Zurich Open Repository and Archive
University of Zurich
Main Library
Strickhofstrasse 39
CH-8057 Zurich
www.zora.uzh.ch

Year: 2014

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

Roth, Sofia ; Spalinger, Marianne R ; Müller, Isabelle ; Lang, Silvia ; Rogler, Gerhard ; Scharl, Michael

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- or IFN- (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- -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- , ICAM-1, and T-bet were lower with BE co-treatment. In contrast, BE enhanced TNF- -mediated p65-NF- B phosphorylation but reduced ERK1/2 phosphorylation. BE co-treatment further increased TNF- -induced mRNA expression and secretion of NF- B 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- -induced signal protein activation, pro-inflammatory gene expression, and cytokine secretion, whereas it enhanced TNF- -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.

DOI: https://doi.org/10.1159/000366055

Posted at the Zurich Open Repository and Archive, University of Zurich
ZORA URL: https://doi.org/10.5167/uzh-104677
Journal Article
Published Version

Originally published at:
Roth, Sofia; Spalinger, Marianne R; Müller, Isabelle; Lang, Silvia; Rogler, Gerhard; Scharl, Michael (2014). Bilberry-derived anthocyanins prevent IFN- -induced pro-inflammatory signalling and cytokine secretion in human THP-1 monocytic cells. Digestion, 90(3):179-189.
DOI: https://doi.org/10.1159/000366055

Original Paper

Digestion

Digestion 2014;90:179–189
DOI: 10.1159/000366055

Received: April 28, 2014
Accepted: July 21, 2014
Published online: November 12, 2014

Bilberry-Derived Anthocyanins Prevent IFN-γ-Induced Pro-Inflammatory Signalling and Cytokine Secretion in Human THP-1 Monocytic Cells

Sofia Roth[a] Marianne R. Spalinger[a] Isabelle Müller[a] Silvia Lang[a] Gerhard Rogler[a, b] Michael Scharl[a, b]

[a]Division of Gastroenterology and Hepatology, University Hospital Zurich, and [b]Zurich Center for Integrative Human Physiology, University of Zurich, Zurich, Switzerland

Key Words
Anthocyanins · TNF-α · NF-κB · JAK/STAT · Inflammatory bowel disease

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. © 2014 S. Karger AG, Basel

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 (*poly*phenols) 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 antho-

KARGER
© 2014 S. Karger AG, Basel
0012–2823/14/0903–0179$39.50/0
E-Mail karger@karger.com
www.karger.com/dig

PD Dr. med. Michael Scharl
Division of Gastroenterology and Hepatology
University Hospital Zurich
Rämistrasse 100, CH–8091 Zurich (Switzerland)
E-Mail michael.scharl@usz.ch

Downloaded by:
Universität Zürich, Zentralbibliothek Zürich
130.60.47.22 - 6/3/2016 2:56:22 PM

cyanins [1, 2]. Berries, red wines, leafy and root vegetables and certain whole grain cereals contain relatively 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 (also called 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, despite standard treatment, an anthocyanin-rich bilberry preparation significantly reduced disease activity, endoscopy scores and faecal calprotectin levels [14]. Hence, anthocyanins represent a potential therapeutic option in inflammatory bowel disease (IBD) patients.

IBD comprising Crohn's disease and ulcerative colitis represents a chronic immune-mediated disorder of the gastrointestinal tract in the genetically susceptible host and is triggered by environmental factors. Though the exact aetiology remains to be determined, there is strong evidence that a dysregulated 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 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, Mass., USA).

Human recombinant IFN-γ was obtained from Sigma (Sigma-Aldrich, St. Louis, Mo., USA). 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, Calif., USA) with additional 10% foetal calf serum (FCS) at an approximate density of $0.5–1 \times 10^6$ cells/ml. Cells were stored in a 5% CO_2 and 95% humidified incubator at 37 °C. For experiments, cells were seeded in 1 ml of FCS-free RPMI 1640 medium plus 1% penicillin/streptomycin per well at $1–1.5 \times 10^6$ cells/ml. Human T84 intestinal epithelial cells (IEC; ATCC, Manassas, Va., USA) were cultured in DMEM medium (Life Technologies, Carlsbad, Calif., USA) with additional 10% FCS. Cells were stored at 37 °C in an incubator with 10% CO_2 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 plus 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

Downloaded by:
Universität Zürich, Zentralbibliothek Zürich
130.60.47.22 - 6/3/2016 2:56:22 PM

(Western blot experiments) or 24 h [quantitative polymerase chain reaction (qPCR) and enzyme-linked immunosorbent assay (ELISA) experiments].

Peripheral Blood Mononuclear Cells

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood using Ficoll-Paque (1.077 g/ml; GE Healthcare, Little Chalfont, UK) density centrifugation. PBMCs (1×10^6) were seeded on 12-well plates in RPMI 1640 medium for 8 h before further treatment in the medium.

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, Ill., USA) 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® 4× 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, Mass., USA). Membranes were blocked during 1 h with blocking solution [3% milk powder (C. Roth GmbH & Co. KG, Karlsruhe, Germany) and 1% bovine serum albumin (GE Healthcare, PAA Laboratories GmbH, Pasching, Austria) in washing buffer (Tris-buffered saline containing 1% Tween 20)]. Primary antibody was diluted in blocking solution (1:1,000 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-labelled secondary anti-mouse- or anti-rabbit-IgG-antibody (1:5,000; Santa Cruz Biotechnologies, Santa Cruz, Calif., USA) in blocking solution was added for 1 h and membranes were washed again for 30 min. Immunoreactive proteins were detected using an enhanced chemiluminescence detection kit (Thermo Scientific, Rockford, Ill., USA) and exposure on X-ray films (GE Healthcare, Little Chalfont, UK). Films were scanned and intensity of the protein bands determined using NIH ImageJ software. Densitometry values from phosphorylated proteins were normalized to the corresponding values from total proteins and to non-treated controls.

