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Abstract: BACKGROUND/AIMS: Anthocyanins are plant-derived dietary components that are highly abundant, for example, in bilberries. We have previously demonstrated that anthocyanins exert anti-inflammatory properties in mouse colitis models and ameliorate disease activity in ulcerative colitis patients. Here, we studied the molecular mechanisms through which anthocyanin-containing bilberry extract (BE) exerts anti-inflammatory effects in human monocytic THP-1 cells. METHODS: THP-1 cells were pre-incubated with BE 20 min prior to TNF-α or IFN-γ (100 ng/ml each) stimulation. Signalling protein activation was studied by Western blotting, mRNA expression by quantitative PCR and cytokine secretion by ELISA. RESULTS: IFN-γ-induced phosphorylation of STAT1 and STAT3 was significantly reduced by BE co-treatment. Consequently, levels of mRNA expression and/or cytokine secretion of MCP-1, IL-6, TNF-α, ICAM-1, and T-bet were lower with BE co-treatment. In contrast, BE enhanced TNF-α-mediated p65-NF-κB phosphorylation but reduced ERK1/2 phosphorylation. BE co-treatment further increased TNF-α-induced mRNA expression and secretion of NF-κB target genes, such as IL-6, IL-8, and MCP-1, while mRNA levels of ICAM-1 were reduced. CONCLUSIONS: BE co-treatment reduced IFN-γ-induced signal protein activation, pro-inflammatory gene expression, and cytokine secretion, whereas it enhanced TNF-α-induced responses. These findings suggest a distinct role for anthocyanins in modulating inflammatory responses that need to be further studied to fully understand anthocyanin-mediated effects.

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Bilberry-derived anthocyanins prevent IFN-γ-induced pro-inflammatory signalling and cytokine secretion in human THP-1 monocytic cells

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Short title: Anthocyanins exert anti-inflammatory effects
Abstract

Background: Anthocyanins are plant-derived dietary components that are highly abundant e.g. in bilberries (Vaccinium myrtillus). We have previously demonstrated that anthocyanins exert anti-inflammatory properties in mouse models of chronic intestinal inflammation and ameliorate disease activity in ulcerative colitis patients. Here, we studied the molecular mechanisms how anthocyanin-containing bilberry extract (BE) modulates IFN-γ- and TNF-α-induced pro-inflammatory signalling and cytokine secretion of in human monocytic THP-1 cells.

Material & Methods: THP-1 cells were pre-incubated with BE 20 minutes prior to TNF-α (100 ng/ml) or IFN-γ (100 ng/ml) stimulation. Cells were analysed for signalling protein activation by Western blot; mRNA expression was assessed by quantitative PCR and cytokine secretion by ELISA.

Results: IFN-γ-induced phosphorylation of signal transducer and activator of transcription (STAT)1+3 was significantly reduced by BE co-treatment. Consequently, levels of mRNA expression and/or cytokine secretion of MCP-1, IL-6, TNF-α, ICAM-1 and T-bet were lower upon BE co-treatment. In contrast, BE enhanced the activation of nuclear factor (NF)-κB by TNF-α (phosphorylation of p65), but reduced TNF-α-mediated phosphorylation of extracellular signal-regulated kinase (ERK) 1/2. BE co-treatment further increased TNF-α-stimulation mRNA expression and secretion of NF-κB target genes, such as interleukin-6 (IL-6), IL-8 and monocyte chemo-attractant protein (MCP)-1, while mRNA levels of intercellular adhesion molecule (ICAM)-1 were reduced.
Conclusions: BE co-treatment resulted in a significant reduction of IFN-γ-induced signal protein activation, pro-inflammatory gene expression and cytokine secretion whereas it enhanced TNF-α-induced responses. These findings suggest a distinct role for anthocyanins in modulating inflammatory responses that need to be further studied to fully understand anthocyanin-mediated effects.

