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RESEARCH ARTICLE

Triggering of dendritic cell apoptosis by xanthohumol

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Xanthohumol, a flavonoid from beer with anticancer activity is known to trigger apoptosis in a variety of tumor cells. Xanthohumol further has anti-inflammatory activity. However, little is known about the effect of xanthohumol on survival and function of immune cells. The present study thus addressed the effect of xanthohumol on dendritic cells (DCs), key players in the regulation of innate and adaptive immunity. To this end, mouse bone marrow-derived DCs were treated with xanthohumol with subsequent assessment of enzymatic activity of acid sphingomyelinase (Asm), ceramide formation determined with anti-ceramide antibodies in FACS and immunohistochemical analysis, caspase activity utilizing FITC conjugated anti-active caspase 8 or caspase 3 antibodies in FACS and by Western blotting, DNA fragmentation by determining the percentage of cells in the sub-G1 phase and cell membrane scrambling by annexin V binding in FACS analysis. As a result, xanthohumol stimulated Asm, enhanced ceramide formation, activated caspases 8 and 3, triggered DNA fragmentation and led to cell membrane scrambling, all effects virtually absent in DCs from gene targeted mice lacking functional Asm or in wild-type cells treated with sphingomyelinase inhibitor amitriptyline. In conclusion, xanthohumol stimulated Asm leading to caspase activation and apoptosis of bone marrow-derived DCs.

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1 Introduction

Xanthohumol, a flavonoid from beer [1–3], has been shown to elicit anti-inflammatory, antiangiogenic, anticancer, antibacterial, antifungal, antimalarial and antiviral effects [4]. It may further favourably influence sleep disorders and menopausal symptoms in women [5]. Xanthohumol can cyclize to form the weak estrogen isoxanthohumol under the acidic conditions of the stomach [6]. In addition, isoxanthohumol can be metabolized by human liver enzymes to form the potent estrogen 8-prenylnaringenin [6]. Therefore, xanthohumol might function as a pro-estrogen, if it is absorbed and transported to the liver following oral administration [7]. However, *in vivo* rodent studies indicate that orally administered xanthohumol is not detected in plasma and that a majority of this dose is excreted unchanged with the faeces [8, 9]. In the Caco-2 cell line, a cell model used for the prediction of intestinal drug absorption in humans [10], xanthohumol was shown to readily enter the cells, but then it becomes trapped and does not exit quickly [7]. The anticarcinogenic effect of xanthohumol is thought to result from inhibition of cell proliferation and stimulation of apoptosis [11–26]. The effect of xanthohumol on the immune response has been attributed to an influence on the function of lymphocytes [18, 27–29] or macrophages [4, 30]. In lymphocytes, immunosuppressive effects of xanthohumol were reported, which include inhibition of T-cell proliferation, cell-mediated cytotoxicity and

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Abbreviations: Asm, acid sphingomyelinase; **AWB,** Annexin washing buffer; **DC,** dendritic cell; **FACS,** fluorescence activated cell sorting; **FCS,** fetal calf serum; **GM-CSF,** granulocyte-macrophage colony-stimulating factor

Th1 cytokine (IL-2, IFN-γ and TNF-α) production, effects attributed to suppression of NF-κB [29]. In contrast, xanthohumol treatment of mouse EL-4 T cells activated with phorbol 12-myristate 13-acetate plus ionomycin significantly increased IL-2 production through the enhancement of IL-2 promoter, NF-AT and AP-1 activity with no effect on NF-κB activity [27]. In LPS- and IFN-γ-stimulated mouse macrophage RAW 264.7 cells xanthohumol inhibited the production of NO by suppressing the expression of inducible NO synthase [30]. In LPS-activated RAW264.7 cells xanthohumol was also shown to reduce the expression of the LPS receptor components such as TLR4 and MD2 resulting in the suppression of NF-κB activation [4]. In the IFN-γ-stimulated RAW264.7 cells, the binding activity of STAT-1α and IRF-1 was inhibited by xanthohumol [4]. Several flavonoids were shown to inhibit dendritic cell (DC) growth and functional differentiation [31], to have an inhibitory effect on the production of cytokines [32–36], formation of ROS and NO, and change in intracellular Ca^{2+} levels [34], to induce apoptosis [37], to suppress cell surface molecule expression and antigen presentation [35, 37, 38] and to enhance antigen capture *via* mannose receptor-mediated endocytosis [35].

At least in theory, xanthohumol may influence the immune system by modifying the survival of DCs, antigen-presenting cells operating at the interface of the innate and adaptive immune system [39–41]. DCs are located throughout the body to capture and internalize invading pathogens, and subsequently process and present antigen on MHC class I and class II molecules to $CD8^+$ and $CD4^+$ T cells, respectively [40]. DCs further counteract potentially harmful immune responses against nonpathogenic antigens [42]. Since DCs are in contact with the intestinal lumen [43], they may be exposed to relatively high nutrient concentrations. An inappropriate immune response to microbial antigens of commensal microorganisms in genetically susceptible individuals may lead to chronic inflammatory diseases, such as Crohn's disease or ulcerative colitis [44].

The present study explored, whether xanthohumol affects DC survival. To this end, bone marrow-derived mouse DCs were exposed to xanthohumol and ceramide formation, caspase activity, DNA fragmentation as well as cell membrane scrambling determined.

