Research Article

Influence of apple polyphenols on inflammatory gene expression

Mathias Jung[1]*, Sven Triebel[2]*, Timm Anke[1], Elke Richling[2] and Gerhard Erkel[1]**

[1] Department of Biotechnology, University of Kaiserslautern, Kaiserslautern, Germany
[2] Department of Food Chemistry and Toxicology, Molecular Nutrition, University of Kaiserslautern, Kaiserslautern, Germany

Apples (*Malus* spp., Rosaceae) and products thereof contain high amounts of polyphenols which show diverse biological activities and may contribute to beneficial health effects, like protecting the intestine against inflammation initiated by chronic inflammatory bowel diseases (IBD). IBD are characterized by an excessive release of several proinflammatory cytokines and chemokines by different cell types which results consequently in an increased inflammatory response. In the present study we investigated the preventive effectiveness of polyphenolic juice extracts and single major constituents on inflammatory gene expression in immunorelevant human cell lines (DLD-1, T84, MonoMac6, Jurkat) induced with specific stimuli. Besides the influence on proinflammatory gene expression, the effect on NF-κB-, IP-10-, IL-8-promoter-, STAT1-dependent signal transduction, and the relative protein levels of multiple released cytokines and chemokines were studied. DNA microarray analysis of several genes known to be strongly regulated during gastrointestinal inflammation, combined with quantitative real-time PCR (qRT-PCR) revealed that the apple juice extract AE04 (100 – 200 μg/mL) significantly inhibited the expression of NF-κB regulated proinflammatory genes (TNF-α, IL-1β, CXCL9, CXCL10), inflammatory relevant enzymes (COX-2, CYP3A4), and transcription factors (STAT1, IRF1) in LPS/IFN-γ stimulated MonoMac6 cells without significant effects on the expression of house-keeping genes. A screening of some major compounds of AE04 revealed that the flavan-3-ol dimer procyanidin B_2 is mainly responsible for the anti-inflammatory activity of AE04. Furthermore, the dihydrochalcone aglycone phloretin and the dimeric flavan-3-ol procyanidin B_1 significantly inhibited proinflammatory gene expression and repressed NF-κB-, IP-10-, IL-8-promoter-, and STAT1-dependent signal transduction in a dose-dependent manner. The influence on proinflammatory gene expression by the applied polyphenols thereby strongly correlated with the increased protein levels investigated by human cytokine array studies. In summary, we evaluated selected compounds responsible for the anti-inflammatory activity of AE04. In particular, procyanidin B_1, procyanidin B_2, and phloretin revealed anti-inflammatory activities *in vitro* and therefore may serve as transcription-based inhibitors of proinflammatory gene expression.

Keywords: Apple polyphenols / Inflammatory gene expression / Phloretin / Procyanidins / Proinflammatory cytokines and chemokines

Received: December 17, 2008; revised: March 24, 2009; accepted: May 15, 2009

1 Introduction

Polyphenols, one of the most common groups of plant secondary metabolites, have received considerable interest over the past few years due to their presumed role in the prevention of various degenerative diseases [1]. Apples (*Malus* spp., Rosaceae) represent an important source of polyphenols and other phytochemicals [2 – 4]. The main structural

Correspondence: Dr. Gerhard Erkel, Department of Biotechnology, University of Kaiserslautern, Paul-Ehrlich-Strasse 23, 67663 Kaiserslautern, Germany
E-mail: erkel@ibwf.de
Fax: +49-0-631-205-2999

Abbreviations: CM, cytokine mixture; **COX,** cyclooxygenase; **FCS,** fetal calf serum; **GAPDH,** glycerinaldehyde-3-phosphate dehydrogenase; **IBD,** inflammatory bowel disease; **IP-10,** IFN-γ-inducible protein-10; **IRF,** INF regular factor; **NF-κB,** nuclear factor-κB; **qRT-PCR,** quantitative real-time PCR; **STAT,** signal transducer and activator of transcription; **TNF,** tumor necrosis factor

* Both authors contributed equally to this work.
** Additional corresponding author: Professor Elke Richling, E-mail: richling@chemie.uni-kl.de

classes include hydroxycinnamic acids, flavonols (quercetin glycosides), flavan-3-ols (catechins and oligomeric procyanidins), dihydrochalcones (phloretin glycosides), as well as triterpenoids in apple peel and anthocyanins depending on the apple variety under study [5 – 7]. Major apple polyphenols are 5-caffeoylquinic acid (chlorogenic acid), procyanidins, and dihydrochalcones such as phloretin 2′-*O*-glucoside (phloridzin) and phloretin 2′-*O*-xyloglucoside [8, 9].

Several lines of evidence suggest that apples and apple products, such as apple juice and extracts, possess a wide range of biological activities which may contribute to health beneficial effects against cardiovascular disease, asthma and pulmonary dysfunction, diabetes, obesity, cancer, and inflammation (reviewed by Boyer and Liu [10]). In this context, apple extracts and their constituents, especially oligomeric procyanidins, have shown to influence multiple mechanisms relevant for cancer prevention in *in vitro* studies. These include anti-mutagenic activity, modulation of carcinogen metabolism, antioxidant activity, anti-inflammatory mechanisms, modulation of signal transduction pathways, anti-proliferative and apoptosis-inducing activity, as well as novel mechanisms on epigenetic events and innate immunity (reviewed by Gerhäuser [11]).

In addition, some polyphenols are known to possess anti-inflammatory activity *in vitro* and *in vivo*, which have been attributed to the modification of signal transduction pathways [12]. Furthermore, some polyphenols have been found to inhibit the arachidonic acid pathway by inhibiting cellular enzymes such as phospholipase A_2 (PLA_2), cyclooxygenase (COX), or lipoxygenase (LOX). For instance, the flavan-3-ols (−)-epigallocatechin (EGC); (−)-gallocatechin (GC); (−)-epicatechin gallate (ECG); (−)-catechin gallat (CG); as well as (−)-epigallocatechin gallate (EGCG) have been shown to inhibit COX-1/COX-2 activity in different human and murine cell lines [13 – 16]. In addition, polyphenols are capable of acting on the cytokine system by affecting the balance between pro- and anti-inflammatory cytokine expression [17]. Quercetin and some catechins, for instance, inhibit TNF-α and IL-1β expression due to the enhancement of the release of IL-10 [18].

One of the most important anti-inflammatory activities of some phenolic compounds is their ability to modulate the activation of nuclear factor-κB (NF-κB). NF-κB plays a major role in coordinating the transcriptional induction of a wide range of genes. Some of these genes encode for several cytokines (*e.g.*, TNF-α, IL-1β, IL-6, IL-2), chemokines like IL-8 and IFN-γ-inducible Protein-10 (CXCL10, IP-10) or proinflammatory enzymes such as COX-2, which are involved in the development and progression of inflammatory processes. Several of these gene products such as TNF-α or IL-1β also directly activate the NF-κB pathway which results in an amplification loop, increasing the length and duration of the inflammatory response. In resting cells, NF-κB is sequestered in an inactive form in the cytoplasm by interaction with inhibitors, like IκB-α and related proteins. In response to stimulation, IκB is phosphorylated on two serine residues by the IκB kinases (IKK) complex. Ubiquitination and degradation of IκB leads to translocation of NF-κB into the nucleus, DNA binding of the transcription factor, and subsequent activation of transcription [19]. The interaction of polyphenols with the NF-κB pathway can thereby occur at multiple steps in the activation process [20, 21]. For instance, EGCG has been shown to block the activation of IKK and therefore the degradation of IκB-α [22, 23], whereas the flavonol quercetin was found to inhibit the recruitment of the NF-κB cofactor CBP/p300, hence inhibiting the TNF-α-induced expression of the proinflammatory cytokine IP-10 in primary murine small intestinal Mode-K epithelial cells [24].

Some of the most important chronic inflammatory diseases influenced by nutritive constituents are represented by inflammatory bowel diseases (IBD). IBD are either characterized by an excessive T helper 1 (T_H1)-cell response that is associated with increased secretion of IL-12, IFN-γ, and TNF-α, or an excessive T_H2-cell response that is associated with increased secretion of IL-4, IL-5, and IL-13 [25]. The two major forms of IBD are the T_H1-mediated Crohn's disease and the T_H2-mediated ulcerative colitis. In addition to intestinal epithelial cells, macrophages also play a decisive role in the inflammatory process of IBD. The antigen presenting macrophages of the intestinal immune system are producing a multitude of proinflammatory cytokines (*e.g.*, IL-1, TNF-α), which results in the activation of T-cells and consequently leads to an increased inflammatory response. Nutritional inhibitors could therefore be useful as anti-inflammatory agents in the treatment of such diseases.

In initial studies we observed an inhibitory effect of the polyphenol enriched apple juice extract AE04 on inflammatory gene expression in LPS/IFN-γ stimulated Mono-Mac6 cells. The objectives of the present study were to elucidate the influence of the major constituents procyanidin B_1, procyanidin B_2, phloretin, and phloridzin on the expression of proinflammatory marker genes in human immunorelevant and epithelial colon carcinoma cell lines using qRT-PCR. Moreover, we studied the influence of the polyphenols on NF-κB-, STAT1-, IP-10 (CXCL10)-, and IL-8-promoter-dependent reporter gene activity as well as their ability to inhibit the production of multiple cytokines and chemokines.

2 Materials and methods

2.1 Cell lines, media, chemicals, and stimuli

T84 cells (ATCC CCL-248), a human colon epithelial cell line, derived from a lung metastasis of colon carcinoma, was obtained from American Type Culture Collection (Rockville, MD, USA). The human colon adenocarcinoma

cell line DLD-1 (DSMZ ACC278), the human leukemia T-cell line Jurkat (DSMZ ACC282), and the human monocytic leukemia cell line MonoMac6 (DSMZ ACC124) were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ Braunschweig, Germany). Cell media DMEM (high glucose), Ham's Nutrient Mixture F12 (Ham's F12) and RPMI 1640, as well as the medium supplements glutamin, penicillin/streptomycin, and fetal calf serum (FCS) were purchased from Invitrogen (Karlsruhe, Germany), catalase (from bovine liver) from Sigma–Aldrich (Taufkirchen, Germany).

All solvents and chemicals used were of analytical grade or in compliance with the standards needed for cell culture experiments. 5-caffeoylquinic acid (chlorogenic acid); (−)-epicatechin; phloretin 2′-*O*-glucoside (phloridzin); procyanidin B_1; procyanidin B_2; phloretin; (+)-catechin; resazurin sodium salt; and saponin were purchased from Sigma–Aldrich. Phloretin 2′-*O*-xyloglucoside was isolated from extracts of apple juice treated with laccase according to Will *et al.* [26].

Human recombinant cytokines TNF-α (Biochrom AG, Berlin, Germany), IL-1β (Axxora, Lörrach, Germany), and IFN-γ (BioVision, CA, USA) were stabilized after resuspension by adding 0.1% BSA (solved in DPBS). LPS, as well as 12-O-tetradecanoylphorbol-13-acetate (TPA) and ionomycine were acquired from Sigma–Aldrich.

Cell culture consumable material (*e.g.*, cell culture flasks, petri dishes, well plates, *etc.*) were purchased from Greiner Bio-One (Essen, Germany).

2.2 Cell culture and stimulation protocols

T84 cells were maintained in 75 cm^2 flasks in DMEM/Ham's F12 (1:1) supplemented with 10% FCS, 100 units/mL penicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine in a 5% CO_2 humidified incubator at 37°C. Initially cells were seeded out in six-well plates in 2 mL fresh medium *per* well at a density of 5×10^5 cells *per* mL. Cells were starved for 16 h in DMEM/Ham's F12 (1:1) with 0.5% FCS after 48 h of cultivation. Later on, cells were pretreated with test compounds dissolved in DMSO in serum-free medium containing 100 units/mL catalase for 1 h. Stimulation with a cytokine mixture (CM) consisting of 10 ng/mL TNF-α, 5 ng/mL IL-1β, and 10 ng/mL IFN-γ was performed for 4 h (qRT-PCR) and 16 h (proteome profiling), respectively. The final DMSO concentration was 0.5%.

The human colon adenocarcinoma cell line DLD-1, the human leukemia T-cell line Jurkat and the human monocytic leukemia cell line MonoMac6 were grown in RPMI 1640 medium containing 25 mM HEPES buffer and 2 mM L-glutamine and supplemented with 10% FCS, 100 units/mL penicillin, and 100 μg/mL streptomycin at 37°C and 5% CO_2. For qRT-PCR analysis, DLD-1 cells were plated into six-well plates at a density of 5×10^5 cells *per* well. After starving in RPMI 1640 medium with 0.5% FCS for 16 h, cells were pretreated with test compounds in serum-free medium containing 100 units/mL catalase for 1 h and subsequently induced with a CM containing 10 ng/mL IFN-γ, 5 ng/mL IL-1β, and 10 ng/mL TNF-α for 5 h. Jurkat and MonoMac6 cells were starved for 16 h in RPMI 1640 medium with 0.5% FCS. Later on, both cell lines were plated into six-well plates at a density of 3×10^6 cells *per* well. After treatment with test compounds for 1 h, both cell lines were induced for 4 h. Jurkat cells were induced with 10 ng/mL 12-O-tetradecanoylphorbol-13-acetate (TPA) and 2.5 μM ionomycine, MonoMac6 cells were induced with 1 μg/mL lipopolysaccharide (LPS) and 10 ng/mL IFN-γ.

