Eur J Nutr (2011) 50:163–172
DOI 10.1007/s00394-010-0125-8

ORIGINAL CONTRIBUTION

Immunoregulatory effects of the flavonol quercetin in vitro and in vivo

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Received: 9 December 2009 / Accepted: 8 July 2010 / Published online: 23 July 2010

Abstract
Purpose Atherosclerosis is known to be an inflammatory disease. Dendritic cells (DCs) are essential for the regulation of the immune system. Up to 10% of the cells in atherosclerotic plaques are DCs. The cardiovascular protective effects of flavonoids (tea, wine) may be mediated by anti-inflammatory mechanisms that affect DC regulation. We aimed to characterize the impact of the flavonol quercetin on DC activity and differentiation in vitro and in vivo.
Methods For the in vitro experiments, we used murine DCs and endothelial cells to study adhesion properties. For all other experiments (DC phagocytosis capacity, DC maturation, DC differentiation (BDCA-1/-2) and NF-kB-activation), human monocyte-derived DCs were used. The cells were incubated with quercetin (10 μmol/L) ± oxLDL (10 μg/mL) between 24 and 48 h. For in vivo experiments, eight healthy male volunteers took 500 mg of quercetin twice daily over 4 weeks, five healthy male volunteers served as control. Before and after intake, blood samples were collected. Peripheral blood leukocytes were isolated (analyses of DC differentiation), and plasma was immediately frozen.
Results Quercetin reduced DC adhesion (−42%; $p < 0.05$) and expression of CD11a (−21%; $p < 0.05$). OxLDL-induced DC differentiation was partially inhibited by quercetin (BDCA-1–29%; BDCA-2–33%; $p < 0.05$). These effects were achieved by compensation of oxLDL-induced up-regulation of NF-kB by quercetin. The 4-week treatment with quercetin resulted in relevant plasma levels (2.47 μmol/L) and reduced BDCA-2 + DCs in the peripheral blood by 42% ($p < 0.05$) as well as systemic levels of the NO-synthase inhibitor asymmetric dimethylarginine (−31%, $p < 0.05$).
Conclusion In vitro, quercetin reduced DC adhesion and oxLDL-induced DC differentiation. In vivo, quercetin reduced circulating plasmacytoid DCs and systemic ADMA-levels. The immunoregulatory effects of quercetin may contribute to the anti-atherosclerotic potential of flavonols.

Keywords Quercetin · Flavonoids · Dendritic cells · Atherosclerosis

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Introduction

Flavonoids are polyphenolic compounds of plants. Over 6,000 flavonoids have been identified, many of which are present in fruits, vegetables, nuts, seeds, tea and wine. They are divided into subclasses based on their connection between the B-ring and the C-ring as well as their oxidation

state and functional groups of the C-ring. Three of the main subclasses are flavonols (e.g., epigallocatechin-3-gallate), flavones (e.g., genistein) and flavonols (e.g., quercetin) [1]. The flavonol quercetin is the main representative of the flavonol class and is especially present in onions, apples and tea. The normal intake is around 23–34 mg/d. The vasculo-protective effects of flavonoids have been attributed clinically to their blood pressure and cholesterol-lowering potential, as well as to their anti-inflammatory properties [2].

Atherosclerosis is an inflammatory and (auto)-immune-mediated disease, which accounts for most deaths in industrial countries [3, 4]. The interaction of lipids and their oxidative products with plaque cells play a central role in the pathogenesis of atherosclerosis. Furthermore, autoantigens like oxidized LDL (oxLDL) and heat shock proteins (HSP) 60/65 are contributing to the progression of atherosclerotic disease [3, 5].

Naderi et al. [6] were able to show in vitro that flavonoids diminish the oxidation of LDL to oxLDL. Besides oxLDL, the potent endogenous and competitive inhibitor of nitric oxide synthase (NOS), asymmetric dimethylarginine (ADMA) was detected as an independent cardiovascular risk factor [7]. Hong-Bo et al. and Li et al. were able to demonstrate a positive impact of soy-products (puerarin) and kaempferol on ADMA-levels in vitro [8–10].

During recent years, the dominant role of the dendritic cells (DCs) in the pathophysiological setting of atherosclerosis became more and more apparent [11]. Vascular DCs are homing in the vessel wall and recognize foreign- and autoantigens. DC-induced T-cell activation further stimulates vascular inflammation and adhesion of monocytes. The number of T cells, monocytes and DCs is strongly up-regulated in the human atherosclerotic plaque [12]. Since DCs control immunity, modulating DC activity is important for atherogenesis.

The aim of this study was to further characterize the impact of quercetin on DC activation with regard to adhesion properties, endocytosis capacity, maturation- and differentiation-processes, and cytokines. Furthermore, we aimed to characterize the impact of quercetin on DC activity, oxLDL and ADMA-levels in vivo.

Methods

Quercetin concentration

Previous studies have shown that quercetin concentrations between 10 and 100 μmol/L have specific effects on different immune and endothelial cells without mediating cytotoxic effects. Based on previous publications, we used 10 μmol/L of quercetin (di-hydroquercetin) for our in vitro studies [13].

Murine DCs and endothelial cell lines

For the adhesion experiments, an autologous murine cell line model was used. The murine cell line originates from C57BL/6 mice (Wesel, Germany, CRL-2279) [14]. The murine myeloid dendritic cell line DC2.4 (Dana Faber Cancer Institute, Boston) also originates from C57BL/6 mice. Both cell lines were cultivated under 5% CO_2 and 37 °C in phenolred-free DMEM (Invitrogen GmbH Karlsruhe, Germany) complemented with 10% fetal calf serum (PAA Laboratories Germany, Coelbe, Germany). Both cell types were used at least 10 days after thawing and for no more than 20 passages [14].