RNA Isolation and Complementary DNA Synthesis

THP-1 cells were washed with ice-cold PBS and disrupted in RLT buffer (Qiagen, Venlo, The Netherlands) and 1 M dithiothreitol solution. Total RNA was isolated using RNeasy Plus Mini Kit (Qiagen) according to the 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, Calif., USA) following the manufacturer's instructions.

Real-Time PCR

Real-time 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 analysed by the ΔΔCT method. The real-time PCR contained 45 cycles consisting of a denaturing (95 °C, 20 s) and an annealing/extending (60 °C, 20 s) step. Gene expression assays were all obtained from Applied Biosystems.

Enzyme-Linked Immunosorbent Assay

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 the 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 the Student-Newman-Keuls post hoc test. p values <0.05 were considered significant.

Results

IFN-γ-Induced STAT1 and STAT3 Phosphorylation Is Decreased in the Presence of BE

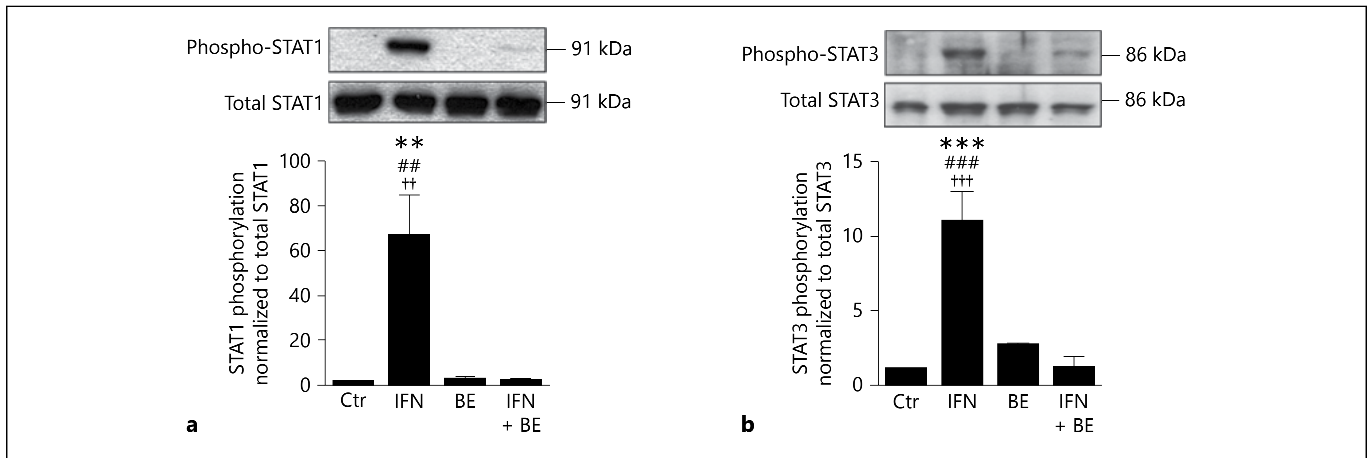
To study the signalling pathways regulated by bilberry-derived anthocyanins, we analysed phosphorylation of STAT1 and STAT3 in response to IFN-γ and/or BE by Western blotting. IFN-γ led to a significant increase in STAT1 (>60-fold) and STAT3 (>10-fold) phosphorylation in THP-1 cells after 20 min (fig.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

BE Did Not Affect IFN-γ Influence on MAP Kinase Pathways

We next investigated the influence of BE on IFN-γ-induced phosphorylation of MAPK isoforms. Co-stimulation with IFN-γ and BE led to a trend towards increased phosphorylation of the MAPK isoforms ERK, JNK, and p38 (online suppl. fig. 1; for all online suppl. material, see www.karger.com/doi/10.1159/000366055). However, these effects were statistically not significant and correlate with our observations in T84 IEC (data not shown).

Digestion 2014;90:179–189
DOI: 10.1159/000366055

Downloaded by:
Universität Zürich, Zentralbibliothek Zürich
130.60.47.22 - 6/3/2016 2:56:22 PM



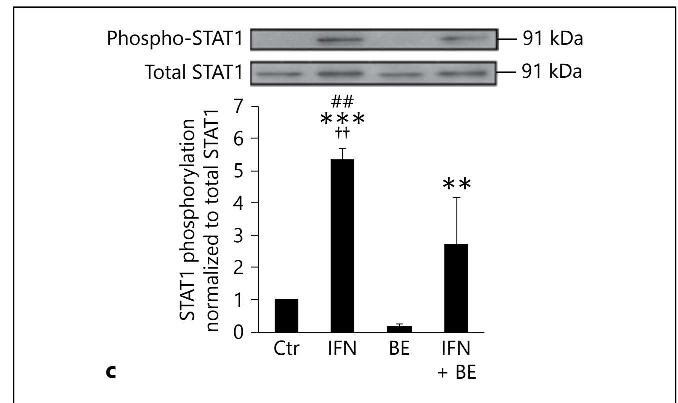


Fig. 1. STAT phosphorylation is significantly reduced when IFN-γ and BE are simultaneously available. IFN leads to considerable activation of both STAT1 (**a**) and STAT3 (**b**). On the other hand, BE prevents STAT activation. Subsequent gene transcription is therefore suppressed. THP-1 cells were pre-stimulated 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 the presence of BE. Error bars depict SEM. Ctr = Control; IFN = IFN-γ. Significant results are marked as follows: ** $p < 0.01$, *** $p < 0.001$, versus control; ## $p < 0.01$, ### $p < 0.001$, versus BE; †† $p < 0.01$, ††† $p < 0.001$, versus IFN and BE (n = 4).

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, 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 and BE was arranged. BE treatment alone did not cause a significant increase of the respective gene expression. BE treatment also diminished the IFN-γ-induced expression of the pro-inflammatory cytokines IL-12 and IL-1β, while it enhanced the expression of the anti-inflammatory cytokine IL-10 (fig. 3a–c). 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 and BE co-stimulation was performed (fig. 4a, b). IL-6 concentrations in these ELISA experiments were below detection levels (data not shown). These data demonstrate that BE is sufficient to ameliorate IFN-γ-induced pro-inflammatory signalling, gene expression, and cytokine secretion, which might critically contribute to its observed anti-inflammatory effects.

