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Dietary fiber and the short-chain fatty acid acetate promote resolution of neutrophilic inflammation in a model of gout in mice

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ABSTRACT

Gout is a disease characterized by the deposition of monosodium urate (MSU) crystals in the joints. Continuous gout episodes may lead to unresolved inflammatory responses and tissue damage. We investigated the effects of a high-fiber diet and acetate, a short-chain fatty acid (SCFA) resulting from the metabolism of fiber by gut microbiota, on the inflammatory response in an experimental model of gout in mice. Injection of MSU crystals into the knee joint of mice induced neutrophil influx and inflammatory hypernociception. The onset of inflammatory response induced by MSU crystals was not altered in animals given a high-fiber diet, but the high-fiber diet induced faster resolution of the inflammatory response. Similar results were obtained in animals given the SCFA acetate. Acetate was effective, even when given after injection of MSU crystals at the peak of the inflammatory response and induced caspase-dependent apoptosis of neutrophils that accounted for the resolution of inflammation. Resolution of neutrophilic inflammation was associated with decreased NF-κB activity and enhanced production of anti-inflammatory mediators, including IL-10, TGF-β, and annexin A1. Acetate treatment or intake of a high-fiber diet enhanced efferocytosis, an effect also observed in vitro with neutrophils treated with acetate. In conclusion, a high-fiber diet or one of its metabolic products, acetate, controls the inflammatory response to MSU crystals by favoring the resolution of the inflammatory response. Our studies suggest that what we eat plays a determinant role in our capacity to fine tune the inflammatory response. Introduction *J. Leukoc. Biol.* 101: 275–284; 2017.

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Gouty arthritis is an inflammatory disease triggered by the deposition of MSU crystals in joints, rapid release of inflammatory cytokines, and inflammatory cell infiltration associated with progressive tissue damage and debilitating pain [1–3]. Neutrophils are the main inflammatory cells recruited to the joint in a CXCR1/2-dependent manner and contribute to the amplification of tissue damage [3, 4]. Therefore, prevention of the influx or clearance of neutrophils from the site of inflammation may prevent or decrease tissue damage and facilitate return to homeostasis after an acute gout attack.

Gout has been known since ancient times; however, it was virtually unknown in many countries before the adoption of Western dietary habits. Epidemiologic studies support a link between gout and Western diet and lifestyle [5, 6]. The prevalence of gout has risen within the past few decades, rendering this disease an important health care issue. In some studies, diet and associated changes in gut microbiota and their metabolites have emerged as factors that influence inflammatory responses [7, 8]. In addition, increased consumption of fiber has been observed to alleviate symptoms of gout [9]. Commensal bacteria possess the appropriate enzymes to metabolize complex plant polysaccharides to SCFAs—mainly acetate, propionate, and butyrate [10, 11]. It has been shown that SCFAs mediate profound anti-inflammatory effects in chronic diseases [12, 13]. Furthermore, administration of SCFAs to mice and humans alleviates certain inflammatory conditions [12, 14, 15].

Thus, in the current study, we sought to investigate the effects of an HF diet and acetate, one of the metabolic products resulting from the metabolism of fiber by gut microbiota on the inflammatory response in an experimental model of gout.

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MATERIALS AND METHODS

Mice

Male C57Bl/6 mice and C57Bl6 GFP Het (8–12 wk) were obtained from the Centro de Bioterismo of the UFMG in Brazil. All mice were supplied with water and food ad libitum. The UFMG Animal Ethics Committee approved all procedures (protocol 165/2008).

In experiments in which mice were given SCFA/sodium acetate (Sigma-Aldrich, St. Louis, MO, USA) before MSU challenge, 150 mM acetate was added to the drinking water of the mice 5 d before the challenge and by oral gavage in experiments whereby SCFA treatment was to be given after the MSU challenge. In the diet experiments, as in earlier studies [16, 17], mice were maintained on an HF (AIN93M with addition of Pectin 10%) and a normal LF (AIN93M) diet (Supplemental Fig. 1), 2 wk before and during MSU challenge.

MSU crystal–induced gout

MSU crystals were prepared by dissolving uric acid (5 mg/ml) in 0.1 M borate buffer (pH 8.5) [3]. The MSU crystals used were endotoxin free, as determined by Limulus amebocyte lysate assay.

Mice were given an i.a. injection of 100 µg of MSU crystals diluted in 10 µl of PBS. Control mice received an i.a. injection of PBS as vehicle. The mice were killed at different time points after MSU challenge. The knee cavity was washed with PBS (2×5 µl) to collect the cells. Periarticular tissues were also removed for evaluation of cytokines, chemokines, and MPO activity. The total number of leukocytes was determined by counting them in a Neubauer chamber after staining with Turk's solution. Differential cell counts were determined with standard morphologic criteria and were performed on May-Grünwald-Giemsa-stained slides.

For histological analysis, knee joints were removed and fixed and the bone decalcified, dehydrated, and embedded in paraffin. Sections of 3 µm were obtained and stained with H&E.

Evaluation of hypernociception

Mechanical hypernociception was tested in the mice [18]. In brief, the mice were placed in acrylic cages with a wire grid floor, and an electronic pressure meter was used (electronic von Frey anesthesiometer; Insight Instruments, São Paulo, Brazil). The electronic pressure–meter apparatus automatically recorded the intensity of the force applied when the paw was withdrawn. Results are expressed as a change in withdrawal threshold (in grams) calculated by subtracting the time-zero mean measurements from the time interval mean measurements.

Assessment of apoptosis

Mice were given an injection of MSU crystals (3 mg in 0.5 ml PBS, i.p.). After 15 h they were killed by CO_2 administration, and cells from the peritoneal cavity were harvested by washing the cavity with 3 ml RPMI 1640 medium containing 10% FBS. The cells were incubated with anti-Gr1-PE (BD Biosciences, Franklin Lakes, NJ, USA), 7AAD, and annexin V-FITC, to allow quantification of apoptotic neutrophils. The cells were acquired on an FACS Canto 1 system (BD Biosciences) and analyzed with FlowJo software (Tree Star, Ashland, OR, USA). Alternatively, neutrophils were isolated from human peripheral blood by using Histopaque gradient (Histopaque 11191 and 10771; Sigma-Aldrich, St. Louis MO, USA). Neutrophils (1×10^6 cell per well) were resuspended in RPMI 1640 medium, seeded in 96-well culture plates (BD Biosciences), and incubated for 18 h at 37°C in a 5% CO_2 atmosphere. Cell viability was determined by trypan blue staining, and the purity of the preparations was 95%. Neutrophils were treated with acetate, and the apoptosis was assessed morphologically. For analysis of apoptotic morphology, cytospin preparations of neutrophils were stained with May-Grünwald-Giemsa. The typical morphologic changes indicating apoptosis, such as cell shrinkage and chromatin condensation-yielding fragments, were recorded in at least 300 cells per slide. The data were reported as the percentage of apoptotic neutrophils.