Key Words: Anthocyanins, TNF-α, NF-κB, JAK/STAT, inflammatory bowel disease
Introduction

Phenols are plant-derived molecules with anti-inflammatory, anti-oxidant, anti-carcinogenic, anti-adipogenic, anti-diabetic and neuroprotective potential (1, 2). Many phenols constitute components of our regular diet. Chemically, they comprise one or more (polyphenols) aromatic ring(s) with at least one hydroxyl group attached. Based on their chemical structure, they are classified into two groups, flavonoids and non-flavonoids. Anthocyanidins represent an important sub-class of dietary flavonoids. They are widespread in fruits and flowers where they account for the blue, purple and red colours. In these plant-derived forms anthocyanidins are commonly conjugated to sugars or organic acids and then named anthocyanins (1, 2). Berries, red wines, leafy and root vegetable and certain whole grain cereals contain relative high amounts of anthocyanins. The health-promoting effects of polyphenols have captured increasing interest in the past few years as epidemiological studies have demonstrated associations between the consumption of polyphenol-rich foods and beverages and the prevention of diseases, i.e. coronary heart disease, certain forms of cancer and inflammatory diseases (2-5). Initially, the benefits of polyphenols were attributed to their anti-oxidant properties. However, in the meantime, it has been postulated that other mechanisms such as direct interference with signalling pathways and gene expression events could be involved (6, 7).

Bilberries (Vaccinium myrtillus L.) have one of the highest natural anthocyanin contents (8). Various studies provide evidence that anthocyanin extracts inhibit inflammatory gene expression in vitro (8-10). Moreover, it has been demonstrated that anthocyanins from bilberries and blueberries decrease pro-inflammatory cytokine serum levels and attenuate the severity of experimental colitis in mice (11-13). In an open label pilot study in patients with mild to moderate ulcerative colitis (UC) despite standard treatment an anthocyanin-rich bilberry preparation significantly reduced disease activity, endoscopy scores and fecal
calprotectin levels (14). Hence, anthocyanins represent a potential therapeutic option in inflammatory bowel disease (IBD) patients.

IBD comprising of Crohn’s disease (CD) and UC represents a chronic immune-mediated disorder of the gastrointestinal (GI) tract in the genetically susceptible host and is triggered by environmental factors. Though the exact etiology remains to be determined, there is strong evidence that a dys-regulated immune response to commensal intestinal microbiota initiates the chronic and relapsing inflammation of the intestinal mucosa or the gut wall (15-17). Family members of affected patients have an increased risk to develop IBD indicating a genetic component in IBD pathogenesis (18-21). Hence, numerous genome-wide association studies (GWAS) have identified variations in more than 160 genes involved in the intestinal immune homeostasis as risk factors for developing IBD. Most variants that have been functionally characterized are associated with the development of an immunological imbalance and inadequate immune response to the commensal flora (17, 22-27). Several genetic variants associated with IBD are also risk factors for other inflammatory or autoimmune disorders, e.g. rheumatoid arthritis or type I diabetes (15-17). Since a significant percentage of IBD patients is not satisfactorily treated with the established treatment options or suffers from therapy-related side effects, the need for new and more effective, but also safe and well-tolerated therapeutic options is obvious (14, 28).

Therefore, the aim of this study was to elucidate the molecular mechanisms underlying the anti-inflammatory potential of anthocyanin-rich bilberry extract (BE). We found that co-treatment of BE together with TNF-α or IFN-γ in human monocytic THP-1 cells had distinct effects on cytokine-induced signal transduction and gene expression. While BE enhanced TNF-α-induced pro-inflammatory signals, it significantly prevented IFN-γ-mediated pro-inflammatory effects.
**Materials and Methods**