BE Reduced IFN-γ-Induced Pro-Inflammatory Signalling and Gene Expression in Human PBMCs

To strengthen the results of our study, we next evaluated the effects of BE on IFN-γ-mediated signalling and gene expression in human PBMCs from healthy control patients. In figure 4, we demonstrate that treatment with BE exerts similar effects in PBMCs as in THP-1 cells. In detail, co-treatment with BE prevented the IFN-γ-induced increase in STAT1 and STAT3 phosphorylation, but enhanced p38 phosphorylation (fig. 5a). Further, BE co-treat-

Downloaded by:
Universität Zürich, Zentralbibliothek Zürich
130.60.47.22 - 6/3/2016 2:56:22 PM

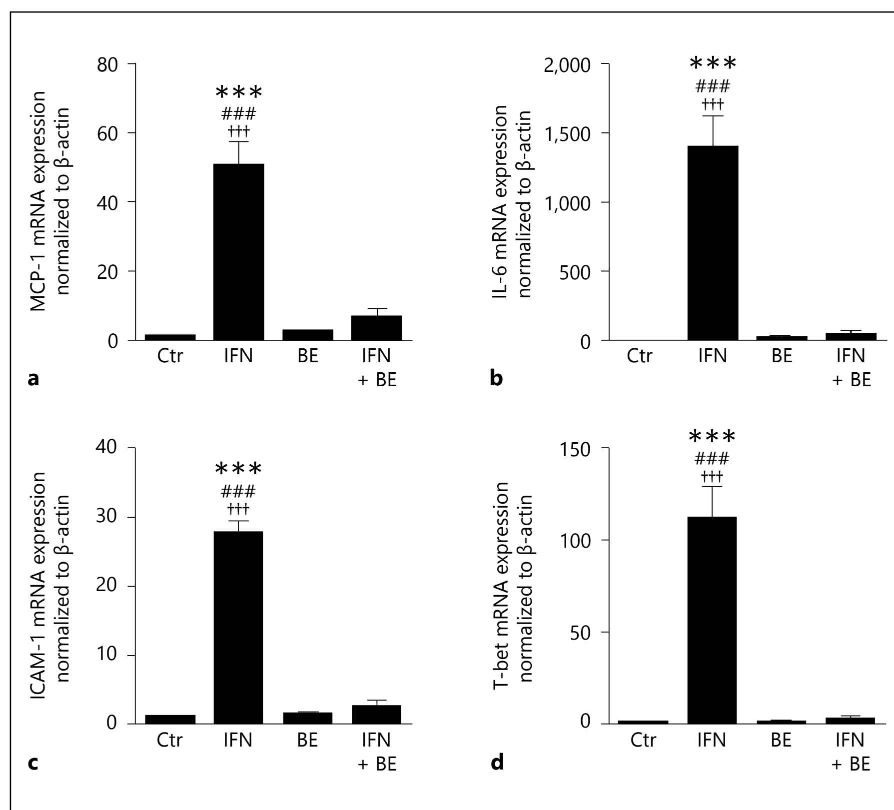


Fig. 2. BE inhibits IFN-γ-mediated activation of gene expression. THP-1 cells were stimulated with 100 ng/ml IFN-γ, 10 μg/ml BE, or both for 24 h. The untreated cells served as control group. In all experiments (**a–d**), BE reduced the inflammatory effect of IFN-γ significantly. mRNA concentration was normalized to β-actin. T-bet is a T-cell transcription factor inducing IFN-γ production. Error bars depict SEM. Ctr = Control; IFN = IFN-γ. Significant results are marked as follows: *** $p < 0.001$, versus control; ### $p < 0.001$, versus IFN and BE; ††† $p < 0.001$, versus BE (n = 4).

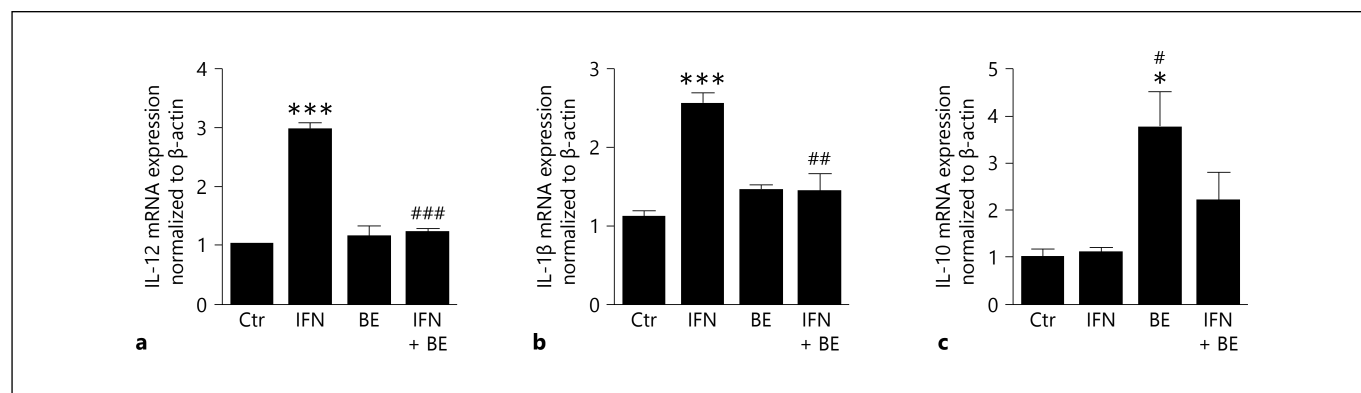


Fig. 3. BE inhibits IFN-γ-induced mRNA expression of IL-12 and IL-1β. THP-1 cells were stimulated with 100 ng/ml IFN-γ, 10 μg/ml BE, or both for 24 h. The untreated cells served as a control group. Histograms demonstrate mRNA levels of IL-12 (**a**), IL-1β (**b**), and IL-10 (**c**). mRNA concentration was normalized to β-actin. Error bars depict SEM. Ctr = Control; IFN = IFN-γ. Significant results are marked as follows: * $p < 0.05$, *** $p < 0.001$, versus control; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, versus IFN (n = 4).

ment completely diminished the IFN-γ-induced up-regulation of ICAM-1, MCP-1, and IL-6 similar as in THP-1 cells (fig. 5b). These data clearly demonstrate that the effects of BE are comparable in THP-1 monocytes and human PBMCs.

