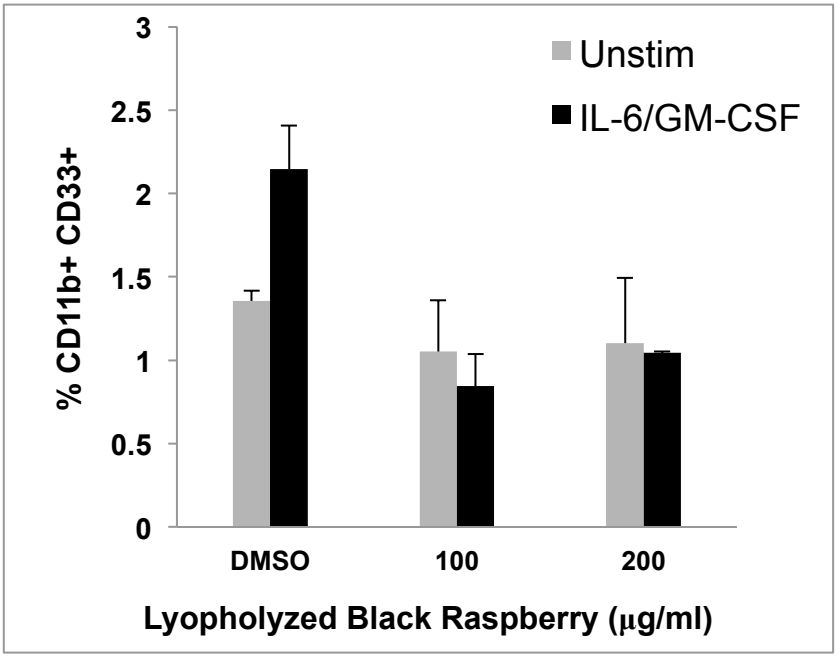

Figure 10B.

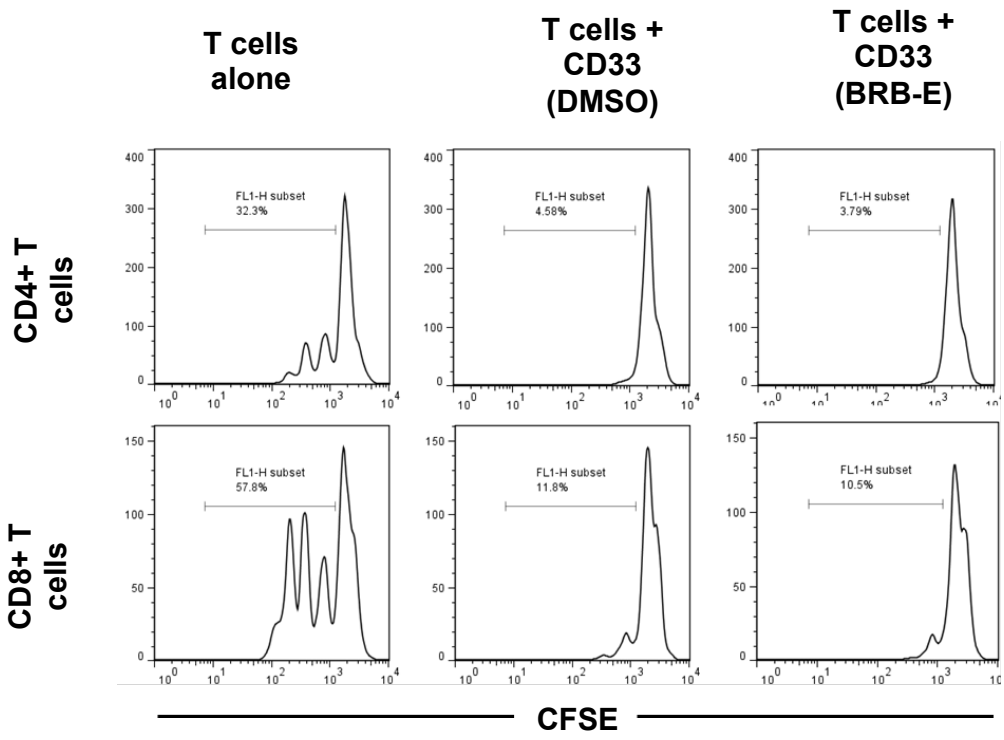

Figure 10C.