**Reagents and Antibodies**

Monoclonal mouse anti-human phospho-p38 mitogen-activated protein kinase (MAPK; Thr^{180}/Tyr^{182}; 28B10), polyclonal rabbit anti-human p38 MAPK, polyclonal rabbit anti-human phospho-ERK1/2 (Thr^{202}/Tyr^{204}), monoclonal rabbit anti-human ERK1/2 (137F5), polyclonal rabbit anti-human phospho-c-Jun N-terminal kinase (JNK; Thr^{183}/Tyr^{185}), polyclonal rabbit anti-human JNK, monoclonal rabbit anti-human phospho-NF-κB p65 (Ser^{536}; 93H1), polyclonal rabbit anti-human NF-κB p65 (Ser^{276}), polyclonal rabbit anti-human phospho-STAT1 (Tyr^{701}), polyclonal rabbit anti-human STAT1, polyclonal rabbit anti-human phospho-STAT3 (Tyr^{705}), and polyclonal rabbit anti-human STAT3 were obtained from Cell Signaling Technologies (Danvers, MA, United States).

Human recombinant IFN-γ was obtained from Sigma (Sigma-Aldrich, St. Louis, MO, United States). Human recombinant TNF-α was purchased at Promokine (Heidelberg, Germany). The BE was manufactured by Kaden Biochemicals, Symrise GmbH & Co (Holzminden, Germany) and was allocated as a powder (25% anthocyanin content). Using this powder a stock solution of 10 mg BE/ml was established. All other reagents were of analytical grade and obtained commercially.

**THP-1 Cell Culture and Stimulation Protocols**

Human monocytic THP-1 cells (Sigma-Aldrich) were cultured in RPMI 1640 medium (Life technologies, Gibco, Carlsbad, CA, United States) with additional 10 % fetal calf serum (FCS) at an approximate density of 0.5 to 1 x 10^6 cells/ml. Cells were stored in a 5 % CO₂ and 95 % humidified incubator at 37°C. For experiments, cells were seeded in 1 ml of FCS-free RPMI 1640 medium + 1 % Penicillin/Streptomycin per well at 1-1.5 x 10^6 cells/ml. Human T84 intestinal epithelial cells (IEC; ATCC, Manassas, VA, United States) were cultured in DMEM medium (Life Technologies, Carlsbad, CA, United States) with additional
10% FCS. Cells were stored at 37°C in an incubator with 10 % CO₂ and 95 % humidity. Cells were seeded 24 h prior to experiments. Pre-treatment with BE solution (composed of BE powder and FCS-free RPMI 1640 medium + 1 % Penicillin/Streptomycin) with a final concentration of 10 µg/ml was conducted 20 min before stimulation. Then, TNF-α or IFN-γ were administered in a concentration of 100 ng/ml for either 20 min (Western blot experiments) or 24 h (qPCR and ELISA experiments).

**Preparation of Whole Cell Lysates**

Cells were washed twice with phosphate buffered saline (PBS) and lysed in M-Per Mammalian protein extraction reagent (Pierce Biotechnology, Rockford, IL, United States) supplemented with protease inhibitors (Roche, Basel, Switzerland) for 45 min. After centrifugation (10 min at 13,000 g), cell lysate supernatants were assayed for protein content using a NanoDrop spectrophotometer (NanoDrop ND1000; Pierce Biotechnology).

**Western Blotting**

Each lysate was mixed with loading buffer (NuPAGE® 4x LDS Sample Buffer (Life technologies), 500 mM dithiothreitol and boiled for 5 min at 95°C. Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes (Millipore, Billerica, MA, United States). Membranes were blocked during 1 h with blocking solution (3 % milk powder (C. Roth GmbH+Co. KG, Karlsruhe, Germany) and 1 % bovine serum albumin (BSA) (GE Healthcare, PAA Laboratories GmbH, Pasching, Austria) in washing buffer (Tris buffered saline containing 1 % Tween 20)). Primary antibody was diluted in blocking solution (1:1000 for all experiments). Membranes were incubated in primary antibody solution overnight at 4°C and then washed with washing buffer for 30 min. Horseradish peroxidase (HRP)-labelled secondary anti-mouse- or anti-rabbit-IgG-antibody (1:5000; Santa Cruz Biotechnologies, Santa Cruz, CA, United States) in blocking solution
was added for 1 hour and membranes were washed again for 30 min. Immunoreactive proteins were detected using an enhanced chemiluminescence detection kit (Thermo Scientific, Rockford, IL, United States).