Generation of human monocytes-derived DCs

Apart from the adhesion experiments, we used human monocytes-derived DCs. Mononuclear cells were isolated from 100 mL of peripheral blood of a healthy human donor by a Ficoll density gradient. The pureness of the monocyte culture was enhanced up to 97% by adhesion on gamma globulin-coated plates. DCs were generated from this monocyte culture by IL-4 and GM-CSF-stimulation according to our recent publication [15]. For the experiments, we started the stimulation between day 4 and day 5 of DC cultivation. This time period was chosen because most DCs are immature at this crucial time point and are starting to differentiate into mature DCs or back to monocytes [16]. The incubation period of 48 h (24 h oxLDL and 24 h quercetin) was chosen in accordance with Obermaier et al. [17].

Influence of quercetin on apoptosis and necrosis of DCs

DCs were incubated 24 h with quercetin (10 μmol/L) or ethanol (15%) as the positive control. After treatment with the chemicals, the DCs were washed with PBS, stained for AnnexinV–FITC (Beckmann Coulter, USA) for apoptosis and propidium iodide (SIGMA, Germany) for necrosis, according to the manufacturer's protocol and analyzed by flow cytometry.

LDL–oxidation

LDL (density = 1.019 to 1.063 g/mL) was isolated from human plasma of normolipidemic healthy volunteers by sequential ultracentrifugation as described and stored in PBS containing 2 mmol/L EDTA [18]. Immediately before oxidation, the EDTA was removed from LDL by passing the lipoprotein through a PD 10 column (Pharmacia, Austria).

LDL was oxidized in Ham's F-10 medium by exposure to 5 μmol/L CuSO4 at 37 °C [18]. All preparations were dialyzed before they were added to the cultured cells. We used a concentration of 10 μg/mL in our experiments. This mirrors oxLDL concentrations in the plaque and has been used in previous studies [15, 19].

Flow cytometer analysis

Cells were incubated with antibodies according to the manufacturers′ instructions and as described in our recent study [20]. Antibodies were matched to iso-controls (Mouse-γ2a-(Fitcs)/-γ1(PE)-FastImmune; BD; USA). To verify the purity of our DC culture, we used CD3 (BD, USA) and CD20 (BD, USA) to rule out T-cell and B-cell contamination (<5%). We characterized the DC morphology by missing CD14 (BD, USA) and high expressions (>90%) of CD80 (BD, USA), CD86 (BD, USA), HLA-DR (BD, USA) and CD209 (DC-Sign; BD, USA). Maturation and differentiation status were performed by analysis of CD83- (BioLegend, USA), CD11a- (BD, USA) and BDCA-1/-2-(Miltenyibiotec,Germany) expression. Antibodies against CD205 (Biozol, USA) and the mannose receptor (Abcam, USA) were used to detect expression of endocytosis/scavenger receptors. Antibodies against CD54 (BD, USA), CD209 (DC-Sign; BD, USA) and CD11a (BD, USA) were further used to detect adhesion molecules.

Adhesion assay of murine DCs (mDCs) on murine endothelial cells (mECs) and blockage of CD11a

For the adhesion experiments, we used an autologous murine cell line model [14]. mECs were distributed in white 96-well plates and grown until confluence (visual control in transparent plates). mDCs were stimulated with quercetin (10 μmol/L Sigma, Germany) for 24 h before incubation with mECs. Next, mDCs were stained with CellTracker Green CMFDA (Invitrogen GmbH Karlsruhe, Germany). Non-stimulated mDCs served as controls. After the co-incubation period (4 h), the plates were measured instantly by a fluorescence reader with 485 nm excitation and 535 nm emission (GENIOS, Tecan, Austria) [15]. For blockage of CD11a, 20 μg/mL of CD11a antibody (BioLegend, San Diego, USA) was added to the DCs for 1 h at 4 °C (1 h before co-incubation of DCs and ECs). To rule out unspecific blocking, control IgG antibodies were used. Following this, DCs were co-cultivated with the ECs, and the attached fraction was quantified using a fluorescence reader.

Endocytosis capacity and receptor expression

The expression analysis of different receptors for endocytosis (DEC205 = CD205, mannose receptor = CD206) on human DCs (6th day of culture) was performed ± a 24 h pre-stimulation with quercetin (10 μmol/L). DC phagocytosis capacity was assessed by fluorescent assays using FITC-dextran (MWr – 60,000; SIGMA, Germany; 1 mg/mL) as a marker of receptor-mediated endocytosis, lucifer-yellow (Molecular Probes, USA) (1 mg/mL) was chosen as a marker for macropinocytosis [20]. Cells were analyzed on a fluorescence reader. FITC-dextran was parallel investigated by confocal microscopy (Vybrant DiD for cell membrane).

Totally activated NF-κB ELISA

DCs were generated as described and incubated with or without quercetin (10 μmol/L) at the 4th day of culture. After 24 h, oxLDL (10 μg/mL) was added to the culture. After another 24 h, the cells were harvested and nuclear extraction was performed. The chemiluminescence-based sandwich-type ELISA (Oxford Biomedical Research, USA) employs an oligonucleotide, containing the DNA binding NF-κB consensus sequence, bound to a 96-well ELISA plate. The transcription factor NF-κB which is present in DC extracts binds specifically to the oligonucleotide coated on the plate. The DNA-bound NF-κB is selectively recognized by the primary antibody (p50 and p105 specific), which in turn is detected by the secondary antibody alkaline phosphatase conjugate. The relative light units (RLU) were measured by a chemiluminescence detector (Tecan Genios Multireader, Tecan, Austria) after addition of alkaline phosphatase substrate.

