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A Role for Gut Microbiota and the Metabolite-Sensing Receptor GPR43 in a Murine Model of Gout

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Objective. **Host–microbial interactions are central in health and disease. Monosodium urate monohydrate (MSU) crystals cause gout by activating the NLRP3 inflammasome, leading to interleukin-1β (IL-1β) production and neutrophil recruitment. This study was undertaken to investigate the relevance of gut microbiota, acetate, and the metabolite-sensing receptor GPR43 in regulating inflammation in a murine model of gout.**

Methods. **Gout was induced by the injection of MSU crystals into the knee joints of mice. Macrophages from the various animals were stimulated to determine inflammasome activation and production of reactive oxygen species (ROS).**

Results. **Injection of MSU crystals caused joint inflammation, as seen by neutrophil influx, hypernociception, and production of IL-1β and CXCL1. These parameters were greatly decreased in germ-free mice, mice treated with antibiotics, and GPR-43–deficient mice. Recolonization or administration of acetate to germ-free mice restored inflammation in response to injection of MSU crystals. In vitro, macrophages produced ROS and assembled the inflammasome when stimulated with MSU. Macrophages from germ-free animals produced little ROS, and there was little inflammasome assembly. Similar results were observed in macrophages from GPR-43–deficient mice. Treatment of germ-free mice with acetate restored in vitro responsiveness of macrophages to MSU crystals.**

Conclusion. **In the absence of microbiota, there is decreased production of short-chain fatty acids that are necessary for adequate inflammasome assembly and IL-1β production in a manner that is at least partially dependent on GPR43. These results clearly show that the commensal microbiota shapes the host's ability to respond to an inflammasome-dependent acute inflammatory stimulus outside the gut.**

Gouty arthritis is an inflammatory disease triggered by the deposition of monosodium urate monohydrate (MSU) crystals in the joints and periarticular tissues. Activation of the NLRP3 inflammasome by crystals leads to the production of interleukin-1β (IL-1β), and these are key molecular events involved in the initiation of gout (1,2). IL-1β stimulates synovial cells and causes the release of chemoattractants, recruitment of inflammatory cells, and progressive tissue damage (3–6).

The inflammasome pathway also plays a beneficial role in intestinal homeostasis and is influenced by products of the gut microbiota (7–9). Germ-free mice, which lack an intestinal microbiota, exhibit altered

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inflammatory responses (10–12). We have shown recently that a normal gut microbiota is essential for the host to mount inflammatory responses, both local and systemic, in germ-free mice (12–14). Thus, the influence of the gut microbiota extends beyond the confines of the gut (15), although the mechanisms whereby gut bacteria might influence inflammasome activation in peripheral immune cells have not been studied.

A number of molecules are important for regulating the delicate interactions between the gut microbiota and the immune system. These include sensing by innate pattern-recognition receptors, including NOD-like receptors, Toll-like receptors (TLRs), and others. Recently, another class of sensors that detect metabolites derived from the microbiota (such as short-chain fatty acids) has emerged. These G protein–coupled receptors have been termed "metabolite-sensing" receptors and include GPR43, GPR41, GPR109A, and GPR120. GPR43 (also known as FFAR2) is highly expressed by polymorphonuclear cells and macrophages (12,16–18). Acetate is the most abundant short-chain fatty acid produced in the gut by commensal microbiota. It reaches high levels in the blood (12) and regulates several inflammatory events, in part, through interaction with GPR43 (12,19,20). We previously showed that inflammatory responses were exacerbated or nonresolving in GPR-43–deficient mice (12).

Thus, given the involvement of the gut microbiota, short-chain fatty acids, and their metabolic sensor GPR43 in inflammation, we assessed the function of these in a model of gut. We used both GPR-43–deficient mice (GPR-43$^{-/-}$) and germ-free mice to show that the initiation of an inflammatory response to MSU crystals in a gout model was intimately connected to the actions of acetate and GPR43. This was in part mediated by immune cell inflammasome activation and IL-1β secretion. Thus, the gut microbiota and their metabolites represent new elements that may influence the pathogenesis of gout.

MATERIALS AND METHODS

Animals. GPR-43/FFAR2 gene–deficient (GPR-43$^{-/-}$) mice were produced as previously described (12), backcrossed onto the C57BL/6 background for 10 generations, and maintained in the animal facilities at Universidade Federal de Minas Gerais (UFMG; Belo Horizonte, Brazil). Wild-type male C57BL/6 mice were obtained from UFMG. Germ-free NIH mice were purchased from Taconic and maintained under germ-free conditions at UFMG. These animals were housed in flexible plastic isolators (Standard Safety Equipment Company). All mice were housed in filter top cages, with sterilized water and food supplied ad libitum, and used for experiments at 8–12 weeks of age. The Garvan Institute of Medical Research/St. Vincent's Hospital and UFMG Animal Ethics Committee approved all procedures.

MSU crystal–induced gout. MSU crystals were prepared under pyrogen-free conditions by dissolving 5 mg/ml uric acid (Sigma-Aldrich) in 0.01*M* NaOH (pH 7.1) solution. The supersaturated uric acid solution was filtered (0.45 μm) and maintained at room temperature for 48 hours. Crystals were washed with 100% ethanol and sonicated to decrease their size. The amount of endotoxin present in the MSU crystals injected was <5 pg (at a dose of 100 μg of MSU), as assessed using a chromogenic *Limulus* amebocyte lysate assay (Endosafe). Mice were given an intraarticular injection of 100 μg of MSU crystals and killed after 0, 6, and 15 hours. The knee cavity was washed with phosphate buffered saline (PBS) and the number of leukocytes was determined. Periarticular tissues were used for evaluation of cytokines and myeloperoxidase (MPO) activity. Total leukocytes were determined in a Neubauer chamber after staining with Turk's solution. Differential cell counts were determined using standard morphologic criteria and were performed on May-Grünwald-Giemsa–stained slides. There were 6–8 animals in each experimental group, and all experiments were performed at least twice.

Acetate treatment. Mice were given sodium acetate (Sigma-Aldrich) for 5 days before the MSU challenge and until they were killed. Acetate was added in the sterile drinking water of mice ad libitum at 150 m*M* and pH was adjusted as needed. The water solution was changed every day to avoid contamination and significant changes.

Conventionalization of germ-free mice (microbiota reposition). Microbiota reposition was achieved by administration of feces from conventionally housed (conventional) mice to germ-free mice (13). Briefly, the feces removed from the large intestines of conventional mice were homogenized in saline (10%), and 100 μl of the feces suspension was administered to germ-free mice by oral gavage. After 21 days, animals were injected with MSU crystals, as described above. A thioglycolate test, which measures aerobic and anaerobic bacteria, showed similar numbers of bacteria in conventionalized and conventional mice (data not shown). Similarly, 16S ribosomal gene quantitative reverse transcriptase–polymerase chain reaction (qRT-PCR) was performed in fecal samples from conventionalized germ-free mice and conventional mice and showed similar 16S gene copies in both groups (data not shown).

Intestinal microbiota depletion using antibiotics. Mice were treated with a cocktail of broad-spectrum antibiotics in their drinking water continuously for 21 days prior to experimentation. Antibiotic treatment started with 3 days of amphotericin B (0.1 mg/ml every 12 hours) given by gavage, and then water flasks were supplemented with an antibiotic cocktail containing 2 gm/liter ampicillin, 2 gm/liter neomycin, 1 gm/liter metronidazole, and 0.5 gm/liter vancomycin. Additionally, all mice received an intraperitoneal injection of ciprofloxacin (0.2 gm/liter). There was no bacterial growth on fecal pellets collected on day 21, which were handled aseptically, and placed into thioglycolate medium (detection limit for this assay was set at 1 colony-forming unit [CFU]/mg feces). Only mice with <1 CFU/mg feces were defined as successfully depleted and used in further experiments.