**RNA Isolation and Complementary DNA Synthesis**

THP-1 cells were washed with ice-cold phosphate buffered saline (PBS) and disrupted in RLT buffer (Qiagen, Venlo, Netherlands) and 1 M dithiothreitol solution. Total RNA was isolated using RNeasy Plus Mini Kit (Qiagen) according to manufacturer's instructions. RNA concentration was measured by absorbance at 260 and 280 nm (NanoDrop ND1000). Complementary DNA (cDNA) synthesis was performed using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, United States) following the manufacturer's instructions.

**Real-time Polymerase Chain Reaction**

Real-time polymerase chain reaction (PCR) was performed using FAST qPCR MasterMix for Taqman Assays (Applied Biosystems) on a Fast 7900HT Real-Time PCR system using SDS Software (Applied Biosystems). Measurements were performed in triplicate, human β-actin was used as endogenous control, and results were analyzed by ΔΔCT method. The real-time PCR contained 45 cycles consisting of a denaturing (95° C, 20 sec) and an annealing/extending (60° C, 20 sec) step. Gene expression assays were all obtained from Applied Biosystems.

**Enzyme-linked Immunosorbent Assay (ELISA)**

Supernatant of THP-1 cells was collected and stored at -20° C. ELISA kits detecting human IL-6, human MCP-1 and human TNF-α were obtained from Promokine, human IL-8 was purchased at SABiosciences (Qiagen). Assays were carried out according to
manufacturer’s recommendations using a sample volume of 100 μl. Absorbance at 450 nm
was detected on a BioTek-Synergy Luminescence Reader using Gen5.1.1.1 Software.
Measurements were performed in duplicates.

Statistical Analysis

Data are presented as means ± SEM for a series of n experiments. Data are expressed
as relative values of the respective control. Statistical analysis was performed by analysis of
variance (ANOVA) followed by Student–Newman–Keuls post hoc test. p values < 0.05 were
considered significant.
Results

IFN-γ-induced STAT1+3 phosphorylation is decreased in presence of BE

To study the signalling pathways regulated by bilberry-derived anthocyanins, we analysed phosphorylation of STAT1+3 in response to IFNγ and/or BE by Western Blotting. IFN-γ led to a significant increase in STAT1 (more than 60-fold) and STAT3 (more than 10-fold) phosphorylation in THP-1 cells after 20 min (Figures 1A+B). Treatment with BE alone had no effect on STAT1+3 phosphorylation. However, co-incubation with BE was able to abrogate the IFN-γ-induced activation in THP-1 cells. The levels of STAT1+3 phosphorylation in the latter were comparable to control cells (Figures 1A+B). Similarly, in T84 IEC, co-treatment of IFN-γ and BE also ameliorated the IFN-γ-induced phosphorylation of STAT1 (Figure 1C).

BE did not affect IFN-γ-influence on MAP-Kinase pathways

We next investigated the influence of BE on IFNγ-induced phosphorylation of MAPK isoforms. Co-stimulation with IFN-γ and BE led to trend towards increased phosphorylation of the MAPK isoforms ERK, JNK and p38 (Suppl. figure 1). However these effects were statistically not significant and correlate with our observations in T84 IEC (data not shown).