Patients

The study was approved by the ethic committee of the Ludwig-Maximillians-University of Munich. Between May 2007 and August 2007, eight healthy men (age: 30.1 ± 1.6 years) without a history of chronic medication intake, without acute infection (normal body temperature (36.6 ± 0.4 °C) and a normal white cell blood count (5.4 ± 0.39 k per l) were treated with 500 mg quercetin twice a day for 4 weeks. This concentration was used based on previous results by Shoskes et al. [21]. Probands were asked to continue their regular diet. Blood samples of each patient were taken at baseline and at follow-up after 4 weeks. Moreover, the quercetin group was compared to five age-matched healthy men (31.5 ± 3.7 years) who underwent blood examination without taking quercetin.

Quercetin (quercetin-dihydrat; Fagron, Germany) was encapsulated by the hospital pharmacy. Once before the start of quercetin intake and once after the 4-week treatment, 40 mL of blood was taken in the morning after a fasting period of 10 h. Of these 40 mL, 18 mL of EDTA blood was used for peripheral blood leukocyte isolation

through a ficoll gradient. The harvested leukocytes were then divided into 2 parts, one part for cytometry analysis and the other part for rt–PCR. Another 18 mL of serum of all eight individuals was isolated from the blood, divided up in several tubes and immediately frozen at −80 °C. Simultaneously, 2 mL of EDTA blood was taken for the leukocyte blood count.

Quercetin serum concentration

For analysis, the conjugates have to be cleaved by enzymatic treatment and were quantified as total quercetin. Without an enzymatic treatment, the amount of free quercetin could be analyzed. This analysis reports solely total quercetin concentrations in serum samples without differentiation of free and conjugated quercetin amounts. Internal standard (flavone) was added to 250 µl of serum sample and diluted with buffer. After addition of glucuronidase/sulfatase, the mixture was treated for 45 min at 37 °C in the dark. The enzymatic treatment was stopped and the analyzed agent extracted twice with aid of a solvent. The filtrated solution was dehydrated with nitrogen, rehydrated in solvent and analyzed by RPHPLC. Detection was accomplished by UV-diode array detection, using two different extracted wavelengths for cross-channel internal standard quantification.

Total RNA isolation and real-time PCR

For isolation of RNA from peripheral blood mononuclear cells (PBMC; isolated from 50 mL blood samples by ficoll gradient), the total RNA isolation RNeasy Mini Kit (Cat.#74104) from Qiagen (Hilden, Germany) was used according to the instructions provided by the manufacturer. Fifty picograms per tube RNA (PBMC) was used. cDNA synthesis and PCR were performed using Omniscript from Qiagen (Hilden, Germany). The two-step quantitative rt–PCR system was applied according to the manufacturers' instructions. The quantitative rt–PCR system provides optimal performance by the use of SYBR Green primers (Qiagen, Germany). Real-time PCR was performed in the ABI PRISMTM 7,700 System (Applied Biosystems, Germany). Data analysis was performed using the delta-delta ct method [22]. The different primer sequences of the analyzed receptors are:

CD83: 5′-CGGTCTCCTGGGTCAAGTTA-3′/5′-AGA ACCATTTTGCCCCTTCT–3′; Mannose-receptor: 5′-CCA CGAGCAGACCTACATCA–3′/5′-TTCTCCTCACTGGG GTTGTC–3′; CD11a: 5′ -AGAGTCCAGGCTTCTGTCC A-3′/5′-GATGGGGATGATGGTA GTGG–3′; BDCA-1: 5′-AGGAGCAACTGGGCACTAAA–3′/5′-GATGATGTC CTGGCC TCCTA-3′; BDCA-2: 5′-ACT GGG ATG CAA TCT TGG AC-3′/5′-GAT CTG ACA GCC CAG AAA A-3′; NF-κB: 5′-GCC TCT AGA TAT GGC CAC CA-3′/5′-TCA GCC AGC TGT TTC ATG TC-3′.

Flow cytometer analysis of peripheral blood mononuclear cells

From 18 mL of EDTA blood, peripheral blood leukocytes were isolated through ficoll gradient. Next, cells were incubated with antibodies according to the manufacturers' instructions and as described earlier. We primarily analyzed the receptors which we studied in vitro. For the maturation and differentiation status, we analyzed CD83- and BDCA-1/-2-expression and CD11a as adhesion receptor. We also analyzed the expression of the mannose receptor.

Lipid status analysis

Plasma tubes were centrifuged, and the plasma was fractionated and frozen at −80 °C. Samples were defrosted, and HDL, triglycerides and cholesterol were directly measured by the ALCYON 300 (Abbott, Germany). The LDL-fraction was calculated by the Friedwald-formula $(\mathrm{LDL-Cholesterol} = \mathrm{total-Cholesterol} - \mathrm{HDL-Cholesterol} - \mathrm{Triglycerins}/2.18)$.

oxLDL-ELISA

OxLDL levels were examined by using a oxLDL-specific ELISA kit according to the manufacturer′s instructions (ImmunDiagnostik, Germany). Before analysis, samples were treated as described earlier. In brief, after washing with wash buffer several times, standard samples and controls were added and incubated for 4 h at room temperature on a horizontal mixer. Thereafter a washing conjugate was added and incubated for 1 h under the conditions as described before. Then, the substrate was added and incubated for 25 min in the dark. Stop solution was added and immediately analyzed by an ELISA reader at 450 nm.