2 Materials and methods

2.1 Mice and cell culture

DCs were obtained from bone marrow of 7–12 wk old acid sphingomyelinase (Asm) knockout ($asm^{-/-}$) mice, completely lacking functional Asm on C57/BL6 genetic background and their age and sex matched wild-type littermates ($asm^{+/+}$). All animal experiments were conducted according to the guidelines of the American Physiological Society as well as the German law for the welfare of animals and were approved by local authorities. The $asm^{-/-}$ mice [45, 46] were a kind gift of Dr. Verena Jendrossek (University of Tübingen, Germany) and were originally obtained from Dr. R. Kolesnick (Sloan Kettering Cancer Memorial Center, NY, USA). DCs were cultured as previously described [47–49] with slight modifications. Briefly, bone marrow cells were flushed out of the cavities from the femur and tibia with PBS (in mM: 155.2 NaCl, 1.5 KH_2PO_4, 2.7 Na_2HPO_4, pH 7.2, GIBCO, Carlsbad). Cells were washed twice with RPMI and seeded out at a density of 2×10^6 cells *per* 60–mm dish. Cells were cultured for 8 days in RPMI 1640 (GIBCO) containing: 10% fetal calf serum (FCS), 1% penicillin/streptomycin, 1% glutamine, 1% non-essential amino acids and 0.05% β-mercaptoethanol. Cultures were supplemented with Granulocyte-macrophage colony-stimulating factor (GM-CSF, 35 ng/mL, Preprotech Tebu) and fed with fresh medium containing GM-CSF on days 3 and 6. Nonadherent and loosely adherent cells were harvested after 8 days of culture. At day 8, >80% of the cells expressed CD11c, which is a marker for mouse DCs. Experiments were performed in the absence or in the presence of different concentrations of xanthohumol (2–50 μM, Sigma-Aldrich, Germany) in the absence and in the presence of amitriptyline (0.5 μM, Sigma-Aldrich, Germany) at day 9.

2.2 Asm activity assay

The activity of the Asm was measured, as previously described [50]. Briefly, 2.5×10^5cells were incubated with xanthohumol (20 μM) for different time spans: 30 min; 1, 2, 5, 8, 20, 22 or 24 h, frozen in liquid nitrogen and kept at −80°C. Then the cells were lysed in 50 μL of ice-cold buffer containing 50 mM sodium acetate (pH 5.0), 1% NP40. After 10 min lysis on ice, the lysates were diluted to 0.1% NP40 in a final volume of 200 μL. The lysates were incubated with 0.02 μCi of [^{14}C]sphingomyelin for 30 min at 37°C. The reaction was stopped by addition of 1 mL v/v of $CHCl_3$:CH_3OH. The substrate was dried before use and resuspended in 50 mM sodium acetate (pH 5.0), 0.1% NP40 followed by 10 min bath sonication to promote the formation of micelles. Phases were separated by 5 min centrifugation at 14 000 rpm, and an aliquot of aqueous phase was applied for liquid scintillation counting. Hydrolysis of [^{14}C]sphingomyelin by sphingomyelinase results in release of [^{14}C]choline chloride into the aqueous phase, whereas ceramide and unreacted [^{14}C]sphingomyelin remain in the organic phase. Therefore, the release of [^{14}C]choline chloride (pmol/10^5 cells/h) serves to determine the activity of the Asm.

2.3 Ceramide formation in FACS

For detection of ceramide formation, mouse DCs were stained for 60 min at 37°C with anti-ceramide antibodies (Mouse IgM, Alexis) at a dilution of 1:10 in PBS containing

0.1% FCS. After three washes with PBS/0.1% FCS, cells were stained with FITC-labelled goat anti-mouse IgG antibody at a dilution of 1:400 (Invitrogen, UK) in PBS/0.1% FCS for 30 min at 37°C. Unbound secondary antibodies were removed by washing the cells with PBS/0.1% FCS. Cells were then analyzed by flow cytometry (FACS Calibur, BD Biosciences).

2.4 Immunocytochemistry for ceramide formation

Untreated or xanthohumol-treated (20 μg/mL, 24 h) DCs were smeared onto glass slides, rinsed in PBS and fixed with 4% formaldehyde in PBS for 15 min at room temperature. After three washing steps with PBS for 5 min, slides were permeabilized and blocked in PBS containing 5% goat serum (Invitrogen, Karlsruhe, Germany) and 0.3% Triton X-100 for 60 min and then incubated overnight at 4°C with anti-ceramide antibodies (Mouse IgM, Alexis) at a dilution of 1:10 in antibody dilution buffer (including PBS, 1% BSA and 0.3% Triton X-100). The slides were washed again three times for 5 min and then incubated with goat anti-mouse IgG-FITC (Invitrogen, Germany) in antibody dilution buffer for 90 min at 1:500 dilution at room temperature in dark. After three washing steps with PBS, nuclei were stained with DRAQ5 (1:1000, BioStatus, Shepshed Leicestershire, UK) in PBS containing 0.5% Triton X-100 for 10 min and washed final two times with PBS. Stained slides were mounted using Prolong® Gold antifade reagent (Invitrogen, Germany). Images were taken on a Zeiss LSM 5 EXCITER Confocal Laser Scanning Microscope (Carl Zeiss MicroImaging GmbH, Germany) with immersion Plan-Neofluar 10_/1.3 NA DIC objective and camera function.

2.5 Caspase 8 and caspase 3 activation assay

Caspase 8 and caspase 3 activity was determined using kits from Biovision according to the manufacturer's instruction. Briefly 1×10^6 cells were washed twice with cold PBS, fixed and permeabilized with ''Cytofix/Cytoperm'' solution and then by washing twice with ''Perm/Wash'' buffer. Then cells were stained with FITC conjugated anti-active caspase 8 or caspase 3 antibody in ''Perm/Wash'' buffer for 60 min. After two washing steps, the cells were analyzed by flow cytometry (FACS Calibur, BD Biosciences).

2.6 DNA fragmentation

In total, 5×10^5 cells were fixed with 2% formaldehyde for 30 min on ice and then incubated with 70% ethanol for 15 min at 37°C. Cells were then treated with RNase A (40 μg/mlL) for 30 min at 37°C, washed and resuspended in 200 μL PI (50 μg/mL, Sigma). The DNA content of the samples was analyzed by flow cytometry (FACS Calibur, BD Biosciences).