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

Downloaded by:
Universität Zürich, Zentralbibliothek Zürich
130.60.47.22 - 6/3/2016 2:56:22 PM

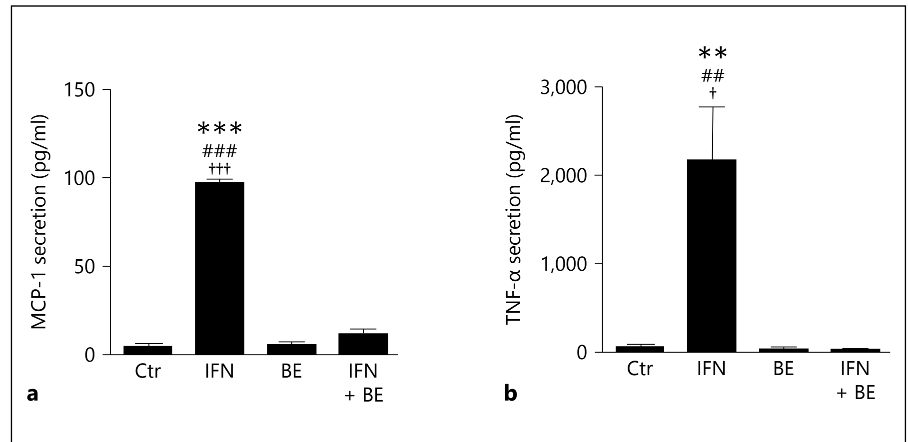


Fig. 4. BE inhibits IFN-γ-induced cytokine secretion in THP-1 cells significantly. THP-1 cells co-stimulated with 100 ng/ml IFN-γ and 10 μg/ml BE secrete significantly lower amounts of MCP-1 (**a**) and TNF-α (**b**) compared to cells stimulated with IFN-γ only. Untreated cells and cells stimulated with BE only secrete similar amounts of MCP-1 and TNF-α, respectively. Stimulation lasted 24 h. Error bars depict SEM. Ctr = Control; IFN = IFN-γ. Significant results are marked as follows: ** $p < 0.01$, *** $p < 0.001$, versus control; $^{\#\#}$ $p < 0.01$, $^{\#\#\#}$ $p < 0.001$, versus BE; † $p < 0.05$, ††† $p < 0.001$, versus IFN and BE (n = 4).

Fig. 5. BE co-treatment prevents IFN-γ-induced signalling and cytokine gene expression in primary human PBMCs. PBMCs were either left untreated, stimulated with 100 ng/ml IFN-γ, 10 μg/ml BE, or both for either 30 min (**a**) or 24 h (**b**). **a** Representative Western blots and respective densitometric analyses are shown for phospho- and total STAT1, phospho- and total STAT3 as well as phospho- and total p38. **b** Histograms demonstrate mRNA levels of ICAM-1, MCP-1, and IL-6. mRNA concentration was normalized to β-actin. Error bars depict SEM. pSTAT = Phospho-STAT; tSTAT = total STAT; p-p38 = phospho-p38; t-p38 = total p38; Ctr = control; IFN = IFN-γ. Significant results are marked as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, versus control; $^{\#}$ $p < 0.05$, versus IFN (n = 4).

Downloaded by:
Universität Zürich, Zentralbibliothek Zürich
130.60.47.22 - 6/3/2016 2:56:22 PM

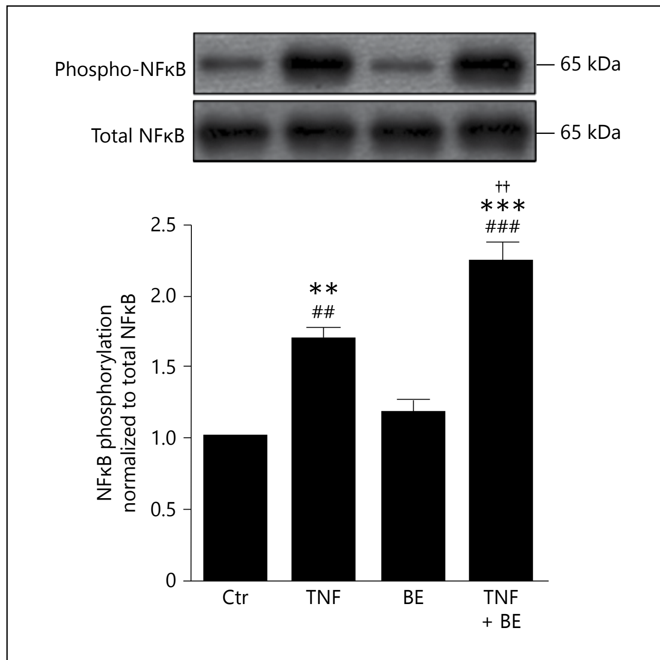


Fig. 6. TNF-α activates NF-κB (NF-κB, p65). However, co-stimulation with BE and TNF-α amplifies NF-κB activation. Thus, anthocyanins from BE seem to reinforce TNF-α-associated effects. Note that sole stimulation with BE did not lead to a considerable increase in NF-κB activation. Experiments were conducted 6-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. Error bars depict SEM. Ctr = Control; TNF = TNF-α. Significant results are marked as follows: ** $p < 0.01$, *** $p < 0.001$, versus control; ## $p < 0.01$, ### $p < 0.001$, versus BE; †† $p < 0.01$, versus TNF (n = 4).