2.3 Cell viability testing

The cytotoxicity against different cell lines used was determined after 4 and 24 h in a resazurin reduction assay (RRA) according to [27], and a XTT-based cell viability assay as previously described by Roehm *et al.* [28].

For RRA, cells were seeded in 48-well plates at a density of 1.5×10^5 (T84, DLD-1) or 2.5×10^4 (MonoMac6, Jurkat) cells *per* well and 0.5 mL medium described in Chapter 2.2. After 24 h cultivation, cells were treated with test compounds in different concentrations. Additionally medium control, solvent control (0.5% DMSO), positive control with 0.1% saponin (hemolytic steroide glucoside), and cell-free control were performed. After 4 h of incubation at 37°C and 5% CO_2, the test medium was removed, cells were washed with PBS, and 0.5 mL resazurin working solution *per* well was added subsequently. Then after 1 h of incubation at 37°C and 5% CO_2, the resazurin solution was analyzed by fluorometric measurements using excitation of 544 nm and emission of 590 nm. Cytotoxicity of test compounds was determined by comparison of treated probes to 100% normalized solvent controls.

2.4 Quantitative real-time PCR analysis (qRT-PCR analysis)

Following stimulation, cells were lysed and total cellular RNA was prepared by using either the RNeasy mini kit obtained from Qiagen (Hilden, Germany) or the Total RNA Isolation Reagent (TRIR) from ABgene® (Hamburg, Germany) according to the manufacturer's protocol. RNA samples were dissolved in DEPC-treated water and stabilized by adding RNA-Later reagent obtained from Invitrogen. Dissolved RNA was immediately stored in a −80°C freezer.

Gene expression was quantified in a two-step reverse transcription PCR using aliquots of total RNA. After determination of the quantity and quality of isolated RNA using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, USA), cDNA was prepared from 1 μg of total cellular RNA. The reverse transcription was done by using either the "RevertAid™ H Minus First Strand cDNA Synthesis kit" (MBI Fermentas, St. Leon-Rot) or the iScript

cDNA synthesis kit from BioRad (Munich, Germany) according to the manufacturer's instructions.

PCR products were synthesized from cDNA (100 or 300 ng) using functionally validated gene expression assays, as the QuantiTec SYBR® Green PCR-Kit obtained from Qiagen or the ABsolute qPCR SYBR® Green fluorescein mix obtained from ABgene subsequently. To analyze the mRNA expression of investigated genes, qRT-PCR was carried out using gene-specific primers for human COX2 (for 5′-TTC AAA TGA GAT TGT GGG AAA ATT GCT-3′, rev 5′-AGA TCA TCT CTG CCT GAG TAT CTT-3′), CXCL3 (for 5′-TGGTCACTGAACTGCGCT -3′, rev 5′-ATGCGGGGTTGAGACAAG -3′), CXCL10 (for 5′-TGAGCCTACAGCAGAGGAA-3′, rev 5′-TACTCCTTGAATGCCACTTAGA-3′), GAPDH (for 5′-CCTCCGGGAAACTGTGG-3′, rev 5′-AGTGGGGACACGGAAG-3′), IL-1β (for 5′-AAGCTGAGGAAGATGCTG-3′, rev 5′-ATCTACACTCTCCAGCTG-3′), IL-2 (for 5′-AACTCACCAGGATGCTCACATTT-3′, rev 5′-TTAGCACTTCCTCCAGAGGTTTG-3′), IL-8 (for 5′-TGCCAAGGAGTGCTAAAG-3′, rev 5′-CTCCACAACCCTCTGCAC-3′), and TNF-α (for 5′-TCTTCTGCCTGCTGCACTTTGG-3′, rev 5′-ATCTCTCAGCTCCACGCCATTG-3′). All primers were manufactured by MWG-Biotech AG (Ebersberg, Germany). A two-step amplification protocol was chosen: initial denaturation step at 95°C for 10 min followed by 45 cycles with 15 s denaturation at 94°C, 30 s annealing at 56°C and 30 s extension at 72°C.

Measurements were done using either the LightCycler or the iCycler (Roche, Mannheim, Germany) detection system according to manufacturer's suggestions. Relative mRNA amounts were determined using the mathematical model for relative quantification in real-time PCR proposed by Pfaffl [29] in which the relative expression ratio is calculated from the real-time PCR efficiencies and the crossing point deviation of an unknown sample *versus* a control, in our case the housekeeping gene glycerinaldehyde-3-phosphate dehydrogenase (GAPDH).

2.5 Reporter gene assays

The 972 bp human IP-10 promoter −875 to +97 relative to the transcription start site) was amplified by PCR from MonoMac6 cell genomic DNA using primers derived from published sequences [30]. The PCR product was cloned into the *KpnI–NheI* site of the pGL3 basic vector (Promega™, Mannheim, Germany) to generate the hIP-10 promotor driven luciferase reporter plasmid. The 1.5 kb human IL-8 promoter driven reporter plasmid, as well as the NFκB driven reporter plasmid has been previously reported [31]. The STAT1 driven plasmid pGAS-TA-Luc, which contains two copies of the STAT1 enhancer element, was obtained from Clontech (Heidelberg, Germany). The plasmids pRL-TK and pRL-CMV for normalizing transfection efficiency were obtained from Promega (Dual-Luciferase-Reporter-Assay).

Transfection of DLD-1 cells was performed by using the liposomal formulation "Lipofectamine 2000" purchased from Invitrogen. 48 h before transfection, 1×10^5 cells/mL in RPMI 1640 medium containing 10% FCS were plated in 24-well plates. 24 h before transfection, the medium was replaced by RPMI 1640 medium containing 0.5% FCS and no antibiotics. On the day of transfection, cells were 90–95% confluent. The lipofection was performed by using 0.8 μg of the indicated plasmid in accordance to the manufacturer's instructions. Luciferase expression was induced with a triple CM containing 10 ng/mL IFN-γ, 5 ng/mL IL-1β, and 10 ng/mL TNF-α for 24 h.

Transfection of Jurkat and MonoMac6 cells was performed by electroporation using a Gene Pulser from Bio-Rad. In case of Jurkat cells, 1×10^8 cells/mL were electroporated in 0.2 mL of 20 mM HEPES buffer together with 50 μg of the indicated plasmids at 200 V and 975 μF. After electroporation, cells were seeded in OptiMEM I medium containing 10% FCS in a 24-well plate with and without test compounds and later on, luciferase expression was induced by adding 10 ng/mL TPA and 2.5 μM ionomycine. 24 h after transfection, the reporter gene activity was measured. MonoMac6 cells were electroporated at a density of 3×10^7 cells/mL in RPMI 1640 medium containing 25 mM HEPES buffer together with 40 μg of the indicated plasmids at 200 V and 975 μF. After electroporation, the cells were seeded in RPMI 1640 medium containing 10% FCS in a 24-well plate with and without test compounds. Luciferase expression was induced for 4 h by adding 1 μg/mL LPS and 10 ng/mL IFN-γ. The reporter gene activity was measured by luminometer using the luciferase assay system acquired from Promega according to the manufacturer's instructions.

2.6 "Proteome profiler™" human cytokine antibody array

To correlate transcriptional data with translation, the influence of selected apple polyphenols on the production of an assortment of cytokines and chemokines was investigated using a "Human Cytokine Array Panel A" array system obtained from R&D Systems GmbH (Wiesbaden-Nordenstadt, Germany). Therefore, MonoMac6 cells were starved for 24 h in RPMI 1640 medium containing 0.5% FCS, pretreated for 1 h with test compounds, and subsequently induced with 1 μg/mL LPS and 10 ng/mL IFN-γ for 16 h. Later on, cells were centrifuged and the supernatant was analyzed according to the manufacturer's instructions. Additionally T84 cells were used for comparative investigations. Therefore, cells were starved for 24 h in DMEM/Ham's F12 (1:1) medium with 0.5% FCS after 48 h of cultivation. Later on, cells were pretreated for 1 h with test compounds solved in DMSO in serum-free medium containing 100 units/mL catalase and induced 16 h with human CM consisting of 10 ng/mL TNF-α, 5 ng/mL IL-1β, and 10 ng/

Table 1. Formation of the human cytokine array (antibodies are spotted in duplicates)

Coordinate	Detected protein	Coordinate	Detected protein
A1, A2	Positive control	C7, C8	IL-13
A3, A4	Complement component 5a	C9, C10	IL-16
A5, A6	CD154	C11, C12	IL-17
A7, A8	G-CSF	C13, C14	IL-17E
A9, A10	GM-CSF	C15, C16	IL-23
A11, A12	CXCL1	C17, C18	IL-27
A13, A14	CCL1	D3, D4	IL-32α
A15, A16	sICAM-1	D5, D6	CXCL10
A17, A18	IFN-γ	D7, D8	CXCL11
A19, A20	Positive control	D9, D10	CCL2
B3, B4	IL-1α	D11, D12	MIF
B5, B6	IL-1β	D13, D14	CCL3
B7, B8	IL-1ra	D15, D16	CCL4
B9, B10	IL-2	D17, D18	PAI-1
B11, B12	IL-4	E1, E2	Positive control
B13, B14	IL-5	E3, E4	CCL5
B15, B16	IL-6	E8, E9	CXCL12
B17, B18	IL-8	E7, E8	TNF-α
C3, C4	IL-10	E9, E10	STREM-1
C5, C6	IL-12 p70	E11, E12	Negative control

mL IFN-γ. Subsequently, the supernatant was used after a short centrifugation step according to the manufacturer's protocol. The formation of the antibody array is shown in Table 1.

2.7 Preparation of the phenolic apple juice extract AE04 and analysis of constituents

Phenolic apple juice extract AE04 was produced from juices of different apple varieties harvested in 2002 and 2003 at Geisenheim Research Center and from local orchards as previously described [32, 33]. Analysis of extract constituents were performed as described recently [34].

2.8 Statistical analysis of data

Statistical evaluations were performed using Student's paired *t*-tests, and differences were considered significant if $p \leq 0.05$. Data are presented as means of triplicate stimulations from three independent experiments ±standard error of mean (±SEM).

3 Results

In recent investigations we found that the polyphenol enriched apple juice extract AE04 significantly inhibited the expression of proinflammatory marker genes in LPS/IFN-γ stimulated MonoMac6 cells at a concentration of 100 – 200 μg/mL. As determined by microarray analysis, the expression of proinflammatory cytokines and chemokines (*e.g.*, IL-1β, CXCL9, CXCL10), enzymes (CYP3A4), and transcription factors like STAT1 or INF regular factor 1 (IRF1) were significantly down-regulated by AE04 at a concentration of 200 μg/mL (unpublished data).

In order to identify the constituents responsible for the anti-inflammatory activity of AE04, we investigated the influence of some major compounds (summarized in Table 2) on the expression of proinflammatory marker genes in immunorelevant and epithelial cell lines using qRT-PCR. Consequently, 5-caffeoylquinic acid (chlorogenic acid); (–)-epicatechin; phloretin 2′-*O*-glucoside (phloridzin); phloretin 2′-*O*-xyloglucoside; and procyanidin B_2 were used in the present study. For structure – activity correlations, we integrated phloretin (aglycone), procyanidin B_1, and the flavan-3-ol monomer (+)-catechin in our investigations.

To evaluate the influence of the compounds on proinflammatory gene expression in DLD-1, T84, Jurkat, and MonoMac6 cells, RNA was isolated at different time points after induction of the cells with various stimuli and used for cDNA probe preparation. This time course revealed that the expression of selected proinflammatory marker genes (TNF-α, IL-1β, CXCL-10, IL-2, IL-8, CXCL3, COX-2) reached maximum levels between 4 and 6 h (data not shown) depending on the cell line. Therefore a four (T84, MonoMac6, and Jurkat) and 5 h (DLD-1) induction time was chosen for further experiments.

Cytotoxicity of the applied compounds was determined by resazurin reduction and a XTT-based cell viability assay as described in Section 2.3. Up to a concentration of 200 μM (treatment for 4 and 24 h), the test compounds possess no significant cytotoxicity in every cell line under study (data not shown).