Evaluation of hypernociception. Evaluation of hypernociception was performed as described previously (21). Animals were tested before and after challenge with MSU.

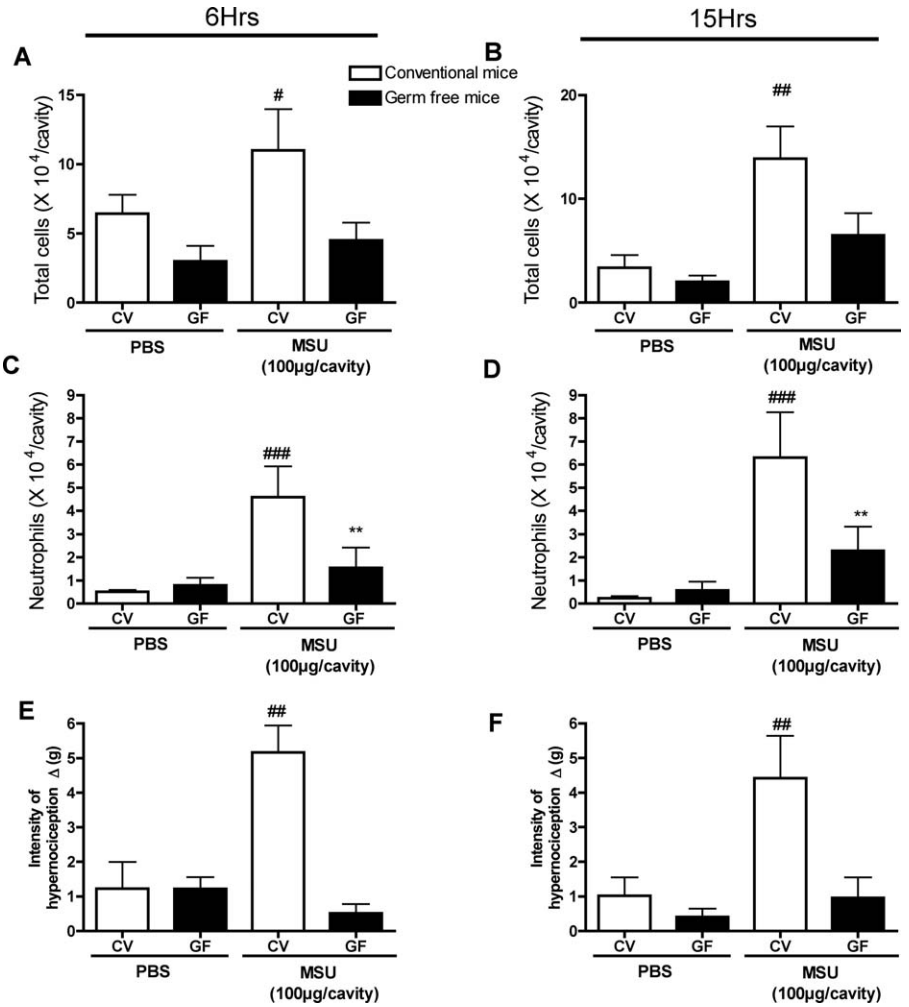

Figure 1. Poor response to injection of monosodium urate monohydrate (MSU) crystals into the knee joint in germ-free (GF) mice. Conventionally housed (conventional [CV]) mice and germ-free mice were injected with MSU crystals (100 μg into the knee joint). **A** and **B,** Total numbers of cells, as determined 6 hours (**A**) and 15 hours (**B**) after injection of MSU crystals. **C** and **D,** Numbers of neutrophils in the articular cavity, as assessed 6 hours (**C**) and 15 hours (**D**) after injection of MSU crystals. **E** and **F,** Intensity of hypernociception in the mouse knee, as determined 6 hours (**E**) and 15 hours (**F**) after injection of MSU crystals. Bars show the mean ± SEM (n = 5–7 mice per group). Each experiment was repeated twice. # = $P < 0.05$; ## = $P < 0.01$; ### = $P < 0.001$, versus mice injected with phosphate buffered saline (PBS); ** = $P < 0.01$ versus conventional mice injected with MSU crystals.

Briefly, mice were adapted to the environment and flexion-elicited withdrawal threshold was used to infer behavioral responses associated with pain using an electronic pressure meter (Insight Instruments). Results are expressed as the change in withdrawal threshold, calculated by subtracting zero-time mean measurements from mean measurements (in grams).

Macrophage culture. Macrophages were harvested from GPR-43$^{-/-}$ or wild-type control mice after intraperitoneal injection of 3% thioglycolate solution. Macrophages were cultured overnight in RPMI (Gibco) with 10% fetal calf serum (FCS; heat-inactivated), HEPES, 50 m*M* β-mercaptoethanol, penicillin/streptomycin, and L-glutamine. Triplicates of each condition were performed, and cells were stimulated in serum-free RMPI as indicated. For inflammasome activation, cells were primed with 1 μg/ml ultrapure lipopolysaccharide (LPS) (*Escherichia coli* serotype O111:B4; Sigma) for 2 hours (or with PBS as a control) and subsequently stimulated with 5 m*M* ATP disodium salt (Sigma) or 300 μg/ml MSU crystals for 4 hours. For Western blot analysis, macrophages were lysed and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed for pro–IL-1β and procaspase 1 with antibodies directed against murine IL-1β (R&D Systems) and caspase 1 (Santa Cruz Biotechnology), respectively. Membranes were stripped and reprobed for β-actin (Cell Signaling Technology). Cell culture supernatants were mixed at a ratio of 1:1 with lysis buffer and heated to 100°C for 5 minutes prior to SDS-PAGE and Western blot analysis for cleaved IL-1β and cleaved caspase 1 with antibodies directed against murine cleaved IL-1β (p17; R&D Systems) and cleaved caspase 1 (p10; Santa Cruz Biotechnology), respectively.

Cytokines and MPO determination. The concentrations of IL-1β and CXCL1/keratinocyte-derived chemokine

(KC) in the periarticular knee tissue and supernatants from macrophage cultures of mice were quantified by enzyme-linked immunosorbent assay (R&D Systems). MPO activity (a quantitative measurement of neutrophil sequestration) in periarticular tissue homogenates was assayed as previously described (22). Briefly, the periarticular tissue was removed from the mouse knee joint and snap-frozen in liquid nitrogen. Upon thawing and processing, the tissue was assayed for MPO activity, and the results were expressed as total number of neutrophils by comparing the optical density (OD) of tissue supernatant with the OD of casein-elicited murine peritoneal neutrophils processed in the same way.

Measurement of messenger RNA (mRNA) expression by real-time RT-PCR. Total RNA was isolated from periarticular tissue using TRIzol reagent (Invitrogen) and from peritoneal macrophage culture using an RNeasy Mini kit (Qiagen). Total RNA was reverse transcribed with SuperScript III (Invitrogen) as described by the manufacturer. Real-time quantitative PCR was performed using an ABI Prism 7000 Sequence Detection System (Applied Biosystems). PCRs of murine GAPDH and GPR43 were performed with SYBR Green PCR Master Mix (Applied Biosystems). The relative level of gene expression was determined by the comparative threshold cycle method as described by the manufacturer, whereby data for each sample were normalized to the GAPDH constitutive gene and expressed as the fold change compared with control.