2.7 Phosphatidylserine translocation

Apoptotic cell membrane scrambling was evidenced from annexin V binding to phosphatidylserine at the cell surface [51]. To this end, the percentage of phosphatidylserine-translocating cells was evaluated by staining with FITC-conjugated Annexin V. In brief 4×10^5 cells were harvested and washed twice with Annexin washing buffer (AWB, 10 mM Hepes/NaOH, pH 7.4, 140 mM NaCl, 5 mM $CaCl_2$). The cell pellet was resuspended in 100 μL of Annexin-V-Fluos labelling solution (Roche) (20 μL Annexin-V-Fluos labelling reagent in 1 mL AWB), incubated for 15 min at room temperature. After washing with AWB, they were analyzed by flow cytometry.

2.8 Immunoblotting

DCs (2×10^6 cells) were washed twice in PBS and then solubilized in lysis buffer (Pierce) containing protease inhibitor cocktail (Sigma-Aldrich, Taufkirchen, Germany). Samples were stored at −80°C until use for Western blotting. Cell lysates were separated by 12% SDS-PAGE and blotted on nitrocellulose membranes. The blots were blocked with 5% nonfat-milk in triethanolamine-buffered saline and 0.1% Tween-20. Then the blots were probed overnight with antibodies detecting caspase 8 or caspase 3 full length and fragments resulting from cleavage (17–18 kDa) or α/β-tubulin (Cell signaling) diluted (1:1000) in blocking buffer, washed five times, probed with secondary antibodies (anti-mouse or anti-rabbit, GE healthcare, München, Germany) diluted 1:5000 for 1 h at room temperature and washed final five times. Antibody binding was detected with the enhanced chemiluminescence kit (Amersham, Freiburg, Germany). Densitometer scans of the blots were performed using Quantity One (BioRad, Munich, Germany).

2.9 Statistics

Data are provided as means ± SEM, n represents the number of independent experiments. Differences were tested for significance using ANOVA. $p < 0.05$ was considered statistically significant.

3 Results

Bone marrow-derived DCs were cultured from either wild-type mice or mice lacking functional acid sphingomyelinase (*Smpd1*$^{-/-}$). The cells were grown in GM-CSF containing

media for 8 days and in the following exposed for 24 h to xanthohumol (20 μM). As illustrated in Fig. 1A–C, administration of xanthohumol within 24 h stimulated ceramide formation in DCs from wild-type mice but not in DCs from $asm^{-/-}$ mice and not in wild-type cells treated with sphingomyelinase inhibitor amitriptyline (Fig. 1C). The observation revealed a stimulating effect of xanthohumol on the acid sphingomyelinase of wild-type mice. The effect on ceramide formation reached statistical significance at 20 μM xanthohumol (Fig. 1D). LPS, which leads to DC activation through TLR4, is known to result in enhanced DC survival by inhibition of DC apoptosis [40]. As shown in Fig. 1D, the production of ceramide was slightly, but significantly, decreased by LPS treatment (100 nM, 24 h, Fig. 1C).