ADMA-ELISA

The plasma was handled as described earlier and analyzed by ELISA, as described by Schulze et al. [23] and in our recent publication [24]. The sensitivity of this ELISA is 0.05–5.0 µM ADMA (dld-diagnostika, Germany).

Statistical analysis

Data are presented as mean ± standard deviation (SD). The boxplots represent the interquartile range (25th to

75th percentile) around the median line in each box. We used non-parametric tests since normal distribution could not be assumed. The Wilcoxon signed Rank Test for not normally distributed paired samples was used to compare baseline with 30 days follow-up samples (paired samples). To compare changes between the groups (independent samples), we used the Mann–Whitney U Test. Since our study population was beyond 20, we calculated exact p-values. Differences between means were considered significant with $p < 0.05$ and highly significant with $p < 0.01$. All in vitro experiments were repeated at least three times with different cells and lipoprotein preparations. SPSS (Version 16, IBM-USA) was used for statistical analysis.

Results

DCs apoptosis and necrosis

The DCs, which were pre-incubated with quercetin, showed an apoptosis rate of 13 ± 6% and a necrosis rate of 29.4 ± 9% compared to the control apoptosis rate of 9.1 ± 4% and the necrosis rate of 27.4 ± 5%. We did not find any significant differences. In contrast, ethanol increased the rates of apoptosis (69.6 ± 5.6%) and necrosis (70.6 ± 14%) compared to quercetin and the control significantly ($p < 0.01$; $p < 0.05$).

DC adhesion molecules

DC adhesion on endothelial cells represents the first step in DC recruitment to the subintimal space. After DC incubation with quercetin, we detected a significant decrease in CD11a positive cells. CD11a was down-regulated from 53 ± 22% to 42 ± 22% (−11%; $p < 0.05$; $n = 7$). For CD54 (from 89% ± 17 to 88 ± 20%; n.s.) and CD209 (from 28 ± 10% to 27 ± 9%; n.s.), our results showed a slight but not significantly decreased expression and antigen density in response to quercetin.

Adhesion assays ± blockage of CD11a

To examine whether CD11a down-regulation correlates with reduced DC adhesion, we performed an adhesion model. We used a murine cell line model to rule out heterogeneity of endothelial cell lines and DCs which would be prevailed in a human model. Compared to the unstimulated DCs and control IgG-antibody, the pre-incubation with quercetin reduced the adhesion of the DCs by −42%, in the case of anti-CD11a-antibodies by −25% and for both quercetin and anti-CD11a by −39%, respectively (all $p < 0.01$). DC adhesion after quercetin and quercetin/anti-CD11a was significantly different compared to DC adhesion in response to anti-CD11a ($p < 0.01$; Fig. 1).

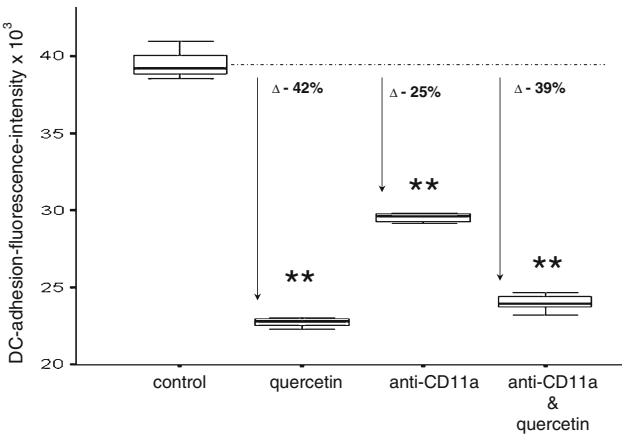

Fig. 1 Adhesion assay with blocking CD11a. CD11a antibodies (20 μg/mL) were incubated with DCs (± quercetin) for 1 h, and adhesion assays were performed. The adhesion fraction on ECs was analyzed using a fluorescence assay. The highest reduction was found for quercetin alone (−42%; $p < 0.01$), followed by the combination of quercetin and CD11a antibody (−39%, $p < 0.01$). There was no significant difference between both approaches. *Wilcoxon-test*: $^{**}p < 0.01$ versus unstimulated control, ($n = 12$)

Endocytosis capacity and expression of endocytosis receptors

DCs have a very high endocytosis capacity. In the subintimal space, DCs take up several substances (like oxLDL) by receptor-related endocytosis and also by macropinocytosis [20].

The receptor-related phagocytosis was significantly reduced by quercetin (−27%, $p < 0.01$; Fig. 2a). The mannose receptor (29 ± 18% expressing cells versus 25 ± 20% with quercetin) and DEC209 (28 ± 10% expressing cells versus 27 ± 10% with quercetin; n.s.), a DC-specific receptor (e.g., relevant for viral antigen uptake), was not affected by quercetin. Lucifer-yellow as a marker for macropinocytosis was reduced by −21% ($p < 0.01$; Fig. 2b).