IL-6 induced STAT3 phosphorylation is reduced by black raspberry and its metabolites

PBMCs from donor blood were isolated using methods described above. These cells were incubated with BRB-E, C3R, Q3R, or the DMSO control for 4 hours. After incubation, the cells were stimulated with IL-6 at 0, 1, or 5 ng/ml concentration for 20 minutes. Next, lysates were made and analyzed via immunoblot. Pre-treatment of cells with BRB-E, C3R, or Q3R was shown to reduce expression of phosphorylated STAT3. Total STAT3 levels were not noted to change. Beta actin was used as a loading control. (**Figure 11**)

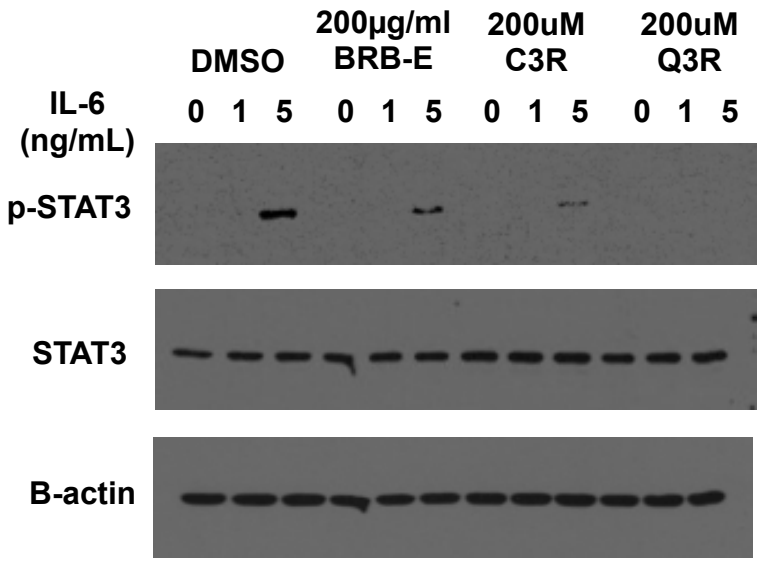

Figure 11.

Metabolites of black raspberry also inhibit MDSC generation and T cell proliferation

(**12A**) Isolated PBMCs, as described above, were cultured with DMSO, BRB-E, C3R, or Q3R and stimulated with IL-6/GM-CSF (10 ng/ml) for 7 days. The presence of black raspberry or its metabolites inhibited MDSC generation as compared to the DMSO control. (**12B**) Isolated T cells, as described above, were cultured with DMSO (control), BRB-E, C3R, or Q3R. Histograms represent flow cytomteric analysis of showing inhibition of CFSE diluted CD4+ T cells by black raspberry and its metabolites.