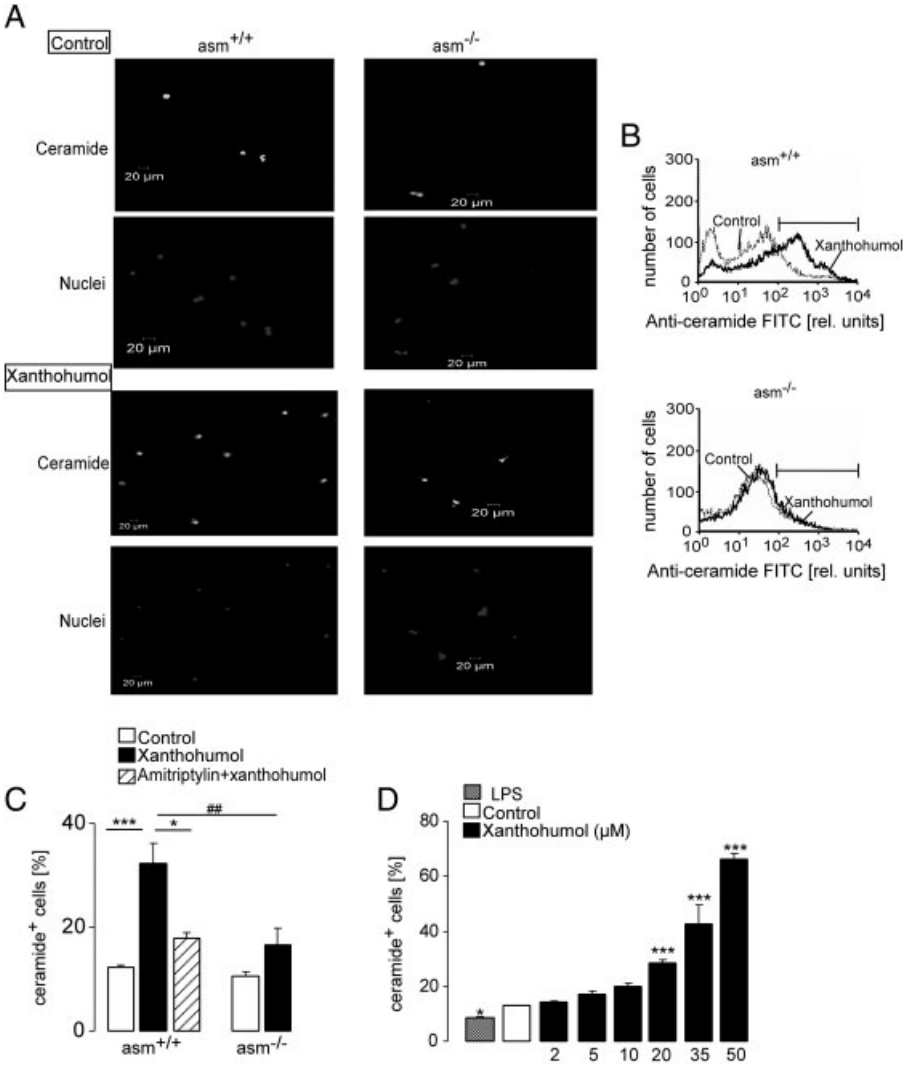

Figure 1. Effect of xanthohumol on ceramide formation in DCs, (A) Immunohistochemistry of anti-ceramide FITC-coupled-antibody binding and nuclei staining in a representative experiment on wild-type ($asm^{+/+}$, left panels) and Asm knockout ($asm^{-/-}$, right panels) DCs either untreated (control, upper panels) or incubated for 24 h with xanthohumol (20 μg/mL, lower panels). (B) Histograms of anti-ceramide FITC-coupled-antibody binding as obtained by FACS analysis in a representative experiment on $asm^{+/+}$ (upper panel) and $asm^{-/-}$ (lower panel) DCs either untreated (control, dotted line) or incubated for 24 h with xanthohumol (20 μM, black line). (C) Arithmetic means ($n = 3$–8) of the percentage of $asm^{+/+}$ (left bars) and $asm^{-/-}$ (right bars) DCs presenting ceramide at the cell surface. Ceramide formation is shown prior to (control, white bars) and 24 h following (black bars) treatment with xanthohumol (20 μM) either in the absence or in wild-type cells also in the presence of amitriptyline (0.5 μM, hatched bars).* $p < 0.05$ and *** $p < 0.001$ represent significant difference from wild-type xanthohumol-treated cells and $p < 0.01$ represents significant difference between xanthohumol-treated $asm^{+/+}$ and $asm^{-/-}$ DCs, ANOVA. (D) Dose-dependent effect of xanthohumol on ceramide formation. Arithmetic means ($n = 4$–6) of the percentage of wild-type DCs presenting ceramide at the cell surface. Ceramide formation is shown in untreated cells (control, white bars) or in DCs either treated with LPS (0.1 μg/mL, 24 h, dotted bars) or xanthohumol (2–50 μM, 24 h, black bars). * $p < 0.05$ and *** $p < 0.001$ represent significant difference from control, ANOVA.

We further determined the enzymatic activity of Asm in wild-type DCs treated with xanthohumol for different time periods. No increase of Asm activity was observed at 30 min, 1 h, 2 h and 5 h of xanthohumol treatment. Activation of Asm could be observed after 8 h and reached its maximum at 22 h of incubation with xanthohumol (8 h: 140 ± 5%, 20 h: 160 ± 11%, 22 h: 175 ± 13%, 24 h: 150 ± 10% of Asm activity in untreated cells, $n = 4$).