Efferocytosis assay

BMDMs were obtained from the tibia and femur of adult C57Bl/6 mice. Cells in RPMI 1640 medium (2.0×10^6 cells/ml) were plated and incubated at 37°C in humidified 5% CO_2 for 7 d. Thioglycollate elicited neutrophils from transgenic mice expressing GFP (C57B6 GFP Het) were collected 18 h after intraperitoneal administration. GFP^+ neutrophils (1.5×10^6 cells/well) were incubated with or without acetate at the final concentration of 25 mM. After 18 h of incubation, the neutrophils were washed with PBS and added to the macrophages at a proportion of 5 neutrophils to 1 macrophage. After 30 min of incubation, nonphagocytosed neutrophils were removed by successive washes with PBS (3 times, 1 ml). The adherent macrophages were harvested with a cell scraper and cold PBS. The cells were then stained with antibody anti-F4/80 allophycocyanin (Biolegend, San Diego, CA, USA). After 20 min of incubation, the cells were washed with PBS and then analyzed by flow cytometry (Gallius; Beckman Coulter, Brea, CA, USA). A total of 10,000 events were recorded, and $F4/80^+$ cells were gated and then evaluated for the presence of GFP. Results are expressed as the percentage of F4/80 and GFP^+ cells in the $F4/80^+$ population. For cytokine production, neutrophils were removed after 1 h of incubation with macrophages and stimulated with LPS (1 µg/ml). The supernatants were collected 24 h later by ELISA (R&D Systems, Minneapolis, MN, USA).

Analysis of NF-κB activation and AnxA1

NF-κB activation was investigated through Western blot analysis of nuclear and cytosolic extracts obtained from inflammatory cells harvested from mice 6 h after intraperitoneal injection of MSU crystals into the peritoneal cavity [19]. The protein concentration of the lysates was determined by Bradford assay reagent (Bio-Rad, Hercules, CA, USA). Samples (30 µg) were separated by electrophoresis on a denaturing 10% SDS-polyacrylamide gel and transferred onto nitrocellulose membranes, blocked overnight, and incubated with rabbit anti-p65/*RelA* (C-20/sc-372; Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse anti-phospho-IκB-α (Cell Signaling Technology, Beverly, MA, USA), or rabbit anti-AnxA1 (Thermo Fisher Scientific, Waltham, MA, USA) or anti-β-actin (Sigma-Aldrich). After they were washed, the membranes were incubated with appropriate horseradish peroxidase–conjugated secondary antibody (Santa Cruz Biotechnology). Immunoreactive bands were visualized by using an enhanced chemiluminescence detection system, as described by the manufacturer (GE Healthcare, Piscataway, NJ, USA). The values of intact AnxA1 were quantified by using densitometric analysis software (ImageJ; National Institutes of Health, Bethesda, MD, USA). Changes in protein levels were estimated, and results are expressed as intact AnxA1, P-IκB-α, or p65/RelA (in arbitrary units), normalized to the levels of β-actin in the same sample.

Acetate measurement

Serum was isolated from blood harvested by heart puncture of mice fed for 14 d on LF or HF diets. Serum acetate measurement was performed by NMR at 20–22°C at the Bio21 at Melbourne University. Serum were dissolved in 0.6 ml of deuterium oxide (Goss Scientific Instruments Ltd., Ingatestone, United Kingdom), and 10 µl D_2O containing 10 mM 3-trimethylsilyltetradeuterosodium propionate (Goss Scientific Instruments) was added as a concentration and chemical shift standard for NMR. Acetate concentrations were calculated after baseline adjustment from the peak height of selected signals relative to the signal height of the internal standard 3-trimethylsilyltetradeuterosodium propionate with correction for the number of protons contributing to each signal.

Cytokines and myeloperoxidase determination

The concentrations of CXCL1/KC, IL-10, and TGF-β in the supernatant of knee tissue were quantified with ELISA kits, in accordance with the manufacturer's instructions (R&D Systems). The knee tissue was homogenized (for each 100 mg of tissue, 1 ml of solution was added) in PBS (0.4 M NaCl and 10 mM $NaPO_4$) containing protease inhibitors (0.1 mM PMSF, 0.1 mM benzethonium chloride, 10 mM EDTA, and 20 KI aprotinin A) and 0.05%

Tween 20. The samples were then centrifuged for 10 min at 3000 g, and the supernatant was immediately used for the ELISA assays. In brief, 50 μl dilutions of cell-free supernatants were added in duplicate to the ELISA plates coated with a specific murine polyclonal antibody against the cyto/chemokine, followed by the addition of a second polyclonal antibody against the cyto/chemokine. After washing to remove any unbound antibody–enzyme reagent, a substrate solution (a 1:1 solution of H_2O_2 and tetramethylbenzidine) was added to the wells. The reaction was terminated with 50 μl/well 1 M H_2SO_4. The plates were read at 492 nm in a spectrophotometer (E_{max}; Molecular Devices, Sunnyvale, CA, USA). Standards were 0.5 $\log_{10}$ dilutions of recombinant murine chemokines from 7.5 to 1000 pg/ml (100 μl). The results are expressed as picograms per milligram of tissue.

Statistical analysis

All results are presented as means ± SEM. Data were analyzed for normal distribution by using Bartlett's test. All experiments were repeated at least 3 times. Data were analyzed by 1-way ANOVA, and differences between groups were assessed using the Student-Newman-Keuls post hoc test. Results were considered significant at $P < 0.05$.

RESULTS

An HF diet decreased MSU-induced inflammation in the joints of mice

The injection of MSU crystals in the knee joint of mice induced an inflammatory response characterized by neutrophil influx, neutrophil-dependent tissue injury, and articular dysfunction [3]

(**Fig. 1**). To examine the effects of dietary fiber on neutrophil influx, mice were fed an HF or LF diet for 2 wk before injection of MSU crystals into the knee joint. As seen in Fig. 1, there was comparable recruitment of total cells/neutrophils at 6 h after MSU challenge in mice given an LF or HF diet (Fig. 1A, B). However, there were significantly fewer neutrophils in the joints of mice fed an HF diet at 15 h after MSU challenge (Fig. 1B). No difference was observed between the groups in the number of mononuclear cells in the joint (Fig.1C). At 15 h, tissue from mice fed an HF diet showed reduction in joint destruction (Fig. 1D) and decreased joint dysfunction, as assessed by measuring hypernociception (Supplemental Fig. 2) and IL-1β production (Fig.1E).