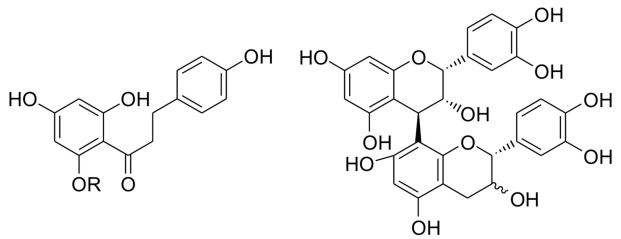
5-caffeoylquinic acid (chlorogenic acid) is the quantitatively most frequent polyphenol in apple juice and apple

Table 2. Polyphenol constituents of AE04 (mg/g extract; analyzed by HPLC-DAD according to Huemmer *et al.* [34]), and resulting concentrations for incubations previous to microarray and qRT-PCR analysis (μg/mL and μM, respectively)

Compound	Concentration in AE04 (mg/g extract)	Incubated concentration [μg/mL] (μM)
5-Caffeoylquinic acid	183.2	36.6 (103)
4-*p*-Coumaroylquinic acid	66.0	13.2 (39)
Phloretin 2′-*O*-xyloglucoside	68.9	13.8 (24)
Phloretin 2′-*O*-glucoside	48.0	9.6 (22)
Procyanidin B_1	n.d.	n.d.
Procyanidin B_2	12.1	2.4 (4)
(+)-Catechin	n.d.	n.d.
(−)-Epicatechin	12.5	2.5 (9)
Quercetin 3-*O*-rutinoside	4.5	0.9 (1)
Quercetin 3-*O*-galactoside	1.8	0.4 (1)
Quercetin 3-*O*-glucoside	1.5	0.3 (1)
Quercetin 3-*O*-xyloside	n.d.	n.d.
Quercetin 3-*O*-rhamnoside	4.3	0.9 (2)
Procyanidin C_1	2.0	0.4 (1)
4-Caffeoylquinic acid	9.2	1.8 (5)
Cumaroyl glucose	11.9	2.4 (7)
3-*p*-Cumaroylquinic acid	9.4	1.9 (6)
5-*p*-Cumaroylquinic acid	39.8	7.9 (23)
p-Cumaric acid	2.6	0.5 (3)
Caffeic acid	7.5	1.5 (8)
Phloretin 2′-*O*-xylogalactosid	4.2	0.8 (1)
Total amount	**489.4 mg/g extract**	**97.5 μg/mL (260 μM)**

n.d., not determined.

extracts, respectively. Up to a concentration of 182 μM, 5-caffeoylquinic acid revealed no significant inhibition of the proinflammatory marker genes (TNF-α, IL-1β, CXCL10, COX-2) in CM induced colonic epithelial DLD-1 and in LPS/IFN-γ induced MonoMac6 cells as determined by qRT-PCR. Furthermore, the flavan-3-ol monomers (−)-epicatechin (150 μM) and (+)-catechin (150 μM), and the dihydrochalcone glucosides phloretin 2′-*O*-xyloglucoside (150 μM) and phloretin 2′-*O*-glucoside (phloridzin) (150 μM) showed no inhibitory effect on the proinflammatory marker gene expression in CM induced colonic epithelial DLD-1 and T84, as well as in LPS/IFN-γ induced MonoMac6 cells. In our investigations the dimeric flavan-3-ols procyanidin B_1 and procyanidin B_2, and the dihydrochalcone aglycone phloretin showed the strongest inhibitory effect on the inducible expression of the proinflammatory marker genes. The chemical structures of the investigated polyphenols are shown in Fig. 1. All experiments were performed at least in triplicates as described in Section 2.



1 2

Figure 1. Chemical structures of phloretin (*R* = H) (1), phloretin 2′-*O*-glucoside (phloridzin, *R* = β-D-glucoside) (1), procyanidin B_1 (−)-epicatechin (4β → 8) (+)-catechin) (2), and procyanidin B_2 (−)-epicatechin (4β → 8) (−)-epicatechin) (2).

3.1 Effect of apple polyphenols on proinflammatory gene transcription

To investigate the effect of the applied apple polyphenols on proinflammatory gene expression, qRT-PCR experiments for selected responsive genes (*e.g.*, TNF-α, IL-8, CXCL 10, CXCL 3, IL-2, IL-1β) and COX-2 in immunorelevant and epithelial cell lines were performed. Following pretreatment with test compounds and stimulation, total cellular RNA was isolated from the CM induced colonic epithelial cell lines DLD-1 and T84, the LPS/IFN-γ induced MonoMac6, and the TPA/Ionomycin stimulated T-cells for cDNA preparations as described in Section 2.4. As shown in Figs. 2–5, values are expressed as ratios (log 2) of relative mRNA content of induced *versus* un-induced cells, and induced and polyphenol treated *versus* induced and untreated cells, each corrected for the constitutive expressed housekeeping gene GAPDH as reference determined in the same sample in parallel. For significant differences in mRNA levels between induced and polyphenol

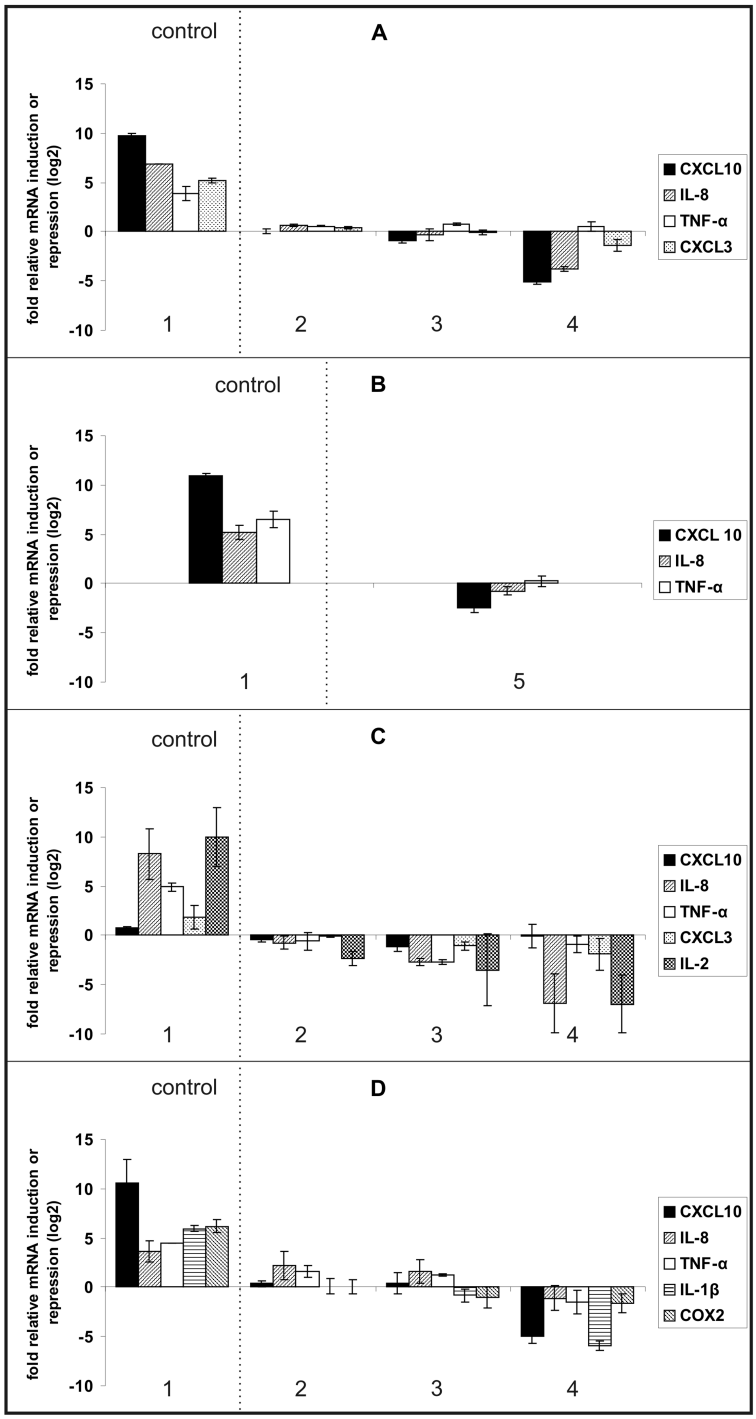


Figure 2. Effect of procyanidin B_1 on mRNA levels of selected proinflammatory genes in IFN-γ/IL-1β/TNF-α stimulated DLD-1 (A) and T84 cells (B), TPA/Ionomycin stimulated Jurkat-cells (C), and LPS/IFN-γ stimulated MonoMac6-cells (D). Values are expressed as ratios (log 2) of relative mRNA levels of stimulated *versus* unstimulated cells as control (1) and procyanidin B_1 pretreated (17 μM (2), 35 μM (3), 86 μM (4), and 50 μM (5)) and stimulated *versus* untreated, stimulated cells, corrected for GAPDH as reference determined in the same sample in parallel. Data are shown as mean values ±SEM of three independent experiments.

treated *versus* induced and untreated samples a range of ±2-fold has been chosen as a basis for significant induction or repression of gene expression. In all experiments, measured mRNA values for GAPDH did not vary significantly upon treatment of the cells with the according stimuli or the applied polyphenols (data not shown).

All investigated genes were found to be significantly up-regulated at least two-fold after stimulation of the used cell lines with specific stimuli (Figs. 2A-D1 – 5A-D1). In Jurkat cells stimulation with TPA/Ionomycin strongly induced IL-8, TNF-α, CXCL3, and IL-2 expression, but only caused weak up-regulation of CXCL10 (Figs. 2C1 – 5C1).

Procyanidin B_1 (86 μM) down-regulated the mRNA levels of CXCL10 in the induced cell lines DLD-1 and MonoMac6 by more than 90% (Figs. 2A4, 2D4). IL-8 expression was down-regulated at least 8-fold in DLD-1 and Jurkat

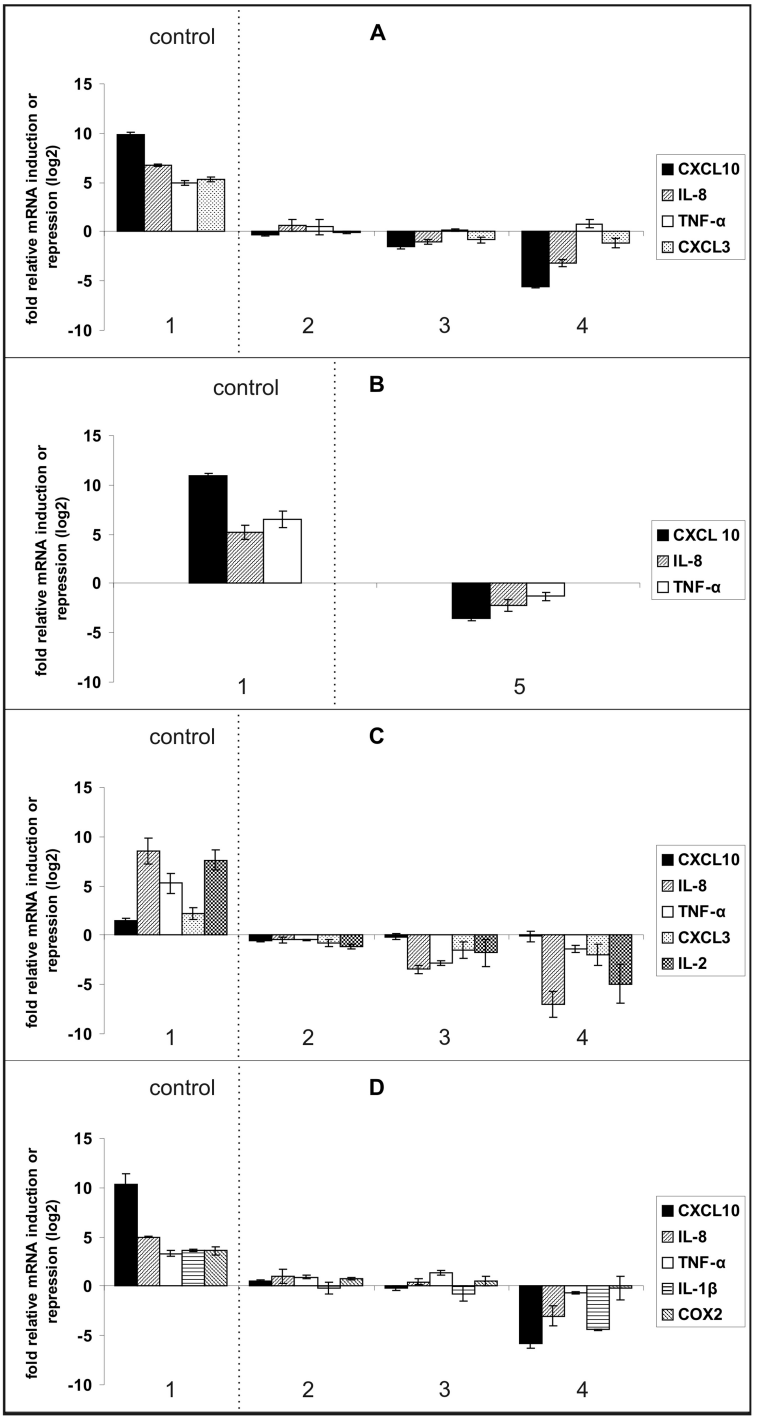


Figure 3. Effect of procyanidin B_2 on mRNA levels of selected proinflammatory genes in IFN-γ/IL-1β/TNF-α stimulated DLD-1 (A) and T84 cells (B), TPA/Ionomycin stimulated Jurkat-cells (C) and LPS/IFN-γ stimulated MonoMac6-cells (D). Values are expressed as ratios (log 2) of relative mRNA levels of stimulated *versus* unstimulated cells as control (1) and procyanidin B_2 pretreated (17 μM (2), 35 μM (3), 86 μM (4), and 50 μM (5)) and stimulated *versus* untreated, stimulated cells, corrected for GAPDH as reference determined in the same sample in parallel. Data are shown as mean values ±SEM of three independent experiments.

cells at a concentration of 86 μM (Figs. 2A4, 2C4). At the same concentration, procyanidin B_1 inhibited the expression of CXCL3 (3-fold) in DLD-1 and Jurkat cells (Fig. 2A4), IL-2 (128-fold) in Jurkat cells (Fig. 2C4), and IL-1β (64-fold) and COX-2 (3-fold) in MonoMac6 cells as well (Fig. 2D4). In epithelial T84 cells, mRNA levels of CXCL10 were significantly down-regulated (5-fold) by procyanidin B_1 at a concentration of 50 μM (Fig. 2B5). As shown in Fig. 3, procyanidin B_2 had a similar effect on gene expression in all tested cell lines. In addition, procyanidin B_2 (50 μM) repressed the IL-8 expression (4-fold) and moderately influenced the expression of TNF-α in epithelial T84 cells (Fig. 3B5).