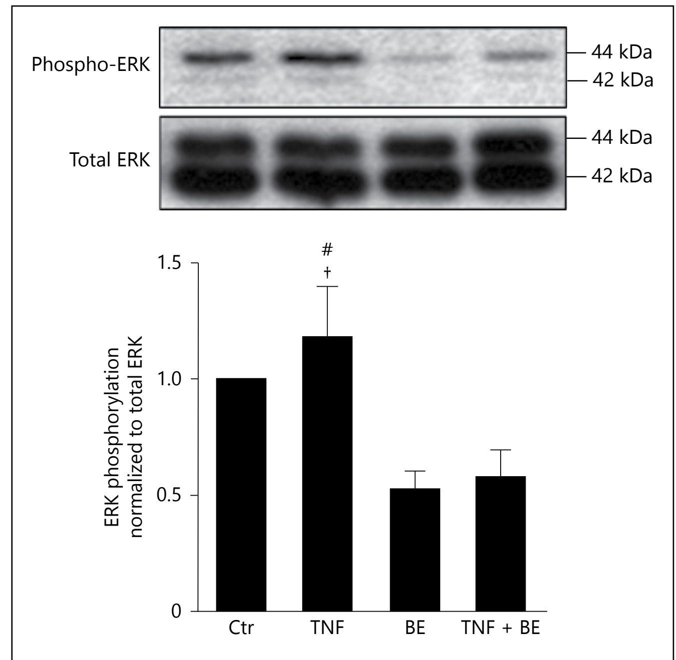


Fig. 7. The MAP kinase ERK1/2 is barely activated by TNF-α, whereas BE tends to evoke decreased activation. In the case of ERK, TNF-α did not induce a potent activation. On the other hand, BE seems to inhibit TNF-α-mediated ERK activation (n = 3). This effect might explain part of the anti-inflammatory properties accredited to BE. Cells were pre-stimulated with BE (10 μg/ml) for 20 min followed by 20 min of stimulation with TNF-α (100 ng/ml). Error bars depict SEM. Ctr = Control; TNF = TNF-α. Significant results are marked as follows: # $p < 0.05$, versus BE; † $p < 0.05$, versus TNF and BE (n = 4).

cells. Incubation with BE alone had no effect on NF-κB phosphorylation. However, co-incubation with TNF-α and BE further enhanced NF-κB phosphorylation (fig. 6). Similar results were obtained in T84 IEC (data not shown).

BE Reduced MAP Kinase Activation in Response to TNF-α

We further analysed 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 (fig. 7). For p38, we did not find significant changes. However, there was a trend for reduction of p38 phosphorylation in cells co-stimulated with TNF-α and BE (online suppl. fig. 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 (fig. 8a–d). IL-6 expression was significantly increased upon single BE stimulation compared to non-stimulated cells. In the 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 ex-

Downloaded by:
Universität Zürich, Zentralbibliothek Zürich
130.60.47.22 - 6/3/2016 2:56:22 PM

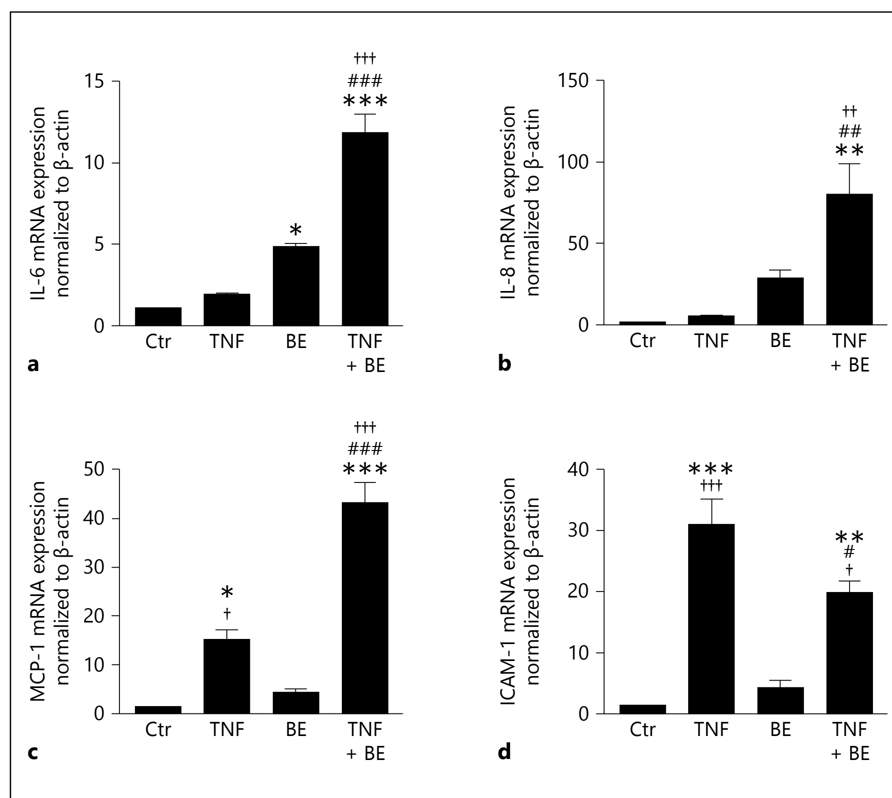


Fig. 8. BE affects TNF-α-induced cytokine mRNA expression. THP-1 cells were stimulated with 100 ng/ml TNF-α, BE (10 μg/ml) containing anthocyanins, or both for 24 h. There was always an untreated control group. mRNA concentration was measured by qPCR and normalized to the constitutively expressed housekeeping gene β-actin. In the case of the pro-inflammatory cytokines IL-6, IL-8, and MCP-1 (**a–c**), increased gene expression was registered in the presence of TNF and BE. For the adhesion molecule ICAM-1, contrary results were found (**d**): gene expression was lowered in co-stimulated cells (TNF and BE). Error bars depict SEM. Ctr = Control; TNF = TNF-α. Significant results are marked as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, versus control; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, versus TNF; † $p < 0.05$, †† $p < 0.01$, ††† $p < 0.001$, versus BE (n = 4).