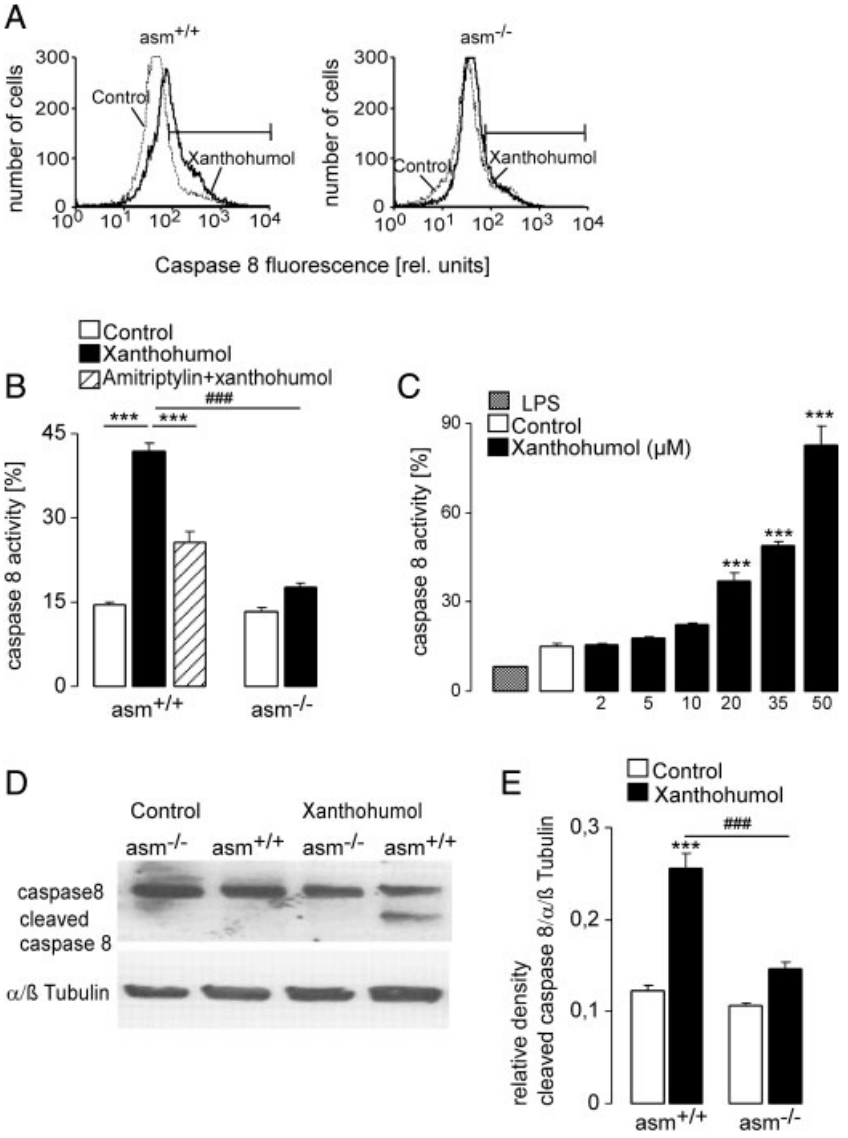

Figure 2. Effect of xanthohumol on caspase 8 activity in DCs, (A) Histograms of caspase 8 activity as obtained by FACS analysis in a representative experiment on *asm*$^{+/+}$ (left panel) and *asm*$^{-/-}$ (right panel) DCs either untreated (control, dotted line) or incubated for 24 h with xanthohumol (20 μM, black line). (B) Arithmetic means ($n = 4$–8) of the percentage of *asm*$^{+/+}$ (left bars) and *asm*$^{-/-}$ (right bars) DCs with activated caspase 8. Caspase activation is shown prior to (control, white bars) and 24 h following (black bars) treatment with xanthohumol (20 μM) either in the absence or in wild-type cells also in the presence of amitriptyline (0.5 μM, striped bars). $^{***}p < 0.001$ represents significant difference from wild-type xanthohumol-treated cells and $^{\#\#\#}p < 0.001$ represents significant difference between xanthohumol -treated *asm*$^{+/+}$ *and asm*$^{-/-}$ DCs, ANOVA. (C) Dose-dependent effect of xanthohumol on caspase 8 activation. Arithmetic means ($n = 4$–6) of the percentage of wild-type DCs with activated caspase 8. Caspase 8 activity is shown in untreated DCs (control, white bars) or DCs treated with either LPS (0.1 μg/mL, 24 h, dotted bars) or xanthohumol (2–50 μM, 24 h, black bars).$^{***}p < 0.001$ represent significant difference from control condition, ANOVA. (D) Original Western blot of DCs from *asm*$^{+/+}$and *asm*$^{-/-}$ mice, which were either treated with xanthohumol (20 μM, 24 h) or left untreated (control). Protein extracts were analyzed by direct Western blotting using antibodies directed against caspase 8. Protein loading was controlled by anti-α/β-tubulin antibody. One representative experiment out of three is shown. (E) Arithmetic mean ± SEM ($n = 3$–5) of cleaved protein of caspase 8 as the ratio of cleaved caspase 8:α/β-tubulin. $^{***}p < 0.001$ indicates significant difference between control and xanthohumol-treated cells, $^{\#\#\#}p < 0.001$ indicates difference between xanthohumol-treated *asm*$^{+/+}$ and *asm*$^{-/-}$ DCs, ANOVA.

As ceramide is known to stimulate suicidal cell death, a second series was performed to elucidate the effect of xanthohumol on caspase activation. The administration of xanthohumol was indeed followed by activation of caspase 8 in wild-type DCs as obvious from FACS analysis (Figs. 2A–C) and Western blotting (Fig. 2D and E). This