Pectin-rich fiber diets (Supplemental Table 1), as used in our studies, are known to at least double systemic levels of acetate and others SCFAs [20, 21]. In this study, mean levels of acetate in the serum of mice fed a diet rich in fiber (90.6 μM) were higher than those found in mice fed an LF diet (22.2 μM). Therefore, we examined the effects of the SCFAs acetate, propionate, and butyrate in the recruitment of neutrophils after MSU challenge. As we observed in Fig. 1F, similar results were obtained with all these 3 SCFAs. Altogether, these results suggest that the onset of the inflammatory response induced by MSU crystals is not altered by an HF diet. In contrast, the HF diet induces faster resolution of the inflammatory response, and the beneficial effect may be related to SCFAs.
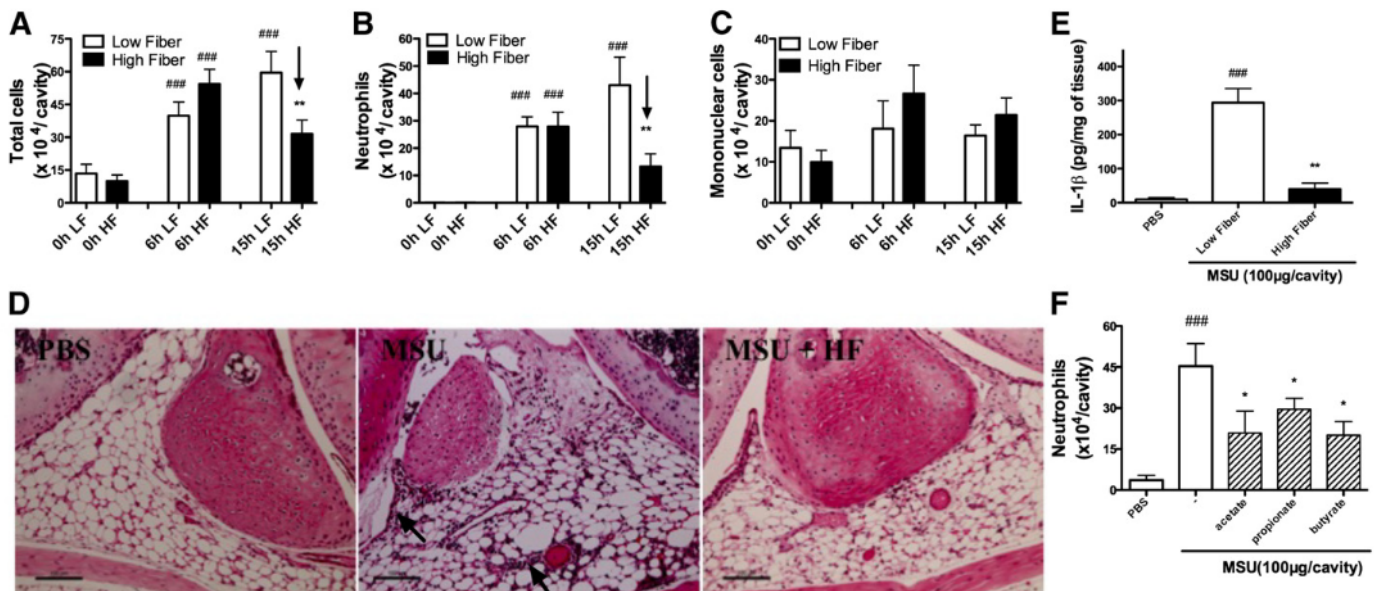

Figure 1. An HF diet decreases knee inflammation and pathology after MSU-induced gout. (A–E) Two groups of mice were fed different diets (HF and LF), 2 wk before MSU crystal injection in the knee joints. The mice were killed 0, 6, and 15 h after i.a. MSU challenge, the knee articular synovial fluid collected, and the number of total cells (A), neutrophils (B), and mononuclear cells (C) counted. (D) Representative photographs of H&E-stained sections of knee joints of animals after 15 h injected with vehicle or MSU crystals (100 μg/joint). Note an increase in leukocyte infiltration and hyperplasia of the synovial membrane after MSU crystal injection indicated by the black arrows. (E) Mice were killed 15 h after i.a. MSU injection, and the knee periarticular tissue was harvested for IL-1β measurement by ELISA. (F) Mice were treated with acetate (150 mM) in the drink water and butyrate (50 mM) or propionate (25 mM) by single oral gavage (per day) during the 5 d before MSU challenged. All groups of mice were killed 15 h after i.a. injection of MSU (100 μg/cavity) and the cells were harvest from the knee cavity and processed for leukocyte counting. Results are means ± SEM of 6 mice in each group in at least 3 independent experiments. *$P < 0.05$ vs. untreated MSU-challenged mice; **$P < 0.01$ HF 15 h vs. LF 15 h; ###$P < 0.001$ vs. the respective group given PBS.

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The SCFA acetate decreases neutrophil accumulation at late stages after injection of MSU crystals

High levels of acetate in the colon and in blood relate to fiber intake, and the presence of certain commensal bacterial species in the gut [22, 23]. Acetate is the most abundant SCFA that reaches high systemic levels. In this study, we observed that oral pretreatment with acetate induced a dose-dependent reduction in MSU crystal-induced neutrophils in the knee cavity (**Fig. 2A**) and reduced hypernociception (Fig. 2B) at 15 h after injection of the stimulus, as compared to mice receiving MSU crystals alone. Treatment with acetate had no significant effects on the number of mononuclear cells recruited to the cavity (Supplemental Fig. 3). Next, we performed experiments to examine closely the effects of acetate treatment on neutrophil influx observed at various times after injection of MSU crystals. Acetate treatment did not decrease significantly the recruitment of neutrophils in the early phase of the inflammatory response (Fig. 2C), but significantly decreased the number of neutrophils at 15 h after injection of the MSU crystals.

In mice, CXCL1 is necessary for the recruitment of neutrophils after injection of MSU crystals into the knee joint [3, 24, 25]. Injection of MSU crystals induced a significant increase in CXCL1 levels in the synovial fluid of MSU crystal–treated control

mice (Fig. 2D). A similar increase in chemokine levels was observed over the first 3 h in acetate-treated mice, but CXCL1 levels declined rapidly at 6 h when compared to levels in the MSU crystal–treated controls (Fig. 2D). Acetate treatment reduced IL-1β levels at 3 h after MSU challenge (Fig. 2E).

To examine the effects of delayed administration of acetate, we gave oral acetate to mice 6 h after MSU challenge, at the peak of CXCL1 production. We showed that delayed treatment with acetate was still able to decrease the number of neutrophils in the knee cavity compared with the number in untreated mice (**Fig. 3A**), without affecting the infiltration of mononuclear cell (Fig. 3B). These results indicate that acetate treatment controls gout inflammation when administered either preventively or therapeutically.