Upon CM stimulation, phloretin significantly inhibited the expression of CXCL10 in both epithelial cell lines by more than 90% (at 182 μM in DLD-1 and 100 μM in T84

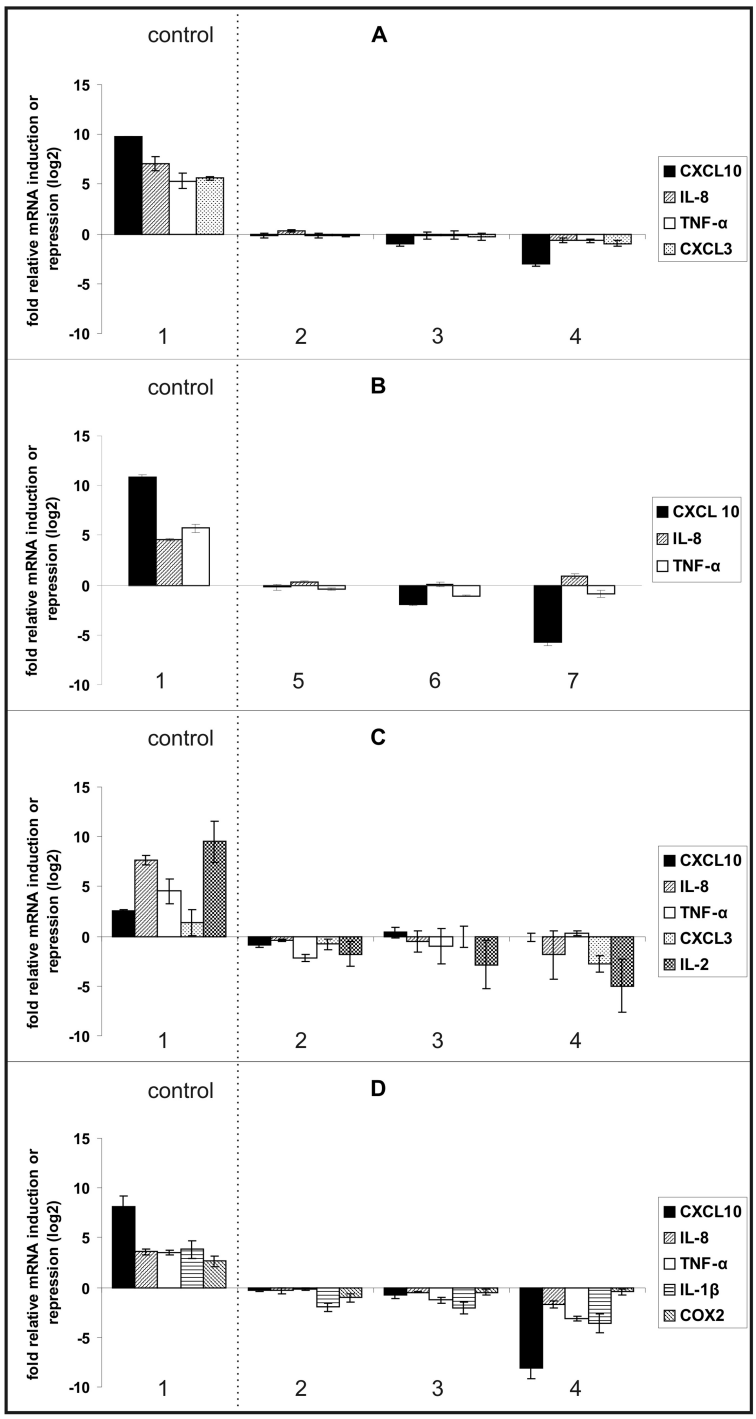


Figure 4. Effect of phloretin on mRNA levels of selected proinflammatory genes in IFN-γ/IL-1β/TNF-α stimulated DLD-1 (A) and T84 cells (B), TPA/Ionomycin stimulated Jurkat-cells (C) and LPS/IFN-γ stimulated MonoMac6-cells (D). Values are expressed as ratios (log 2) of relative mRNA levels of stimulated *versus* unstimulated cells as control (1) and phloretin pretreated (36 μM (2), 73 μM (3), 182 μM (4), 25 μM (5), 50 μM (6), and 100 μM (7)) and stimulated *versus* untreated, stimulated cells, corrected for GAPDH as reference determined in the same sample in parallel. Data are shown as mean values ±SEM of three independent experiments.

cells (Figs. 4A4, 4B7)). The dihydrochalcone also dose-dependently suppressed the expression of CXCL10 in LPS/IFN-γ stimulated MonoMac6 cells by more than 90% (Figs. 4D2–4). In addition, phloretin down-regulated the mRNA levels of IL-8 (3-fold) in Jurkat and MonoMac6 cells, as well as TNF-α (8-fold) in MonoMac6 cells and CXCL3 (6-fold) in Jurkat cells at 182 μM (Figs. 4C4, 4D4). Furthermore the transcription of IL-2 in Jurkat and IL-1β in MonoMac6 cells was strongly suppressed by more than 90% (Figs. 4C4, 4D4). In contrast, phloretin 2′-*O*-glucoside (phloridzin) had no significant effect on mRNA levels in all cell lines investigated (Fig. 5).

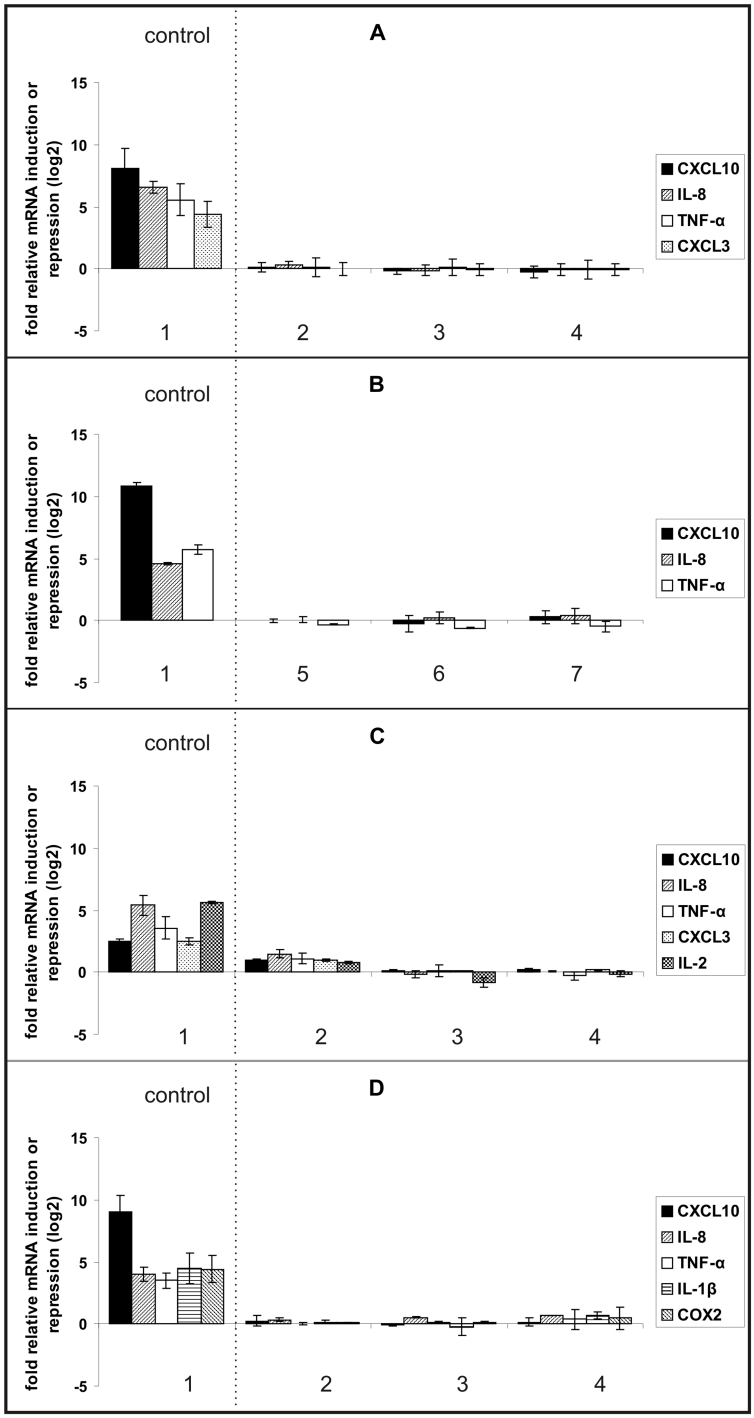


Figure 5. Effect of phloretin 2′-*O*-glucoside (phloridzin) on mRNA levels of selected proinflammatory genes in IFN-γ/IL-1β/TNF-α stimulated DLD-1 (A) and T84 cells (B), TPA/Ionomycin stimulated Jurkat-cells (C) and LPS/IFN-γ stimulated MonoMac6-cells (D). Values are expressed as ratios (log 2) of relative mRNA levels of stimulated *versus* unstimulated cells as control (1) and phloridzin pretreated (42 μM (2), 106 μM (3), 212 μM (4), 25 μM (5), 50 μM (6), and 100 μM (7)) and stimulated *versus* untreated, stimulated cells, corrected for GAPDH as reference determined in the same sample in parallel. Data are shown as mean values ±SEM of three independent experiments.

3.2 NF-κB, STAT1, IP-10-, and IL-8-promoter-dependent reporter gene activities

To elucidate the underlying anti-inflammatory mechanism of the applied apple polyphenols, we investigated the influence on IP-10- and IL-8-promoter activity in DLD-1, MonoMac6, and Jurkat cells.

Transfection of the cells with a hIP-10 promoter driven luciferase reporter plasmid and stimulation with CM (DLD-1), TPA/Ionomycin (Jurkat), or LPS/IFN-γ (MonoMac6) increased the luciferase activity 5–10-fold compared to non-stimulated cells. The inducible hIP-10 promoter activity was dose-dependently inhibited by all compounds investigated (IC_{50} values are summarized in Table 3). Procyanidin B_1 and procyanidin B_2 (Fig. 6) elicited the strongest inhibitory effect with IC_{50} values of 7–9 μM. Compared to phloretin (Fig. 7), phloridzin revealed only

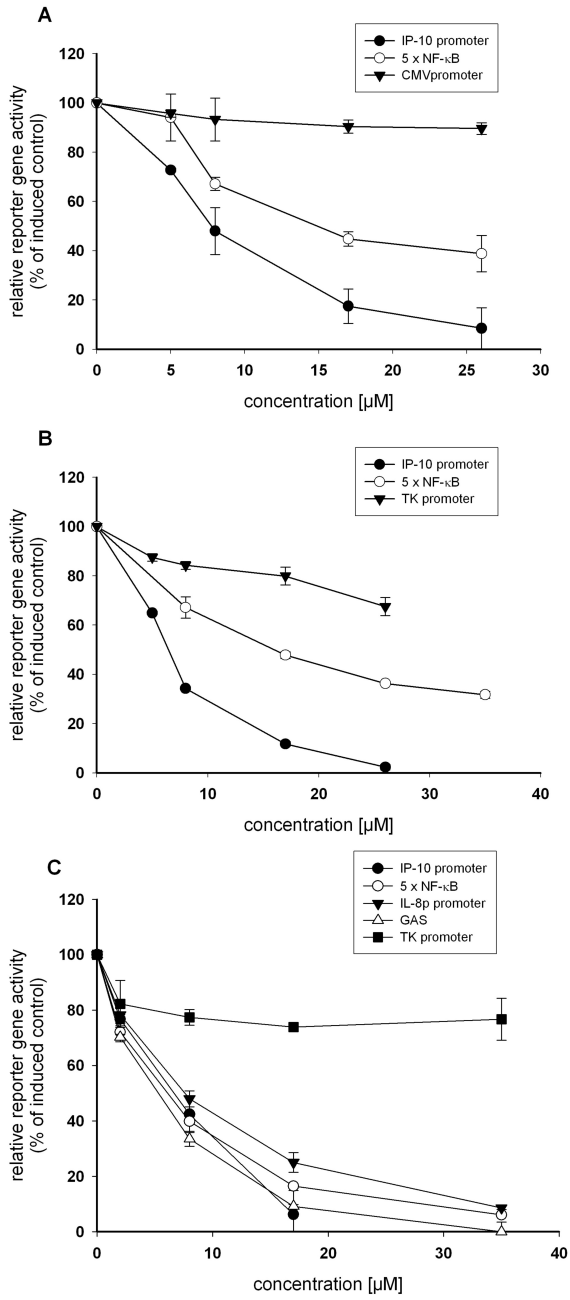


Figure 6. Effect of procyanidin B_2 on NFκB, hIP-10, GAS, hIL-8, and CMV/TK promoter driven reporter gene expression in DLD-1 (A), Jurkat (B), and MonoMac6 cells (C). Cells were transiently transfected with NFκB and hIP-10 promoter dependent reporter constructs. MonoMac6 cells were additionally transfected with GAS and hIL-8 promoter dependent reporter constructs. Furthermore, cells were transiently transfected with the constitutive CMV-(DLD-1) or TK-promoter dependent reporter constructs (Jurkat and MonoMac6). Stimulation was done with IFN-γ/IL-1β/TNF-α (DLD-1) for 24 h, TPA/Ionomycin (Jurkat) for 24 h, or LPS/IFN-γ (MonoMac6) for 4 h with or without procyanidin B_2. Control (100%) means stimulation only. The expression of the reporter genes was determined as described in Section 2. Data presented are the means of at least three independent experiments.