Reactive oxygen species (ROS) assay. Peritoneal macrophages were cultured in 96-well plates (0.5×10^6 cells/well) using RPMI medium without phenol red (Gibco) supplemented with 10% heat-inactivated FCS (Gibco) for 24 hours in a humidified incubator at 37°C with 5% CO_2. Cells were then loaded for 30 minutes with the ROS-specific fluorescent probe H2DCFDA (2′,7′-dichlorofluorescein diacetate; 20 μM final concentration) (Sigma-Aldrich), washed twice with preheated medium, and exposed to MSU (150 μg). Fluorescence was assessed at 5-minute intervals for 95 minutes with a spectrofluorometer (Synergy 2 BioTek) with a fluorescein isothiocyanate filter (excitation 485 nm; emission 538 nm). Eight repeats were performed for each experimental condition described in the Results section.

Phagocytosis assay. For in vitro assays, heat-inactivated *Staphylococcus aureus* conjugated to fluorescent BODIPY FL (fluorescein-like dye) (BioParticles; Molecular Probes) were used in order to assess phagocytosis. Twenty-five microliters of peripheral blood from wild-type or GPR-$43^{-/-}$ mice was incubated for 30 minutes at 37°C with 1×10^6 *S aureus* particles. Cells were then put on ice and costained with Ly6G&C, Ly6G (BD PharMingen), and CD11b (eBioscience). Cells were then fixed with 4% paraformaldehyde, and red blood cells were lysed with hypotonic solution. Phagocytosis was determined by flow cytometry (FACSCalibur; Becton Dickinson). The mean fluorescence intensity (MFI) of phagocytosis (by BODIPY fluorescence) was determined for macrophages, Ly6G&C+Ly6G−CD11b+. Analysis was done using FlowJo (Tree Star).

For in vivo assay, wild-type or GPR-$43^{-/-}$ mice were injected with 1×10^6 *S aureus* particles in PBS. After 1 hour, mice were killed and peritoneal cells were harvested, counted, and then stained for Ly6G&C, Ly6G (BD PharMingen), and CD11b (eBioscience). Phagocytosis was then determined by flow cytometry. The numbers of peritoneal macrophages were determined (relative to the amount of lavage fluid obtained) by gating on the Ly6G&C+Ly6G−CD11b+ population. The MFI of BODIPY (FITC channel) of this population was then determined as a measure of phagocytosis of the *S aureus* particles.

Statistical analysis. Data are presented as the mean ± SEM. Data were analyzed for normal distribution using Bartlett's test. Normalized data were compared by using analysis of variance followed by a Student-Newman-Keuls post hoc analysis. *P* values less than 0.05 were considered significant.

RESULTS

Initiation of responses to MSU crystals in a gout model requires microbiota or short-chain fatty acids. Injection of MSU crystals into the knee joints of germ-free mice yielded significantly less cell recruitment, especially neutrophil recruitment, than in conventional mice (Figure 1). MSU induced no macrophage recruitment in either conventional or germ-free mice 6 hours after MSU crystal injection (data not shown) or 15 hours after MSU crystal injection (mean ± SEM $4.6 \pm 1.1 \times 10^5$ cells/cavity in conventional mice treated with PBS, $4.6 \pm 1.8 \times 10^5$ cells/cavity in conventional mice treated with MSU, $3.7 \pm 0.5 \times 10^5$ cells/cavity in germ-free mice treated with PBS, and $3.9 \pm 1.3 \times 10^5$ cells/cavity in germ-free mice treated with MSU). Mechanical hypernociception, an index of pain and measurement of articular dysfunction, followed a pattern that was similar to cell recruitment, with levels of hypernociception in germ-free mice decreased 6 and 15 hours after crystal injection (Figures 1E and F).

To clarify if the phenotype observed in germ-free mice after MSU stimuli was dependent on the gut microbiota, we depleted conventional mice of their gut microbiota by providing antibiotic therapy (23) for 4 weeks prior to MSU stimulation. Treatment with antibiotics also decreased recruitment of leukocytes in response to MSU challenge (see Supplementary Figure 1, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.39107/abstract). Taken together, these results suggested that products of the gut microbiota were necessary for optimal leukocyte recruitment by MSU crystals.

Because MSU crystals stimulate innate immune cells within the joint, the most likely explanation is that a product of the gut microbiota somehow influences peripheral inflammatory responses. Acetate is the most abundant systemically distributed short-chain fatty acid (24). Measurement of acetate in serum from germ-free mice confirmed that the levels were significantly reduced compared with those in conventional mice (mean ± SEM acetate levels 106 ± 20 μM in conventional mice and 9 ± 2 μM in germ-free mice; n = 5 mice per group) ($P < 0.001$). To determine whether the