Acetate treatment induces caspase-dependent neutrophil apoptosis

Apoptosis is essential for the safe clearance of inflammatory cells, including neutrophils, during the resolution of inflammation [26–28]. In addition, as shown in **Fig. 4A** and previously [12], acetate induced apoptosis of human and murine neutrophils in vitro. To evaluate the effects of acetate on neutrophil apoptosis in vivo, we used a model of MSU crystal–induced inflammation in
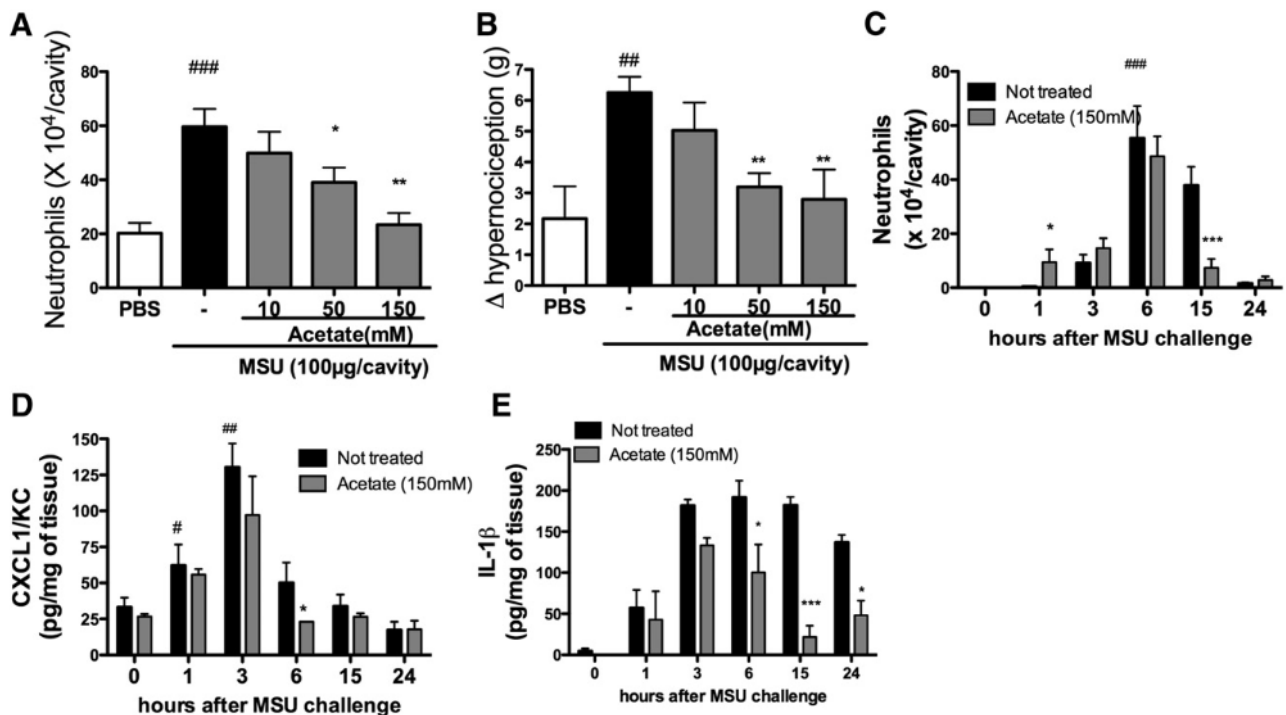

Figure 2. High concentrations of acetate (SCFA) reduced MSU-induced articular inflammation and dysfunction and resolved neutrophilic inflammation. (A, B) Mice were injected with MSU crystals (100 μg/joint) and the inflammatory parameters assessed 15 h later. Different doses of acetate (mM) were added to the drinking water of mice 5 d before MSU (100 μg/ joint) injection. Tissue inflammation was accompanied by neutrophil recruitment (A) and joint dysfunction (B), as noted by the increased nociceptive responses of animals to mechanical stimulation after MSU injection (horizontal line). (C, D) Mice received 150 mM of acetate in the drinking water 5 d before MSU and until death. (C) The number of neutrophils in the synovial cavity was assessed at different time points (0–24 h) after injection of MSU. CXCL1/KC (D) and IL-1β (E) levels were measured in the periarticular knee tissue by ELISA. Results are expressed as means ± SEM of 6–8 mice per group in at least 3 independent experiments. #P < 0.05; ##P < 0.01; ###P < 0.001 vs. animals injected with PBS or at time zero. *P < 0.05; **P < 0.01; ***P < 0.001 acetate-treated group vs. untreated group injected with MSU.
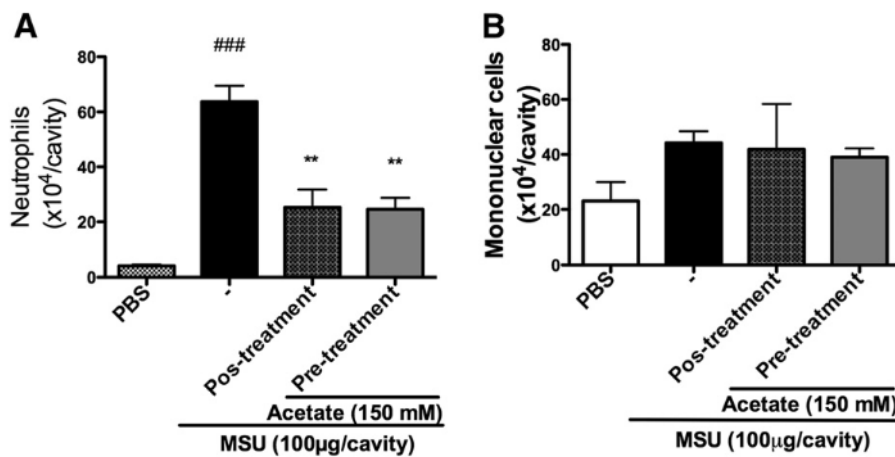
Figure 3. Both pre- and postacetate treatment decreased the number of neutrophils in the knee cavity induced by MSU injection and increased macrophages uptake of MSU crystals. Neutrophils (A) and mononuclear cells (B) were harvested from the knee cavity 15 h after MSU i.a. injection and counted. Posttreatment with acetate was performed by oral gavage 6 h after MSU injection. Acetate-pretreated mice received acetate (150 mM) in the drinking water 5 d before MSU injection and until they were killed. Results are means ± SEM of 6 mice per group in at least 2 independent experiments. $^{###}P < 0.001$ vs. PBS-injected mice; $^{**}P < 0.01$ vs. MSU-injected mice.

the peritoneum, which allowed sampling of more cells for apoptosis assays and for local evaluation of mediators of inflammation. Mice treated with acetate and then challenged with MSU crystals showed a significant increase in the number of neutrophils undergoing apoptosis when compared with MSU-challenged mice given vehicle (Fig. 4B, C). The capacity of acetate to resolve inflammation and induce neutrophil apoptosis was not restricted to using MSU crystals as an inflammatory stimulus, as acetate also resolved inflammation and induced neutrophil apoptosis when given at the peak of LPS-induced inflammation (Supplemental Fig. 4).