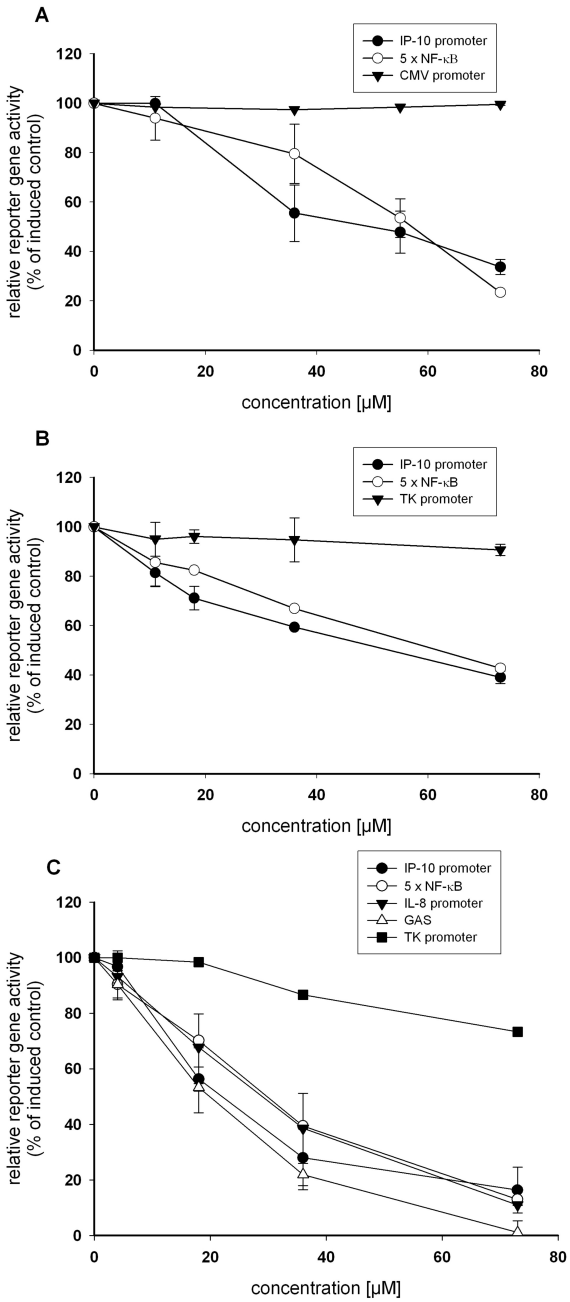


Figure 7. Effect of phloretin on NFκB, hIP-10, GAS, hIL-8, and CMV/TK promoter driven reporter gene expression in DLD-1 (A), Jurkat (B), and MonoMac6 cells (C). Cells were transiently transfected with NFκB and hIP-10 promoter dependent reporter constructs. MonoMac6 cells were additionally transfected with GAS and hIL-8 promoter dependent reporter constructs. Furthermore, cells were transiently transfected with the constitutive CMV-(DLD-1) or TK-promoter dependent reporter constructs (Jurkat and MonoMac6). Stimulation was done with IFN-γ/IL-1β/TNF-α (DLD-1) for 24 h, TPA/Ionomycin (Jurkat) for 24 h or LPS/IFN-γ (MonoMac6) for 4 h with or without phloretin. Control (100%) means stimulation only. The expression of the reporter genes was determined as described in Section 2. Data presented are the means of at least three independent experiments.

Table 3. Inhibition of selected reporter gene activities (NF-κB, IP-10, IL-8, STAT1) by different polyphenols in DLD-1, Jurkat, and MonoMac6 (MM6) cells (IC_{50} values (μM))

	Procyanidin B_1			Procyanidin B_2			Phloretin			Phloridzin		
	DLD-1	Jurkat	MM6	DLD-1	Jurkat	MM6	DLD-1	Jurkat	MM6	DLD-1	Jurkat	MM6
NF-κB	17	–	10	15	15	5	55	62	29	–	–	74
IP-10	9	8	7	8	7	7	47	53	23	53	106	80
IL-8	n.a.	n.a.	12	n.a.	n.a.	7	n.a.	n.a.	29	n.a.	n.a.	21
STAT1	n.a.	n.a.	10	n.a.	n.a.	5	n.a.	n.a.	18	n.a.	n.a.	85

n.a., not analyzed.

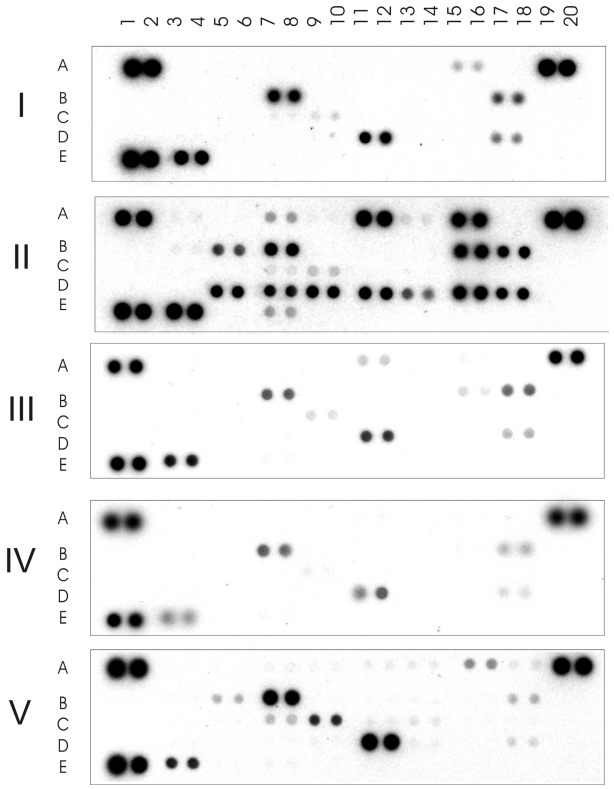


Figure 8. Effect of procyanidin B_1, procyanidin B_2, and phloretin on the cytokine- and chemokine-production in LPS/IFN-γ stimulated MonoMac6 cells. MonoMac6 cells were induced with 1 μg/mL LPS and 10 ng/mL IFN-γ for 16 h. Cells were pretreated with 86 μM procyanidin B_1, 86 μM procyanidin B_2, and 182 μM phloretin for 1 h prior to stimulation. The supernatant was used according to the manufacturer's instructions (Proteome Profiler–Human Cytokine Array Panel A, R&D Systems). Cells were left uninduced (I), induced with LPS/IFN-γ for 16 h (II), induced and treated with 86 μM procyanidin B_1 (III), 86 μM procyanidin B_2 (IV), or 182 μM phloretin (V). The formation of the antibody array is shown in Table 1.

moderate influence on hIP-10 promoter activity. In addition, the influence on IL-8 promoter activity was determined, which is mainly regulated at the transcriptional level by NF-κB and AP-1 [35]. The IL-8 dependent induction of luciferase activity in MonoMac6 cells was increased 10–13-fold after stimulation with 1 μg/mL LPS and 10 ng/mL IFN-γ. The hIL-8 promoter activities were dose-dependently inhibited by all compounds with IC_{50} values of 7–21 μM. All IC_{50} values are summarized in Table 3.

Because the transcription factor NF-κB plays a pivotal role in the inducible transcription of the IP-10 and IL-8 genes, we investigated the influence of the test compounds using a NF-κB transcriptional reporter in DLD-1, Jurkat, and MonoMac6 cells. Cell transfection with the transcriptional NF-κB reporter and induction with the indicated stimuli increased the luciferase activity 15–20-fold compared to non-stimulated cells. The inducible NF-κB driven reporter gene expression was dose-dependently inhibited by procyanidin B_2 with IC_{50} values of 5–15 μM (Fig. 6). In comparison, procyanidin B_1 had a similar effect on NF-κB activation, whereas in Jurkat cells the reporter gene activity was still around 60% at a concentration of 35 μM. Phloretin (Fig. 7) dose-dependently repressed the NF-κB activation with IC_{50} values of 29–62 μM. Phloridzin only weakly inhibited the NF-κB reporter gene activity in MonoMac6 cells (IC_{50} of 74 μM), whereas it was not influenced in DLD-1 and Jurkat cells.

Beside NF-κB, STAT1 represents another important transcription factor involved in IP-10 transcriptional regulation. Therefore the influence of the polyphenols on STAT1-dependent reporter gene activity was analyzed. Transfection of MonoMac6 cells with the STAT1 transcriptional reporter and stimulation with LPS/IFN-γ resulted in a 20–24-fold luciferase activity compared to the non-stimulated cells. Procyanidin B_1, procyanidin B_2 (Fig. 6C), and phloretin (Fig. 7C) inhibited STAT1-driven reporter gene expression with IC_{50} values of 5–18 μM, whereas phloridzin showed only a slight inhibitory effect with an IC_{50} value of 85 μM (Table 3).

To determine whether the repression of the transcriptional reporter is a result of the inhibition of general transcription factors, the effects of the phenolic compounds on a constitutive promoter in DLD-1, Jurkat, and MonoMac6 cells were analyzed. Therefore the plasmid pRL-TK (Jurkat and MonoMac6 cells) containing the thymidine kinase promoter and the plasmid pRL-CMV (DLD-1) containing the cytomegalovirus promoter were applied. Both promoters were fused to renilla luciferase as described in Section 2.

All compounds showed no inhibition of the constitutive promoter activity up to a concentration of 26 μM procyanidin B_1 and procyanidin B_2 (Fig. 6), 73 μM phloretin (Fig. 7), and 106 μM phloridzin, respectively.

3.3 Relative protein levels of an assortment of multiple released cytokines and chemokines

To correlate the inhibitory activity seen on the transcriptional level with the synthesis and excretion of proinflammatory mediators, we investigated the influence of procyanidin B_1, procyanidin B_2, and phloretin on the inducible synthesis of selected cytokines and chemokines in the human cell lines MonoMac6 and T84 with an antibody array.

The cells were pretreated with test compounds for 1 h and subsequently stimulated with LPS/IFN-γ (MonoMac6) or CM (T84) for 16 h. As shown in Fig. 8 II, stimulation of MonoMac6 cells with LPS/IFN-γ resulted in the induction of expression and secretion of various cytokines and chemokines. In particular, ILs (*e.g.*, IL-1β, IL-1ra, IL-6, IL-8, IL-13, IL-13), "plasminogen activator inhibitor type-1" (PAI-1), "intercellular adhesion molecule-1" (sICAM-1), as well as several chemokines (*e.g.*, CXCL10, CXCL11, CCL2, CCL3, CCL4, and CCL5), and the proinflammatory cytokine TNF-α were induced. As shown in Fig. 8 III, the synthesis of chemokines (CXCL10, CXCL11, CCL2, CCL4), cytokines (IL-1β, TNF-α), and adhesion molecules (sICAM-1) were completely inhibited by procyanidin B_1 at a concentration of 86 μM. The production of CXCL1, IL-1ra, IL-6, IL-8, and PAI-1 was only partially repressed. In contrast, procyanidin B_2 (86 μM) completely inhibited the release of almost all induced chemokines and cytokines (Fig. 8 IV). Interestingly, the amount of the constitutive expressed chemokines CCL5 and "macrophage migration inhibitory factor" (MIF) were not significantly affected by both compounds.

As shown in Fig. 8 V, phloretin (182 μM) strongly repressed the production of chemokines, like CXCL1, CXCL10, CXCL11, CCL2, and CCL4. The synthesis of cytokines, like IL-6, IL-8, and TNF-α were also inhibited.