DC maturation and differentiation in vitro

Mature DCs express high numbers of co-stimulatory molecules like CD83 and CD86. By this, they can stimulate and prime T cells and also induce different kinds of immune answers by differentiating into special subtypes (BDCA-1/-2-positive DCs), which induce Th1- or Th2-cell answers. Quercetin had no influence on the unstimulated maturation (CD83 up-regulation) or differentiation (BDCA-1/-2 up-regulation) process (data not shown). The oxLDL (10 μg/mL; 24 h) induced enhanced maturation

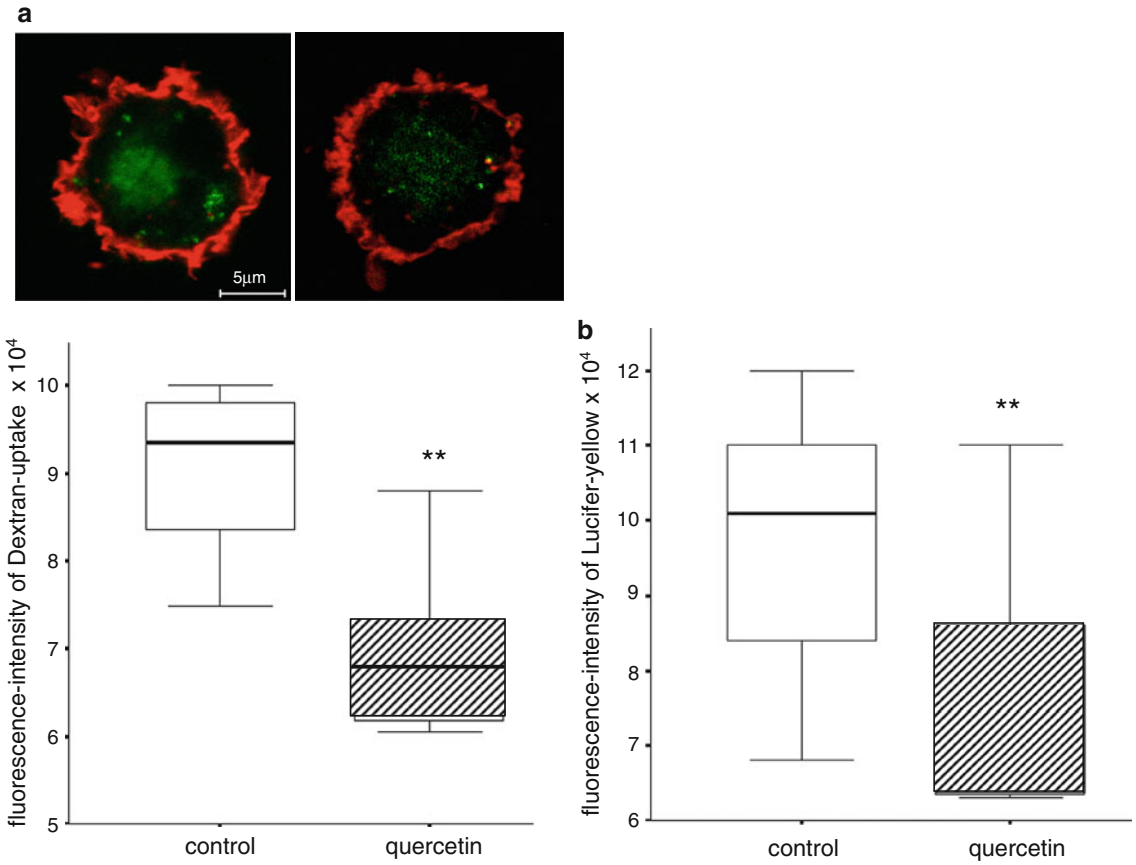

Fig. 2 Endocytosis-capacity. **a** The receptor-related phagocytosis was significantly reduced by quercetin (−27%). At the top of the *graphs,* representative images from confocal microscopy (63× amplification) are shown. *Red color* cell membrane (Vybrant DiD) and *green color* Fitcs-Dextran (scale 5 µm). **b** Lucifer-yellow as a marker for macropinocytosis was reduced by −21%; *Wilcoxon-test*: $^{**}p < 0.01$ versus unstimulated control ($n = 5$)

process was not significantly blocked by quercetin. When we measured the oxLDL-enhanced differentiation process, quercetin could partially inhibit this process. We found a significantly reduced expression of the differentiation marker BDCA-2 (−33%; $p < 0.05$) and BDCA-1 (−29%; $p < 0.05$) compared to the oxLDL-control (Fig. 3a, b).

NF-κB-ELISA

We focused on the NF-κB transcription factor, because of its important role in DC-mediated inflammatory processes [25]. Using a chemiluminescence-based sandwich-type ELISA, we demonstrated that quercetin (10 µmol/L) reduces the total NF-κB activity by −45% ($p < 0.01$). Moreover, quercetin diminished the oxLDL-induced increase in NF-κB activity by −52% ($p < 0.05$) compared to the oxLDL-control (Fig. 4).

Quercetin serum concentration

In the control group, prior to regular quercetin intake, no baseline quercetin levels of 0.06 µmol/L and higher were achieved (except for one vegetarian person with a baseline level of 1.1 µmol/L). After 30 days of quercetin intake, all subjects showed a significant quercetin increase to levels of 2.47 µmol/L and above 4 h after intake (Table 1).

rt–PCR and flow cytometer analyses of peripheral blood leukocytes

With regard to the low numbers of circulating BDCA1/2 expressing cells in human blood, we decided to measure protein and gene expression of various markers. Only a positive change detected by both methods was validated as significant. BDCA-2 protein expression was significantly reduced after a 4-week treatment with 1 g quercetin/day (−42 ± 40% versus baseline; $n = 8$; $p < 0.05$), whereas for BDCA-1 (−32 ± 56%), CD83 (−21 ± 115%), CD11a (−27 ± 83%) and the mannose receptor (−26 ± 82%), no significant changes were found.

rt–PCR confirmed a reduced gene expression only for BDCA-2 (−33 ± 46% versus baseline; $p < 0.05$). Again BDCA-1 (32 ± 140%), CD83 (13 ± 62%), CD11a