Part of the effects of acetate may occur via activation of the G-protein coupled receptor, Gpr43. However, we have recently demonstrated that MSU-induced inflammation in Gpr43-deficient mice is impaired [29]. Therefore, it was not possible to study the relevance of Gpr43 for the resolution of MSU crystal–induced inflammation. Nonetheless, we collected peritoneal neutrophils after 6 h of MSU challenge and evaluated these cells for apoptosis (Fig.4C). Acetate increased the percentage of apoptotic neutrophils in wild-type mice, but this effect was not observed in Gpr43-deficient mice challenged with MSU and treated with acetate. In an attempt to determine whether Gpr43 activation may be involved in the effects of an HF diet on the resolution of inflammation, we used the model in which LPS is injected into the pleural cavity. This model allows for better recovery of cells and would confirm the proresolving effects of acetate in other vascular beds. Feeding an HF diet for 14 d reduced LPS-induced neutrophil influx in the pleural cavity of mice but increased the number of apoptotic cells (Supplemental Fig. 5). Of note, the HF diet did not impair the recruitment of neutrophils; we observed a similar number of neutrophils in the pleural cavity at 4 h after LPS injection in mice given an LF or HF diet (Supplemental Fig. 6). The effects of an HF diet were not observed in Gpr43-deficient mice (Supplemental Fig. 5), suggesting that the proresolving effects of the HF diet are, in great part, GPR43 dependent.

Neutrophil apoptosis is controlled by a complex network of signaling pathways, including activation of the caspase family of proteases [28, 30]. In the current study, acetate induced neutrophil apoptosis and administration of the broad-spectrum caspase inhibitor z-VAD-fmk prevented acetate-induced apoptosis of neutrophils (Fig. 4B). These results indicate that apoptosis of neutrophils induced by acetate is caspase dependent.

The transcription factor NF-κB is a crucial regulator of granulocyte apoptosis mediation of cell survival, and thus, their timely modulation contributes to the resolution of inflammation [19, 31, 32]. To investigate the importance of NF-κB to mediate neutrophil accumulation/survival in this gout model, we used gliotoxin, a nonspecific NF-κB, inhibitor largely known to prevent IκB degradation (Supplemental Fig. 7). Treatment with gliotoxin 6 h after MSU injection (*i.e.*, after inflammation had been established) reduced neutrophil accumulation in the knee cavity and increased neutrophil apoptosis. Gliotoxin treatment had no significant effects on the number of mononuclear cells (Supplemental Fig. 7). Next, we examined whether acetate-induced resolution was associated with NF-κB modulation. Cells harvested 6 h after MSU crystal peritoneal challenge showed decreased p65 nuclear translocation and IκBα phosphorylation in animals treated with acetate (Fig. 4D). Thus, increased apoptosis after acetate treatment was associated with down-modulation of NF-κB.

Acetate treatment induces mediators of anti-inflammation and resolution

AnxA1, a glucocorticoid-regulated protein, promotes resolution of inflammation by inducing apoptosis of neutrophils [33, 34] and increasing efferocytosis, two important events in the resolution program. In the present study, the expression of the intact form of AnxA1 increased markedly in cells recovered from the peritoneal cavity of mice treated with acetate 6 h after MSU challenge, compared with those from untreated mice (**Fig. 5A, B**).