BE reduced IFN-γ-induced pro-inflammatory gene expression and cytokine secretion

We next evaluated whether co-administration of BE might inhibit IFN-γ-induced pro-inflammatory gene expression and cytokine secretion. We studied gene expression in THP-1 cells stimulated with 100 ng/ml IFN-γ and/or 10 µg/ml BE for 24 h. mRNA expression levels of IL-6 and MCP-1, ICAM-1 and T-bet transcription factor were normalized to the housekeeping gene β-actin. As shown in Figure 2A-D, stimulation with IFN-γ induced mRNA levels of MCP-1, IL-6, ICAM-1 and T-bet compared to non-simulated cells. Of note,
mRNA expression of all of the four markers was significantly reduced when co-stimulation with IFN-γ+BE was arranged. BE treatment alone did not cause a significant increase of the respective gene expression. We next investigated whether BE was also able to inhibit IFN-γ-induced cytokine secretion. THP-1 cells stimulated with IFN-γ for 24 h secreted increased levels of MCP-1 and TNF-α. Single BE exposure did not lead to enhanced MCP-1 and TNF-α secretion. Interestingly, cytokine secretion was significantly reduced in cells where IFN-γ+BE co-stimulation was performed (Figures 3A+B). IL-6 concentrations in this ELISA experiments were below detection levels (data not shown). These data demonstrate that BE are sufficient to ameliorate IFN-γ-induced pro-inflammatory signalling, gene expression and cytokine secretion what might critically contribute to their observed anti-inflammatory effects.

**BE enhanced TNF-α-induced NF-κB phosphorylation**

We next studied the effects of BE with respect to TNF-α-induced signalling, gene expression and cytokine secretion. As expected, incubation with 100 ng/ml TNF-α for 20 min induced NF-κB phosphorylation in THP-1 cells. Incubation with BE alone had no effect on NF-κB phosphorylation. However, co-incubation with TNF-α+BE further enhanced NF-κB phosphorylation (Figure 4). Similar results were obtained in T84 IEC (data not shown).

**BE reduced MAP-Kinase activation in response to TNF-α**

We further analyzed the impact of TNF-α (100 ng/ml) and BE (10 µg/ml) on phosphorylation of ERK-, p38- and JNK-MAPK in THP-1 cells. TNF-α induced ERK phosphorylation only to a small extent compared to untreated cells. BE was able to reduce ERK phosphorylation significantly in comparison to unstimulated control cells. Co-stimulation with TNF-α and BE resulted in a similar reduction of ERK phosphorylation as for
BE alone (Figure 5). For p38 we did not find significant changes. However, there was a trend
for reduction of p38 phosphorylation in cells co-stimulated with TNF+BE (Suppl. Figure 2).
Results for JNK phosphorylation were also not significant, but also showed a similar trend
(data not shown). Altogether, BE tends to reduce TNF-α-mediated MAPK activation.

BE amplified pro-inflammatory cytokine expression and secretion in TNF-α-stimulated cells

We next investigated the effects of BE on mRNA transcription levels of pro-
inflammatory TNF-α target genes. We quantified mRNA expression of the pro-inflammatory
cytokines IL-6, IL-8 and MCP-1 and of the adhesion molecule ICAM-1 in THP-1 cells.
mRNA levels were normalized to β-actin. TNF-α induced mRNA expression of IL-6, IL-8,
MCP-1 and ICAM-1 significantly (Figures 6A-D). IL-6 expression was significantly
increased upon single BE stimulation compared to non-stimulated cells. In case of IL-8,
MCP-1 and ICAM-1 BE did not affect gene expression compared to control cells. However,
MCP-1 and ICAM-1 mRNA levels were significantly lower when cells were exposed to BE
compared to TNF-α. THP-1 cells exposed to TNF-α+BE showed further significant
enhancement in IL-6, IL-8 and MCP-1 expression (Figures 6A-C), whereas co-stimulation led
to reduction of ICAM-1 expression when compared to TNF-α treatment alone (Figure 6D).

We further investigated IL-8 and MCP-1 secretion quantified by ELISA after 24 h
stimulation. Concentrations of IL-8 were significantly increased after co-stimulation with
TNF-α and BE (Figure 7A) confirming the mRNA expression data (see Figure 4B). Likewise,
THP-1 cells exposed to both, TNF-α and BE, showed a significant increase in MCP-1
secretion compared to TNF-α stimulation only (Figure 7B) again confirming the mRNA
expression data.
Discussion

Here, we have demonstrated that BE is able to ameliorate IFN-\(\gamma\)-induced STAT1 and STAT3 activation, pro-inflammatory cytokine gene expression and secretion as well as TNF-\(\alpha\)-induced ERK activation in human monocytic cells (THP-1). In contrast BE induced NF-\(\kappa\)B activation and cytokines regulated by NF-\(\kappa\)B in response to TNF-\(\alpha\).