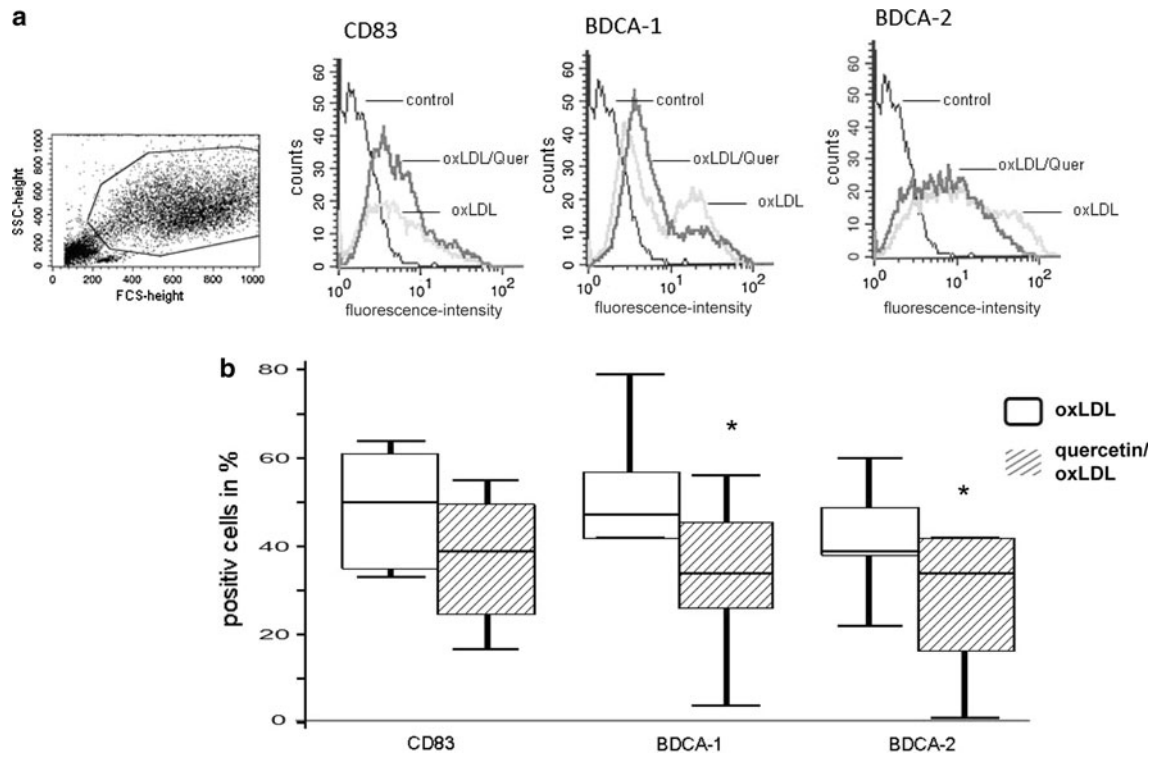

Fig. 3 Quercetin modulates oxLDL-induced maturation and differentiation of DCs. Human DCs were incubated with oxLDL (10 μg/mL) ± quercetin (10 μmol/L). The influence of quercetin on the oxLDL-induced maturation- and differentiation-process was analyzed by flow cytometer analysis. For DC differentiation (but not DC maturation), we found a significant reduction in the expression of the differentiation markers BDCA-1 (−29%; $p < 0.05$) and BDCA-2 (−33%; $p < 0.05$) compared to the oxLDL-controls. **a** Representative histograms of FACS scans of DCs (6th day of culture) expressing CD83, BDAC-1 and BDCA-2 (oxLDL-stimulated; ± quercetin). First *curve* represents always control, 2nd *curve* (*bright gray*) shows expression of CD83, BDCA-1 and BDCA-2 by oxLDL, and the 3rd *curve* (*dark gray*) represents expression of the according antibodies by oxLDL during quercetin stimulation. **b** BDCA-1- and BDCA-2-expression were down-regulated by quercetin, whereas CD83 was not affected (positive cells versus unstimulated control; FACS-analysis). *Wilcoxon-test:* *$p < 0.05$; versus oxLDL stimulated control ($n = 6$)

(132 ± 200%) and the mannose receptor (−57 ± 106%) were not significantly affected by the treatment with quercetin. Accordingly, DC marker expression did not significantly change in the control group during follow-up (BDCA-1 58 ± 67%, BDCA-2 5 ± 32%, CD83 −38 ± 64%, CD11a 32 ± 36% and mannose receptor 26 ± 68%; all n.s.), whereas a significant reduction ($p < 0.05$) for BDCA-2 was found between both groups.

rt–PCR of NF-κB

NF-κB is the crucial transcription factor involved in different immunomodulatory responses. There is evidence that especially flavonols are able to inhibit NF-κB-activity [26]. In our study, NF-κB gene expression was not significantly changed (0.98 ± 0.2 fold increase $n = 8$; $p =$ n.s.) in response to quercetin supplementation (data analysis was performed using the delta-delta ct method [22]). We did not find a significant change (1.2 ± 0.66 fold increase $n = 5$; $p =$ n.s) in the control group, respectively, between both groups regarding NF-κB gene expression.