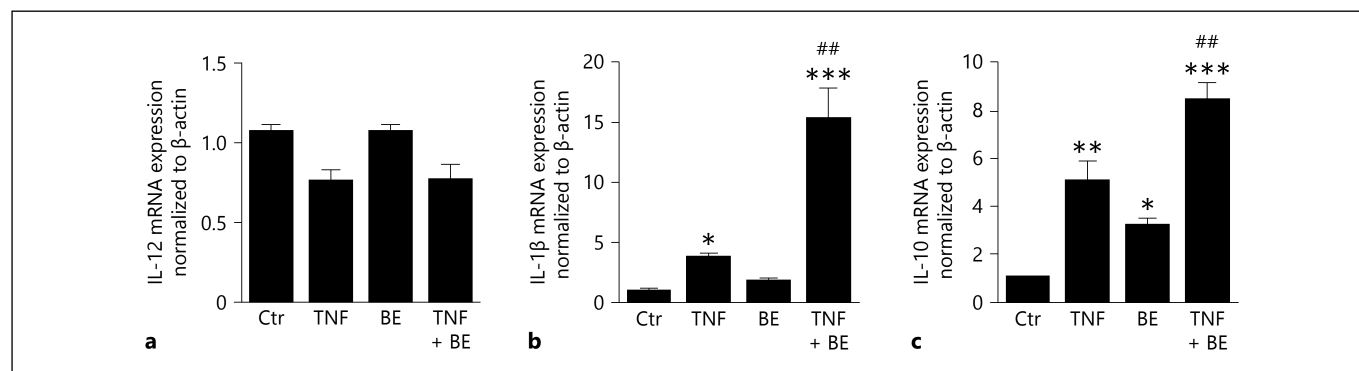


Fig. 9. BE inhibits TNF-α-induced mRNA expression of IL-1β. THP-1 cells were stimulated with 100 ng/ml TNF-α, 10 μg/ml BE, or both for 24 h. The untreated cells served as a control group. Histograms demonstrate mRNA levels of IL-12 (**a**), IL-1β (**b**), and IL-10 (**c**). mRNA concentration was normalized to β-actin. Error bars depict SEM. Ctr = Control; TNF = TNF-α. Significant results are marked as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, versus control; ## $p < 0.01$, versus TNF (n = 4).

posed to BE compared to TNF-α. THP-1 cells exposed to TNF-α and BE showed further significant enhancement in IL-6, IL-8, and MCP-1 expression (fig. 8a–c), whereas co-stimulation led to a reduction of ICAM-1 expression when compared to TNF-α treatment alone (fig. 8d). BE treatment enhanced the TNF-α-induced expression of the pro-inflammatory cytokine IL-1β, but also of the anti-inflammatory cytokine IL-10. Neither TNF-α nor BE treatment had any effect on mRNA expression of IL-12 in THP-1 cells (fig. 9a–c).

We further investigated IL-8 and MCP-1 secretion quantified by ELISA after 24-hour stimulation. Concentrations of IL-8 were significantly increased after co-stimulation with TNF-α and BE (fig. 10a), confirming the

Downloaded by:
Universität Zürich, Zentralbibliothek Zürich
130.60.47.22 - 6/3/2016 2:56:22 PM

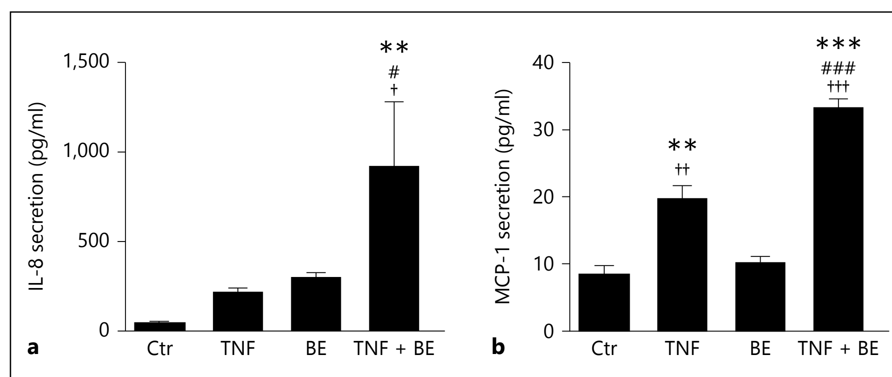


Fig. 10. BE stimulates cytokine secretion reinforcing the pro-inflammatory effects of TNF-α. THP-1 cells stimulated with both 100 ng/ml TNF-α and 10 μg/ml BE for 24 h showed a significantly higher secretion of the pro-inflammatory cytokines (**a**, **b**). Cytokine secretion was measured applying the ELISA method. Error bars depict SEM. Ctr = Control; TNF = TNF-α. Significant results are marked as follows: ** $p < 0.01$, *** $p < 0.001$, versus control; # $p < 0.05$, ### $p < 0.001$, versus TNF; † $p < 0.05$, †† $p < 0.01$, ††† $p < 0.001$, versus BE (n = 4).

mRNA expression data (fig. 8b). 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 (fig. 10b), again confirming the mRNA expression data.

Discussion

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

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-γ and TNF-α play a central role in IBD pathogenesis [29]. IFN-γ 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 of IFN-γ-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 and TNF-α). 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-γ-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 a 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. However, there was a trend towards increased MAPK activation in cells co-stimulated with BE and IFN-γ. Again, this result matches 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

Downloaded by:
Universität Zürich, Zentralbibliothek Zürich
130.60.47.22 - 6/3/2016 2:56:22 PM

THP-1 cells and T84 IEC. It is known that NF-κB is an important regulator of pro-inflammatory gene 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 but also amplified gene expression of MCP-1 and induced gene expression of IL-6 and IL-8 in THP-1 cells (fig. 6a–c). Accordingly, BE enhanced TNF-α-induced IL-8 and MCP-1 secretion (fig. 7). It could be argued that the used BE contained lipopolysaccharides, which would explain the increased NF-κB phosphorylation. Yet, the addition of 1% penicillin/streptomycin to the cell medium should have prevented lipopolysaccharide 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 present in black rice) on NF-κB activation in RAW 264.7 macrophage cells was reported [48]. However, it is not clear 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 leucocyte recruitment and is an MAPK target gene [39]. BE suppressed TNF-α-induced ICAM-1 expression, which confirms ERK inhibition. Altogether, these data demonstrate that BE exerts pro- as well as anti-inflammatory properties in response to TNF-α in human immune cells.