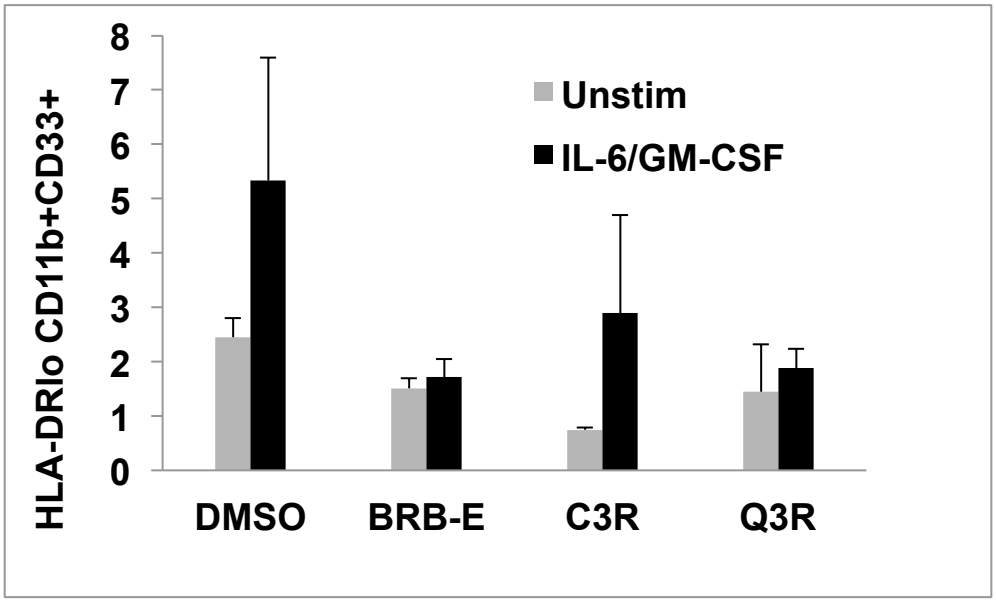

Figure 12A.