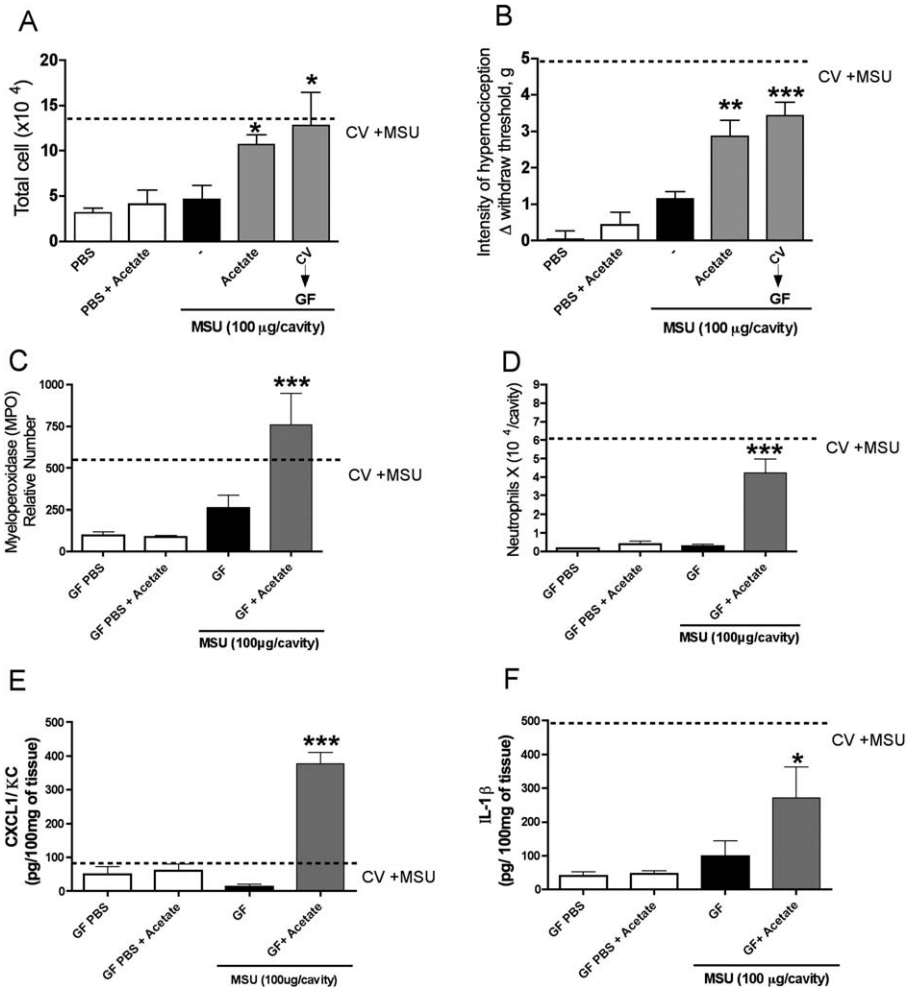

Figure 2. Microbiota and metabolic products of commensal bacteria are essential for initiating responses to MSU crystals in the mouse knee joint. **A** and **B**, Total number of cells in the synovial cavity (**A**) and hypernociception (**B**) in control germ-free mice that received an intraarticular injection of PBS, acetate-treated germ-free mice that received an intraarticular injection of PBS, germ-free mice that received an intraarticular injection of MSU crystals, acetate-treated germ-free mice that received an intraarticular injection of MSU crystals, and germ-free mice that received oral gavage of feces from conventional mice 14 days prior to challenge with MSU crystals. **C** and **D,** Myeloperoxidase level (**C**) and number of neutrophils in the synovial cavity (**D**) in the mouse articular knee tissue. **E** and **F,** Levels of CXCL1/keratinocyte-derived chemokine (KC) (**E**) and interleukin-1β (IL-1β) (**F**) in the mouse periarticular knee tissue, determined by enzyme-linked immunosorbent assay. Broken lines represent values found in conventional mice injected with MSU crystals and killed 15 hours after injection. Bars show the mean ± SEM (n = 5–7 mice per group). Experiments were repeated twice. $* = P < 0.05$; $** = P < 0.01$; $*** = P < 0.001$, versus germ-free mice injected with MSU crystals. See Figure 1 for other definitions.

lack of microbiota and short-chain fatty acids were responsible for the reduced recruitment of inflammatory cells, feces from conventional mice or acetate was reintroduced under sterile conditions into germ-free mice. Microbiota reposition and acetate treatment reversed the hypoinflammatory responsiveness of germ-free mice, as shown by increased total cell recruitment to the knee joint and increased hypernociception in the germ-free mice (Figures 2A and B). Furthermore, the treatment of germ-free mice with acetate induced increases in MPO levels (Figure 2C) and neutrophil infiltration (Figure 2D) in the periarticular tissue and synovial cavities, as well as increases in inflammatory mediators, such as the chemokine CXCL1 (KC) (Figure 2E) and IL-1β (Figure 2F), to levels similar to those found in conventional mice challenged with MSU.

Treatment of conventional mice with acetate did not significantly alter leukocyte recruitment 6 hours after MSU injection (mean ± SEM $4 \pm 1 \times 10^5$ cells/cavity in mice treated with PBS, $87 \pm 17 \times 10^5$ cells/cavity in mice treated with MSU, $6 \pm 1 \times 10^5$ cells/cavity in mice treated with acetate, and $69 \pm 5 \times 10^5$ cells/

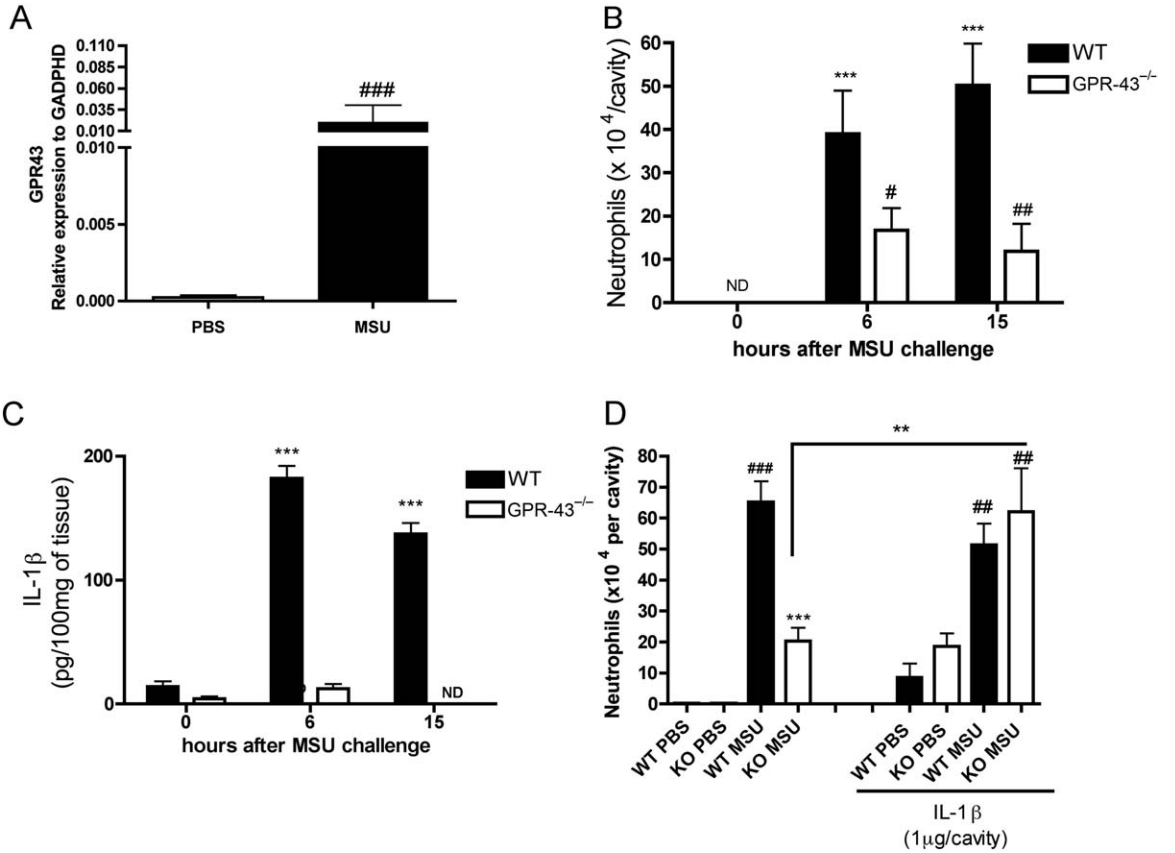

Figure 3. Role of GPR43 in gouty arthritis in mice. **A,** Quantitative reverse transcription–polymerase chain reaction analysis of GPR43 in the articular knee tissue of mice killed 15 hours after MSU challenge. **B** and **C,** Number of neutrophils in the synovial cavity (**B**) and production of interleukin-1β (IL-1β) in periarticular knee tissue (**C**) in wild-type (WT) and GPR-43$^{-/-}$ mice, assessed 0, 6, and 15 hours after injection of 100 μg of MSU. **D,** Number of neutrophils in the synovial fluid in wild-type and GPR-43$^{-/-}$ knockout (KO) mice that received an intraarticular injection of PBS or MSU crystals and either received no further treatment or were treated with recombinant IL-1β (1 μg per cavity). Mice were killed 15 hours after injection, and neutrophils were harvested from the synovial fluid and counted. Bars show the mean ± SEM (n = 6 mice per group). ** = $P < 0.01$; *** = $P < 0.001$ versus wild-type mice at 0 hours or wild-type mice treated with PBS; # = $P < 0.05$; ## = $P < 0.01$; ### = $P < 0.001$, versus wild-type mice at the same time point or mice of the same strain treated with PBS. ND = not detected (see Figure 1 for other definitions).

cavity in mice treated with acetate plus MSU; n = 6 mice per group). Thus, gut microbes and one of their major metabolites, acetate, contribute to the initiation of the inflammatory response to MSU crystals.