In mice injected with MSU crystals and treated with acetate, there was also a significant increase in the levels in the knee tissue of two anti-inflammatory cytokines: TGF-β and IL-10 (Fig. 5C, D). The ability of acetate to enhance production of both IL-10 and TGF-β may contribute to the capacity of this SCFA to resolve inflammation, but further studies are necessary to indicate exact sources and relevance of these mediators.
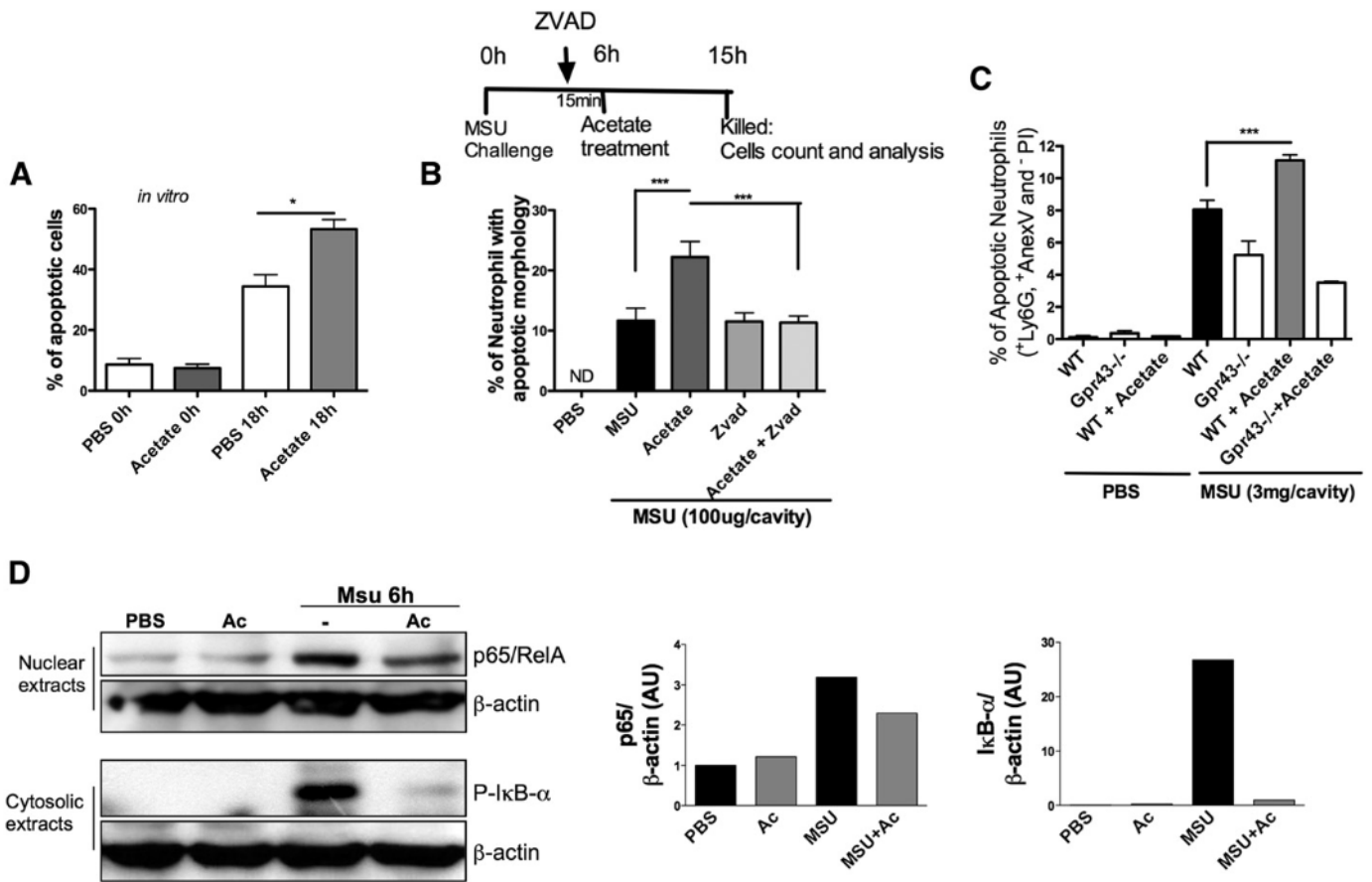
Figure 4. Acetate induces caspase-mediated neutrophil apoptosis associated with NF-κB inhibition in MSU-challenged mice. (A) Percentages of apoptotic neutrophils were identified by morphologic changes after 0 and 18 h of neutrophils isolated from PBMCs from human donors and after incubation at 37° and 5% CO_2. The cells were treated with PBS or acetate at 25 mM. (B) Mice received an i.a. knee injection with MSU and 6 h later, received a 100 μl oral gavage of acetate (150 mM). The pan caspase inhibitor z-VAD-fmk (1 mg/kg, i.p.) was given 15 min before the acetate treatment. The percentage of apoptotic neutrophils was evaluated by morphology, and the cells were harvested 15 h after MSU challenge. (C) Mice were injected with MSU crystals (3 mg/cavity) intraperitoneally. After 15 h, peritoneal cells were harvested and stained for Gr-1, 7AAD, and annexin V and analyzed by flow cytometry. Percentages of apoptotic neutrophils were identified as GR-1^{high} and annexin V$^+$. (D) Cells were harvested from the peritoneal cavity of mice 6 h after MSU (3 mg/cavity) injection in the peritoneum. Nuclear and cytosolic extracts were fractionated on 10% SDS-polyacrylamide gels, transferred onto nitrocellulose membranes, and then probed with anti-p65 or anti-phospho-IκB-α antibodies, respectively. Membranes were reprobed with anti-β-actin for loading control. Blots were performed of pooled cells from at least 6 animals per group. Results for in vivo experiments are means ± SEM of 6 biologic samples from each group and represent at least 2 independent experiments. For the in vitro apoptosis assay, pooled cells from at least 6 animals per group were used and replicates of 5 wells were analyzed. *$P < 0.05$; ***$P < 0.001$.

Efferocytosis is enhanced in mice treated with both acetate and an HF diet

An increase in neutrophil apoptosis without efficient efferocytosis of cell remnants may perpetuate inflammation. In HF diet-fed mice, the percentage of efferocytosis after MSU injection was increased as compared with that in mice fed LF (**Fig. 6A**). In mice treated with acetate and then challenged with MSU crystals, there was also a significant increase in percentage of efferocytosis, compared with that in untreated mice (Fig. 6B). A representative example of efferocytosis is shown in Fig. 6, with the black arrow indicating an apoptotic neutrophil inside the macrophage. In vitro, engulfment of neutrophils by macrophages was higher when the neutrophils had been exposed to acetate (Fig. 6C). Furthermore, macrophages that engulfed neutrophils treated with acetate produced lower levels of IL-1β (Fig. 6D) but increased levels of IL-10 (Fig. 6E) after stimulation

with LPS. These effects on cytokine production were not observed in macrophages incubated with acetate or the supernatant of neutrophils incubated with acetate and stimulated with LPS (Supplemental Fig. 8). These findings suggest that the modulatory effect of acetate on macrophages depends on neutrophil internalization. Thus, in addition to promoting neutrophil apoptosis, SCFAs also enhanced the efferocytosis of apoptotic neutrophils by macrophages.

DISCUSSION

The major findings of our study can be summarized as follows: (1) an HF diet or acetate did not affect the onset of gout (and LPS-induced) inflammation, but resolved inflammation by inducing caspase-dependent neutrophil apoptosis; (2) apoptosis of neutrophils was associated with inhibition of NF-κB and
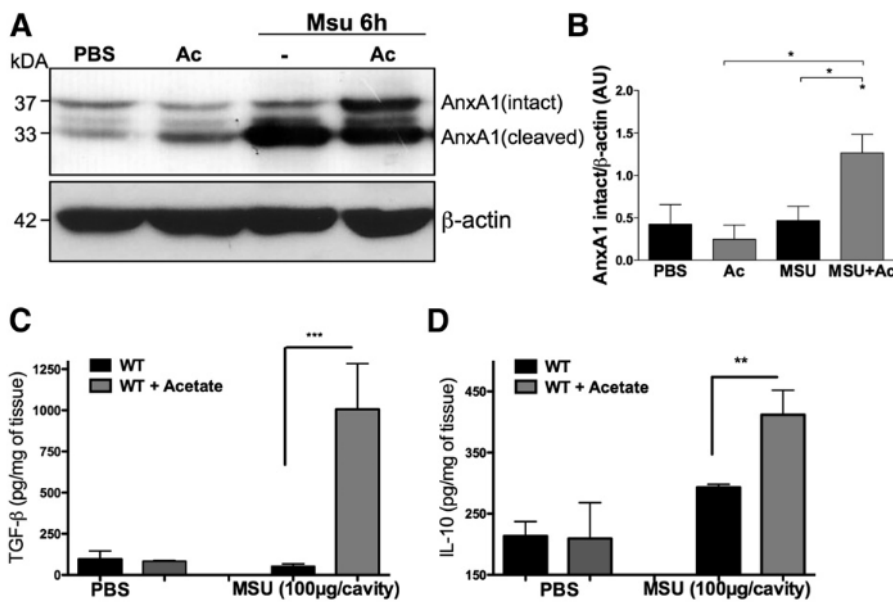
Figure 5. Acetate treatment increases proresolution mediators after injection of MSU crystals. (A) Cells were harvested from the knee cavity of mice 6 h after MSU injection. Cell extracts were fractionated on 10% SDS-polyacrylamide gels, transferred onto nitrocellulose membranes, and probed with anti-AnxA1. Membranes were reprobed with anti-β-actin for loading control and (B) densitometric analyses were performed. (C, D) TGF-β and IL-10 cytokines were measured by ELISA of the periarticular tissue of mice 15 h after MSU injection. Results are means ± SEM of 6 biologic samples from each group in at least 2 independent experiments. The blot is representative of 3 independent experiments conducted on pooled cells from at least 5 animals in each experiment. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

induction of mediators of anti-inflammation/resolution, including IL-10, annexin-1, and TGF-β and (3) enhanced efferocytosis.