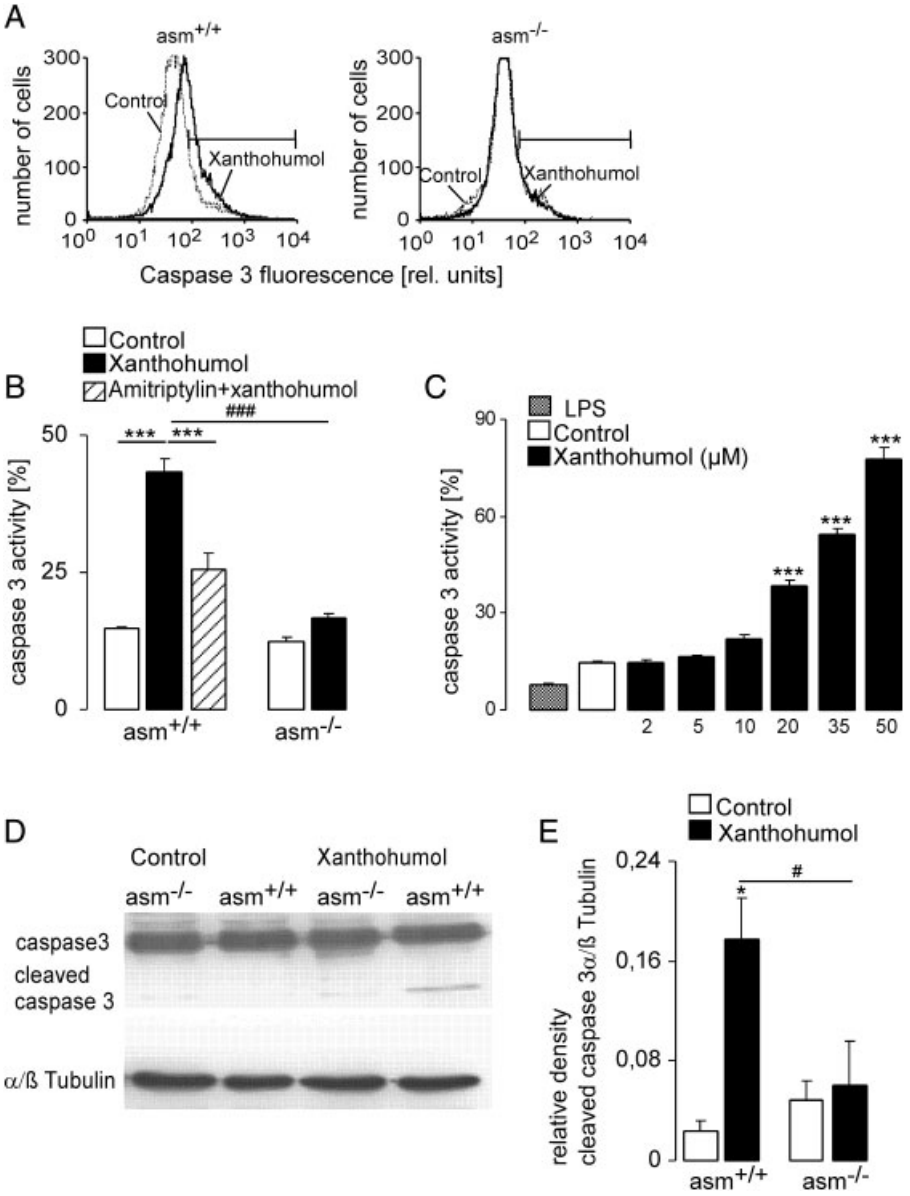

Figure 3. Effect of xanthohumol on caspase 3 activity in DCs, (A) Histograms of caspase 3 activity as obtained by FACS analysis in a representative experiment on *asm*$^{+/+}$ (left panel) and *asm*$^{-/-}$ (right panel) DCs either untreated (control, dotted line) or incubated for 24 h with xanthohumol (20 μM, black line). (B) Arithmetic means ($n = 3$–8) of the percentage of *asm*$^{+/+}$ (left bars) and *asm*$^{-/-}$ (right bars) DCs with activated caspase 3. Caspase activation is shown prior to (control, white bars) and 24 h following (black bars) treatment with xanthohumol (20 μM) either in the absence or in wild-type cells also in the presence of amitriptyline (0.5 μM, striped bars). $^{***}p<0.001$ represents significant difference from wild-type xanthohumol-treated cells and $^{\#\#\#}p<0.001$ represents significant difference between xanthohumol-treated *asm*$^{+/+}$ *and asm*$^{-/-}$ DCs, ANOVA. (C) Dose-dependent effect of xanthohumol on caspase 3 activation. Arithmetic means ($n = 4$–6) of the percentage of wild-type DCs with activated caspase 3. Caspase 3 activity is shown in untreated DCs (control, white bars) or DCs treated with either LPS (0.1 μg/mL, 24 h, dotted bars) or xanthohumol (2–50 μM, 24 h, black bars). $^{***}p<0.001$ represents significant difference from control condition, ANOVA. (D) Original Western blot of DCs from *asm*$^{+/+}$and *asm*$^{-/-}$ mice, which were either treated with xanthohumol (20 μM, 24 h) or left untreated (control). Protein extracts were analyzed by direct Western blotting using antibodies directed against caspase 3. Protein loading was controlled by anti-α/β-tubulin antibody. One representative experiment out of three is shown. (E) Arithmetic mean ± SEM ($n = 3$–5) of cleaved protein of caspase 3 as the ratio of cleaved caspase 3:α/β-tubulin $^{*}p<0.05$ indicates significant difference between control and xanthohumol-treated cells, $^{\#}p<0.05$ indicates difference between xanthohumol-treated *asm*$^{+/+}$and *asm*$^{-/-}$ DCs, ANOVA.

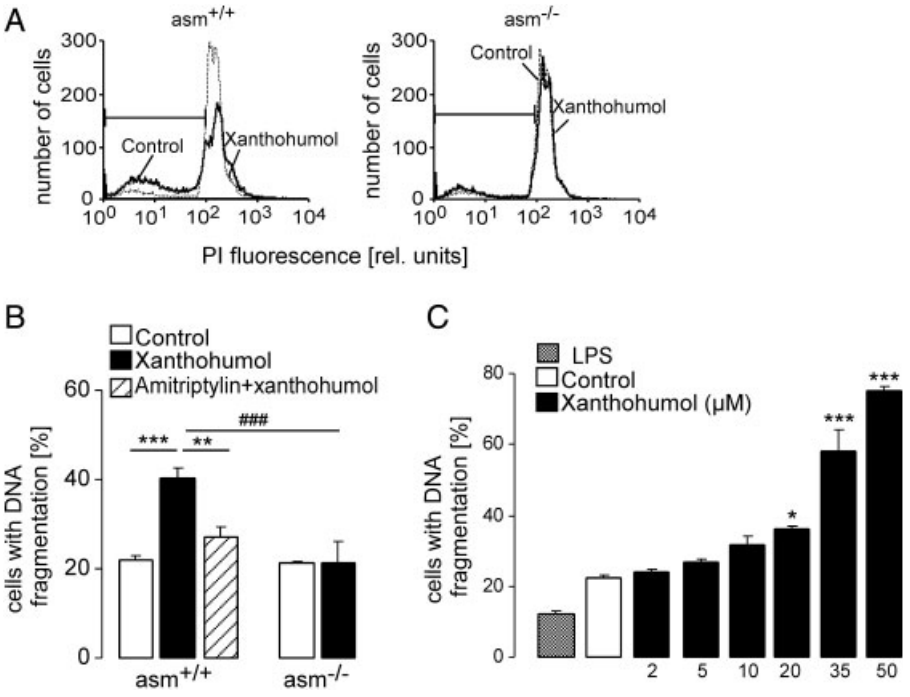

Figure 4. Effect of xanthohumol on DNA fragmentation in DCs, (A) Histograms of DNA content in sub-G1 fraction as obtained by FACS analysis in a representative experiment on *asm*$^{+/+}$ (left panel) and *asm*$^{-/-}$ (right panel) DCs, which remained either untreated (control, dotted line) or were incubated for 24 h with xanthohumol (20 μM, black line). (B) Arithmetic means ($n = 3$–8) of the percentage of *asm*$^{+/+}$ (left bars) and *asm*$^{-/-}$ (right bars) DCs with fragmented DNA. DNA fragmentation is shown prior to (control, white bars) and 24 h following (black bars) treatment with xanthohumol (20 μM) either in the absence or in wild-type cells also in the presence of amitriptyline (0.5 μM, striped bars). $^{**}p<0.01$ and $^{***}p<0.001$ represent significant difference from wild-type xanthohumol-treated cells and $^{\#\#}p<0.01$ represents significant difference between xanthohumol-treated *asm*$^{+/+}$ and *asm*$^{-/-}$ DCs, ANOVA. (C) Dose-dependent effect of xanthohumol on DNA fragmentation. Arithmetic means ($n = 4$–6) of the percentage of wild-type DCs with fragmented DNA. DNA fragmentation is shown in untreated DCs (control, white bars) or DCs treated with either LPS (0.1 μg/mL, 24 h, dotted bars) or xanthohumol (2–50 μM, 24 h, black bars) treated DCs. $^{**}p<0.05$, and $^{***}p<0.001$ represent significant difference from control condition, ANOVA.