Effect of the short-chain fatty acid receptor GPR43 on neutrophil recruitment in the MSU model of gout. GPR43 is a receptor that binds short-chain fatty acids, particularly acetate, and is expressed on neutrophils and macrophages (12,17). As expected, expression of GPR43 was high in the knee joints of wild-type mice challenged with MSU crystals (Figure 3A), and this is compatible with GPR43 being expressed abundantly on infiltrating neutrophils. To better explore the role of GPR43 and its effects on gout-induced inflammatory response, we investigated the course of MSU-induced gout in wild-type and GPR-43$^{-/-}$ mice. GPR-43$^{-/-}$ mice showed significantly less MSU crystal–induced neutrophil recruitment throughout the course of the inflammatory response (Figure 3B).

As seen in Figure 3C, GPR-43$^{-/-}$ mice produced much lower levels of IL-1β in the periarticular tissue in response to injection of MSU crystals. We have previously shown the importance of inflammasome activation and IL-1β production for induction of CXCL1/KC and CXCR2-dependent neutrophil recruitment to the knee in this gout model (25). To confirm that absence of IL-1β production was responsible for the failure of neutrophil recruitment after MSU injection in GPR-43$^{-/-}$ mice, we injected recombinant IL-1β together with MSU into the knees of both wild-type and GPR-43$^{-/-}$ mice. Fifteen hours after injection of MSU plus IL-1β, we observed that recombinant IL-1β restored

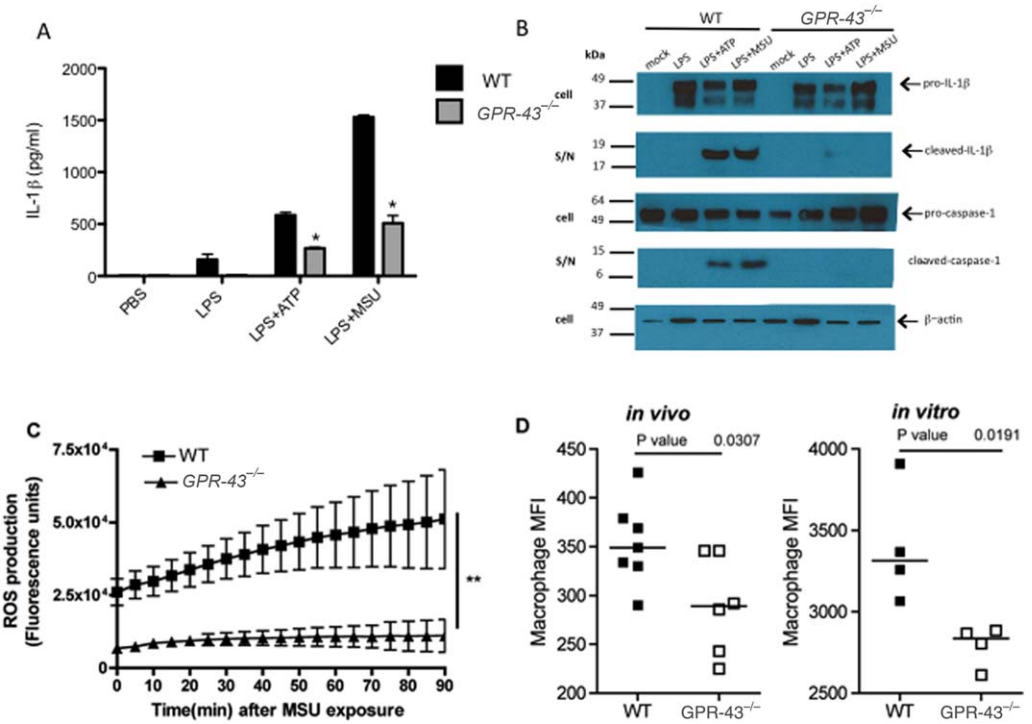

Figure 4. Impaired inflammasome activation in immune cells from GPR-43$^{-/-}$ mice. **A,** Interleukin-1β (IL-1β) levels in wild-type (WT) and GPR-43$^{-/-}$ mice. Macrophages isolated from GPR-43$^{-/-}$ or wild-type mice were primed with phosphate buffered saline (PBS) or pure lipopolysaccharide (LPS; 1 μg/ml) for 2 hours and subsequently stimulated with ATP or monosodium urate monohydrate (MSU) crystals for 4 hours. Cell culture supernatants were assayed for mouse IL-1β by enzyme-linked immunosorbent assay. **B,** Procaspase 1 and pro–IL1-β in cell lysates, analyzed by Western blotting, and cleaved caspase 1 and cleaved IL-1β in supernatants (S/N). **C,** Reactive oxygen species (ROS) production in nonprimed macrophages from wild-type and GPR-43$^{-/-}$ mice stimulated with MSU. **D,** Phagocytosis of *Staphylococcus aureus* bacteria by peritoneal macrophages from wild-type and GPR-43$^{-/-}$ mice injected intraperitoneally with heat-inactivated *S aureus*. Mean fluorescence intensity (MFI) is shown as a measure of phagocytosis. Symbols represent individual mice; horizontal lines show the mean. Results are representative of 3 independent experiments. In **A** and **C,** values are the mean ± SEM (n = 6 mice per group). $* = P < 0.01$; $** = P < 0.01$, versus wild-type mice.

neutrophil recruitment to the knee cavity of GPR-43$^{-/-}$ mice (Figure 3D). These results suggest that there is no intrinsic defect in neutrophil recruitment in this model, but there is a lack of adequate production of inflammasome-dependent mediators of inflammation that is needed for neutrophils to be recruited.

Necessity of GPR43 for optimal immune cell inflammasome activation and inflammatory responses to MSU crystals. To assess the importance of GPR43 for inflammasome activation in immune cells, we primed thioglycolate-elicited peritoneal macrophages from GPR-43$^{-/-}$ or wild-type control mice with LPS, and then stimulated these cells with ATP or MSU crystals to activate the inflammasome pathway. ATP- or MSU-induced IL-1β secretion was significantly reduced in macrophages from GPR-43$^{-/-}$ mice compared with wild-type controls (Figure 4A). Western blot analysis showed that wild-type macrophages activated caspase 1 and produced large amounts of cleaved IL-1β upon ATP or MSU exposure (Figure 4B), whereas macrophages lacking GPR43 produced almost no cleaved caspase 1 or IL-1β in response to ATP or MSU challenge (Figure 4B).

GPR43 signaling is known to regulate the production of ROS (12), and ROS can activate the NLRP3 inflammasome (26). Addition of MSU crystals to wild-type macrophages induced rapid production of ROS, whereas macrophages from GPR-43$^{-/-}$ mice showed a significant decrease in MSU crystal–induced production of ROS (Figure 4C). ROS are generated in large amounts by NADPH oxidase after microbial and/or particle phagocytosis. Consistent with this, GPR-43$^{-/-}$ macrophages exhibited marked reduction in capacity for phagocytosis of *S aureus* bacteria both in vitro and in vivo (Figure 4D). Thus, defects in phagocytosis, impaired production of ROS, and inefficient activation of the inflammasome pathway in GPR-43$^{-/-}$ macrophages is likely responsible for the observed inability of GPR-43$^{-/-}$ mice to fully develop MSU crystal–induced gout. A defect in response to LPS by GPR-43$^{-/-}$ macrophages could have been an alternative explanation for the lack of inflammasome assembly.