Dietary fiber has been reported to be beneficial in numerous diseases, although the mechanisms behind the action of fiber are still poorly understood [21, 35]. In our system, treatment of mice with an HF diet decreased inflammatory responses to MSU crystals. The HF diet used in our experiments is known to enhance levels of various SCFAs, including acetate [21]. The effects of the administration of an HF diet were similar to those of acetate treatment: there was no change in the onset of inflammation but faster resolution of inflammatory responses. This result is in line with studies showing that SCFAs modify the function of the immune system [20, 35, 36].

We could not formally prove that acetate and other SCFAs were indeed the contributing factor for the beneficial effects of diet. However, our experiments did show that Gpr43, a receptor for SCFAs, contribute to the beneficial effects of the diet. Indeed, the proresolving effects of both acetate and HF diet were decreased in Gpr43-deficient mice stimulated with LPS. It was not possible to evaluate the relevance of Gpr43 in the context of gout, as inflammasome assembly is defective in the gut and joint of these animals [21, 29]. Indeed, Gpr43 sensing of SCFAs provides a complementary set of priming signals necessary for inflammasome assembly in the joint of germ-free mice [21, 29], but experiments in LPS-injected mice clearly showed the partial relevance of GPR43. Therefore, it is clear that HF diet and acetate decrease inflammation induced by MSU crystals or LPS, by inducing resolution of inflammation, but not by preventing the influx of cells. SCFAs may bind to other G-protein-coupled receptors in addition to Gpr43, including Gpr41 and -109A. GPR41 selectively couples with Gαi, which inhibits adenylyl cyclase, so that its activation by SCFAs decreases the intracellular concentration of cAMP, which it is also important to promote cell apoptosis [19, 28]. In addition, butyrate-induced GPR109a activation inhibits colon cancer growth by inducing apoptosis via increased MCT-1 expression and subsequent increase of butyrate transport SMCT-1

into the cell [37]. The relevance of the latter two receptors for the proresolving effects of diet was not investigated in this study, but our system showed a contribution of Gpr43.

These findings add to the concept that SCFAs are important regulators of immune cell function and inflammatory responses [12, 13, 38]. Most of our experiments have been conducted using acetate, as this is the SCFA that reaches the highest concentration in blood. Butyrate and propionate are other SCFAs produced in response to an HF diet, and both molecules were found to decrease the number of neutrophils after injection of MSU crystals. Therefore, it is not possible to pinpoint the single most relevant SCFA that is accountable for the effects of diet on the resolution of inflammation. Our results, however, showed that the effects of diet are in part Gpr43-dependent, suggesting that acetate, because of its higher levels and affinity for Gpr43, is the most relevant SCFA.

Resolution of inflammation is now widely recognized as an active process regulated by various factors [39, 40]. Timely removal of neutrophils is thought to be essential for resolution of inflammation and avoidance of tissue injury and dysfunction [41, 42]. In the present study, acetate induced apoptosis of neutrophils in vitro, an effect that is consistent with other studies showing that acetate is able to induce apoptosis of bone marrow–derived mouse neutrophils by activation of Gpr43 [12]. Our results also showed that acetate can induce apoptosis of neutrophils in vivo and that apoptosis contributes to the ability of acetate to resolve the inflammatory response. Akin with this finding, SCFAs, such as butyrate, possess antitumor activity by inducing caspase-3-mediated apoptotic cell death [43, 44]. Therefore, the capacity of acetate to resolves inflammation was associated with the induction of caspase-dependent neutrophil apoptosis.

In addition, pharmacological inhibitors of NF-κB, such as gliotoxin, induced resolution of inflammation in our system by causing apoptosis of neutrophils, an effect also reported in other systems [31, 32]. Treatment with acetate reduced translocation of the NF-κB p65/*RelA* subunit to the nucleus, an effect consistent with
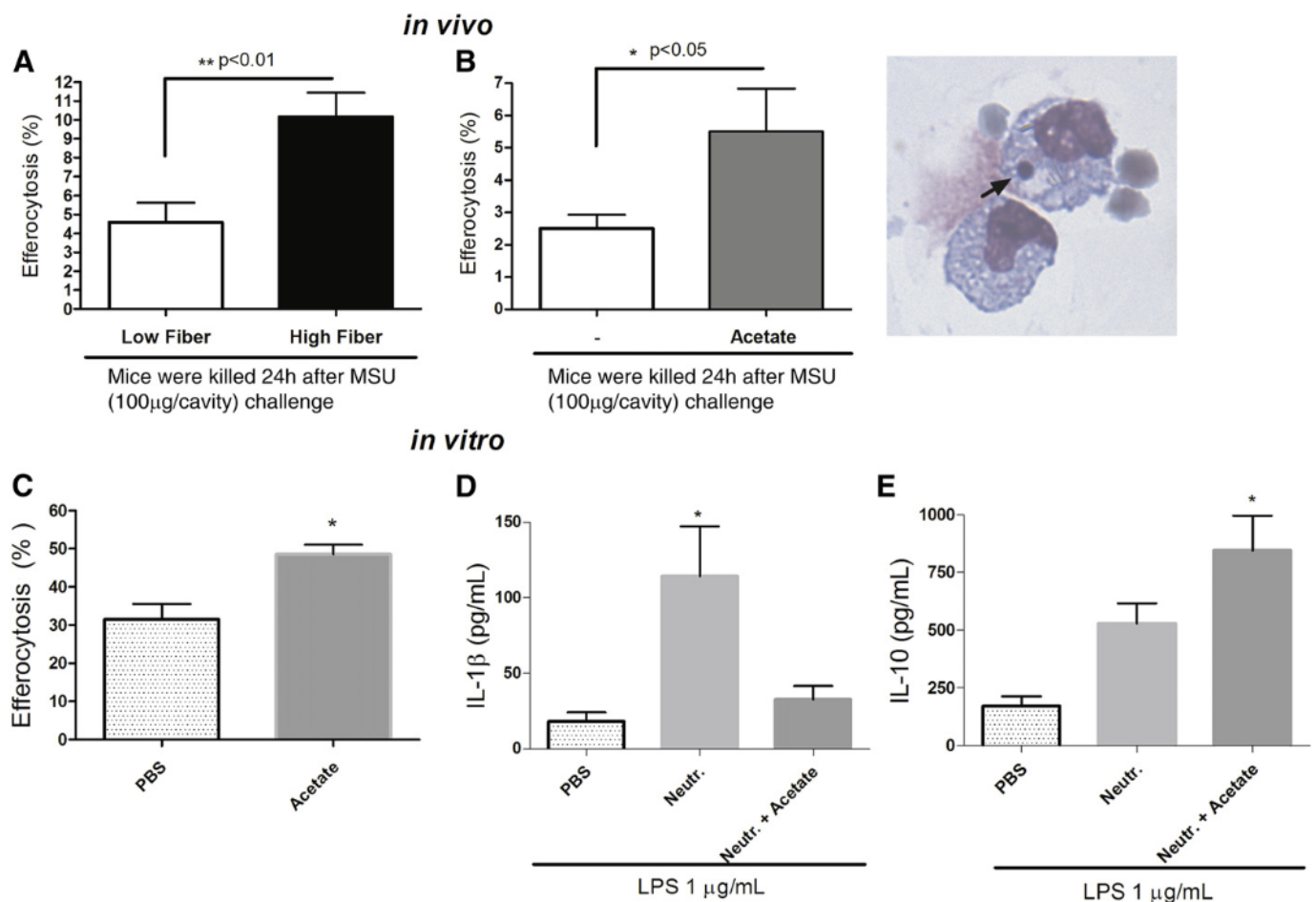
Figure 6. Acetate and HF diet improves efferocytosis by macrophages. (A, B) Macrophages with distinctive efferocytic morphology (presence of apoptotic bodies inside macrophages) were evaluated 24 h after an i.a. MSU injection in the knee. Image shows macrophages with apoptotic bodies inside (arrow). (C) Percentage of BMDMs with apoptotic neutrophils. Macrophage engulfment of neutrophils previously incubated with acetate or PBS was analyzed by flow cytometry (D, E) and production of IL-1β (D) and IL-10 (E) by efferocytic macrophages stimulated with LPS for 24 h. Results are presented as means ± SEM of 5–8 mice per group and in at least 2 independent experiments.