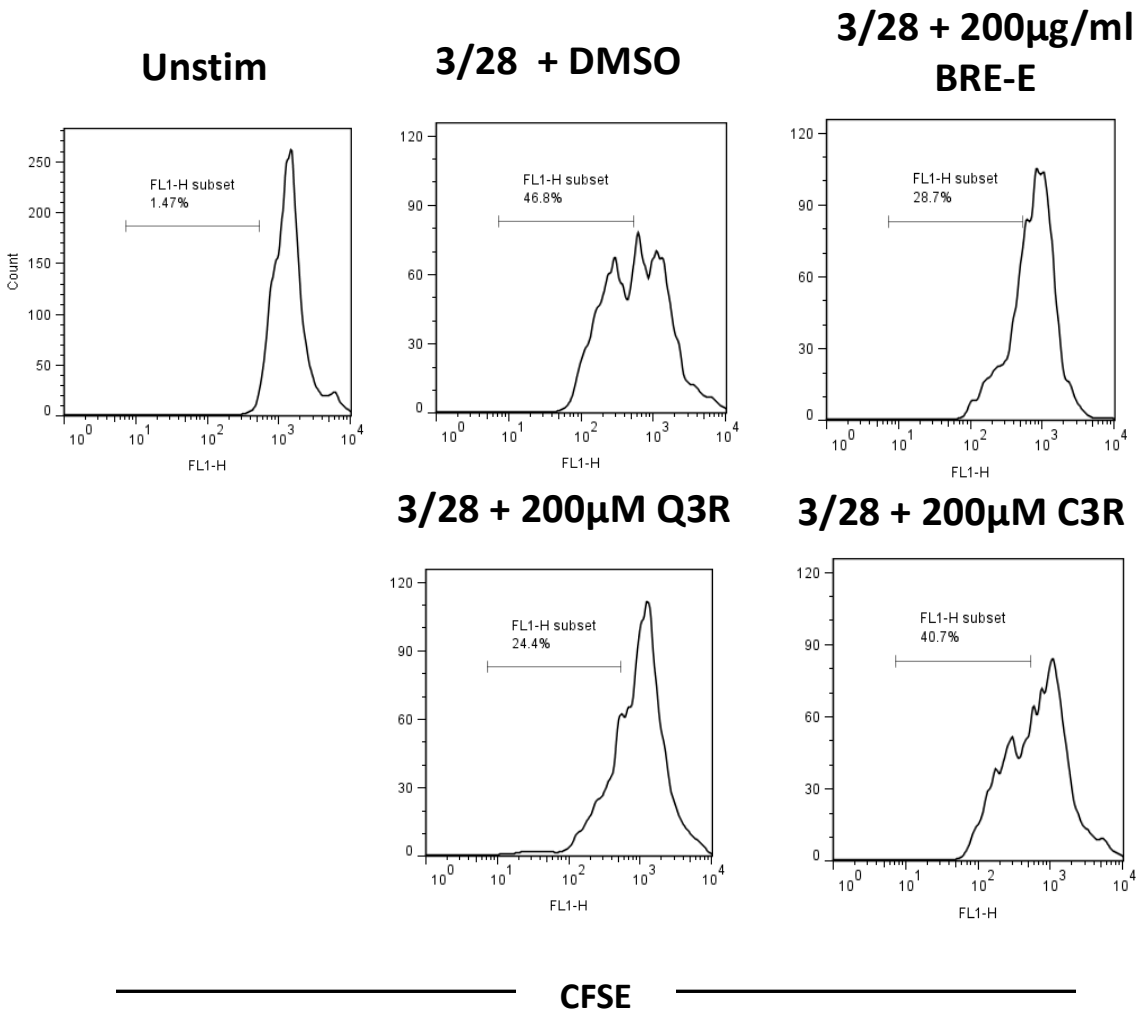

Figure 12B.

DISCUSSION

Our findings suggest that an ethanol based black raspberry extract and its metabolites, cyanidin-3-rutinoside and quercetin-3-rutinoside have an immunomodulatory effect on T lymphocyte function and MDSC generation. Black raspberries are a natural, food-based approach to inhibit carcinogenesis that is gaining interest based on data in vitro and in animal models.[18,2] Prior studies have shown that black raspberry can inhibit proliferation and promote apoptosis in malignant cells.[3] However few, if any, studies to date have explored how they might act on the immune system. Our current results are important because they present some of the immune mechanisms by which black raspberry can act. These mechanisms could then be directed towards regulating diseases involving chronic inflammation such as cancer, atherosclerosis, and autoimmune diseases.[19] Thus, this area of research has the potential to be used in a variety of applications.

We found that BRB-E had interesting effects on T lymphocytes in vitro. We saw a significant increase in T lymphocyte chemotaxis in the presence of BRB-E. We also saw an upregulation in the CXCR3 receptor expression in both CD4+ and CD8+ cells. Enhanced T cell migration could be beneficial in anti-cancer responses. However, this may also have the potential to aggravate the progression of the disease depending on the T cell subset. For example, if the ability of T regulatory cells to reach the tumor was enhanced, this could promote tumor progression. Inhibition of T cell proliferation by BRB-E could also be beneficial or harmful depending on the environment.[20] Thus, these observations need to be further studied to fully understand the significance of BRB-E.

In addition to effects on T lymphocyte function, we also found that BRB-E modulates MDSC differentiation. Elevated MDSC are found at sites of inflammation and cancer. They act to suppress immune responses and can thus exacerbate the pathology of the disease.[10] We also observed that the presence of BRB-E inhibited STAT3 phosphorylation, an important transcription factor required for MDSC differentiation. This suggests a mechanism by which BRB-E inhibits MDSC differentiation.

Furthermore, we investigated the bioactive metabolites within black raspberry as this could be important for future development of drugs that may modulate immune function. Both anthocyanin and quercetin compounds have been shown to have anti-cancer effects.[5] We found that both cyanidin-3-rutinoside and quercetin-3-rutinoside had similar effects to BRB-E in modulating T cell function and MDSC generation. These specific compounds also have the potential to be isolated and used in therapy of diseases such as cancer.

Black raspberry has the potential to be used as a beneficial dietary intervention but its effects require further investigation. Digestion, metabolism, and absorption of black raspberry may alter the bioactive compounds studied. In addition, genetic differences among individuals may affect its metabolism. Metabolite processing may also vary in individuals depending on their oral and intestinal microbiota.[21] The effects of black raspberry thus need to be further evaluated in vivo models and clinical trials to understand how it can be implemented to benefit patients. In conclusion, our results show that black raspberries and its metabolites can modulate immune function by influencing T cell function and MDSC generation. These findings have various potential applications such as dietary intervention or drug development in diseases such as cancer.

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