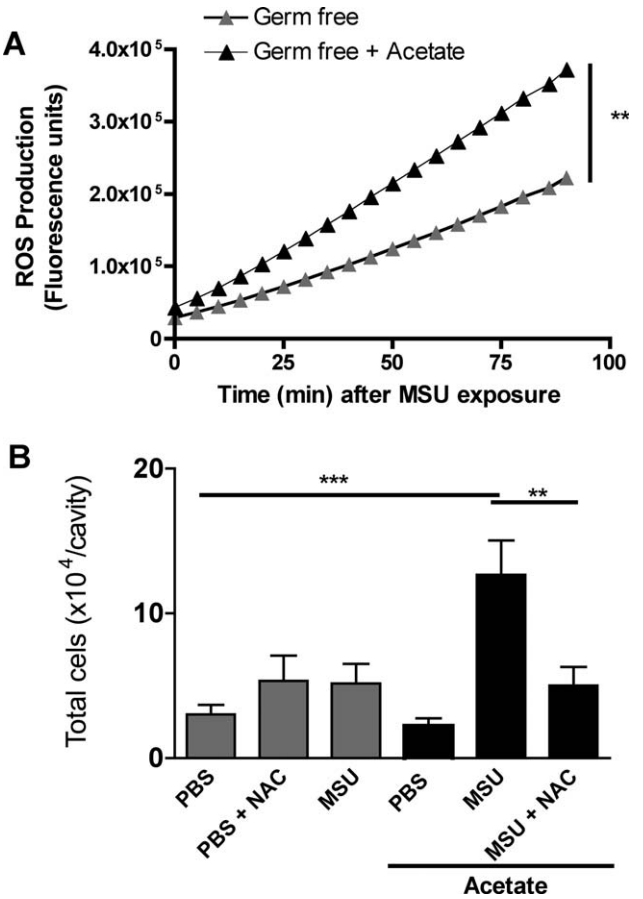

Figure 5. Acetate induces reactive oxygen species (ROS) production in macrophages from germ-free mice, and treatment with an ROS scavenger reverses the acetate-treated germ-free phenotype after monosodium urate monohydrate (MSU) injection. **A,** ROS production in nonprimed peritoneal macrophages isolated from germ-free mice or acetate-treated germ-free mice. Values are the mean. **B,** Total number of cells in the synovial cavity in germ-free mice 15 hours after phosphate buffered saline (PBS) or MSU challenge. The antioxidant *N*-acetyl-L-cysteine (NAC; 500 mg/kg) was administered intraperitoneally 1 hour after MSU challenge to germ-free mice that were previously left untreated or treated with acetate. Results are representative of 2 independent experiments. Bars show the mean ± SEM (n = 6 mice per group). ** = $P < 0.01$; *** = $P < 0.001$.

However, pro–IL-1β levels (Figure 4) and ROS production (see Supplementary Figure 2, available on the Arthritis & Rheumatology web site at http://onlinelibrary.wiley.com/doi/10.1002/art.39107/abstract) in response to LPS were similar in wild-type and GPR-43$^{-/-}$ mice.

In germ-free mice, LPS induced a 20-fold increase in pro–IL-1β mRNA above baseline (not detected in PBS-treated germ-free macrophages; mean ± SEM 31 ± 22 arbitrary units in LPS-treated germ-free macrophages; n = 3 mice per group) ($P < 0.05$), but this was not associated with the secretion of IL-1β (not detected in PBS-treated germ-free macrophages or in LPS-treated germ-free macrophages; n = 3 mice per group) ($P > 0.05$). In addition, there was decreased production of ROS by germ-free macrophages stimulated with MSU crystals (Figure 5A). Treatment with acetate enhanced ROS production (Figure 5A). In vivo, acetate restored inflammatory responses to MSU crystals, an effect that was prevented by the administration of the antioxidant *N*-acetyl-L-cysteine (an ROS scavenger) (Figure 5B). These data suggest that acetate could induce inflammatory responses after MSU stimulation by inducing increased levels of ROS in macrophages.

DISCUSSION

The major findings of our study can be summarized as follows: the short-chain fatty acid receptor GPR43 is necessary for effective activation of the inflammasome in vivo and in macrophages; GPR43 contributes to the respiratory burst in response to inflammasome activators and consequent inflammasome assembly; and in the absence of the microbiota, there are reduced levels of short-chain fatty acids, reduced inflammasome assembly, and reduced inflammatory responses to MSU crystals. This is reversed by administration of acetate in a manner dependent on the respiratory burst. Therefore, acetate and GPR43 contribute significantly to inflammasome assembly in response to MSU crystals, suggesting that these molecules contribute to the development of gout.

The inflammasome is a multimeric protein platform that regulates the cleavage and secretion of proinflammatory cytokines such as IL-1β, and hence plays an important role in host defense (27). Direct stimulation of NLRP3 by diverse molecular structures, including MSU crystals, may trigger the inflammasome directly. However, NLRP3 activation may also occur indirectly. Although we have not shown any evidence of direct influence of GPR43 activation or its downstream events on inflammasome activation, we do show that GPR43 and the products of gut microbiota are required for proper activation of caspase 1 and IL-β secretion, and the ensuing inflammatory response to MSU-induced gout. The latter finding is consistent with our observations that IL-β drives CXCR2-dependent neutrophil influx and hypernociception into the joint after injection of MSU crystals (25).

Caspase 1 activation induced by ATP stimulation is dependent on the formation of ROS (28), and ROS blockade by chemical scavengers suppresses inflammasome activation (29,30). Additionally, as we have shown previously in a model of acute gout, activation of the inflammasome is ROS dependent, and acetate stimulates ROS production in neutrophils in a GPR43-dependent manner (12,25). In the present study, phagocytosis of particles was

defective in GPR43-deficient leukocytes and so was subsequent production of ROS. Therefore, poor activation of the NLRP3 inflammasome in GPR-43$^{-/-}$ mouse macrophages appears to be related to their impaired ability to cause phagocytosis of particles and to produce ROS following MSU stimulation. Hence, GPR43/acetate-induced ROS production in immune cells represents a possible mechanism for regulation of the NLRP3 inflammasome.

Our findings provide an explanation of the findings of a previous study (31) that revealed a link between commensal microbiota and inflammasome-dependent cytokine activation at a remote site, the lung. Although the mechanism(s) whereby gut commensal bacteria could support inflammasome activation in remote sites was not addressed, the authors speculated that products of commensal bacteria might stimulate leukocytes systemically, through unknown pattern recognition receptors. We propose that the binding of acetate to GPR43 is an important systemic signal for inflammasome activation in immune cells.

The gut microbiota appears to have profound systemic effects on leukocytes, as evidenced by the finding that neutrophils and macrophages from germ-free mice display reduced phagocytosis, microbicidal activity, and production of superoxide anions (32–34). Indeed, decreased inflammatory responsiveness of germ-free mice to inflammatory stimuli is accompanied by a state of active IL-10–mediated hyporesponsiveness (35). Thus, germ-free mice are more susceptible to infections because they fail to mount an appropriate inflammatory response (35,36). Because germ-free leukocytes have never encountered pathogens, they respond less to many stimuli, but the molecular mechanisms have been poorly understood. A previous study found that neither TLR-2 nor TLR-4 was required for immune responses to MSU crystals, suggesting that LPS was not essential for priming gouty inflammation (37). This was consistent with our finding that germ-free mice responded well to MSU crystals once acetate was added to the drinking water, independent of any priming with other TLR-related bacterial products.