A number of studies showed that polyphenols attenuate disease activity in IBD patients (11-14). The aim of this cell culture model was to provide more information about the cellular mechanisms underlying the protective and anti-inflammatory properties of anthocyanins. It is well known that inflammatory pathways induced by IFN-\(\gamma\) and TNF-\(\alpha\) play a central role in IBD pathogenesis (29). IFN-\(\gamma\) effects are elicited through activation of intracellular signalling pathways like the JAK-STAT pathway (30). This pathway leads to STAT1 phosphorylation resulting in target gene expression. Similar molecular mechanisms are valid for STAT3 (31, 32). In our experiments, BE featured significant inhibition on IFN-\(\gamma\)-induced STAT1 and STAT3 activation. These findings correlate with anthocyanin-mediated inhibition of STAT1 phosphorylation in the human intestinal HT-29 cell line described in the literature (6). STAT1 and STAT3 expression and activation are increased in IBD patients (33-35). We demonstrated that not only STAT1 and STAT3 phosphorylation was inhibited by BE but also target gene expression (IL-6, MCP-1, ICAM-1 and T-bet) and cytokine secretion (MCP-1, TNF-\(\alpha\)). Our data correlate with Triebel et al (10) who evaluated effects of BE in human colon epithelial cells T84. In our experiments, we confirmed that BE prevents IFN-\(\gamma\)-induced pro-inflammatory signalling in THP-1 mononuclear cells and T84 IEC.

We suggest that the STAT inhibition could either be due to receptor antagonism (BE might impede correct receptor clustering and therefore initiation of the JAK/STAT pathway) or altered activation of phosphatases and kinases, respectively. Another possible explanation postulated for the decreased STAT phosphorylation is a BE-mediated induction of the SOCS
family proteins. They are responsible for the negative feedback mechanism of STAT recruitment (6). For better understanding of these molecular mechanisms further investigations are necessary.

MAPK signalling is important in cellular processes, such as proliferation, differentiation, apoptosis and inflammatory signalling cascades (36-38). In the mucosa of inflamed gut tissue MAPK phosphorylation is increased (38-41). In our experiments, IFN-γ-induced MAPK signalling pathways in THP-1 cells and T84 IEC were not significantly affected by BE. Though, there was a trend towards increased MAPK activation in cells co-stimulated with BE+IFN-γ. Again, this result matches with the finding of Serra et al (6) who did not find reduced p38 phosphorylation upon anthocyanin and cytokine stimulation in HT-29 cells. On the other hand, it has been reported that polyphenols reduce MAPK activation in human basophilic cells (42). There are more investigations needed to clarify the role of BE in MAPK signalling pathways.

In contrast to the experiments with IFN-γ as inflammatory stimulus, BE was not able to ameliorate TNF-α-induced cellular reactions. In our assay conditions, BE strengthened TNF-α-induced NF-κB phosphorylation in THP-1 cells and T84 IEC. It is known that NF-κB is an important regulator of pro-inflammatory genes expression, such as IL-6, IL-8 and MCP-1 (43-46). Moreover, there is evidence that NF-κB is induced in IBD tissue (47). We demonstrated that anthocyanins from BE reinforced not only NF-κB phosphorylation, they also amplified gene expression of IL-6, IL-8 and MCP-1 in THP-1 cells (Figure 6A-C). Accordingly, BE enhanced TNF-α-induced IL-8 and MCP-1 secretion (Figure 7). It could be argued that the used BE contained lipopolysaccharids (LPS), which would explain increased NF-κB phosphorylation. Yet, the addition of 1% Penicillin/Streptomycin to the cell medium should have prevented LPS occurrence. These results are in accordance with Serra et al (6) who did not observe a prevention of NF-κB activation by anthocyanins in HT-29 cells either.
On the other hand, polyphenols suppressed NF-κB activation in human basophilic cells KU812 (42). Furthermore, inhibitory effects of a typical dietary anthocyanin (cyanidin-3-glycoside, mainly existent in black rice) on NF-κB activation in RAW 264.7 macrophage cells was reported (48). However, it is not sure whether this specific anthocyanin is contained in our BE.