the inhibition of NF-κB function in inflammatory cells from mice challenged with MSU crystals. Therefore, control of NF-κB translocation may contribute to the proresolving effects of acetate.

Efficient resolution of inflammation requires not only the shutting down of proinflammatory pathways but also active production of proresolving and anti-inflammatory molecules, such as AnxA1, TGF-β, and IL-10 [45, 46]. The production of these molecules may in turn be enhanced after the clearance of apoptotic cells by phagocytes. We had shown that strategies that cause neutrophil apoptosis and consequent resolution of inflammation can increase levels of proresolving mediators in mice [28, 33]. Elevated levels of TGF-β are found in synovial fluid from patients with gout and related with spontaneous resolution of gouty arthritis [47]. In addition, phagocytosis of apoptotic neutrophils by macrophages is commonly associated with a switch of macrophage phenotype to increased production of IL-10 and TGF-β, the cytokines associated with resolution and tissue repair [48–50]. In vivo treatment with acetate resulted in the release of AnxA1, IL-10, and TGF-β in response to the injection of MSU crystals. In vitro, treatment with acetate also induced the

production of the anti-inflammatory molecule IL-10. The relevance of individual molecules for the effects of acetate were not investigated, but our results clearly suggest that exposure to acetate favors the production of mediators of anti-inflammation/resolution and, consequently, a proresolving environment.

Efferocytosis by macrophages is an important step in the resolution of inflammation and return of tissue to homeostasis. Indeed, defective efferocytosis may be associated with persistent tissue inflammation (40). Acetate treatment not only increased neutrophil apoptosis but also induced phagocytic clearance of these cells. Although it was not shown in this study, we observed that acetate increases the RNA expression (by a 4-fold difference) of the M2 macrophages markers Arginase1 and Fizz1 (data not shown) in thioglycollate-elicited peritoneal macrophages. This result suggests that modulation of M2 macrophages by acetate reduces inflammation by increasing the phagocytic ability of the macrophages. Furthermore, efferocytosis is closely linked with the generation of a proresolving environment, suggesting that this effect contributes significantly to the overall benefits of acetate treatment in our system.

Our experiments clearly show that acetate, in part via Gpr43, induced apoptosis of neutrophils and resolution of inflammation in vivo. In contrast, we have recently shown that Gpr43-deficient mice were hyporesponsive to the injection of MSU crystals into the knee joint [29]. Indeed, acetate by acting on Gpr43 contributed to NALP3 inflammasome activation. Therefore, as discussed before [51], SCFAs have opposing roles in MSU-induced inflammasome-dependent inflammation: SCFAs are necessary for inflammasome assembly and consequent tissue inflammation [21, 29, 51], but also contribute to the resolution at the later stages of inflammation by inducing caspase-dependent apoptosis of neutrophils (as shown in our study). Under normal conditions, the amount of SCFAs is sufficient for inflammasome assembly in response to MSU crystals [21, 29]. In germ-free mice, availability of SCFA is limiting and, hence, inflammasome assembly is deficient. In these mice, gout inflammation in the knee is impaired, and this may be perceived as beneficial [29]. In the gut, absence of microbiota and SCFAs lead to impaired inflammasome assembly in epithelial cells, leading to inability to deal with noxious stimuli, such as dextran sodium sulfate [21]. In mice given excess SCFAs (oral acetate or HF diet), inflammasome assembly is in great part comparable to that of mice fed a normal diet, although we did see a minor facilitation of neutrophil influx in the joint after MSU crystal injection (see Fig. 1). However, in presence of excess SCFA, resolution of inflammation is greatly facilitated, as shown here. Therefore, the net effects of the availability of SCFAs will depend on the tissue involved and the amount of SCFAs available in the system, which in turn depend on our diet.

In conclusion, our results clearly show that the SCFA acetate regulates acute inflammation by promoting caspase-dependent neutrophil apoptosis, efferocytosis, and consequent resolution of inflammation. These results place acetate as a novel mediator of resolution of inflammation [26]. Similar proresolving effects were attained in animals treated with an HF diet known to induce elevated levels of SCFAs. Therefore, these studies suggest that what we eat plays a determinant role in our capacity to fine tune the inflammatory response and that acetate-mediated resolution of inflammation contributes to these effects.

AUTHORSHIP

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DISCLOSURES

The authors declare no conflicts of interest.

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