Stimulation of T84 cells with CM, induced the synthesis of proinflammatory cytokines, like CXCL10, TNF-α, and IL-8, as well as RANTES, IL-1β, I-TAC, GRO-α, and sICAM-1 (Fig. 9 II). As shown in Fig. 9 III, pretreatment with procyanidin B_1 (100 μM) caused a complete inhibition of the synthesis of CXCL10, TNF-α, RANTES, IL-1β, I-TAC, GRO-α, and sICAM-1, and a partial repression of IL-8 production. In contrast, procyanidin B_2 completely blocked the synthesis of almost all induced chemokines and cytokines at 100 μM (Fig. 9 IV). Additionally, the production of CXCL10, RANTES, I-TAC, GRO-α, and sICAM-1 was completely repressed by phloretin at a concentration of 100 μM, whereas the release of IL-8 and TNF-α was only moderately influenced (Fig. 9 V).

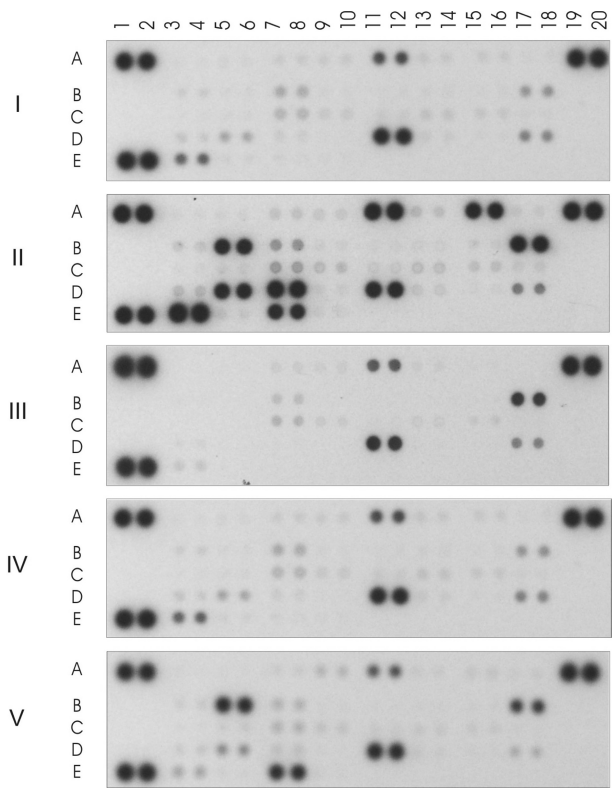


Figure 9. Effect of procyanidin B_1, procyanidin B_2, and phloretin on the cytokine- and chemokine-production in IFN-γ/IL-1β/TNF-α stimulated T84 cells. T84 cells were induced with 10 ng/mL IFN-γ, 5 ng/mL IL-1β, and 10 ng/mL TNF-α for 16 h. Cells were pretreated with 100 μM procyanidin B_1, 100 μM procyanidin B_2, and 100 μM phloretin for 1 h prior to stimulation. The supernatant was used according to the manufacturer's instructions (Proteome Profiler – Human Cytokine Array Panel A, R&D Systems). Cells were left uninduced (I), induced with IFN-γ/IL-1β/TNF-α for 16 h (II), induced and treated with 100 μM procyanidin B_1 (III), 100 μM procyanidin B_2 (IV), or 100 μM phloretin (V). The formation of the antibody array is shown in Table 1.

4 Discussion

Constitutively active, dysregulated expression of proinflammatory cytokines, chemokines, cell adhesion molecules, and enzymes has been implicated in many inflammatory and autoimmune diseases such as IBD [25]. Cytokines and chemokines are extracellular signaling molecules that mediate cell–cell communication. They are released from cells and have critical roles in many biological processes such as cellular growth, differentiation, gene expression, migration, immunity, and inflammation. In most biological processes, multiple cytokines operate in a large network, where the action of one cytokine is regulated by the presence or absence of other cytokines. As a disorder of the gastrointestinal tract, inflammatory processes in IBD could be positively affected by nutrition factors. In the present study

we therefore evaluated the influence of selected apple polyphenols on inflammatory gene expression.

Initial experiments revealed an inhibitory effect of the apple juice extract AE04 on proinflammatory gene expression in LPS/IFN-γ stimulated monocytic macrophages. In addition to transcription factors (STAT1, IRF1) and enzymes (COX-2, CYP3A4), AE04 significantly down-regulated the mRNA levels of several proinflammatory cytokines and chemokines (*e.g.*, TNF-α, IL-1β, CXCL9, CXCL10), especially the induction of the CXC chemokine IFN-γ-inducible protein 10 (IP-10, CXCL10) was strongly repressed.

IP-10 is produced by several cell types, such as endothelial cells [36], macrophages, keratinocytes [37], eosinophils [38], and neutrophils [39]. Furthermore, it is also constitutively expressed by normal human colon epithelium [40]. CXCL10 is a ligand for the CXCR3 receptor and serves as chemoattractant for activated type-1 T-lymphocytes and natural killer cells [30]. An increased expression of IP-10 has been observed in patients with COPD [41], asthma [42], and in acute inflammation in response to pathogens [43]. In addition, IP-10 is permanently over-expressed in patients with IBD [44, 45].

In order to identify the constituents responsible for the anti-inflammatory activity of AE04, the influence of some major compounds of AE04 on gene expression of selected proinflammatory mediators was investigated in cell lines playing a decisive role in the inflammatory process of IBD. In our study, the flavan-3-ol dimers procyanidin B_1 and procyanidin B_2, and the dihydrochalcone aglycone phloretin had the strongest effect on proinflammatory gene expression.

Investigations were performed using the human epithelial colon adenocarcinoma cell lines DLD-1 and T84. Intestinal epithelial cells are the first immunologic barrier of the organism on the villus surface. Furthermore, colonic epithelial cells are mainly involved in the characteristic infiltration of selected populations of leukocytes in IBD by producing a multitude of chemokines [46, 47]. For stimulation, DLD-1 and T84 cells were induced with IFN-γ, IL-1β, and TNF-α (CM). There is a functional synergy between TNF-α and IFN-γ in promoting CXCL10 gene expression, that depends on the co-existence of TNF-α responsive NF-κB binding sites and IFN-γ responsive signal transducers and activators of transcription (STAT) protein-binding elements within the gene promoter [30, 48 – 52]. Stimulation with IL-1β additionally activates MAPK- and NF-κB-pathways [53].

In addition, investigations were performed in TPA/Ionomycin stimulated human T-cells (Jurkat) which are mainly responsible for mucosal inflammation due to an excessive T_H1-cell response associated with increased secretions of IL-12, IFN-γ, and TNF [25]. Stimulation with TPA and Ionomycin mimics the T-cell receptor activation and therefore the transcription factors NF-κB and NF-AT are induced [54, 55].

Furthermore, the anti-inflammmatory effect of applied polyphenols was studied in LPS/IFN-γ stimulated cells of the monocytic system (macrophages, monocytes), which plays a major role in the colonic immune defence by presenting processed antigens to T-cells, phagocytosis of foreign material, and coordination of the immune response by secretion of cytokines. As a constituent of gram-negative bacteria cell walls, LPS is a potent macrophage-activating stimulus which induces the expression of many genes necessary for immune defence function [56 – 58]. After interaction with toll-like receptor 4 (TLR4), LPS stimulated gene expression is mainly induced by myeloid differentiation protein (MyD88) dependent pathways which involve the activation of NF-κB [59 – 62]. Moreover, STAT1 is essential for maximum LPS-induced IP-10 expression [51]. Further stimulation of MonoMac6 cells with the type II interferon-γ results in an additional activation of the JAK/STAT signaling pathway with a subsequent activation of STAT1α homodimers.

In our investigations, procyanidin B_1, procyanidin B_2, and phloretin down-regulated the mRNA levels of most genes under study. For instance, CXCL10 expression was significantly inhibited at a concentration of 86 μM (procyanidins) or 182 μM (phloretin) in all cell lines. In contrast, the glucoside phloridzin had no inhibitory effect. In Jurkat cells the stimulation of CXCL10 expression with TPA/Ionomycin was only weak (Figs. 2C1 – 5C1).

To evaluate effects of the applied polyphenols on IP-10 promoter activity, a hIP-10 promoter driven luciferase reporter plasmid was used. Procyanidin B_1, procyanidin B_2, and phloretin repressed the IP-10 promoter activity in a dose-dependent manner in all cell lines under study (Figs. 6, 7). On the transcriptional level, inducible IP-10 expression is regulated by the transcription factors NF-κB, IRF3, and STAT1 [48, 59]. Therefore, we determined the effect of the polyphenols on NF-κB and STAT1 dependent promoter activities. Both signaling pathways were inhibited by the procyanidins B_1, B_2, and phloretin in a dose-dependent manner. IP-10 expression is therefore synergistically repressed by blocking NF-κB and STAT1 dependent signaling. Unlike phloretin, the glucoside showed only a weak inhibitory effect on NF-κB, STAT1, and IP-10-promoter dependent reporter gene expression. In DLD-1 and Jurkat cells, phloridzin had no effect on NF-κB activation (Table 3).

The ability to modulate the activation of NF-κB is one of the most important anti-inflammatory activities of some phenolic compounds. For instance, green tea catechins have been found to be potent inhibitors of NF-κB [63]. Procyanidin B_2 has been shown to affect the NF-κB signaling pathway by preventing DNA-binding of the activated NF-κB transcription factor [64]. In addition, some polyphenols are known to interact with components of the JAK/STAT signaling pathway. For example, green tea polyphenols blocked the tyrosine phosphorylation of the STAT1α protein resulting in an inhibition of the STAT1α DNA binding activity [65].

Furthermore, the effect of the compounds on IL-8 mRNA levels in DLD-1, T84, Jurkat, and MonoMac6 cells, and their influence on IL-8 promoter activity in LPS/IFN-γ stimulated MonoMac6 cells was investigated. IL-8 is released in response to mucosal adherent *E. coli* in the inflammatory process of IBD [66]. On the transcriptional level, procyanidin B_1 significantly down-regulated the mRNA levels of IL-8 in DLD-1 and Jurkat cells, whereas procyanidin B_2 repressed the mRNA levels of IL-8 in all cell lines under study (Figs. 2, 3). Phloretin significantly inhibited the mRNA expression of IL-8 in LPS/IFN-γ stimulated MonoMac6 cells at a concentration of 182 μM (Fig. 5D4). The glucoside had no inhibitory influence on IL-8 mRNA expression (Fig. 5). All compounds had a similar effect inhibiting the IL-8 promoter activity in a dose dependent manner (Table 3). Two *cis*-acting elements are known to play a functional role in the regulation of the IL-8 promoter. Besides a distal promoter element comprised of an activator protein-1 (AP-1) binding site, there is a proximal promoter element containing binding sites for NF-IL-6 and NF-κB [67]. Inhibition of IL-8 expression is possibly correlated with inhibition of NF-κB dependent signal transduction or additional repression of AP-1 signaling. The AP-1 complex consists of either homo- or heterodimers of the JUN and FOS protein family members [68]. For instance, PMA-induced IL-8 release in U937 cells was repressed by resveratol due to inhibition of AP-1 activation [69].

The transcriptional activation of the TNF-α gene highly depends on NF-κB [70]. Inhibition of the activation of the transcription factor NF-κB and subsequent TNF-α release in macrophage-like cell lines have been suggested to account for the anti-inflammatory properties of several natural compounds, including gliotoxin, aspirin, curcumin, and myricetin [71]. TNF-α is known to be highly expressed in IBD [72]. In the present study, procyanidin B_2 (50 μM) slightly down-regulated the expression of TNF-α in T84 cells (Fig. 3B5). In LPS/IFN-γ stimulated MonoMac6 cells, phloretin (182 μM) significantly blocked the mRNA expression of TNF-α (Fig. 4D4). Phloridzin had no effect on TNF-α expression up to a concentration of 212 μM (Fig. 5).

Furthermore, IL-1β is known to be another key mediator in the pathogenesis of IBD. Functional NF-κB, AP-1, and C/EBP-sites have been found within the promoter region of IL-1β, which is comparably regulated to TNF-α [73, 74]. In our study both procyanidins (86 μM) and phloretin (182 μM) significantly down-regulated the mRNA levels of IL-1β in LPS/IFN-γ stimulated MonoMac6 cells (Figs. 2D4, 3D4, 4D4).

Additionally, CXCL3 expression was slightly repressed by the procyanidins in CM stimulated DLD-1 and TPA/Ionomycin stimulated Jurkat cells (Figs. 2A4, 2C4, 3A4, 3C4). CXCL3 (GROγ) is found to be highly expressed in inflamed mucosa [75]. Among NF-κB and AP-1, additionally STAT signaling pathways are involved in regulation of CXCL3 expression [76, 77].

Furthermore, both procyanidins and phloretin significantly down-regulated the mRNA levels of IL-2 in TPA/Ionomycin stimulated Jurkat cells. Increased levels of IL-2 have been observed in the intestinal mucosa of IBD patients [78]. In consideration of putative transcription factor binding sites, the inhibition could be due to an intervention in NF-κB, AP-1, or NF-AT pathways [79].