Interestingly, BE reduced TNF-α-induced ERK phosphorylation in THP-1 cells. We could not confirm this finding for p38 and JNK. Other groups reported polyphenol-/anthocyanin-induced inhibition of ERK, p38 and JNK (42, 48). ICAM-1 plays an important role in leukocyte recruitment and is a MAPK target gene (39). BE suppressed TNF-α-induced ICAM-1 expression, which confirms ERK-inhibition. All in all, these data demonstrate that BE exert pro- as well as anti-inflammatory properties in response to TNF-α in human immune cells.

In conclusion, our data demonstrate that BE exert anti-inflammatory effects in cell models of inflammation. However, these effects seem to be dependent on the respective stimulus. Our observations suggest also that BE might enhance, at least to some extent, TNF-α-induced inflammation. This might have an impact on the clinical utility of BE in treating IBD patients. Further studies are clearly needed to further define these mechanisms and to evaluate whether BE might work or not for example in patients responding or not responding to anti-TNF antibodies.
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Competing interests: The authors declare there are no competing interests.

Author contributions: SR, IM, MRS and SL performed experiments and analyzed data. MS and GR conceived the experimental study. MS supervised the project. All authors wrote, corrected and approved the manuscript.
References


Figure 1: STAT phosphorylation is significantly reduced when IFN-γ (IFN) and BE are simultaneously available. IFN leads to considerable activation of both STAT1 (A) and STAT3 (B). On the other hand, BE prevents STAT activation. Subsequent gene transcription is therefore suppressed. THP-1 cells were pre-stimulated with BE (10 μg/ml) for 20 min. Subsequent stimulation with IFN (100 ng/ml) lasted 20 min too. C: In T84 cells STAT1 activation was similarly suppressed in presence of BE. Asterisks represent significant results (**=p<0.01; ***=p>0.001; ###=p<0.01; ####=p<0.001; ••=p<0.01; •••=p<0.001). Columns marked with asterisks are to compare to other columns as described in the following: * = versus Ctr; # = versus BE; • = versus IFN+BE.

Figure 2: TNF-α activates nuclear factor-κB (NF-κB, p65). However, co-stimulation with BE and TNF-α amplifies NF-κB activation. Thus, anthocyanins from BE seem to reinforce TNF-α-associated effects. Note that sole stimulation with BE did not lead to considerable increase in NF-κB activation. Experiments were conducted six fold (n=6). THP-1 cells were pre-stimulated 20 min with BE (10 μg/ml) and subsequently exposed to TNF (100 ng/ml) for another 20 min depending on the subgroup. Asterisks represent significant results (°°=p<0.01; **=p<0.01; ***=p<0.001; ###=p<0.01; ####=p<0.001). Each column marked with an asterisk is to be compared with another column: ° = versus TNF; * = versus Ctr; # = versus BE.
Figure 3: The MAP Kinase ERK1/2 is barely activated by TNF-α, whereas BE tends to evoke decreased activation. In case of ERK, TNF-α did not induce a potent activation. On the other hand, BE seems to inhibit TNF-α-mediated ERK activation (n=3). This effect might explain part of the anti-inflammatory properties accredited to BE. Cells were pre-stimulated with BE (10 µg/ml) for 20 min followed by 20 min stimulation with TNF-α (100 ng/ml). Asterisks show significant results (#=p<0.05; •=p<0.05). The column marked with asterisks is to be compared to another column: # = versus BE; • = versus BE+TNF.