We believe that an important means by which commensal bacteria influence inflammation at a remote peripheral site such as the joints is through production of factors that distribute systemically. One of the major factors that is absent from the colon and blood of germ-free mice but is produced in abundance by bacteria in conventional mice are short-chain fatty acids, particularly acetate. Propionate and butyrate may also contribute to or control inflammatory responses, particularly in inflammatory bowel diseases (37,38). Hence, these molecules could also be contributing to responses to MSU crystals in our system. However, when compared with acetate, the systemic distribution of propionate and butyrate is significantly lower (39), suggesting that the contribution of acetate is presumably greater in conventional mice.

In our experiments, absence of gut microbiota, which was studied through the use of germ-free mice and antibiotic treatment, resulted in inflammatory hyporesponsiveness to injection of MSU crystals in the joints of mice. There is, however, no intrinsic defect of cell recruitment in germ-free mice, as evidenced by normal recruitment of neutrophils to CXCL1 (38), suggesting that inflammasome assembly in response to MSU was deficient in these animals. Interestingly, administration of acetate to germ-free mice was sufficient to restore their ability to assemble the inflammasome and to respond to MSU crystals. Moreover, inhibition of ROS reversed the phenotype of germ-free mice given acetate.

Short-chain fatty acids may induce neutrophil recruitment (an effect that is dependent on activation of GPR43) in vitro (16,39,40). The relevance of the latter finding for our in vivo observations is still not clear, but the findings do show that short-chain fatty acids can stimulate various functions on leukocytes, including contribution to the assembly of the inflammasome. Therefore, the absence of microbiota is associated with decreased levels of short-chain fatty acids, such as acetate, and decreased inflammasome assembly, which is dependent on the activation of GPR43, a receptor for short-chain fatty acids. GPR41 and GPR109A are also short-chain fatty acid receptors that contribute to inflammatory bowel disease and epithelium integrity (12,41). We cannot exclude a role for these receptors in our system, but we do show that activation of GPR43 is necessary for inflammasome assembly and that administration of acetate alone was sufficient to promote inflammatory responses to MSU crystals in germ-free mice by facilitating inflammasome assembly. GPR43 is believed to be a preferential receptor for acetate (17).

Some inflammatory conditions may be exacerbated in germ-free mice (10–12). However, the MSU gout model was clearly dependent on gut commensal microbes. This is consistent with previous studies by our group (13,14,42) showing that most inflammatory responses depend on the presence of gut microbiota. The reason some inflammatory responses are exacerbated and others are attenuated in germ-free mice is still unclear. It may be that inflammasome-related responses, which rely on ROS production, are attenuated in germ-free mice. One alternative explanation is based on the observation that inflammatory responses that are greater in germ-free mice are usually associated with enhanced

tissue damage and decreased capacity to resolve (14). Further studies are clearly needed to understand these issues in greater detail.

Recent studies have shown that short-chain fatty acids negatively modulate chronic diseases in conventional mice (43–45). We previously observed that both germ-free and GPR43-deficient mice showed exacerbated immune responses and nonresolving inflammation in chronic diseases, such as colitis, arthritis, and asthma (12). Moreover, treatment with short-chain fatty acids ameliorated these conditions in conventional mice (43–45). Here, we show that short-chain fatty acids are necessary for acute inflammasome-dependent inflammatory responses. Therefore, it appears that short-chain fatty acids are central modulators of inflammation by contributing to the onset, the intensity, and the duration of inflammatory responses.

In conclusion, this study demonstrated that the inability of germ-free mice to respond to MSU challenge is due in part to the absence of a microbial-derived metabolite, the short-chain fatty acid acetate. The main acetate receptor, the metabolic sensor GPR43, facilitated short-chain fatty acid effects in the MSU gout model. Hence, future research will require a deeper understanding of the complex interactions between the gut microbiota, their metabolic products, nutrition, and modulation of immune responses. This may also allow new approaches to the prevention or treatment of gouty arthritis.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Drs. Teixeira and Mackay had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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REFERENCES

1. Shi Y, Mucsi AD, Ng G. Monosodium urate crystals in inflammation and immunity. Immunol Rev 2010;233:203–17.
2. Martinon F, Petrilli V, Mayor A, Tardivel A, Tschopp J. Gout-associated uric acid crystals activate the NALP3 inflammasome. Nature 2006;440:237–41.
3. Terkeltaub RA, Ginsberg MH. The inflammatory reaction to crystals. Rheum Dis Clin North Am 1988;14:353–64.
4. Hachicha M, Naccache PH, McColl SR. Inflammatory microcrystals differentially regulate the secretion of macrophage inflammatory protein 1 and interleukin 8 by human neutrophils: a possible mechanism of neutrophil recruitment to sites of inflammation in synovitis. J Exp Med 1995;182:2019–25.
5. Martin WJ, Walton M, Harper J. Resident macrophages initiating and driving inflammation in a monosodium urate monohydrate crystal–induced murine peritoneal model of acute gout. Arthritis Rheum 2009;60:281–9.
6. Roberge CJ, Grassi J, De Medicis R, Frobert Y, Lussier A, Naccache PH, et al. Crystal-neutrophil interactions lead to interleukin-1 synthesis. Agents Actions 1991;34:38–41.
7. Elinav E, Strowig T, Kau AL, Henao-Mejia J, Thaiss CA, Booth CJ, et al. NLRP6 inflammasome regulates colonic microbial ecology and risk for colitis. Cell 2011;145:745–57.
8. Zaki MH, Boyd KL, Vogel P, Kastan MB, Lamkanfi M, Kanneganti TD. The NLRP3 inflammasome protects against loss of epithelial integrity and mortality during experimental colitis. Immunity 2010;32:379–91.
9. Hirota SA, Ng J, Lueng A, Khajah M, Parhar K, Li Y, et al. NLRP3 inflammasome plays a key role in the regulation of intestinal homeostasis. Inflamm Bowel Dis 2011;17:1359–72.
10. Chervonsky AV. Influence of microbial environment on autoimmunity. Nat Immunol 2010;11:28–35.
11. Herbst T, Sichelstiel A, Schar C, Yadava K, Burki K, Cahenzli J, et al. Dysregulation of allergic airway inflammation in the absence of microbial colonization. Am J Respir Crit Care Med 2011;184:198–205.
12. Maslowski KM, Vieira AT, Ng A, Kranich J, Sierro F, Yu D, et al. Regulation of inflammatory responses by gut microbiota and chemoattractant receptor GPR43. Nature 2009;461:1282–6.
13. Souza DG, Vieira AT, Soares AC, Pinho V, Nicoli JR, Vieira LQ, et al. The essential role of the intestinal microbiota in facilitating acute inflammatory responses. J Immunol 2004;173:4137–46.
14. Fagundes CT, Souza DG, Nicoli JR, Teixeira MM. Control of host inflammatory responsiveness by indigenous microbiota reveals an adaptive component of the innate immune system. Microbes Infect 2011;13:1121–32.
15. McFall-Ngai M, Hadfield MG, Bosch TC, Carey HV, Domazet-Loso T, Douglas AE, et al. Animals in a bacterial world, a new imperative for the life sciences. Proc Natl Acad Sci U S A 2013;110:3229–36.
16. Le Poul E, Loison C, Struyf S, Springael JY, Lannoy V, Decobecq ME, et al. Functional characterization of human receptors for short chain fatty acids and their role in polymorphonuclear cell activation. J Biol Chem 2003;278:25481–9.
17. Brown AJ, Goldsworthy SM, Barnes AA, Eilert MM, Tcheang L, Daniels D, et al. The orphan G protein-coupled receptors GPR41 and GPR43 are activated by propionate and other short chain carboxylic acids. J Biol Chem 2003;278:11312–9.
18. Nilsson NE, Kotarsky K, Owman C, Olde B. Identification of a free fatty acid receptor, FFA_2R, expressed on leukocytes and activated by short-chain fatty acids. Biochem Biophys Res Commun 2003;303:1047–52.
19. Vinolo MA, Rodrigues HG, Hatanaka E, Sato FT, Sampaio SC, Curi R. Suppressive effect of short-chain fatty acids on production of proinflammatory mediators by neutrophils. J Nutr Biochem 2011;22:849–55.
20. Masui R, Sasaki M, Funaki Y, Ogasawara N, Mizuno M, Iida A, et al. G protein-coupled receptor 43 moderates gut inflammation through cytokine regulation from mononuclear cells. Inflamm Bowel Dis 2013;19:2848–56.
21. Sachs D, Coelho FM, Costa VV, Lopes F, Pinho V, Amaral FA, et al. Cooperative role of tumour necrosis factor-α, interleukin-1β and neutrophils in a novel behavioural model that concomitantly demonstrates articular inflammation and hypernociception in mice. Br J Pharmacol 2011;162:72–83.
22. Coelho FM, Pinho V, Amaral FA, Sachs D, Costa VV, Rodrigues DH, et al. The chemokine receptors CXCR1/CXCR2 modulate