COX-2 expression is also involved in the susceptibility of IBD [80]. In LPS/IFN-γ stimulated MonoMac6 cells, COX-2 mRNA levels were significantly down-regulated by procyanidin B_1 at a concentration of 86 μM. The binding of transcription factors to the NF-κB, NF-IL-6, and CRE promoter elements within the first 327 base pairs in the 5′-flanking region induce the expression of COX-2 in human macrophages/monocytes. It has been shown that at least two of these *cis*-acting elements are necessary to achieve maximum transcription by LPS induction. Several studies revealed that inhibition of NF-κB activation or interfering with MAPK signaling by pharmacological inhibitors or natural compounds decreases COX-2 transcription and expression [81]. Procyanidin B_2 has been found to suppress the expression of COX-2 in LPS stimulated human monocytic THP-1 cells resulting in a decreased activation of ERK, JNK, and p38 MAPK. These kinases are upstream enzymes known to regulate COX-2 expression in many cell types. Furthermore, procyanidin B_2 has been found to suppress NF-κB activation by stabilization of IκB proteins [82]. In our study, only procyanidin B_1 slightly down-regulated the mRNA levels of COX-2 in LPS/IFN-γ stimulated MonoMac6 cells. In contrast, procyanidin B_2 revealed no inhibitory effect on COX-2 expression.

To correlate the protein levels of the investigated proinflammatory mediators with transcriptional data, the relative levels of selected cytokines and chemokines were analyzed by human cytokine array.

In LPS/IFN-γ stimulated MonoMac6 cells, the synthesis of basically NF-κB regulated chemokines, like CXCL10, CCL2, or CCL4 [83, 84] was inhibited by procyanidin B_1 (86 μM). The transcriptional inhibition of cytokines like IL-1β or TNF-α could be confirmed on the protein level, as well. In addition to chemokines and cytokines, procyanidin B_1 was able to inhibit the production of adhesion molecules, like sICAM-1, which is also involved in inflammatory processes of the bowel [85] (Fig. 8 III). In addition, procyanidin B_2 completely blocked the synthesis of almost all induced chemokines and cytokines at 86 μM (Fig. 8 IV). In CM stimulated T84 cells, procyanidin B_1 (100 μM) in particular inhibited the production of CXCL10, TNF-α, CCL5 (RANTES), IL-1β, CXCL11 (I-TAC), CXCL1 (GRO-α), and sICAM-1 (Fig. 9 III). As shown in MonoMac6 cells, procyanidin B_2 (100 μM) completely averts the production of almost all induced chemokines and cytokines in T84 cells (Fig. 9 IV).

Furthermore, the synthesis of chemokines, like CXCL1, CCL2, and CCL4 were inhibited by phloretin at a concen-

tration of 182 μM. The NF-κB pathway was shown to be an essential transcriptional modulator of these chemokines [83, 84]. In addition to chemokines and cytokines, phloretin was able to repress the production of adhesion molecules, like intercellular adhesion molecule-1 (ICAM-1) (Fig. 8 V). In previous studies it was shown that, phloretin suppresses the expression of ICAM-1 in TNF-α and IL-1β stimulated human umbilical vein endothelial cells by inhibition of IRF1 activation [86]. In our study, an inhibition of ICAM-1 release could be confirmed in CM stimulated T84 cells (Fig. 9 V). Furthermore, phloretin repressed the synthesis of CCL5 (RANTES), CXCL11 (I-TAC), and CXCL1 (GROα). The effect on mRNA expression of investigated proinflammatory genes by the applied polyphenols procyanidin B_1, procyanidin B_2, and phloretin, therefore, strongly correlated with the released protein levels evaluated by the human cytokine array.

In summary, we evaluated selected compounds responsible for the anti-inflammatory activity of AE04. In particular, procyanidin B_1, procyanidin B_2, and phloretin revealed inhibitory potential on inflammatory gene expression *in vitro*. These apple polyphenols, therefore, may serve as transcription-based inhibitors of proinflammatory gene expression.

The study was performed as a part of NutritionNet, supported by the German Federal Ministry of Education and Research (BMBF), Project no. 01EA0501. This work was additionally supported by the state of Rhineland-Palatinate (Projekt ‚Wissen schafft Zukunft'). The authors thank Prof. Dr. Schrenk and his collaborators for providing real-time PCR equipment.

The authors have declared no conflict of interest.

5 References

[1] Scalbert, A., Manach, C., Morand, C., Rémésy, C., Jiménez, L., Dietary polyphenols and the prevention of diseases, *Crit. Rev. Food Sci. Nutr.* 2005, *45*, 287 – 306.

[2] Scalbert, A., Williamson, G., Dietary intake and bioavailability of polyphenols, *J. Nutr.* 2000, *130*, 2073S – 2085S.

[3] Thielen, C., Will, F., Zacharlas, J., Dietrich, H., Jacob, H., Polyphenols in apples: Distribution of polyphenols in apple tissue and comparison of fruit and juice, *Deutsche Lebensmittel-Rundschau* 2004, *100*, 389 – 398.

[4] Lata, B., Tomala, K., Apple peel as a contributor to whole fruit quantity of potentially healthful bioactive compounds. Cultivar and year implication, *J. Agric. Food Chem.* 2007, *55*, 10795 – 10802.

[5] Vrhovsek, U., Rigo, A., Tonon, D., Mattivi, F., Quantitation of polyphenols in different apple varieties, *J. Agric. Food Chem.* 2004, *52*, 6532 – 6538.

[6] Manach, C., Scalbert, A., Morand, C., Rémésy, C., Jimenéz, L., Polyphenols: Food source and bioavailability, *Am. J. Clin. Nutr.* 2004, *79*, 727 – 747.

[7] Kahle, K., Kraus, M., Richling, E., Polyphenol profiles of apple juices, *Mol. Nutr. Food Res.* 2005, *49*, 797 – 806.

[8] Alonso-Salces, R.-M., Ndjoko, K., Queiroz, E.-F., Ioset, J.-R. *et al*., On-line characterization of apple polyphenols by liquid chromatography coupled with mass spectrometry and ultraviolet absorbance detection, *J. Chromatogr., A* 2004, *1046*, 89 – 100.

[9] Tsao, R., Yang, R., Young, J.-C., Zhu, H., Polyphenolic profiles in eight apple cultivars using high-performance liquid chromatography (HPLC), *J. Agric. Food Chem.* 2003, *51*, 6347 – 6353.

[10] Boyer, J., Liu, R.-H., Apple phytochemicals and their health benefits, *Nutr. J.* 2004, *3*, 5.

[11] Gerhäuser, C., Cancer chemopreventive potential of apples, apple juice, and apple components, *Planta Med.* 2008, *74*, 1608 – 1624.

[12] Santangelo, C., Varì, R., Scazzocchio, B., Di Benedetto, R., Filesi, C., *et al.*, Polyphenols, intracellular signalling and inflammation, *Ann. Ist. Super Sanità.* 2007, *43*, 394 – 405.

[13] Hong, J., Smith, J., Ho, C.-T., August, D.-A., Yang, S., Effects of purified green and black tea polyphenols on cyclooxygenase- and lipoxygenase-dependent metabolism of arachidonic acid in human colon mucosa and colon tumor tissues, *Biochem. Pharmacol.* 2001, *62*, 1175 – 1183.

[14] Kundu, J.-K., Na, H.-K., Chun, K.-S., Kim, Y.-K., *et al.*, Inhibition of phorbol ester-induced COX-2 expression by epigallocatechin gallate in mouse skin and cultured human mammary epithelial cells, *J. Nutr.* 2003, *133*, 3805 – 3810.

[15] Seeram, N.-P., Zhang, Y., Nair, M.-G., Inhibition of proliferation of human cancer cells and cyclooxygenase enzymes by anthocyanidins and catechins, *Nutr. Cancer* 2003, *46*, 101 – 106.

[16] Gerhäuser, C., Klimo, K., Heiss, E., Neumann, I., *et al.*, Mechanism-based in vitro screening of potential cancer chemopreventive agents, *Mutat. Res., Fundam. Mol. Mech. Mutagen.* 2003, *523–524*, 163 – 172.

[17] Lyu, S.-Y., Park, W.-B., Production of cytokines and NO by RAW 264.7 macrophages and PBMC in vitro incubation with flavonoids, *Arch. Pharmacal Res.* 2005, *28*, 573 – 581.

[18] Visioli, F., Galli, C., The effect of minor constituents of olive oil on cardiovascular disease: New findings, *Nutr. Rev.* 1998, *56*, 142 – 147.

[19] Yamamoto, Y., Gaynor, R.-B., IkappaB kinases: Key regulators of the NF-kappaB pathway, *Trends Biochem. Sci.* 2004, *29*, 72 – 79.

[20] Rahman, I., Biswas, S.-K., Kirkham, P.-A., Regulation of infammation and redox signaling by dietary polyphenols, *Biochem. Pharmacol.* 2006, *72*, 1439 – 1452.

[21] Rahman, I., Marwick, J., Kirkham, P., Redox modulation of chromatin remodeling: Impact on histone acetylation and deacetylation, NF-kappaB and pro-infammatory gene expression, *Biochem. Pharmacol.* 2004, *68*, 1255 – 1267.

[22] Wheeler, D.-S., Catravas, J.-D., Odoms, K., Denenberg, A., *et al.*, Epigallocatechin-3-gallate, a green tea-derived polyphenol, inhibits IL-1 beta-dependent proinfammatory signal transduction in cultured respiratory epithelial cells, *J. Nutr.* 2004, *134*, 1039 – 1044.

[23] Aneja, R., Hake, P.-W., Burroughs, T.-J., Denenberg, A.-G., *et al.*, Epigallocatechin, a green tea polyphenol, attenuates myocardial ischemia reperfusion injury in rats, *Mol. Med.* 2004, *10*, 55 – 62.

[24] Ruiz, P.-A., Braune, A., Holzlwimmer, G., Quintanilla-Fend, L., *et al.*, Quercetin inhibits TNF-induced NF-kappaB transcription factor recruitment to pro-infammatory gene promoters in murine intestinal epithelial cells, *J. Nutr.* 2007, *137*, 1208 – 1215.

[25] Bouma, G., Strober, W., The immunological and genetic basis of inflammatory bowel disease. *Nat. Rev. Immunol.* 2003, *3*, 521 – 533.

[26] Will, F., Bauckhage, K., Dietrich, H., Apple pomace liquefaction with pectinases and cellulases: Analytical data of the corresponding juices, *Eur. Food Res. Technol.* 2000, *211*, 291 – 297.

[27] O'Brien, J., Wilson, I., Orton, T., Pognan, F., Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity, *Eur. J. Biochem.* 2000, *267*, 5421 – 5426.

[28] Roehm, N.-W., Rodgers, H., Hatfield, S.-M., Glasebrook, A.-L., An improved colometric assay for cell proliferation and viability utilising the tetrazolium salt XTT, *J. Immunol. Methods* 1991, *142*, 257 – 265.

[29] Pfaffl, M.-W., A new mathematical model for relative quantification in real-time RT-PCR, *Nucleic Acid Res.* 2001, *29*, 2002 – 2007.

[30] Spurrell, J., Wiehler, S., Zaheer, R.-S., Sanders, S.-P. *et al.*, Human airway epithelial cells produce IP-10 (CXCL10) in vitro and in vivo upon rhinovirus infection, *Am. J. Physiol. Lung Cell Mol. Physiol.* 2005, *289*, 85 – 95.

[31] Erkel, G., Wisser, G., Anke, T., Influence of the fungal NF-κB inhibitor panepoxydone on inflammatory gene expression in MonoMac6 cells, *Int. Immunopharmacol.* 2007, *7*, 612 – 624.

[32] Will, F., Bauckhage, K., Dietrich, H., Apple pomace liquefaction with pectinases and cellulases: Analytical data of the corresponding juices, *Eur. Food Res. Technol.* 2000, *211*, 291 – 297.

[33] Schaefer, S., Baum, M., Eisenbrand, G., Dietrich, H., *et al.*, Polyphenolic apple juice extracts and their major constituents reduce oxidative damage in human colon cell lines, *Mol. Nutr. Food Res.* 2006, *50*, 24 – 33.

[34] Huemmer, W., Dietrich, H., Will, F., Schreier, P., Richling, E., Content and mean polymerization degree of procyanidins in extracts obtained from clear and cloudy apple juices, *Biotechnol. J.* 2008, *3*, 234 – 243.

[35] Hoffmann, E., Dittrich-Breiholz, O., Holtmann, H., Kracht, M., Multiple control of Interleukin 8 gene expression, *J. Leukoc. Biol.* 2002, *72*, 847 – 855.

[36] Marx, N., Mach, F., Sauty, A., Leung, J.-H., *et al*., Peroxisome proliferator-activated receptor-gamma activators inhibit IFN-gamma-induced expression of the T cell-active CXC chemokines IP-10, Mig, and I-TAC in human endothelial cells, *J. Immunol.* 2000, *164*, 6503 – 6508.

[37] Boorsma, D.-M., Flier, J., Sampat, S., Ottevanger, C., *et al.*, Chemokine IP-10 expression in cultured human keratinocytes, *Arch. Dermatol. Res.* 1998, *290*, 335 – 341.