It has been previously demonstrated that anthocyanins from bilberries decrease pro-inflammatory cytokine serum levels and attenuate the severity of experimental colitis in mice [11–13]. Here, we have shown that BE clearly inhibits the pro-inflammatory effects of IFN-γ in human THP-1 monocytes as well as in human PBMCs. These observations clearly support the observed anti-inflammatory effects of BE in vivo. With respect to TNF, we found that BE promotes TNF-induced NF-κB signalling and secretion of certain pro-inflammatory cytokines, such as IL-6 or IL-8. On the other hand, BE clearly enhanced TNF-induced expression of the anti-inflammatory IL-10. Therefore, in the complex in vivo mouse model, it seems that the anti-inflammatory effects of BE outweigh the pro-inflammatory effects and are, therefore, able to inhibit colitis symptoms in vivo.

In conclusion, our data demonstrate that BE exerts 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. Additional 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.

Acknowledgements

This research was supported by a grant from the Fonds zur Förderung des akademischen Nachwuchses (FAN) of the Zürcher Universitätsverein (ZUNIV) to M.S., a research grant from the Swiss Philanthropy Foundation to M.S., a research credit from the University of Zurich to M.S., a research grant from the Swiss National Science Foundation (SNF) to M.S. (grant No. 314730-146204) and G.R. (grant No. 310030-120312), the Swiss IBD Cohort (grant No. 3347CO-108792), and a grant from the Zurich Centre for Integrative Human Physiology (ZIHP) of the University of Zurich to M.S.

Disclosure Statement

The authors declare that there are no competing interests.

References

1 Crozier A, Jaganath IB, Clifford MN: Dietary phenolics: chemistry, bioavailability and effects on health. Nat Prod Rep 2009;26:1001–1043.
2 Cardona F, Andrés-Lacueva C, Tulipani S, et al: Benefits of polyphenols on gut microbiota and implications in human health. J Nutr Biochem 2013;24:1415–1422.
3 Scalbert A, Williamson G: Dietary intake and bioavailability of polyphenols. J Nutr 2000; 130:2073S–2085S.
4 Jennings A, Welch AA, Fairweather-Tait SJ, et al: Higher anthocyanin intake is associated with lower arterial stiffness and central blood pressure in women. Am J Clin Nutr 2012;96: 781–788.
5 Hooper L, Kay C, Abdelhamid A, et al: Effects of chocolate, cocoa, and flavan-3-ols on cardiovascular health: a systematic review and meta-analysis of randomized trials. Am J Clin Nutr 2012;95:740–751.
6 Serra D, Paixão J, Nunes C, et al: Cyanidin-3-glucoside suppresses cytokine-induced inflammatory response in human intestinal cells: comparison with 5-aminosalicylic acid. PLoS One 2013;8:e73001.
7 Mauray A, Felgines C, Morand C, et al: Nutrigenomic analysis of the protective effects of bilberry anthocyanin-rich extract in apo E-deficient mice. Genes Nutr 2010;5:343–353.

Downloaded by:
Universität Zürich, Zentralbibliothek Zürich
130.60.47.22 - 6/3/2016 2:56:22 PM