antigen-induced arthritis by regulating adhesion of neutrophils to the synovial microvasculature. Arthritis Rheum 2008;58:2329–37.
23. Reikvam DH, Erofeev A, Sandvik A, Grcic V, Jahnsen FL, Gaustad P, et al. Depletion of murine intestinal microbiota: effects on gut mucosa and epithelial gene expression. PLoS One 2011;6:e17996.
24. McOrist AL, Abell GC, Cooke C, Nyland K. Bacterial population dynamics and faecal short-chain fatty acid (SCFA) concentrations in healthy humans. Br J Nutr 2008;100:138–46.
25. Amaral FA, Costa VV, Tavares LD, Sachs D, Coelho FM, Fagundes CT, et al. NLRP3 inflammasome–mediated neutrophil recruitment and hypernociception depends on leukotriene B_4 in a murine model of gout. Arthritis Rheum 2012;64:474–84.
26. Dostert C, Petrilli V, Van Bruggen R, Steele C, Mossman BT, Tschopp J. Innate immune activation through Nalp3 inflammasome sensing of asbestos and silica. Science 2008;320:674–7.
27. Gross O, Thomas CJ, Guarda G, Tschopp J. The inflammasome: an integrated view. Immunol Rev 2011;243:136–51.
28. Martinon F. Signaling by ROS drives inflammasome activation. Eur J Immunol 2010;40:616–9.
29. Cruz CM, Rinna A, Forman HJ, Ventura AL, Persechini PM, Ojcius DM. ATP activates a reactive oxygen species-dependent oxidative stress response and secretion of proinflammatory cytokines in macrophages. J Biol Chem 2007;282:2871–9.
30. Zhou L, Aon MA, Almas T, Cortassa S, Winslow RL, O'Rourke B. A reaction-diffusion model of ROS-induced ROS release in a mitochondrial network. PLoS Comput Biol 2010;6:e1000657.
31. Ichinohe T, Pang IK, Kumamoto Y, Peaper DR, Ho JH, Murray TS, et al. Microbiota regulates immune defense against respiratory tract influenza A virus infection. Proc Natl Acad Sci U S A 2011;108:5354–9.
32. Ohkubo T, Tsuda M, Tamura M, Yamamura M. Impaired superoxide production in peripheral blood neutrophils of germfree rats. Scand J Immunol 1990;32:727–9.
33. Oliveira MR, Tafuri WL, Afonso LC, Oliveira MA, Nicoli JR, Vieira EC, et al. Germ-free mice produce high levels of interferon-γ in response to infection with Leishmania major but fail to heal lesions. Parasitology 2005;131:477–88.
34. Kanther M, Tomkovich S, Xiaolun S, Grosser MR, Koo J, Flynn EJ III, et al. Commensal microbiota stimulate systemic neutrophil migration through induction of serum amyloid A. Cell Microbiol 2014;16:1053–67.
35. Souza DG, Fagundes CT, Amaral FA, Cisalpino D, Sousa LP, Vieira AT, et al. The required role of endogenously produced lipoxin A_4 and annexin-1 for the production of IL-10 and inflammatory hyporesponsiveness in mice. J Immunol 2007;179:8533–43.
36. Fagundes CT, Amaral FA, Vieira AT, Soares AC, Pinho V, Nicoli JR, et al. Transient TLR activation restores inflammatory response and ability to control pulmonary bacterial infection in germfree mice. J Immunol 2012;188:1411–20.
37. Chen CJ, Shi Y, Hearn A, Fitzgerald K, Golenbock D, Reed G, et al. MyD88-dependent IL-1 receptor signaling is essential for gouty inflammation stimulated by monosodium urate crystals. J Clin Invest 2006;116:2262–71.
38. Fagundes CT, Souza DG, Nicoli JR, Teixeira MM. Control of host inflammatory responsiveness by indigenous microbiota reveals an adaptive component of the innate immune system. Microbes Infect 2011;13:1121–32.
39. Vinolo MA, Ferguson GJ, Kulkarni S, Damoulakis G, Anderson K, Bohlooly YM, et al. SCFAs induce mouse neutrophil chemotaxis through the GPR43 receptor. PLoS One2011;6:e21205.
40. Sina C, Gavrilova O, Forster M, Till A, Derer S, Hildebrand F, et al. G protein-coupled receptor 43 is essential for neutrophil recruitment during intestinal inflammation. J Immunol 2009;183:7514–22.
41. Singh N, Gurav A, Sivaprakasam S, Brady E, Padia R, Shi H, et al. Activation of Gpr109a, receptor for niacin and the commensal metabolite butyrate, suppresses colonic inflammation and carcinogenesis. Immunity 2014;40:128–39.
42. Amaral FA, Sachs D, Costa VV, Fagundes CT, Cisalpino D, Cunha TM, et al. Commensal microbiota is fundamental for the development of inflammatory pain. Proc Natl Acad Sci U S A 2008;105:2193–7.
43. Trompette A, Gollwitzer ES, Yadava K, Sichelstiel AK, Sprenger N, Ngom-Bru C, et al. Gut microbiota metabolism of dietary fiber influences allergic airway disease and hematopoiesis. Nat Med 2014;20:159–66.
44. Chang PV, Hao L, Offermanns S, Medzhitov R. The microbial metabolite butyrate regulates intestinal macrophage function via histone deacetylase inhibition. Proc Natl Acad Sci U S A 2014;111:2247–52.
45. Arpaia N, Campbell C, Fan X, Dikiy S, van der Veeken J, deRoos P, et al. Metabolites produced by commensal bacteria promote peripheral regulatory T-cell generation. Nature 2013;504:451–5.