Lipid status analysis

Controversial data have been published with regard to the effects of flavonoids on lipid levels [27]. We investigated LDL and HDL concentrations in response to quercetin. LDL (base-line 124.3 ± 33.5 mg/dL) plasma levels did not change during follow-up, whereas HDL (base-line 58.6 ± 13.3 mg/dL) increased after 4 weeks of treatment with quercetin (11.7%; 6.5 mg/dL; $p < 0.05$). However, in between the groups, no significant difference was detectable (Fig. 5).

oxLDL-ELISA

Because of numerous in vitro data regarding the antioxidative potential of quercetin [27], we examined the effect of quercetin on circulating oxLDL-levels. OxLDL (base-line 73.2 ± 58.5 ng/dL) was significantly decreased after 4-week quercetin treatment (−27.7%; 27.1 ng/dL; $p < 0.05$). Compared to the control group, this decrease tended to be of significance ($p = 0.07$; Fig. 5).

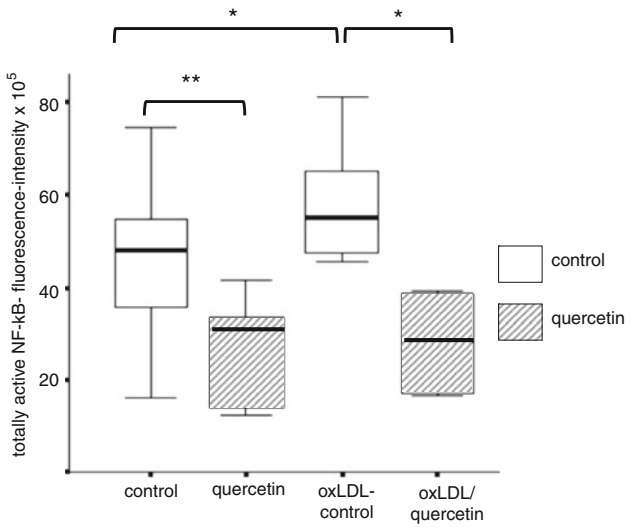

Fig. 4 NF-κB-ELISA. We used a chemiluminescence-based sandwich-type ELISA for NF-κB transcription factor analysis; DCs were generated with or without quercetin (10 µmol/L) and oxLDL (10 µg/mL). Quercetin reduced the total NF-κB activity by −45% ($p < 0.01$) and nullified the oxLDL induced increased NF-κB-activity (+52% compared to oxLDL-control). *Wilcoxon-test*: $*p < 0.05$ versus unstimulated control, ($n = 11$)

Table 1 Quercetin serum levels

	Before quercetin intake	After 30 days of quercetin intake
Proband 1	n.d.	0.65 µmol/L
Proband 2	n.d.	2.89 µmol/L
Proband 3	n.d.	6.14 µmol/L
Proband 4	n.d.	1.34 µmol/L
Proband 5	n.d.	0.54 µmol/L
Proband 6	n.d.	1.65 µmol/L
Proband 7	n.d.	1.25 µmol/L
Proband 8	1.10 µmol/L	3.37 µmol/L
Average ($n = 8$)		2.47 ± 1.8 µmol/L
Control 1–5	n.d.	n.d.

Before and after 30 days of quercetin (500 mg BID) intake, serum levels were measured. In contrast to the baseline and the control group after 30 days of intake, a average quercetin level of 2.47 µmol/L was detected

n.d. non detectable (<0.06 µmol/L)

ADMA-ELISA

ADMA is a circulating marker of endothelial dysfunction in human plasma and accumulates in the presence of metabolic changes [7] such as hypercholesterolemia. Although we looked at a healthy young study population, we found a significant decrease in plasma ADMA concentration. The average ADMA plasma concentration

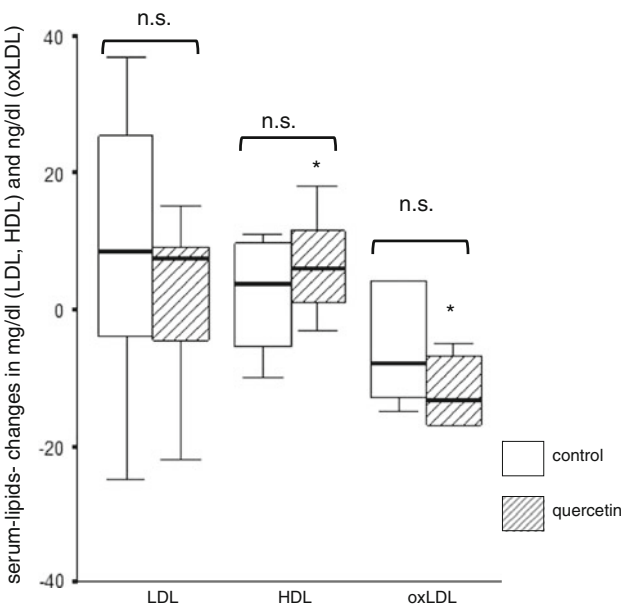

Fig. 5 Lipid serum levels. Plasma tubes from healthy controls were defrosted, and HDL and cholesterol levels were directly measured. The LDL-fraction was calculated by the Friedwald-formula. OxLDL levels were examined by using a specific ELISA kit. We found no significant change for LDL but an increase of 11.7% for HDL (*white bars*; Wilcoxon-test: $p < 0.05$; $n = 8$). OxLDL decreased significantly by −27% compared to baseline (Wilcoxon-test: $p < 0.05$; $n = 8$). Both changes for HDL ($p = 0.37$; Mann–Whitney U Test) and oxLDL ($p = 0.07$; Mann–Whitney U Test) missed the significance when compared to control (*white bars*; $n = 5$)

decreased from 0.32 ± 0.07 µM to 0.22 ± 0.06 µM (−31%, $p < 0.05$) after 30 days of quercetin intake compared to the time point before quercetin intake. In contrast, ADMA-levels in the control group did not change significantly over time (from 0.48 ± 0.02 µM to 0.5 ± 0.02 µM; +4.2%, n.s.). The decrease in ADMA in the quercetin group after follow-up was significant compared to the ADMA change in the control group ($p < 0.01$). However, the detected ADMA-levels in both groups were in the normal range compared to published data [7].