8 Chu W, Cheung SCM, Lau RAW, Benzie IFF: Bilberry (*Vaccinium myrtillus* L.); in Benzie IFF, Wachtel-Galor S (eds): Herbal Medicine: Biomolecular and Clinical Aspects. Boca Raton, CRC Press, 2011, pp 55–72. http://www.ncbi.nlm.nih.gov/books/NBK92770/.
9 Chen J, Uto T, Tanigawa S, et al: Expression profiling of genes targeted by bilberry *(Vaccinium myrtillus)* in macrophages through DNA microarray. Nutr Cancer 2008;60(suppl 1):43–50.
10 Triebel S, Trieu HL, Richling E: Modulation of inflammatory gene expression by a bilberry (*Vaccinium myrtillus* L.) extract and single anthocyanins considering their limited stability under cell culture conditions. J Agric Food Chem 2012;60:8902–8910.
11 Osman N, Adawi D, Ahrné S, et al: Probiotics and blueberry attenuate the severity of dextran sulfate sodium (DSS)-induced colitis. Dig Dis Sci 2008;53:2464–2473.
12 Piberger H, Oehme A, Hofmann C, et al: Bilberries and their anthocyanins ameliorate experimental colitis. Mol Nutr Food Res 2011; 55:1724–1729.
13 Wu LH, Xu ZL, Dong D, et al: Protective effect of anthocyanins extract from blueberry on TNBS-induced IBD model of mice. Evid Based Complement Alternat Med 2011;2011: 525462.
14 Biedermann L, Mwinyi J, Scharl M, et al: Bilberry ingestion improves disease activity in mild to moderate ulcerative colitis – an open pilot study. J Crohns Colitis 2013;7:271–279.
15 Xavier RJ, Podolsky DK: Unravelling the pathogenesis of inflammatory bowel disease. Nature 2007;448:427–434.
16 Cho JH: The genetics and immunopathogenesis of inflammatory bowel disease. Nat Rev Immunol 2008;8:458–466.
17 Khor B, Gardet A, Xavier RJ: Genetics and pathogenesis of inflammatory bowel disease. Nature 2011;474:307–317.
18 Orholm M, Binder V, Sørensen TI, et al: Concordance of inflammatory bowel disease among Danish twins. Results of a nationwide study. Scand J Gastroenterol 2000;35:1075–1081.
19 Halfvarson J, Bodin L, Tysk C, et al: Inflammatory bowel disease in a Swedish twin cohort: a long-term follow-up of concordance and clinical characteristics. Gastroenterology 2003;124:1767–1773.
20 Thompson NP, Driscoll R, Pounder RE, et al: Genetics versus environment in inflammatory bowel disease: results of a British twin study. BMJ 1996;312:95–96.
21 Binder V: Genetic epidemiology in inflammatory bowel disease. Dig Dis 1998;16:351–355.
22 Cho JH: Inflammatory bowel disease: genetic and epidemiologic considerations. World J Gastroenterol 2008;14:338–347.
23 Kobayashi KS, Chamaillard M, Ogura Y, et al: Nod2-dependent regulation of innate and adaptive immunity in the intestinal tract. Science 2005;307:731–734.
24 Hedl M, Li J, Cho JH, et al: Chronic stimulation of Nod2 mediates tolerance to bacterial products. Proc Natl Acad Sci USA 2007;104: 19440–19445.
25 Economou M, Trikalinos TA, Loizou KT, et al: Differential effects of NOD2 variants on Crohn's disease risk and phenotype in diverse populations: a metaanalysis. Am J Gastroenterol 2004;99:2393–2404.
26 Duerr RH, Taylor KD, Brant SR, et al: A genome-wide association study identifies IL23R as an inflammatory bowel disease gene. Science 2006;314:1461–1463.
27 Hampe J, Franke A, Rosenstiel P, et al: A genome-wide association scan of nonsynonymous SNPs identifies a susceptibility variant for Crohn disease in ATG16L1. Nat Genet 2007;39:207–211.
28 Feagan BG, Macdonald JK: Oral 5-aminosalicylic acid for maintenance of remission in ulcerative colitis. Cochrane Database Syst Rev 2012;10:CD000544.
29 Coskun M, Salem M, Pedersen J, et al: Involvement of JAK/STAT signaling in the pathogenesis of inflammatory bowel disease. Pharmacol Res 2013;76:1–8.
30 Blouin CM, Lamaze C: Interferon gamma receptor: the beginning of the journey. Front Immunol 2013;4:267.
31 Gough DJ, Levy DE, Johnstone RW, et al: IFNgamma signalling – does it mean JAK-STAT? Cytokine Growth Factor Rev 2008;19: 383–394.
32 Hu X, Ivashkiv LB: Cross-regulation of signaling pathways by interferon-gamma: implications for immune responses and autoimmune diseases. Immunity 2009;31:539–550.
33 Musso A, Dentelli P, Carlino A, et al: Signal transducers and activators of transcription 3 signaling pathway: an essential mediator of inflammatory bowel disease and other forms of intestinal inflammation. Inflamm Bowel Dis 2005;11:91–98.
34 Schreiber S, Rosenstiel P, Hampe J, et al: Activation of signal transducer and activator of transcription (STAT) 1 in human chronic inflammatory bowel disease. Gut 2002;51:379–385.
35 Li Y, de Haar C, Peppelenbosch MP, et al: New insights into the role of STAT3 in IBD. Inflamm Bowel Dis 2012;18:1177–1183.
36 Yoshioka K: Scaffold proteins in mammalian MAP kinase cascades. J Biochem 2004;135: 657–661.
37 Chen Z, Gibson TB, Robinson F, et al: MAP kinases. Chem Rev 2001;101:2449–2476.
38 Broom OJ, Widjaya B, Troelsen J, et al: Mitogen activated protein kinases: a role in inflammatory bowel disease? Clin Exp Immunol 2009;158:272–280.
39 Scaldaferri F, Sans M, Vetrano S, et al: The role of MAPK in governing lymphocyte adhesion to and migration across the microvasculature in inflammatory bowel disease. Eur J Immunol 2009;39:290–300.
40 Roy PK, Rashid F, Bragg J, et al: Role of the JNK signal transduction pathway in inflammatory bowel disease. World J Gastroenterol 2008;14:200–202.
41 Waetzig GH, Seegert D, Rosenstiel P, et al: p38 mitogen-activated protein kinase is activated and linked to TNF-alpha signaling in inflammatory bowel disease. J Immunol 2002; 168:5342–5351.
42 Rasheed Z, Akhtar N, Anbazhagan AN, et al: Polyphenol-rich pomegranate fruit extract (POMx) suppresses PMACI-induced expression of pro-inflammatory cytokines by inhibiting the activation of MAP Kinases and NF-kappaB in human KU812 cells. J Inflamm (Lond) 2009;6:1.
43 Ueda A, Okuda K, Ohno S, et al: NF-kappa B and Sp1 regulate transcription of the human monocyte chemoattractant protein-1 gene. J Immunol 1994;153:2052–2063.
44 Libermann TA, Baltimore D: Activation of interleukin-6 gene expression through the NF-kappa B transcription factor. Mol Cell Biol 1990;10:2327–2334.
45 Kunsch C, Rosen CA: NF-kappa B subunit-specific regulation of the interleukin-8 promoter. Mol Cell Biol 1993;13:6137–6146.
46 Vanden Berghe W, Vermeulen L, De Wilde G, et al: Signal transduction by tumor necrosis factor and gene regulation of the inflammatory cytokine interleukin-6. Biochem Pharmacol 2000;60:1185–1195.
47 Rogler G, Brand K, Vogl D, et al: Nuclear factor kappaB is activated in macrophages and epithelial cells of inflamed intestinal mucosa. Gastroenterology 1998;115:357–369.
48 Min SW, Ryu SN, Kim DH: Anti-inflammatory effects of black rice, cyanidin-3-O-beta-D-glycoside, and its metabolites, cyanidin and protocatechuic acid. Int Immunopharmacol 2010;10:959–966.

Downloaded by:
Universität Zürich, Zentralbibliothek Zürich
130.60.47.22 - 6/3/2016 2:56:22 PM