effect reached statistical significance at the concentration of xanthohumol ≥20 μM (Fig. 2C). In contrast, little stimulation of caspase activation was observed in DCs derived from *asm*$^{-/-}$ mice (Figs. 2A, B, D and E) or in wild-type DCs treated with amitriptyline (Fig. 2B), indicating that the effect of xanthohumol on caspase activation was secondary to stimulation of ceramide formation.

Similar to caspase 8, caspase 3 activity was activated by administration of xanthohumol in wild-type DCs. Again, xanthohumol at concentrations above 20 μM stimulated the caspase 3 only in wild-type DCs but not in DCs from *asm*$^{-/-}$ mice (Fig. 3). Amitriptyline treatment resulted in a significant reduction of caspase 3 activation by xanthohumol in wild-type cells (Fig. 3B). Thus, activation of caspase 3, similar to that of caspase 8, was significantly stimulated by xanthohumol by a mechanism requiring functional acid sphingomyelinase.

As activation of caspases is known to trigger suicidal cell death, additional experiments were performed to determine the effect of xanthohumol on DNA fragmentation, a hallmark of apoptosis. Xanthohumol treatment at concentrations ≥20 μM was indeed followed by an increase of cells in the sub-G1 phase, a known marker for fragmented DNA (Fig. 4). In contrast, administration of xanthohumol did not elicit DNA fragmentation in DCs from *asm*$^{-/-}$ mice (Figs. 4A and B) or in wild-type amitriptyline-treated DCs (Fig. 4B).

Apoptosis is further known to stimulate cell membrane scrambling with subsequent phosphatidylserine exposure at the cell surface. Quantitative assessment of phosphatidylserine exposure was accomplished by determination of annexin V binding. As shown in Fig. 5, xanthohumol at concentrations ≥10 μM stimulated annexin V binding only in wild-type DCs but not in DCs from *asm*$^{-/-}$ mice (Figs. 5A and B) and to a much lesser extent in wild-type cells treated with amitriptyline (Fig. 5B).

4 Discussion

According to the present study, xanthohumol stimulates the acid sphingomyelinase (Asm) in DCs leading to ceramide formation, caspase activation and stimulation of suicidal cell death. The sequence of events in xanthohumol-induced apoptosis could be the following: DC stimulation with xanthohumol leads to translocation of Asm from an