Discussion

A large part of the published data regarding anti-atherosclerotic effects of flavonoids has so far focused on its antioxidant capacity [28]. Since atherosclerosis is an immune-mediated disease, we investigated the ability of quercetin to modify DCs, the most potent antigen-presenting cells. By means of this study, we were able to demonstrate that quercetin modifies several steps in DC recruitment and DC activation and diminishes the immune-activation potential of oxLDL.

Quercetin not only reduced the DC adhesion on ECs, but also inhibited the phagocytosis capacity of DCs. We were further able to demonstrate that quercetin decreased the DC differentiation process induced by oxLDL. These results can, in part, be explained by a reduction of the oxLDL-induced NF-κB activation in DCs.

In addition to these in vitro data, our in vivo approach demonstrates that quercetin has a tendency to lower oxLDL serum levels and to decrease the circulating plasmacytoid DC fraction and systemic ADMA-levels.

Adhesion of DCs to vascular ECs is a critical step in the inflammatory response and involves recruitment of DCs to the site of tissue injury or lesion formation. These processes are mediated by adhesion molecules on ECs and DCs. For CD54 (counterpart for CD11a), Kobuchi et al. [29] found a down-regulation by quercetin on ECs, resulting in a decreased adhesion of leukocytes (DCs not investigated).

In our study, we found a down-regulation of CD11a but not of CD54 or CD209 (DC-SIGN) on human DCs. Including our previous results in which we identified CD11a [15] not only as an adhesion receptor but also as an important factor for DC transmigration, we conclude that quercetin inhibits key steps in leukocyte recruitment to inflammatory regions. We detected effects of quercetin on adhesion after 24 h. In vivo experiments have shown that continuous quercetin consumption quickly (1–3 h) leads to a steady state of 3–10 μmol/L [30], which corresponds to our measured quercetin levels of 2.47 μmol/L (4 h after intake).

One of the characteristic properties of DCs is their phagocytosis capacity. In addition to micropinocytosis, DCs are able to phagocyte different kinds of antigens by macropinocytosis and receptor-mediated endocytosis. We were able to demonstrate, for the first time, that quercetin reduces the macropinocytosis and the receptor-mediated endocytosis. The decrease in receptor-mediated endocytosis did not correlate with a down-regulation of the mannose receptor and was not caused by induction of a maturation process.

Flavonoids can at least in part inhibit the oxLDL-induced effects with regard to DC differentiation. OxLDL induces differentiation and maturation of the DCs via NF-κB up-regulation. Quercetin offsets the oxLDL-mediated NF-κB up-regulation as shown in the present study. In vivo we did not detect a significant reduction of NF-κB-gene expression in the PBMCs of our probands treated with quercetin. However, oxLDL levels at baseline were very low, and separated DC populations were not investigated. Even if we did not find a general NF-κB mRNA-reduction in vivo, this does not rule out a potential influence of quercetin especially in areas like the subintimal space where high oxLDL concentrations might occur.

The demonstrated effects on circulating DC subpopulations are difficult to interpret. There are some studies on DC subpopulations in patients with clinically manifested atherosclerosis. These studies describe a decrease in BDCA-1 and BDCA-2 expressing cells in the peripheral blood, which correlates with disease progression.

We only found a reduction in gene and protein expression in BDCA-2 cells in response to the 4-weeks treatment with quercetin. Recalling the pronounced role of BDCA-2 positive cells in autoimmune diseases [31], modification of BDCA-2 differentiation induced by flavonols could represent a cardiovascular protection and thus therapeutic option.

Flavonoids have been shown to influence circulating lipids [32]. OxLDL is a very important antigen and inflammatory stimulus especially in the context of atherosclerosis. OxLDL levels are directly associated with major cardiac events [33]. OxLDL increases DC adhesion [15], induces CD11a expression and induces DC maturation and DC differentiation [20]. Several in vitro studies have described the protective capacity of quercetin for LDL oxidation. For the first time, we are able to demonstrate that quercetin reduces oxLDL-mediated DC differentiation. Moreover, oral quercetin treatment tended to reduce circulating oxLDL levels in healthy subjects. Certainly, larger studies of high-risk patients are required to assess whether the oxLDL-lowering effect leads to better outcome.

The observed protection of quercetin for oxLDL-induced differentiation processes of inflammatory cells in combination with the reduced ADMA-levels detected in vivo suggests that flavonols may stabilize the inflammatory atherosclerotic plaque.

Limitations

Sample size in our in vivo study was limited. However, blood samples of each patient were taken at baseline and at follow-up after 4 weeks. The use of sorted DCs for the in vivo experiment would be a proper approach to more specifically address the impact of flavonoids on different DC subtypes. The quercetin concentration of 10 μmol/L is higher than the quercetin plasma levels (up to 7 μmol/L), which have been described in the literature. However, relevant metabolites like quercetin-3′-O-glucuronide, 3′-O-methylquercetin-3-O-glucuronide and quercetin-3′-O-sulfate [30] may reach much higher plasma concentrations (2–4 fold) [30, 34]. Even though the health benefits of fruits and vegetables are well acknowledged, the pathways which can explain these effects are still unclear. The present investigation describes one relevant immune pathway, which may warrant further detailed studies.

Conflicts of interest statement None.

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