Figure 3: Influence of BE on gene expression. THP-1 cells were stimulated with 100 ng/ml TNF-α (TNF), BE (10 µg/ml) containing anthocyanins or both (TNF+BE) for 24 h. There always was an untreated control group (Ctr). mRNA concentration was measured by qPCR and normalized to the constitutively expressed housekeeping gene β-actin. In case of the pro-inflammatory cytokines IL-6, IL-8 and MCP-1 (A-C) increased gene expression was registered in presence of TNF+BE. For the adhesion molecule ICAM-1 contrary results were found (D): gene expression was lowered in co-stimulated cells (TNF+BE). Asterisks denote significant differences (*=p<0.05; **=p<0.01; ***=p<0.001; #=p<0.05; ##=p<0.01; ###=p<0.001; •=p<0.05; ••=p<0.01; •••=p<0.001). Columns marked with an asterisk are meant to be compared to another column (* = versus Ctr; # = versus TNF; • = versus BE).
Figure 4: BE stimulates cytokine secretion reinforcing the pro-inflammatory effects of TNF-α. THP-1 cells stimulated with both, 100 ng/ml TNF-α and 10 µg/ml BE (TNF+BE) for 24 h showed significantly higher secretion of the pro-inflammatory cytokines (A, B). Cytokine secretion was measured applying the ELISA method. Asterisks represent significant differences (**=p<0.01; ***=p<0.001; #=p<0.05; ##=p<0.01; •=p<0.05; ••=p<0.01; •••=p<0.001). Asterisked columns are meant to be compared to another column: * = versus Ctr; # = versus TNF; • = versus BE.

Figure 6: BE inhibits IFN-γ-mediated activation of gene expression. THP-1 cells were stimulated with 100 ng/ml IFN-γ (IFN), 10 µg/ml BE or both of them (IFN+BE) for 24 h. The untreated cells served as control group (Ctr). In all experiments (A-D) BE reduced significantly the inflammatory effect of IFN-γ. mRNA concentration was normalized to β-actin. T-bet is a T-cell transcription factor inducing IFN-γ production. Asterisks denote significant differences (*=p<0.05; **=p<0.01; ***=p<0.001; #=p<0.05; ##=p<0.01; ###=p<0.001; •=p<0.05; ••=p<0.01; •••=p<0.001). Columns marked with an asterisk are intended to be compared to another column (* = versus Ctr; # = versus IFN+BE; • = versus BE).

Figure 7: BE inhibits IFN-γ-induced cytokine secretion in THP-1 cells significantly. THP-1 cells co-stimulated with 100 ng/ml IFN-γ and 10 µg/ml BE (IFN+BE) secrete significantly lower amounts of MCP-1 (A) and TNF-α (B) compared to cells
stimulated with IFN-γ only. Untreated cells (Ctr) and cells stimulated with BE only secrete similar amounts of MCP-1 and TNF-α, respectively. Stimulation lasted 24 h. Asterisks demonstrate significant differences (\(*=p<0.01\); \#=#=p<0.01\); \###=p<0.001\); \(\bullet=\)p<0.05; \(\bullet\bullet\bullet=p<0.001\). Asterisked columns are supposed to be compared to another column (* = versus Ctr; # = versus BE; • = versus IFN+BE).

**Supplement figure 1: MAP-Kinases tend to be induced in presence of BE.** The results in these experiments (n=6) were not significant, incoherent and thus difficult to interpret. IFN-γ did not have a strong impact on MAP-Kinase signalling pathways whereat IFN-γ and BE rather induced MAP-Kinase phosphorylation. Cells were pre-stimulated with BE (10 μg/ml) for 20 min and subsequent IFN-γ stimulation (100 ng/ml) lasted another 20 min.

**Supplement figure 2: TNF-α stimulation leads to p38 activation whereas BE rather inhibits it.** This experiment was conducted six times (n=6). Pre-stimulation with BE (10 μg/ml) lasted 20 min and stimulation with TNF-α (TNF, 100 ng/ml) another 20 min before cells were lysed. According to the results of ERK in Figure 2, BE tends to inhibit TNF-activated MAP-Kinase pathways.