[38] Dajotoy, T., Andersson, P., Bjartell, A., Lofdahl, C.-G., *et al.*, Human eosinophils produce the T cell-attracting chemokines MIG and IP-10 upon stimulation with IFN-gamma, *J. Leukoc. Biol.* 2004, *76*, 685 – 691.

[39] Gasperini, S., Marchi, M., Calzetti, F., Laudanna, C., *et al.*, Gene expression and production of the monokine induced by IFN-gamma (MIG), IFN-inducible T cell alpha chemoattractant (I-TAC), and IFN-gamma-inducible protein-10 (IP-10) chemokines by human neutrophils, *J. Immunol.* 1999, *162*, 4928 – 4937.

[40] Dwinell, M.-B., Lugering, N., Eckmann, L., Kagnoff, M.-F., Regulated production of interferon-inducible T-cell chemoattractants by human intestinal epithelial cells, *Gastroenterology* 2001, *120*, 49 – 59.

[41] Saetta, M., Mariani, M., Panina-Bordignon, P., Turato, G., *et al.*, Increased expression of the chemokine receptor CXCR3 and its ligand CXCL10 in peripheral airways of smokers with chronic obstructive pulmonary disease, *Am. J. Respir. Crit. Care Med.* 2002, *165*, 1404 – 1409.

[42] Miotto, D., Christodoulopoulos, P., Olivenstein, R., Taha, R., *et al.*, Expression of IFN-γ-inducible protein, monocyte chemotactic proteins1, 3, and 4, and eotaxin in TH1- and TH2-mediated lung diseases, *J. Allergy Clin. Immunol.* 2001, *107*, 664 – 670.

[43] Yeruva, S., Ramadori, G., Raddatz, D., NF-κB-dependent synergistic regulation of CXCL10 gene expression by IL-1β and IFN-γ in human intestinal epithelial cell lines, *Int. J. Colorectal Dis.* 2008, *23*, 305 – 317.

[44] Banks, C., Bateman, A., Payne, R., Johnson, P., Sheron, N., Chemokine expression in IBD. Mucosal chemokine expression is unselectively increased in both ulcerative colitis and crohn's disease, *J. Pathol.* 2003, *199*, 28 – 35.

[45] Uguccioni, M., Gionchetti, P., Robbiani, D.-F., Rizzello, F., *et al.*, Increased expression of IP-10, IL-8, MCP-1, and MCP-3 in ulcerative colitis, *Am. J. Pathol.* 1999, *155*, 331 – 336.

[46] Sierro, F., Dubois, B., Coste, A., Kaiserlian, D. *et al.*, Flagellin stimulation of intestinal epithelial cells triggers CCL20-mediated migration of dendritic cells, *Proc. Natl. Acad. Sci. USA* 2001, *98*, 13722 – 13727.

[47] Brand, S., Sakaguchi, T., Gu, X., Colgan, S.-P., Reinecker, H.-C., Fractalkine-mediated signals regulate cell-survival and immune-modulatory responses in intestinal epithelial cells, *Gastroenterology* 2002, *122*, 166 – 177.

[48] Majumder, S., Zhou, L., Chaturvedi, P., Babcock, G., *et al.*, p48/stat-1α-containing complexes play a predominant role in induction of IFN-inducible protein 10 kda (IP 10) by IFN alone or in synergy with TNF-α, *J. Immunol.* 1998, *98*, 4736 – 4744.

[49] Czech, W., Krutmann, J., Budnik, A., Schopf, E., Kapp, A., Induction of intercellular adhesion molecule 1 (ICAM-1) expression in normal human eosinophils by inflammatory cytokines, *J. Invest. Dermatol.* 1993, *100*, 417 – 423.

[50] Lee, A.-H., Hong, J.-H., Seo, Y.-S., Tumour necrosis factor-alpha and interferon-gamma synergistically activate the RANTES promoter through nuclear factor kappaB and interferon regulatory factor 1 (IRF-1) transcription factors, *Biochem.* 2000, *350*, 131 – 138.

[51] Ohmori, Y., Schreiber, R.-D., Hamilton, T.-A., Synergy between interferon-gamma and tumor necrosis factor-alpha in transcriptional activation is mediated by cooperation between signal transducer and activator of transcription 1 and nuclear factor kappa B, *J. Biol. Chem.* 1997, *272*, 14899 – 14907.

[52] Pine, R., Convergence of TNF-α and IFN-γ signalling pathways through synergistic induction of IRF-1/ISGF-2 is mediated by a composite GAS/kappaB promoter element, *Nucleic Acids Res.* 1997, *25*, 4346 – 4354.

[53] Karin, M., Greten, F.-R., NF-κB: Linking inflammation and immunity to cancer development and progression, *Nat. Rev. Immunol.* 2005, *5*, 749 – 759.

[54] Rao, A., Luo, C., Hogan, P.-G., Transcription factors of the NF-AT family: Regulation and function, *Annu. Rev. Immunol.* 1997, *15*, 707 – 747.

[55] Serfling, E., Berberich-Siebelt, F., Avots, A., Chuvpilo, S., *et al.*, NF-AT and NF-κB factors – the distant relatives. *Int. J. Biochem. Cell Biol.* 2004, *36*, 1166 – 1170.

[56] Tracey, K.-J., Cerami, A., Tumor Necrosis Factor: A pleiotropic cytokine and therapeutic target, *Annu. Rev. Med.* 1993, *9*, 317 – 343.

[57] Johnson, D.-R., Pober, J.-S., HLA class I heavy-chain gene promoter elements mediating synergy between tumor necrosis factor and interferons, *Mol. Cell. Biol.* 1994, *14*, 1322 – 1332.

[58] Barker, J., Sarma, V., Mitra, R.-S., Dixit, V.-M., Nickoloff, B.-J., Marked synergism between tumor necrosis factor-alpha and interferon-gamma in regulation of keratinocyte-derived adhesion molecules and chemotactic factors, *J. Clin. Invest.* 1990, *85*, 605 – 608.

[59] Doyle, S.-L., O'Neill, L.-A., Toll-like receptors: From the discovery of NF-kB to new insights into transcriptional regulations in innate immunity, *Biochem. Pharmacol.* 2006, *72*, 1102 – 1113.

[60] Kawai, T., Adachi, O., Ogawa, T., Takeda, K., Akira, S., Unresponsiveness of MyD88-decient mice to endotoxin, *Immunity* 1999, *11*, 115 – 122.

[61] Yamamoto, M., Sato, S., Hemmi, H., Sanjo, H., *et al.*, Essential role for TIRAP in activation of the signalling cascade shared by TLR2 and TLR4, *Nature* 2002, *420*, 324 – 329.

[62] Yamamoto, M., Sato, S., Mori, K., Hoshino, K., *et al.*, Cutting edge: A novel Toll/IL-1 receptor domain-containing adapter that preferentially activates the IFN-beta promoter in the Toll-like receptor signaling, *J. Immunol.* 2002, *169*, 6668 – 6672.

[63] Yang, F., Oz, H.-S., Barve, S., Villiers, W.-J. *et al.*, The green tea polyphenol (–)-epigallocatechin-3-gallate blocks nuclear factor-kappa B activation by inhibiting Ikappa B kinase activity in the intestinal epithelial cell line IEC-6, *Mol. Pharmacol.* 2001, *60*, 528 – 533.

[64] Mackenzie, G., Adamo, A.-M., Decker, N.-P., Oteiza, P.-I., Dimeric procyanidin B2 inhibits constitutively active NF-κB in Hodgkin's lymphoma cells independently of the presence of IκB mutations, *Biochem. Pharmacol.* 2008, *75*, 1461 – 1471.

[65] Tedeschi, E., Menegazzi, M., Yao, Y., Suzuki, H., *et al.*, Green tea inhibits human inducible nitric oxidase expression by down-regulation signal transducer and activator of transcription-1alpha activation, *Mol. Pharmacol.* 2004, *65*, 111 – 120.

[66] Subramanian, S., Rhodes, J.-M., Hart, C.-A., Tam, B., *et al.*, Characterization of epithelial IL-8 response to inflammatory bowel disease mucosal E. coli and its inhibition by mesalamine, *Inflamm. Bowel Dis.* 2008, *14*, 162 – 175.

[67] Hipp, M.-S., Urbich, C., Mayer, P., Wischhusen, J., *et al.*, Proteasome inhibition leads to NF-κB-independent IL-8 transactivation in human endothelial cells through induction of AP-1, *Eur. J. Immunol.* 2002, *32*, 2208 – 2217.

[68] Eferl, R., Wagner, E.-F., AP-1: A double-edged sword in tumorigenesis, *Nat. Rev. Cancer* 2003, *3*, 859 – 868.

[69] Shen, F., Chen, S.-J., Dong, X.-J., Zhong, H., *et al.*, Suppression of IL-8 gene transduction by resveratrol in phorbol ester treated human monocytic cells, *J. Asian Nat. Prod. Res.* 2003, *5*, 151 – 157.

[70] Swantek, J.-L., Christerson, L., Cobb, M.-H., Lipopolysaccharide-induced tumor necrosis factor-α promoter activity is inhibitor of nuclear factor-κB kinase dependent, *J. Biol. Chem.* 1999, *274*, 11667 – 11671.

[71] Habtemariam, S., Natural inhibitors of tumor necrosis factor-α production, secretion and function, *Planta Med.* 2000, *4*, 303 – 13.

[72] Andoh, A., Yagi, Y., Shioya, M., Nishida, A., *et al.*, Mucosal cytokine network in inflammatory bowel disease, *World J. Gastroenterol.* 2008, *14*, 5154 – 5161.

[73] Segal, P.-B., Regulation of IL 6 gene expression, *Res. Immunol.* 1992, *143*, 724 – 734.

[74] Ludwiczek, O., Vannier, E., Borggraefe, I., Kaser, A., *et al.*, Imbalance between interleukin-1 agonists and antagonists: Relationship to severity of inflammatory bowel disease, *Clin. Exp. Immunol.* 2004, *138*, 323 – 329.

[75] Okahara, S., Arimura, Y., Yabana, T., Kobayashi, K., *et al.*, Inflammatory gene signature in ulcerative colitis with cDNA microarray analysis, *Aliment. Pharmacol. Ther.* 2005, *21*, 1091 – 1097.

[76] Gouwy, M., Struyf, S., Proost, P., Van Damme, J., Synergy in cytokine and chemokine networks amplifies the inflammatory response, *Cytokine Growth Factor Rev.* 2005, *16*, 561 – 580.

[77] Kracht, M., Saklatvala, J., Transcriptional and post-transcriptional control of gene expression in inflammation, *Cytokine* 2002, *20*, 91 – 106.

[78] Kmiec, Z., Cytokines in inflammatory bowel disease, *Arch. Immunol. Ther. Exp.* 1998, *46*, 143 – 155.

[79] Shapiro, V.-S., Mollenauer, M.-N., Greene, W.-C., Weiss, A., C-rel regulation of IL-2 gene expression may be mediated through activation of AP-1, *J. Exp. Med.* 1996, *184*, 1663 – 1669.

[80] Cox, D.-G., Crusius, J.-B., Peeters, P.-H., Bueno-de-Mesquita, H.-B., *et al.*, Haplotype of prostaglandin synthase 2/cyclooxygenase 2 is involved in the susceptibility to inflammatory bowel disease, *World J. Gastroenterol.* 2005, *11*, 6003 – 6008.

[81] Surth, Y.-J., Chun, K.-S., Cha, H., Han, S., *et al.*, Molecular mechanisms underlying chemopreventive activities of anti-inflammatory phytochemicals: down-regulation of COX-2 and iNOS through suppression of NF-κB activation, *Mutat. Res.* 2001, *480–481*, 243 – 268.

[82] Zhang, W., Liu, H., Xie, K., Yin, L., *et al.*, Procyanidin dimer B2 [epicatechin-(4β-8)-epicatechin] suppresses the expression of cyclooxygenase-2 in endotoxin-treated monocytic cells, *Biochem. Biophys. Res. Commun.* 2006, *345*, 508 – 515.

[83] Heller, R.-A., Schena, M., Chai, A., Shalon, D., *et al.*, Discovery and analysis of inflammatory disease related genes using cDNA microarrays, *Proc. Natl. Acad. Sci. USA* 1997, *94*, 2150 – 2155.

[84] Harant, H., Eldershaw, S.-H., Lindley, I., Human macrophage inflammatory protein-3α/ CCL20/ LARC/ Exodus/ SCYA20 is transcriptionally upregulated by tumor necrosis factor-α via non-standard NF-κB site, *FEBS Lett.* 2001, *509*, 439 – 445.

[85] Goggins, M.-G., Goh, J., O'Connell, M.-A., Weir, D.-G., *et al.*, Soluble adhesion molecules in inflammatory bowel disease, *Ir. J. Med. Sci.* 2001, *170*, 107 – 111.

[86] Stangl, V., Lorenz, M., Ludwig, A., Grimbo, N., *et al.*, The Flavonoid phloretin suppresses stimulated expression of endothelial adhesion molecules and reduces activation of human platelets, *J. Nutr.* 2005, *135*, 172 – 178.