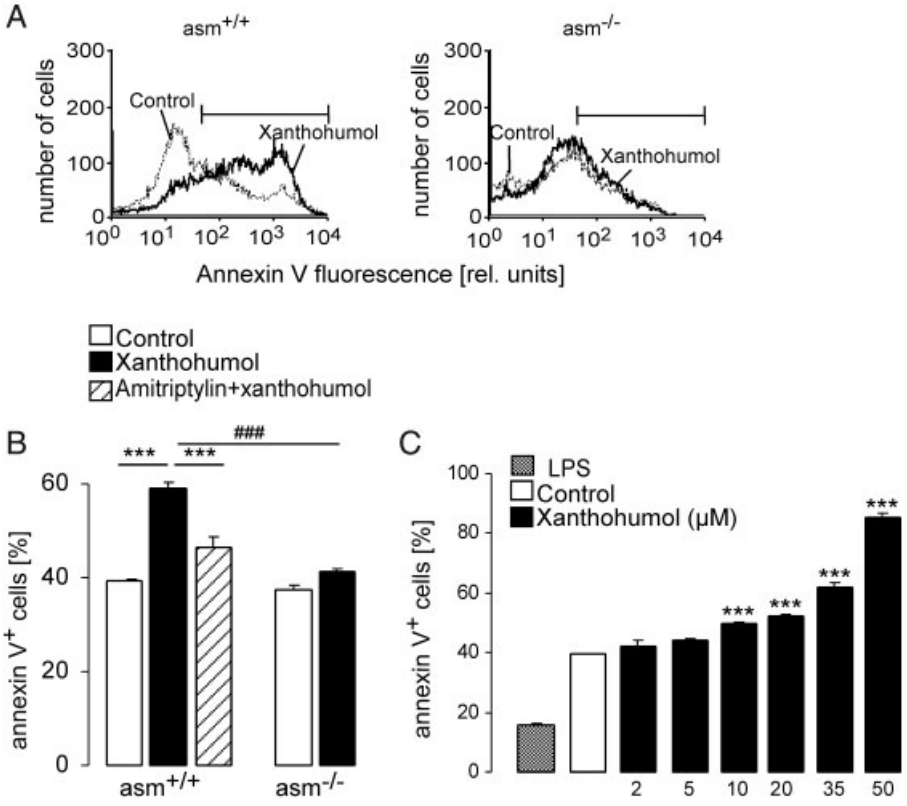

Figure 5. Effect of xanthohumol on cell membrane scrambling in DCs, (A) Histograms of annexin V binding as obtained by FACS analysis in a representative experiment on *asm*$^{+/+}$ (left panel) and *asm*$^{-/-}$ (right panel) DCs either untreated (control, dotted line) or incubated for 24 h with xanthohumol (20 μM, black line). (B) Arithmetic means ($n = 3$–8) of the percentage of *asm*$^{+/+}$ (left bars) and *asm*$^{-/-}$ (right bars) DCs with annexin V binding. Annexin V binding is shown prior to (control, white bars) and 24 h following (black bars) treatment with xanthohumol (20 μM) either in the absence or in wild-type cells also in the presence of amitriptyline (0.5 μM, striped bars). $^{***}p < 0.001$ represents significant difference from wild-type xanthohumol-treated cells and $^{\#\#\#}p < 0.001$ represents significant difference between xanthohumol-treated *asm*$^{+/+}$ and *asm*$^{-/-}$ DCs, ANOVA. (C) Dose-dependent effect of xanthohumol on annexin V binding. Arithmetic means ($n = 4$–6) of the percentage of wild-type DCs with annexin V binding is shown in untreated (control, white bars) or either LPS- (0.1 μg/mL, 24 h, dotted bars) or xanthohumol- (2–50 μM, 24 h, black bars) treated DCs. $^{***}p < 0.001$ represent significant difference from control condition, ANOVA.

intracellular compartment onto the cell surface. In lymphocytes, electron microscopy studies localized Asm in intracellular vesicles of unstimulated cells, which appeared to fuse with the cell membrane upon stimulation with the Fas ligand [52]. This resulted in exposure of the Asm on the extracellular membrane leaflet and subsequent formation of ceramide. Ceramide formation results in caspase 8 autocatalysis that initiates apoptosis induction. Caspase 8 can then directly activate caspase 3. The activation of caspase 3 executes apoptosis by triggering DNA fragmentation and proteolysis of intracellular proteins.

A wide variety of stimuli, including Fas ligand, TNF-α, γ-irradiation, anti-tumor reagents and heat shock trigger the release of ceramide [53]. Most of these stimuli activate the acid sphingomyelinase, which belongs to a class of enzymes that hydrolyze sphingomyelin and thus generate ceramide [53]. However, ceramide can be also generated by ceramide synthase, a key enzyme involved in *de novo* sphingolipid biosynthesis [54]. Moreover, neutral and alkaline sphingomyelinases have been also described [53]. Ceramide formation in DCs obtained from *asm*$^{-/-}$ mice could be thus mediated by these enzymes.

Xanthohumol has previously been shown to trigger apoptosis in a wide variety of cells including adipocytes [55, 56], preadipocytes [57], leukemia cells [12, 13, 18, 21], breast cancer cells [14, 17, 23, 24, 26], prostate cancer [11], hepatocellular carcinoma cells [19], colon cancer cells [25], Bcr/Abl-transformed cells [22], fibrosarcoma [16] and Kaposi's sarcoma [20]. An effect of xanthohumol on DC survival has never been reported.

Xanthohumol is partially effective through activation of the death receptor- and mitochondrial pathway [25], nuclear factor-κB NFκB and p53 modulation [11, 18, 22, 58]. Moreover, xanthohumol has been shown to inhibit diacylglycerol acyltransferase [16] and to upregulate the function of the E-cadherin/catenin complex [26]. Xanthohumol may increase the cellular content of reactive oxidant species but at the same time may exert antioxidant activity [59]. Moreover,

xanthohumol has been shown to upregulate the detoxification enzyme NADPH-quinone oxidoreductase [60]. An involvement of sphingomyelinase and/or ceramide in the effects of xanthohumol has never been published. Ceramide is a well known stimulator of apoptosis in a variety of cell types [61–66] and may thus participate in the stimulation of apoptosis by xanthohumol in other cell types as well.

In contrast to its proapoptotic effect on nucleated cells, xanthohumol protects erythrocytes against suicidal cell death [67], which is, similar to apoptosis of nucleated cells, stimulated following activation of sphingomyelinase and subsequent formation of ceramide [51, 64, 68–70]. The mechanisms accounting for the differences between nucleated cells and erythrocytes during suicidal death remain to be elucidated. Clearly, the suicidal death of erythrocytes lacking nuclei is not expected to be under genomic regulation by transcription factors such as NFκB [64].

The xanthohumol-induced apoptosis may suppress the immune response, an effect, which, at least in theory, may contribute to the anti-inflammatory action of the nutrient [4]. This does, of course, not rule out additional mechanisms contributing to the anti-inflammatory effect of xanthohumol.

Xanthohumol is a component of regular beer, although in very low amounts (100 μg/L). Whether levels of xanthohumol used in our study *in vitro* are achievable *in vivo* is not clear. In leukaemia cells, apoptotic effects of xanthohumol were observed at concentrations 5–10 μM [12]. Xanthohumol potentiates TNF-induced apoptosis several-fold at the concentration of 50 μM in both myeloid and leukaemic cells [18]. It was shown that xanthohumol could at a concentration of 25 μM inhibit the growth of human endothelial cells in culture [71]. Comparable dosages (20 μM in the drinking water), when given orally to mice, inhibited the angiogenesis and tumor growth in mice [71]. In another study xanthohumol administration in concentrations of 500 μM (in the drinking water) for 4 wk did not affect any major organ functions in mice [72].

Reduced apoptosis in DCs matured in the presence of LPS fits well to the previous studies, where it was shown that LPS and TNF-α can increase DC survival through upregulation of Bcl-XL and Bcl-2 [73]. Moreover, mature DCs highly express the caspase 8 inhibitory protein cFLIP, whereas only low levels are detected in immature DCs [74]. Though DCs at all stages of maturation express death receptors, the death ligand sensitivity was shown to depend on DC maturation and inversely correlated with expression levels of cFLIP [74].

In conclusion, xanthohumol stimulates ceramide formation and apoptotic death of DCs. The effect is expected to affect the immune response.

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